UNIVERSITY OF MOLISE

Department of Biosciences and Territory (DiBT)

DOCTORAL THESIS

"BIOLOGY AND APPLIED SCIENCES"
Curriculum BIOLOGY
XXXVII Cycle

Molecular, phylogenetic and functional analysis of species of the genus *Quercus* (subgenus *Quercus* and subgenus *Cerris*)

S.S.D. BIOS-01/B

Doctoral coordinator:

Professor Filippo Santucci De Magistris

Supervisor:

Professor Paola Fortini Paola Fortini

Co-Supervisor:

Professor Piera Di Marzio Piera Di Marzio

PhD Student

Luca Quaranta

Luca Quaranta

Academic year 2023/2024

Doctoral thesis title:

Molecular, phylogenetic and functional analyses of species of the genus *Quercus* (subgenus *Quercus* and subgenus *Cerris*)

PhD candidate:

Luca Quaranta

PhD Program Coordinator:

Professor Filippo Santucci de Magistris

Supervisor:

Professor Paola Fortini

University of Molise - Department of Bioscience and Territory (DiBT)

Pesche (IS), Italy

Co-supervisor:

Professor Piera Di Marzio

University of Molise - Department of Bioscience and Territory (DiBT)

Pesche (IS), Italy

Reviewers:

Dr. Katharina Birgit Budde

Northwest German Forest Research Institute

Hann Mueden, Germany

Professor Carlos Vila-Viçosa

Research Center in Biodiversity and Genetic Resources

University of Porto

Vairão, Portugal

Table of contents

ABSTRACT 5

INTRODUCTION AND OBJECTIVES **7**

CHAPTER 1 Taxonomic and Phylogeographic diversity of white oaks **12**

1.1 Dissecting the continuum and unravelling the phylogeographic knot of plastid DNA in European white oaks (Quercus sect. Quercus): ancient signatures and multiple diversity reservoirs (Fortini et al. 2024 – contributing author) 17

1.2 Chloroplast Haplotype Diversity in the White Oak Populations of the Italian Peninsula, Sicily, and Sardinia (Di Pietro et al. 2024 – contributing author) 38

CHAPTER 2 Ecological adaptation of Quercus cerris L. (Turkey oak) **69**

2.1 Analysis of the functional traits of *Quercus cerris* L. seedlings in the Molise region (southern Italy) (Quaranta et al. 2023) 76

2.2 *Quercus cerris* leaf traits analysis to assess urban forests health status for expeditious analysis in a Mediterranean European contest (Quaranta et al. 2025 – corresponding author) 90

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES 112

ADDITIONAL ACTIVITIES DURING THE PHD **113**

ACKNOWLEDGMENTS **205**

Abstract

This thesis aimed to investigated some of the phylogeographic, taxonomic, and ecological aspects of the genus *Quercus*, one of the most significant genera in terms of distribution and ecological importance globally, in Italy and in Europe, Specifically, the thesis was structured around two distinct research lines. The first line concerned population genetics studies at the European and Italian levels, based on the analysis of plastid DNA using microsatellite markers of species belonging to the subgenus *Quercus* section *Quercus*, a section that maintains several critical points in terms of taxonomic classification, which remain a subject of interest and debate in the scientific community and require, unlike the *Cerris* section, further investigation. The second research line focused on studies regarding responses to environmental variables and health status in natural and urban environments, through the analysis of leaf traits, in *Quercus cerris* L., a representative species of the subgenus *Cerris* section *Cerris*. From a phylogenetic perspective, this is the first European study that investigated DNA sequence polymorphism of two plastid loci in 270 individuals representing 13 species/taxa of white oaks. Several hundred sequences of the same two DNA regions were retrieved from GenBank to fill in the gaps and contribute to a better understanding of the diversity and evolution of white oaks. The "modern" European species of *Quercus* sect. *Quercus* show poorly differentiated plastid DNA, despite its highly diversified current taxonomy The main results were that twenty-nine haplotypes were identified in the Euro-Mediterranean and Near Eastern regions, including two ancestral variants subdivided into Western and South-Eastern groups. Other high- and low-frequency haplotypes also exhibited a well-structured geographic distribution, consistent with the current phytogeographic framework of Europe. The second study, conducted in the Italian Peninsula, focused on potential haplotype structures distributed across the Italian Peninsula, Sicily, and Sardinia. The chloroplast DNA of 60 populations belonging to *Q. frainetto*, *Q. robur*, *Q. petraea* and *Q. pubescens* complexes was analyzed by combining five chloroplast Simple Sequence Repeat (cpSSR) markers. A total of 28 haplotypes were detected. Central and southern Italy displayed the highest variability (14 and 10 haplotypes, respectively), followed by northern Italy (7), Sardinia (7), and Sicily (5). A complex geographical structure of haplotype distribution emerged, highlighting: (i) a high number of low-frequency haplotypes; (ii) the marked isolation of Sardinia; (iii) the occurrence of haplotypes widely distributed throughout the Italian Peninsula; (iv) the idiosyncrasy of Sicily, which exhibits exclusive haplotypes, as well as haplotypes shared with Sardinia and the rest of the Italian Peninsula. The haplotype distribution was also found to be partially related to the taxonomic identity of the specimens, with the following features emerging: a geographic separation between *Q. frainetto* populations from central and southern Italy, an unexpected discontinuity between the Calabrian and Sicilian *Q. petraea* subsp. *austrotyrrhenica* populations, and the absence of the most common haplotype among the *Q. pubescens* populations of central and southern Italy.

The second research line focused on *Q. cerris* and its ecological importance in the Italian Peninsula, where it has been selected as a target species for urban reforestation under the National Recovery and Resilience Plan (PNRR). This research line was structured into two different studies aimed at gaining new knowledge on the ecophysiology of *Q. cerris*.

The first study focused on *Q. cerris* seedlings from three forest communities under different management practices, applying various Plant Functional Traits (PFTs) to assess the health status of the seedlings. The results showed that seedlings from different sampling sites exhibited similar values for all phenotypic parameters. Statistically significant differences, however, were observed in the PFTs. These findings suggested that the different adaptation strategies implemented by the seedlings are linked to the physical environment of the sampling sites and to the different forest structures and woodland management. The forest stand that exhibited better growth conditions for seedlings, as evidenced by higher values of Specific Leaf Area, Chlorophyll content, and lower values of Leaf Dry Matter Content and Leaf Thickness, was the Adult High Forest (90 year) in a substrate of varicoulored clay (0% slope) to which in 2006-2007 high thinning (grade: heavy) silvicultural treatment was applied. These results were interpreted as a greater investment in carbon production for rapid development and renewal of the seedling, rather than in carbon storage for ensuring leaf longevity. The second study on *Q. cerris* was conducted on mature individuals from three forest stands with varying degrees of naturalness, in terms of green fragmentation and green cover, and called Urban forest (UF), Peri-Urban forest (PUF), and Natural forest (NF). The results demonstrated that *Q. cerris* experiences significant water stress in urban forests due to the combined effects of drought and high temperatures. To identify strategies for mitigating this stress, differences in leaf traits such as specific leaf area, thickness, and contents of chlorophyll, anthocyanins, and flavonols in urban and natural forests were analysed. Our findings highlight the high adaptability of *Q. cerris* to diverse climatic and environmental conditions providing a practical method for rapidly assessing tree species' responses to climate change.

These studies are part of a broader and interdisciplinary line of research, dedicated to shedding light on the morphological, ecological and biomolecular characteristics of the *Quercus* genus in Europe, with a particular focus in Italy, carried out by the Laboratory of Botany and Systematic Floristics of the University of Molise.

In summary, this thesis provided new insights into the phylogeny and biogeography of white oaks at both the Italian and European levels and shed light on the ecology and physiology of *Q. cerris*, laying the groundwork for future studies on this species and other species and communities of interest. It also offers valuable information for reforestation efforts at the national level.

Distribution of the genus *Quercus*

The genus *Quercus* (Fagaceae) is one of the most cosmopolitan and ecologically significant genera of woody plants, comprising over 400 species (Govaerts and Frodin 1998; Denk et al. 2017), distributed predominantly across the Boreal Hemisphere, from the Americas to the Euro-Asian continent, occupying a wide range of habitats, from arid deserts and Mediterranean maquis to subtropical rainforests and temperate deciduous forests (Cavender-Bares et al. 2016; Denk et al. 2017; Kremer and Hipp 2020). The most recent comprehensive taxonomic revision by Denk et al. (2017) has reorganized *Quercus* into two main subgenera: *Quercus* (white oaks) and *Cerris* (Figure 1). The subgenus *Quercus*, is globally distributed and includes five sections: *Lobatae*, *Protobalanus*, *Virentes*, *Ponticae* and *Quercus*. The first three are uniquely American, while *Ponticae* and *Quercus* are North American and Eurasian. The subgenus *Cerris* instead, is exclusively present in the Eurasian continent and is divided into three sections: *Ilex*, *Cerris* and *Cyclobalanopsis*.

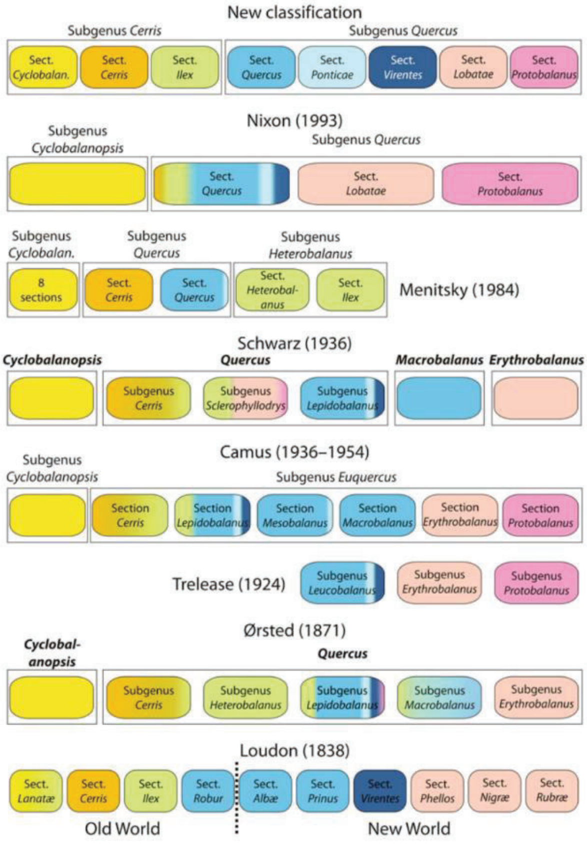


Figure 1 History of classifications of Oaks. Adapted from the work of Denk et al., 2017.

These subgenera are particularly diverse in North America, which harbors approximately 220 species, and in Asia, with notable richness in China and the Himalayas (Manos et al., 2001; Cannon & Manos, 2003). In Europe, although oaks dominate extensive forested landscapes from boreal forests to Mediterranean scrublands (Mucina et al. 2016; Manos and Hipp 2021), they are little diversified in terms of species, six for the subgenus *Cerris* and four for the subgenus *Quercus* (Figure 2).

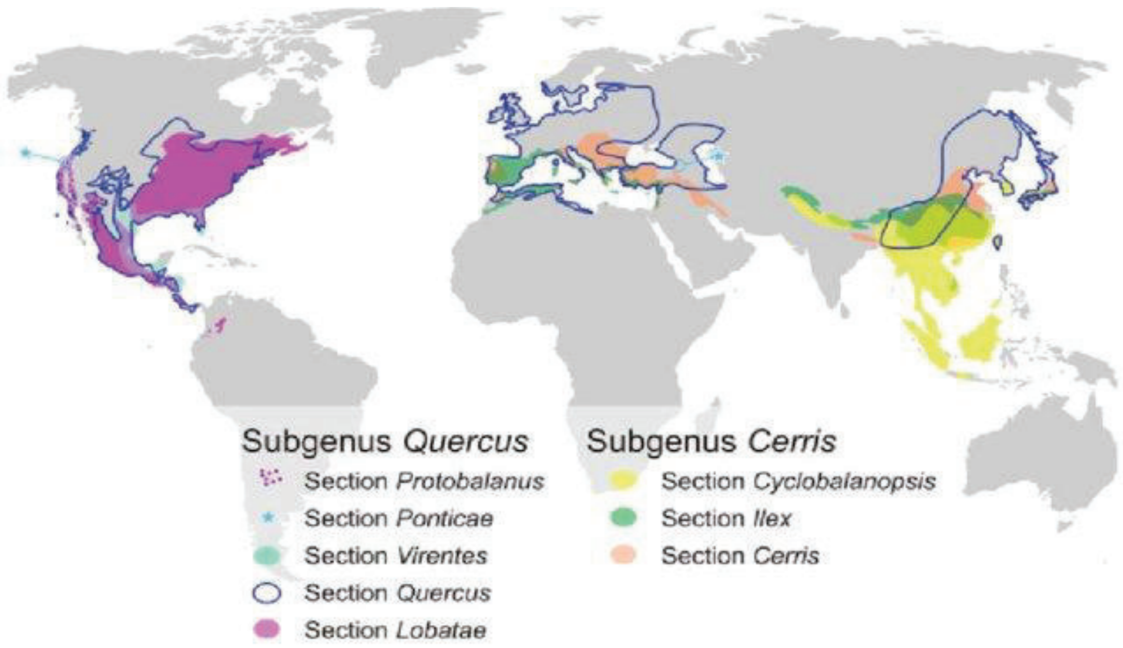


Figure 2 Geographic distribution of the eight sections of Quercus. Distribution data from Browicz and Zieliński (1982), Menitsky (1984), Costa Tenorio et al. (2001), Deng (2007), Fang et al. (2009), and Manos (2016) . Adapted from the work of Denk et al. 2017

In Italy, these subgenera are present with three sections *Quercus*, *Cerris* and *Ilex*. Italy's woodlands are diverse ecosystems shaped by geographical, climatic, and historical factors, spanning from the Alpine regions of the North to the Mediterranean landscapes of the South and varying from evergreen and deciduous species at altitudes ranging from 0 to 1200-1400 meters above sea level. The territory of the peninsula is mainly characterized by mixed woodlands dominated by *Q. ilex* L. along the coast, and extensive mixed oak woods (*Quercus cerris* L., *Q. frainetto* Ten., *Q. petraea* (Matt.) Liebl., *Q. pubescens* Willd., *Q. robur* L.) (Blasi et al. 1982; Blasi and Di Pietro 1998; Blasi et al. 2004) from the lowlands to the hilly and sub-mountainous belt, up to the turkey oaks of the mountain belt (Blasi et al 2005; Di Pietro and Tondi 2015).

The genus *Quercus* plays a fundamental role in ecosystems worldwide. Despite comprising relatively few species, it shapes the physiognomy of forests and numerous habitats in Europe and Italy. From a taxonomic perspective, many principal gaps have been filled, leading to a clearer understanding at the genus and some sub-genus levels. However, significant uncertainties remain in the taxonomy of the subgenus *Quercus*, particularly within section *Quercus*. This has resulted in two contrasting evolutionary viewpoints among taxonomists: one camp has described new species within groups such as *Quercus pubescens* and *Quercus petraea* over the decades, while the other camp attributes the observed variability to substantial intra-specific differences rather than to distinct new species.

Furthermore, although the phylogeography of the *Cerris* section (within the subgenus *Cerris*) has been thoroughly investigated, its distribution across Italian and European territories, combined with the Mediterranean region's vulnerability to climate change and global warming, underscores the ecological importance of *Quercus cerris* in this basin. In fact, Turkey oak, due to its remarkable phenotypic plasticity and its ability to grow on acidic soils in dry, water-poor conditions (Blasi et al 2004), has been selected, along with other species, for afforestation and reforestation initiatives under the National Recovery and Resilience Plan (NRPP). For these reasons, there is a pressing need for new studies on the adaptation and functionality of this species, which would enhance our understanding of its autecology and its response to the impacts of climate change and global warming.

Objectives

Based on the importance of the genus *Quercus* from a phylogenetic, taxonomic and ecological point of view, this thesis considers all these topics. In a wide-ranging study of the genus *Quercus*, the objectives are on the one hand to deepen phylogeographical knowledge on the white oak in Italy and Europe, and on the other hand to provide monitoring tools to assess the health status of the turkey oak. Specifically, the objectives of the thesis are:

Phylogeographical issue

- *reconstruct phylogenetic relationships of the West Eurasian white oaks plastid DNA.*
- *improve our understanding of the phylogeography of white oaks species, European populations and areas;*
- *capture rare genetic variants that could evidence divergent evolutionary lineages*
- *The possibility to correlate the distribution of haplotypes in natural white oak forests in Italy with biogeographical patterns*
- *The possible presence of hotspots of genetic diversity and rare or divergent genetic variants worthy of special management and conservation measures*
- *The identification of a possible haplotype structure linked to the taxonomical identity of the Italian white oak populations*

Ecological issues

- *To study the response of Turkey oak seedlings to environmental conditions*
- *To test and detect the selected leaf functional traits to study the response of tree species in urban forests;*
- *To detect the main climatic variables that may affect urban forests;*
- *To compare the health status of Quercus cerris in urban forests versus natural forests during different seasons;*
- *To propose expedited and practical tools for monitoring the response of the trees in different urban forest conditions.*

References

1. Govaerts, R.; Frodin, D.G. World Checklist and Bibliography of Fagales; Royal Botanic Gardens, Kew, **1998**.
2. Denk, T.; Grimm, G.W.; Manos, P.S.; Deng, M.; Hipp, A.L. An Updated Infrageneric Classification of the Oaks: Review of Previous Taxonomic Schemes and Synthesis of Evolutionary Patterns. In Oaks Physiological Ecology. Exploring the Functional Diversity of Genus Quercus L.; Gil-Pelegrín, E., Peguero-Pina, J.J., Sancho-Knapik, D., Eds.; Tree Physiology; Springer International Publishing: Cham, **2017**; pp. 13–38 ISBN 978-3-319-69099-5.
3. Govaerts, R.; Nic Lughadha, E.; Black, N.; Turner, R.; Paton, A. The World Checklist of Vascular Plants, a Continuously Updated Resource for Exploring Global Plant Diversity. Sci. Data **2021**, 8, 215, doi:10.1038/s41597-021-00997-6.
4. Cavender-Bares, J.; Ackerly, D.D.; Hobbie, S.E.; Townsend, P.A. Evolutionary Legacy Effects on Ecosystems: Biogeographic Origins, Plant Traits, and Implications for Management in the Era of Global Change. Annu. Rev. Ecol. Evol. Syst. **2016**, 47, 433–462, doi:10.1146/annurev-ecolsys-121415-032229.
5. Kremer, A.; Hipp, A.L. Oaks: An Evolutionary Success Story. New Phytol. **2020**, 226, 987–1011, doi:10.1111/nph.16274.
6. Cannon, C.H.; Manos, P.S. Phylogeography of the Southeast Asian Stone Oaks (Lithocarpus). J. Biogeogr. **2003**, 30, 211–226, doi:10.1046/j.1365-2699.2003.00829.x.
7. Manos, P.S.; Cannon, C.H.; Oh, S.-H. Phylogenetic Relationships and Taxonomic Status Of the Paleoendemic Fagaceae Of Western North America: Recognition Of A New Genus, Notholithocarpus. *Madroño* **2008**, 55, 181–190, doi:10.3120/0024-9637-55.3.181.
8. Mucina, L.; Bültmann, H.; Dierßen, K.; Theurillat, J.-P.; Raus, T.; Čarni, A.; Šumberová, K.; Willner, W.; Dengler, J.; García, R.G.; et al. Vegetation of Europe: Hierarchical Floristic Classification System of Vascular Plant, Bryophyte, Lichen, and Algal Communities. Appl. Veg. Sci. **2016**, 19, 3–264, doi:10.1111/avsc.12257.
9. Manos, P.S.; Hipp, A.L. An Updated Infrageneric Classification of the North American Oaks (Quercus Subgenus Quercus): Review of the Contribution of Phylogenomic Data to Biogeography and Species Diversity. *Forests* **2021**, 12, 786, doi:10.3390/f12060786.
10. Blasi, C.; Feoli, E.; Avena, G.C. Due Nuove Associazioni Dei Quercetalia Pubescentis Dell'appennino Centrale. **1982**.
11. Blasi, C.; Di Pietro, R.; Filesi, L. Syntaxonomical Revision of Quercetalia Pubescenti-Petraeae in the Italian Peninsula. *Fitosociologia* **2004**, *41*, 87–164.
12. Blasi, C.; Fortini, P.; Grossi, G.; Presti, G. Faggete e Cerrete Mesofile Nell'Alto Molise. *Fitosociologia* **2005**, *42*, 67–81.
13. Di Pietro, R.; Tondi, G. A New Mesophilous Turkey-Oak Woodland Association From Laga Mts. (Central Italy). *Hacquetia* **2015**, *4/2*: 5–25.

Pignatti et al. (2017) describe the genus *Quercus* as taxonomically complex, primarily due to the influence of gene flow, introgression, and hybridization. These factors significantly impact species differentiation and classification within the genus. For this reason the classification of the different subgenera within the genus Quercus has undergone several changes until the last revision by Denk et al. (2017). The previous classification was that of Nixon (1993) in which proposed a classification of *Quercus* that primarily relied on morphological characters, dividing the genus into two subgenera, *Cyclobalanopsis* and *Quercus* including the group later recognized as white oaks (subgenus *Quercus*) with evergreen oaks and cerris oaks. However, the subsequent molecular phylogenetic studies by Hipp (2015) and then Denk et al. (2017) revealed that Nixon's system did not fully capture the evolutionary relationships among oak lineages. These studies demonstrated that the white oaks form a distinct, monophyletic group characterized by a rapid acorn maturation process completed within a single growing season and by less ornamented cupules compared to their *Cerris* counterparts.

White oaks, display a remarkable and varied distribution across Europe with a broad ecological range and taxonomic complexity, shaped by climatic gradients, soil conditions, and historical land-use patterns. In Eurasia, species such as *Quercus robur* (Figure 3B) and *Q. mongolica* dominate temperate forests, extending into boreal taiga regions and cold steppe biomes (Kubitzki, 1993; Menitsky, 2005)., while *Quercus petraea* (Figure 3D) is predominantly associated with upland regions characterized by well-drained, nutrient-poor soils (Denk et al., 2017). Additionally *Quercus frainetto* (Hungarian oak) (Figure 3C), native to southeastern Europe including Hungary and the Balkan Peninsula and thrives in warmer, well-drained sites; its occurrence in isolated stands in regions where Mediterranean and continental climates converge further enriches the European oak mosaic (Petit et al., 2002). Similarly, xerothermic species like *Q. pubescens* (Figure 3A) thrive in thermophilous Mediterranean forests, often found near thermomediterranean shrublands (Brullo and Marcenò 1985; Blasi and Di Pietro 1998). Southern Europe, particularly the Italian Peninsula, served as a critical glacial refuge during the Quaternary. This contributed to the persistence and diversification of thermophilous forests and white oak populations (Brewer et al., 2002; Blasi et al., 2004; Magri et al., 2006; Guarino et al., 2015).

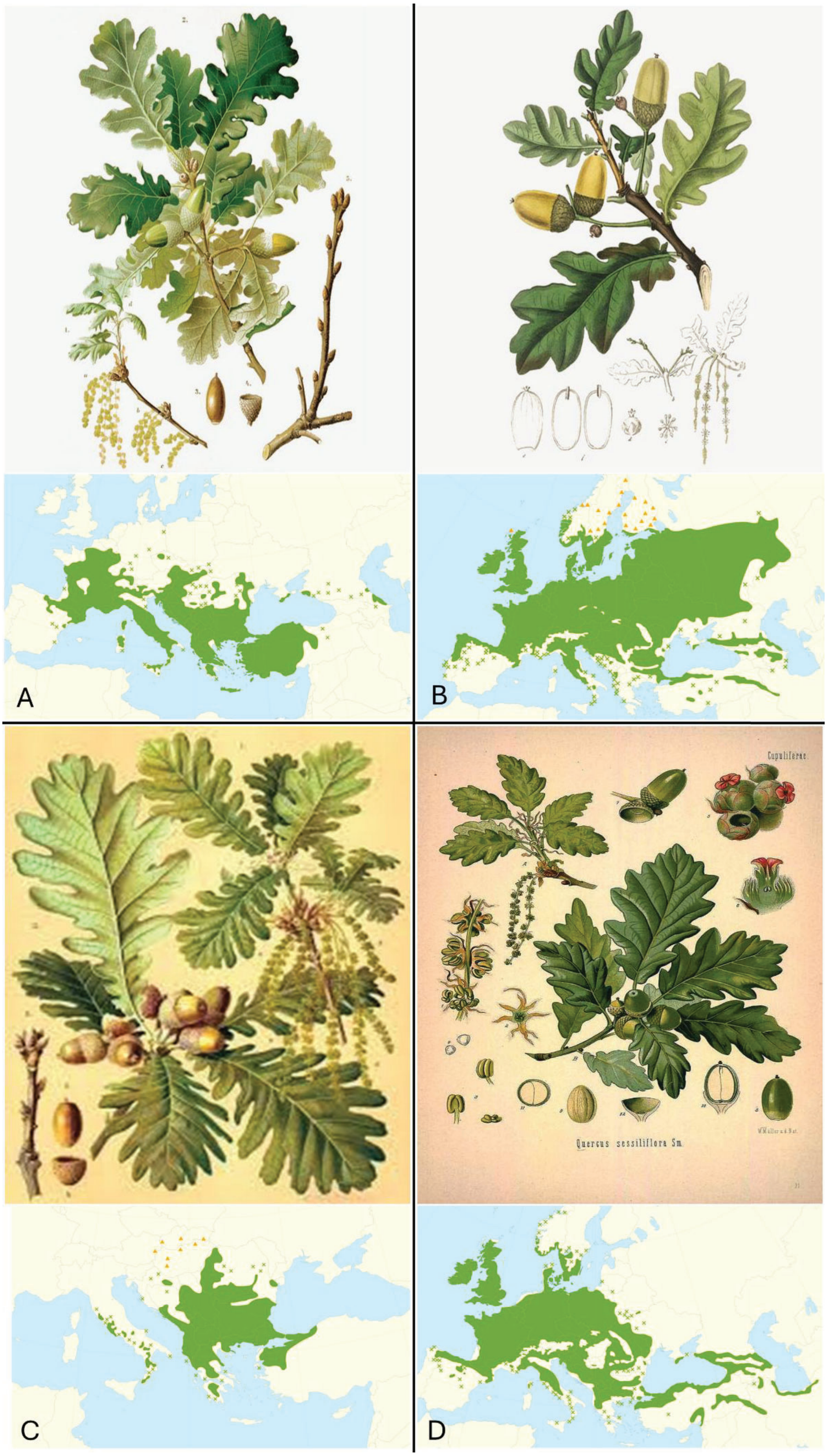


Figure 3 Distribution and diagnostics characteristics of the four species of white oaks analysed: A - Quercus pubescens; B - Quercus robur; C - Quercus frainetto; D - Quercus petraea

In fact, in the Italian Peninsula, the distribution of white oaks reflects the country's geomorphological variety and climatic diversity. *Quercus robur* is primarily found in the northern regions, flourishing in riparian forests and lowland areas where moisture is abundant (Blasi et al., 2004). In contrast, *Quercus petraea* is more common in the upland and montane forests of northern and central Italy, where cooler and more humid microclimates prevail (Petit et al., 2002). *Quercus pubescens* is typically encountered in the warmer, drier regions of central and southern Italy, often forming mixed stands with other Mediterranean species. Meanwhile, *Quercus frainetto*, though less widespread, occurs in localized areas particularly in transitional zones between Mediterranean and continental climates, thereby contributing to the genetic and ecological diversity of Italian oak forests (Blasi et al., 2004). This mosaic of species distributions highlights the ecological versatility of white oaks and reinforces their role in maintaining forest structure, resilience, and ecosystem services across Europe and Italy (Livesley et al. 2016).

From a taxonomical point of view, within the white oaks, the *Q. pubescens* and *Q. petraea* groups have undergone the most substantial classification changes over the decades. These groups, which include several species identified across Italian and European territories, still present unresolved taxonomic uncertainties. Taxonomic debates surrounding white oaks underscore the challenges posed by their high eco-morphological variability and interfertility within the genus (Burger 1975; Di Pietro et al. 2016). In Italy, this variability is further reflected in endemic species such as *Q. amplifolia*, *Q. ichnusae*, and *Q. leptobalana*. These endemics highlight the need for detailed taxonomic and biogeographical studies to unravel the complex evolutionary and ecological dynamics of these taxa (Amaral-Franco 1990; Fortini et al. 2015; Piredda et al. 2021).

Chloroplast DNA (cpDNA) studies have significantly advanced our understanding of the evolutionary history and biogeography of European white oaks. The foundational work of Dumolin-Lapègue et al. (1997), followed by the key contributions of Petit et al. (2002) and Fineschi et al. (2002) in Italy, laid the groundwork for investigating the phylogeographic relationships of white oaks. Using PCR-RFLP methods, these studies identified six major cpDNA lineages across Europe, with three lineages preserved in Southern Italy and the major islands during the Quaternary (Dumlin-Lapègue et al. 1997). Subsequent studies, including those by Fineschi et al. (2002), found additional haplotypes and highlighted the Italian Peninsula as a hotspot of cpDNA diversity. The shift from PCR-RFLP to cpSSR markers has enhanced the resolution of genetic analyses, allowing for the detection of more intricate phylogeographic patterns in Southern European refugia (Hubert et al., 2014; Vitelli et al., 2017). Despite advancements, the plastid genomes of European white oaks remain underexplored compared to their North American and East Asian counterparts, leaving key gaps in the understanding of their evolutionary history (Simeone et al. 2013; Li et al. 2022). Addressing these gaps is essential for refining phylogeographic patterns and developing effective conservation strategies.

References

1. Pignatti, S.; Guarino, R.; La Rosa, M. *Flora d'italia*; 2nd ed.; Edagricole: Bologna, 2017; Vol. 2; ISBN 88-506-5242-9.
2. Denk, T.; Grimm, G.W.; Manos, P.S.; Deng, M.; Hipp, A.L. An Updated Infrageneric Classification of the Oaks: Review of Previous Taxonomic Schemes and Synthesis of Evolutionary Patterns. In Oaks Physiological Ecology. Exploring the Functional Diversity of Genus Quercus L.; Gil-Pelegrín, E., Peguero-Pina, J.J., Sancho-Knapik, D., Eds.; Tree Physiology; Springer International Publishing: Cham, **2017**; pp. 13–38 ISBN 978-3-319-69099-5.
3. Nixon K.C Infrageneric classification of *Quercus* (Fagaceae) and typification of sectional names. *Ann Sci For* **1993** 50:25s–34s
4. Hipp, A.L. Should hybridization make us skeptical of the oak phylogeny. *Int Oaks* **2015**, 26:9–17
5. Kubitzki, K. Fagaceae. In *Flowering Plants · Dicotyledons: Magnoliid, Hamamelid and Caryophyllid Families*; Kubitzki, K., Rohwer, J.G., Bittrich, V., Eds.; The Families and Genera of Vascular Plants; Springer: Berlin, Heidelberg, 1993; pp. 301–309 ISBN 978-3-662-02899-5.
6. Menitsky, Y.L. Oaks of Asia (Translated from Russian), Science Publishes. *Inc Enfield N. H. USA* **2005**, 549.
7. Brullo, S.; Marcenò, C. Contributo Alla Conoscenza Della Classe Quercetea Ilicis in Sicilia. *Not. Fitosociologico* **1985**, *19*, 183–229.
8. Blasi, C.; Di Pietro, R. Two New Phytosociological Types of Quercus Pubescens s.l. Woodland Communities in Southern Latium. *Plant Biosyst. - Int. J. Deal. Asp. Plant Biol.* **1998**, *132*, 207–223, doi:10.1080/11263504.1998.10654205.
9. Brewer, S.; Cheddadi, R.; de Beaulieu, J.L.; Reille, M. The Spread of Deciduous Quercus throughout Europe since the Last Glacial Period. *For. Ecol. Manag.* **2002**, *156*, 27–48, doi:10.1016/S0378-1127(01)00646-6.
10. Blasi, C.; Di Pietro, R.; Filesi, L. Syntaxonomical Revision of Quercetalia Pubescenti-Petraeae in the Italian Peninsula. *Fitosociologia* **2004**, *41*, 87–164.
11. Magri, D. Lineamenti Della Vegetazione Tardoglaciale in Italia Peninsulare e in Sicilia. In *Il Tardiglaciale in Italia–Lavori in Corso*; Archaeopress, 2008; Vol. 1859, pp. 1–8 ISBN 1-4073-0342-2.
12. Guarino, R.; Bazan, G.; Paura, B. Downy-Oak Woods of Italy: Phytogeographical Remarks on a Controversial Taxonomic and Ecologic Issue. In *Warm-Temperate Deciduous Forests around the Northern Hemisphere*; Box, E.O., Fujiwara, K., Eds.; Geobotany Studies; Springer International Publishing: Cham, 2015; pp. 139–151 ISBN 978-3-319-01261-2.

13. Petit, R.J.; Csaikl, U.M.; Bordács, S.; Burg, K.; Coart, E.; Cottrell, J.; van Dam, B.; Deans, J.D.; Dumolin-Lapègue, S.; Fineschi, S.; et al. Chloroplast DNA Variation in European White Oaks: Phylogeography and Patterns of Diversity Based on Data from over 2600 Populations. *For. Ecol. Manag.* **2002**, *156*, 5–26, doi:10.1016/S0378-1127(01)00645-4.

14. Livesley, S. J., McPherson, E. G., & Calfapietra, C. (2016). The urban forest and ecosystem services: Impacts on urban water, heat, and pollution cycles at the tree, street, and city scale. Journal of Environmental Quality, 45(1), 119–124

15. Burger, W.C. The Species Concept in Quercus. *TAXON* **1975**, *24*, 45–50, doi:10.2307/1218998.

16. Di Pietro, R.; Di Marzio, P.; Medagli, P.; Misano, G.; Silletti, G.N.; Wagensommer, R.P.; Fortini, P. Evidence from Multivariate Morphometric Study of the Quercus Pubescens Complex in Southeast Italy. *Bot. Serbica* **2016**, *40*, 83–100.

17. Amaral-Franco, J. Quercus. En: Castroviejo, S. et Al. *Flora Ibérica II CSIC Madr.* **1990**.

18. Fortini, P.; Di Marzio, P.; Di Pietro, R. Differentiation and Hybridization of Quercus Frainetto, Q. Petraea, and Q. Pubescens (Fagaceae): Insights from Macro-Morphological Leaf Traits and Molecular Data. *Plant Syst. Evol.* **2015**, *301*, 375–385, doi:10.1007/s00606-014-1080-2.

19. Piredda, R.; Grimm, G.W.; Schulze, E.-D.; Denk, T.; Simeone, M.C. High-Throughput Sequencing of 5S-IGS in Oaks: Exploring Intragenomic Variation and Algorithms to Recognize Target Species in Pure and Mixed Samples. *Mol. Ecol. Resour.* **2021**, *21*, 495–510, doi:10.1111/1755-0998.13264.

20. Dumolin-Lapègue, S.; Demesure, B.; Fineschi, S.; Le Come, V.; Petit, R.J. Phylogeographic Structure of White Oaks Throughout the European Continent. *Genetics* **1997**, *146*, 1475–1487, doi:10.1093/genetics/146.4.1475.

21. Fineschi, S.; Taurchini, D.; Grossoni, P.; Petit, R.J.; Vendramin, G.G. Chloroplast DNA Variation of White Oaks in Italy. *For. Ecol. Manag.* **2002**, *156*, 103–114, doi:10.1016/S0378-1127(01)00637-5.

21. Hubert, F.; Grimm, G.W.; Jousselin, E.; Berry, V.; Franc, A.; Kremer, A. Multiple Nuclear Genes Stabilize the Phylogenetic Backbone of the Genus Quercus. *Syst. Biodivers.* **2014**, *12*, 405–423, doi:10.1080/14772000.2014.941037.

22. Vitelli, M.; Vessella, F.; Cardoni, S.; Pollegioni, P.; Denk, T.; Grimm, G.W.; Simeone, M.C. Phylogeographic Structuring of Plastome Diversity in Mediterranean Oaks (Quercus Group Ilex, Fagaceae). *Tree Genet. Genomes* **2017**, *13*, 3, doi:10.1007/s11295-016-1086-8.

23. Simeone, M.C.; Piredda, R.; Papini, A.; Vessella, F.; Schirone, B. Application of Plastid and Nuclear Markers to DNA Barcoding of Euro-Mediterranean Oaks (Quercus, Fagaceae): Problems, Prospects and Phylogenetic Implications. *Bot. J. Linn. Soc.* **2013**, *172*, 478–499, doi:10.1111/boj.12059.

24. Li, Y.; Wang, T.-R.; Kozlowski, G.; Liu, M.-H.; Yi, L.-T.; Song, Y.-G. Complete Chloroplast Genome of an Endangered Species Quercus Litseoides, and Its Comparative, Evolutionary, and Phylogenetic Study with Other Quercus Section Cyclobalanopsis Species. *Genes* **2022**, *13*, 1184, doi:10.3390/genes13071184.

European Journal of Forest Research (2024) 143:107–127
https://doi.org/10.1007/s10342-023-01610-8

ORIGINAL PAPER

Dissecting the continuum and unravelling the phylogeographic knot of plastid DNA in European white oaks (*Quercus* sect. *Quercus*): ancient signatures and multiple diversity reservoirs

Paola Fortini[1] · **Romeo Di Pietro**[2] · **Elisa Proietti**[1] · **Simone Cardoni**[3] · **Luca Quaranta**[1] ·
Marco Cosimo Simeone[4]

Received: 14 May 2023 / Revised: 9 August 2023 / Accepted: 14 September 2023 / Published online: 7 October 2023

Abstract

Available knowledge on the European white oaks (*Quercus*, sect. *Quercus*) plastome still exhibits large gaps, mostly in respect of detailed phylogeny and information from southern Europe and adjacent Near East. We investigated DNA sequence polymorphism at two plastid loci on 270 individuals representing 13 white oak species/taxa and retrieved several hundred sequences of the same two DNA regions from GenBank to fill the above gaps, and contribute to a better understanding of white oak diversity and evolution. The modern European sect. *Quercus* species exhibits a poorly differentiated plastid DNA, despite its current highly diversified taxonomy. Twenty-nine haplotypes were identified across the Euro-Mediterranean and Near East regions, including two ancestral variants that are westerly and south-easterly partitioned. Other high- and low-frequency haplotypes also showed a well-structured geographic distribution consistent with the current phytogeographic framework of the European continent. The climatic and geological events that characterized the end of the Tertiary and Quaternary periods played a crucial role in triggering haplotype mixing, isolation, and in offering niche opportunities for more recent diversification. Euro-Mediterranean southern territories host a high and as yet poorly studied genetic variation; the role of the Italian Peninsula as a crossroad and threshold for haplotype diversity and distribution clearly emerges from this study.

Keywords Chloroplast DNA · *Quercus* · White oaks · Evolution · Phylogeography · Mediterranean

Introduction

The genus *Quercus* is one of the main woody components of the forests in the boreal hemisphere. Its ecological dominance and the remarkable heterogeneity and biodiversity asset of its habitats endorse the importance of multidisciplinary studies to integrate ecology and evolution for a better comprehension of community assembly and adaptation processes in a changing world (Cavender-Bares et al. 2016; Kremer and Hipp 2020).

One of the *Quercus* major clades, section *Quercus* (the white oaks), includes nearly 150 species distributed

Communicated by Oliver Gailing.

✉ Romeo Di Pietro
romeo.dipietro@uniroma1.it

Paola Fortini
fortini@unimol.it

Elisa Proietti
elisaproietti91@gmail.com

Simone Cardoni
s.cardoni@unitus.it

Luca Quaranta
l.quaranta1@studenti.unimol.it

Marco Cosimo Simeone
mcsimeone@unitus.it

[1] Department of Biosciences and Territory, University of Molise, Via Hertz S.N.C., Pesche (Is), Italy

[2] Department of Planning, Design, Technology of Architecture (PDTA), Sapienza University of Roma, Via Flaminia 70, 00196 Roma, Italy

[3] CNR - Research Institute On Terrestrial Ecosystems (IRET), Viale Guglielmo Marconi 2, 05010 Porano (Tr), Italy

[4] Department of Agricultural and Forestry Science (DAFNE), University of Tuscia, Via S. Camillo de' Lellis, 01100 Viterbo, Italy

throughout North America, western Eurasia, East Asia, and North Africa (Denk et al. 2017). In the American continent, where over 100 species occur, the white oaks exhibit an extremely wide morpho-physiological variation including sclerophyllous shrubby species inhabiting desert zones and dry savannahs, and lobe-leaved large dominant tree species in alluvial flatwoods, bottomlands and cold intermontane woodlands (Manos and Hipp 2021).

In Eurasia as well, shrubby and tree oak species do occur on extremely diversified niches and act as guide species in a very high number of syntaxa within all the ranks of phytosociological classification (Mucina et al. 2016). For instance, species that are more widespread, such as *Quercus robur* or *Q. mongolica*, are dominant throughout the majority of the temperate broad-leaved deciduous forest Biome and extend to the boundary of Taiga boreal forest or cold steppe Biomes (Kubitzki 1993; Menitsky 2005). Instead, xerothermic forms belonging to the *Q. pubescens s.l.* complex give rise to thermophilous forests adjacently to primary *Oleo-Ceratonion* thermo-Mediterranean shrublands (Brullo and Marcenò 1985; Blasi and Di Pietro 1998).

In addition to this great ecological amplitude, West Eurasian white oaks are characterized by a significant taxonomic complexity. In fact, the debate about the makeup of the white oaks' species list and the definition of a shared taxonomic framework based on morpho-ecological descriptors is still very lively, especially as regards the *Q. pubescens* and Q. *petraea* collective species groups (Trinajstić 2007; Fortini et al. 2009, 2015a, 2022; Di Pietro et al. 2016; Denk et al. 2017). In addition, longstanding partially unsolved nomenclatural issues (see Amaral-Franco 1990; Di Pietro et al. 2012) and the limits that affect the concept of biological species in such a notoriously interfertile genus (Burger 1975; Antonecchia et al. 2015; Hipp 2015), greatly complicate the assessment of taxonomic and syntaxonomic frameworks at national and international scales (Wellstein and Spada 2015; Pasta et al. 2016; Grossoni et al. 2021; Kaplan et al. 2022). What is beyond doubt is that the divisive and sometimes inhomogeneous taxonomic classification and the consequent nomenclatural complexity characterizing the West Eurasian white oaks have negative repercussions for studies of living matter at various level (gene, species, population, ecosystem) involving diverse fields of research (genetics, ecology phytosociology, landscape planning and design, conservation etc.). Taxonomic contradictions and consequent nomenclatural disputes are therefore to be viewed as outcomes of the difficulties in classifying the peculiar molecular, morphological and ecological variability that white oak species and communities express at present, especially in the Euro-Mediterranean zone (Guarino et al. 2015; Fortini et al. 2015b; Piredda et al. 2021).

Biogeographic history and evolutionary legacies had a strong influence on oak species differentiation and local adaptation worldwide (Cavender-Bares 2019). It is generally assumed that the complex palaeogeographic and palaeoclimatic vicissitudes that affected western Eurasia during the Miocene and throughout the 'turbulent' Quaternary played a key role in setting the scene for the high white oaks' diversity we can presently observe. It remains to be established the extent to which the aforementioned vicissitudes influenced the original amount of genetic diversity and how they combined in the different lineages and territories to drive diversification to produce such a varied and puzzling species group.

In this view, robust plastid phylogenies are essential to reveal phylogeographic patterns in closely related species, highlighting complex evolutionary phenomena, gene pools deserving attention and offering the opportunity to correlate the obtained data with the historical reconstruction of biomes, niche evolution and future landscape planning (Cavender-Bares et al. 2016; Blair 2023). However, any direct involvement of plastid phylogenies could be challenged by inaccurate methodologies (Blair 2023). For instance, when only one or few representatives of each species are investigated, thus providing little insight into the multifold issues pertinent to a species' diversity (Backs and Ashley 2021).

Studies on the plastid DNA of the European white oaks began in the closing decade of the last millennium (Dumolin et al. 1995) and culminated with the fundamental research of Petit et al. (2002b) where six main PCR–RFLP lineages were identified across a wide extent of the European continent. From the general results presented in that large-scale research, further insights were subsequently derived, focusing on single European countries or well-defined geographic areas (e.g. Cottrell et al. 2002; Fineschi et al. 2002; Olalde et al. 2002, for Great Britain, Italy and Spain, respectively; Csaikl et al. 2002 for the Alpine Region). Since then, the majority of phylogenetic research has concentrated on the genus nucleome, with the European white oaks being only partially included (e.g. Hubert et al. 2014; see also McVay et al. 2017; Hipp et al. 2020; Denk et al. 2023), while plastid DNA investigation has been relegated to DNA barcoding projects or small-scale phylogeographic studies (e.g. Simeone et al. 2013; Ekhvaia et al. 2018; Douaihy et al. 2020). Curiously, complete plastid genome sequences of the West Eurasian white oaks has also received little attention in phylogenetic investigations. Instead, they have lagged well behind the large amount of data gathered on North American and East Asian oaks (e.g. Pham et al. 2017; Pang et al. 2019; Liu et al. 2021; Li et al. 2022). Furthermore, in contrast to sects. *Ilex* and *Cerris*, the white oaks do not even have a tentative framework phylogeny to direct well-aimed samplings for deeper genomic studies. This information gap represents a serious limitation for any attempts to outline the evolutionary history of European white oaks and propose phylogeographic patterns that might explain the highly variable taxonomical and phytosociological patterns of white oak communities. Such a limitation takes on greater significance if we consider that the majority of the quaternary glacial refugia for the temperate forest vegetation and the highest coenological diversity for the white oaks are concentrated in southern Europe (Blasi et al. 2004; Mucina et al. 2016). All this reinforces the opinion that full genome sequencing from one or just a few individuals per species should not eclipse studies conducted using few but well-defined marker regions, especially if the latter are phylogenetically informative and the sampling design is well aimed and dense. For example, all the major intra- and interclade relationships that have recently been highlighted by means of complete plastome sequence of sect. *Ilex* oaks (Yang et al. 2021; Zhou et al. 2022) were already disclosed in previous studies based on just two markers (trnH-psbA, trnK-matK) and exhaustive samplings (Simeone et al. 2016; Vitelli et al. 2017).

In this work, we have tried to tackle this issue by maximizing taxonomic and geographic sampling in order to: (i) reconstruct phylogenetic relationships of the West Eurasian white oaks plastid DNA; (ii) improve our understanding of the phylogeography of species, populations and areas; (iii) capture rare genetic variants that could subtend divergent evolutionary lineages. With this aim, we have selected two marker regions that proved their efficacy in previous oak studies and partially compensating for the absence of wider samplings taking advantage of the data available in the GenBank repository, thereby expanding the taxonomic and biogeographic breadth of the investigated dataset.

Materials and methods

Sampling design

The study was carried out over a vast area of the Euro-Mediterranean region with some extensions in central Europe and North Africa (Fig. 1). Largely focused on Italy, the sampling ('primary dataset') also included ten additional countries: Austria, Bulgaria, Croatia, Czech Republic, France, Greece, Romania, Serbia, Spain and Morocco. Where possible, the collection sites were selected prioritizing stands of taxonomic or biological relevance (e.g. *loci classici*, protected areas and sites where

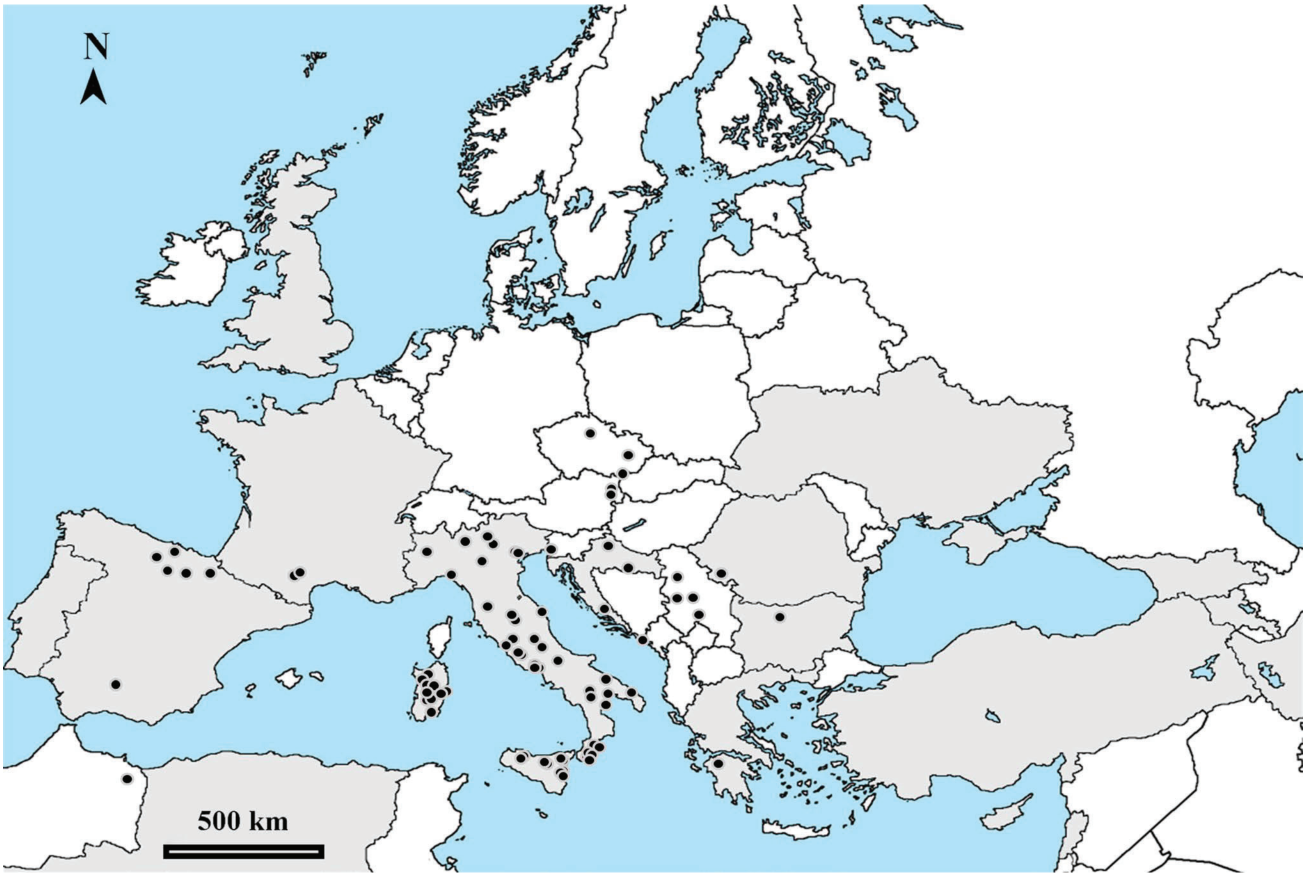


Fig. 1 Geographical distribution of the collection sites of white oaks populations (black dots). Countries from which accessible sequences on GenBank were retrieved and used in our analyses are filled in light grey colour (China, Japan and S-Korea are not reported in this map)

phytosociological descriptions of forest vegetation were available). The following accepted (www.ipni.org; www.powo.science.kew.org) oak species were investigated: *Quercus robur* L., *Q. petraea* (Matt.) Liebl. (including *Q. petraea* subsp. *austrotyrrhenica* Brullo, Guarino & Siracusa), *Q. frainetto* Ten., *Q. pubescens* Willd., *Q. dalechampii* Ten., *Q. faginea* Lam., *Q. pyrenaica* Willd., *Q. congesta* C.Presl and *Q. ichnusae* Mossa, Bacch. & Brullo. In addition three other species were included in our dataset although these are considered as synonyms of other taxa in POWO. These are *Quercus banatus* P.Kucera (name recently proposed as replacing the name *Q. dalechampii* Ten. in SE Europe in the collective group of *Q. petraea*), *Q. virgiliana* (Ten.) Ten. (name largely used in the, national floras checklists, phytosociological descriptions and syntaxonomic frameworks of several S European countries) and *Q. leptobalana* Guss (accepted as valid name in Pignatti et al. 2017 last edition of Flora of Italy and guide species of the association *Quercetum leptobalanae* Brullo & Marcenò 1985). In fact, diverse papers published in the last decade (Bock and Tison 2012; Di Pietro et al. 2012, 2016, 2020b; Von Raab-Straube and Raus 2013; Kučera 2018; Fortini et al. 2022) aimed at a critical analysis of the taxonomy of *Q. pubescens* and *Q. petraea* collective groups, exhibited a tendency towards a reduction in the number of oak taxa. On the other hand, the taxonomic arrangement within these two collective groups is far from being fully defined. For this reason, in order not to lose information on local oak forest diversity, we have preferred to make reference to the names currently reported in the national floras or in phytosociological synthesis already published in the sites of collection or in surrounding areas (see Table 2). However a detailed description of the investigated dataset together with a direct reference to the IPNI/POWO nomenclature is reported in supplementary file 1. Three oak individuals per population collected at least 30 m apart from each other and 90 total populations were sampled. These populations are divided into the following taxa: *Quercus banatus* (1), *Q. congesta* (9), *Q. dalechampii* (8), *Q. faginea* (3), *Q. frainetto* (6), *Q. ichnusae* (3), *Q. leptobalana* (1), *Q. petraea* (12), *Q. petraea* subsp. *austrotyrrhena* (2), *Q. pubescens* (26), *Q. pyrenaica* (1), *Q. robur* (11), *Q. virgiliana* (7). The collected specimens were identified through the use of analytical keys present in the national floras. In the case of dubious or particularly critical specimens, reference was made to already published floristic-phytosociological papers concerning the collection sites (where present) and the expert knowledge of the authors. Voucher specimens (ID number reported in Supplementary file S1) are deposited at the herbarium of the University of Molise (IS; Thiers 2016).

DNA extraction, sequencing and editing

DNA (270 total samples) was extracted with the NucleoSpin™ Plant II Kit (Macherey–Nagel) from silica gel dried leaves, following the manufacturer's instructions. TrnH-psbA intergenic spacer and a portion of the trnK-matK region (3' intron and partial gene sequence) were chosen because of their high number of accessible sequences on GenBank, and the variability displayed in previous studies (e.g. Okaura et al. 2007; Manos et al. 2008; Simeone et al. 2013, 2016). Primers and PCR conditions were as in Piredda et al. (2011). PCR products were purified with Illustra GFX PCR DNA Purification Kit (GE Healthcare) and standardized aliquots were sent to Macrogen Europe (https://www.macrogen-europe.com/) for bi directional sequencing. Electropherograms were edited with Chromas 2.6.2 (https://www.technelysium.com.au) and checked visually. Multiple alignments of the single and combined plastid regions were generated with MEGA X (Kumar et al. 2018) and adjusted manually.

In order to set our data in a broader diversity and phylogenetic context, GenBank was explored for all members of subgenus *Quercus* and West Eurasian subgen. *Cerris* sequenced with trnH-psbA and trnK-matK. Only single individuals sequenced with both markers were retrieved and used in the downstream analyses, with the exception of the oaks of sect. *Cerris*, for which the scarcity of trnK-matK sequences did not allow the creation of a consistent dataset. Haplotype lists and the main diversity parameters of the investigated markers were computed with DnaSP v.6 (Rozas et al. 2017).

Finally, the generated haplotypes were blasted against 244 *Quercus* complete chloroplast genomes available on GenBank (accessed on Jan. 31, 2023).

Data analyses

Phylogenetic tree inference and bootstrap analyses were performed under maximum likelihood with RAxML v.8.2.11 (Stamatakis 2014). We used the GTR + CAT approximation model and the 'extended majority-rule consensus' criterion as bootstopping option (Pattengale et al. 2009), with up to 1000 bootstrap (BS) pseudoreplicates to assess branch support (BS). The CAT model is a computational work–around for the widely used General Time Reversible model of nucleotide substitution under the Gamma model of rate heterogeneity (GTR-Γ). Compared to GTR-Γ, it has the advantage of significantly lower memory consumption, faster inference times and superior likelihood values in the obtained trees (Stamatakis 2006, 2014). The output (78-tip tree, after the removal of the identical sequences) included six accessions of subgen. *Cerris* (sects. *Cerris*, *Cyclobalanopsis* and *Ilex;* this latter including all the currently identified main lineages:

East Asian, West Asia–Himalaya–East Asia ('WAHEA') and Euro-Med; Simeone et al. 2016), was rooted between the two subgenera following Zhou et al. (2022) and imported in iTOL (www.itol.embl.de) for visualization and labelling. A planar (equal angle, parameters set to default) split graph was generated with the Neighbor-net (NNet) algorithm (Bryant and Moulton 2004) implemented in SplitsTree4 (Huson and Bryant 2006), based on the pairwise uncorrected-p ('Hamming') distance matrix estimated with the same program. Median Joining (MJ) haplotype networks were run with Network 4.6.1.1 (http://www.fluxus-engineering.com/), treating gaps as 5th state and using the MJ algorithm with default parameters (equal weight of transversion/transition).

Results

trnH-psbA and trnK-matK sequence data

The primary data (Supplementary file S1) comprised 270 individuals belonging to the Euro-Mediterranean sect. *Quercus* newly sequenced with trnH-psbA and trnK-matK. Sequence quality was high for both marker regions and unambiguous electropherograms were obtained for 100% of the investigated samples. GenBank searches extended the West Eurasian sect. *Quercus* dataset to 425 individuals (22 species, see supplementary files S1, S2) sequenced with the same markers, including 89 Caucasian (Ekhvaia et al. 2018), 23 Lebanese (Douaihy et al. 2020) and further 43 European, Mediterranean and Near East accessions from works mostly focused on DNA barcoding or phylogenetic studies on other *Quercus* sections (Simeone et al. 2013, 2016).

A wider evolutionary contextualization of the white oaks was achieved by expanding the phylogenetic analyses with 81 GenBank accessions of Asian sect. *Quercus*, eight Caucasian samples belonging to sect. *Ponticae* and 31 accessions of American members of subgen. *Quercus* (sects. *Quercus*, *Lobatae*, *Protobalanus*, *Virentes* and *Ponticae*; Simeone et al. 2016; Ekhvaia et al. 2018; Yang et al. 2020). Over 500 trnH-psbA and trnK-matK GenBank sequences of West Eurasian sects. *Cerris* and *Ilex* oaks (14–16 species) were retrieved to compare the molecular differentiation estimates found in the Eurasian white oaks' dataset.

All generated multiple alignments were straightforward. The two-marker combination in the primary dataset was 1147 bp long. Trimming uneven GenBank sequence ends and coding indels longer than 1 bp as single binary characters produced a 1068 bp long matrix in the Eurasian oaks of sect. *Quercus*. A trnH-psbA inversion of 34 bp occurring in 14 North American oak individuals was replaced with its reverse complementary sequence and a binary character was inserted to keep record of it. The final alignment including all members of subgen. *Quercus* and six accessions of Eurasian subgen. *Cerris* was 1252 bp long.

The newly sequenced Euro-Mediterranean white oak dataset produced 14 haplotypes, scaling up to 29 after the inclusion of the GenBank West Eurasian samples. Eleven haplotypes out of the total were inter-specifically shared (up to 13 species), 16 were singlets (12 derived from GenBank) and only two were restricted to one or more populations of a single species (*Q. pubescens* from Croatia) or species complex (*Q. ichnusae, Q. virgiliana, Q. congesta* from Sardinia). The relative haplotype frequency was very variable: besides the 16 singlets, five haplotypes appeared in between 46 and 94 individuals each. The East Eurasian oaks of sect. *Quercus* produced 14 haplotypes, two of which were in common with the West Eurasian white oaks, four were inter-specifically shared (up to six species) and eight were intra-specifically shared. In total, 41 trnH-psbA + trnK-matK haplotypes were detected in the Eurasian sect. *Quercus*. Members of North American subgen *Quercus*, Eurasian sect. *Ponticae* and subgen. *Cerris* generated 37 additional haplotypes. All GenBank accessions, taxonomic identities, geographic origins, and haplotypes are reported in Supplementary file S2.

Phylogenetic setting of the Eurasian white oaks

The 78-tip RAxML tree (Fig. 2), rooted between subgen. *Cerris* (here represented by members of sections *Cyclobalanopsis*, *Cerris* and *Ilex*) and subgen. *Quercus*, reports the well-acknowledged sectional and lineage differentiation within each subgenus. In subgen. *Cerris*, the two Euro-Med members of sect. *Ilex* (*Q. ilex* and *Q. coccifera* from Spain and North Africa) are included in the Euro-Med lineage, slightly diverging from the other members of the same section (*Q. alnifolia* and *Q. baroni*, belonging to the WAHEA and the East Asian lineages, respectively), and from the two members of sect. *Cerris* (*Q. cerris*) and sect. *Cyclobalanopsis* (*Q. acuta*) forming a minor subclade (BS = 57–62). In subgen. *Quercus*, five clades are produced, with medium high support (BS = 60–100). Four clades include all North American oaks of subgen. *Quercus* (sects. *Quercus*, *Lobatae*, *Protobalanus*, *Virentes*, *Ponticae*) and the last one includes their Eurasian counterparts (sects. *Quercus* and *Ponticae*).

Besides the major geographic split within sects. *Quercus* and *Ponticae*, some sectional misplacements emerged. These involve a few members of sect. *Virentes* and *Lobatae* (placed in the sect. *Quercus* subclade), and the two only American and Eurasian surviving members of sect. *Ponticae* (respectively inserted in sects. *Protobalanus* and Eurasian *Quercus*). The Eurasian white oak clade (BS = 99) is highly unresolved: with the only exceptions of five minor subclades including *Q. pontica* (BS = 96, 58), two East Asian oaks (BS = 80), and two local species groups from Lebanon and

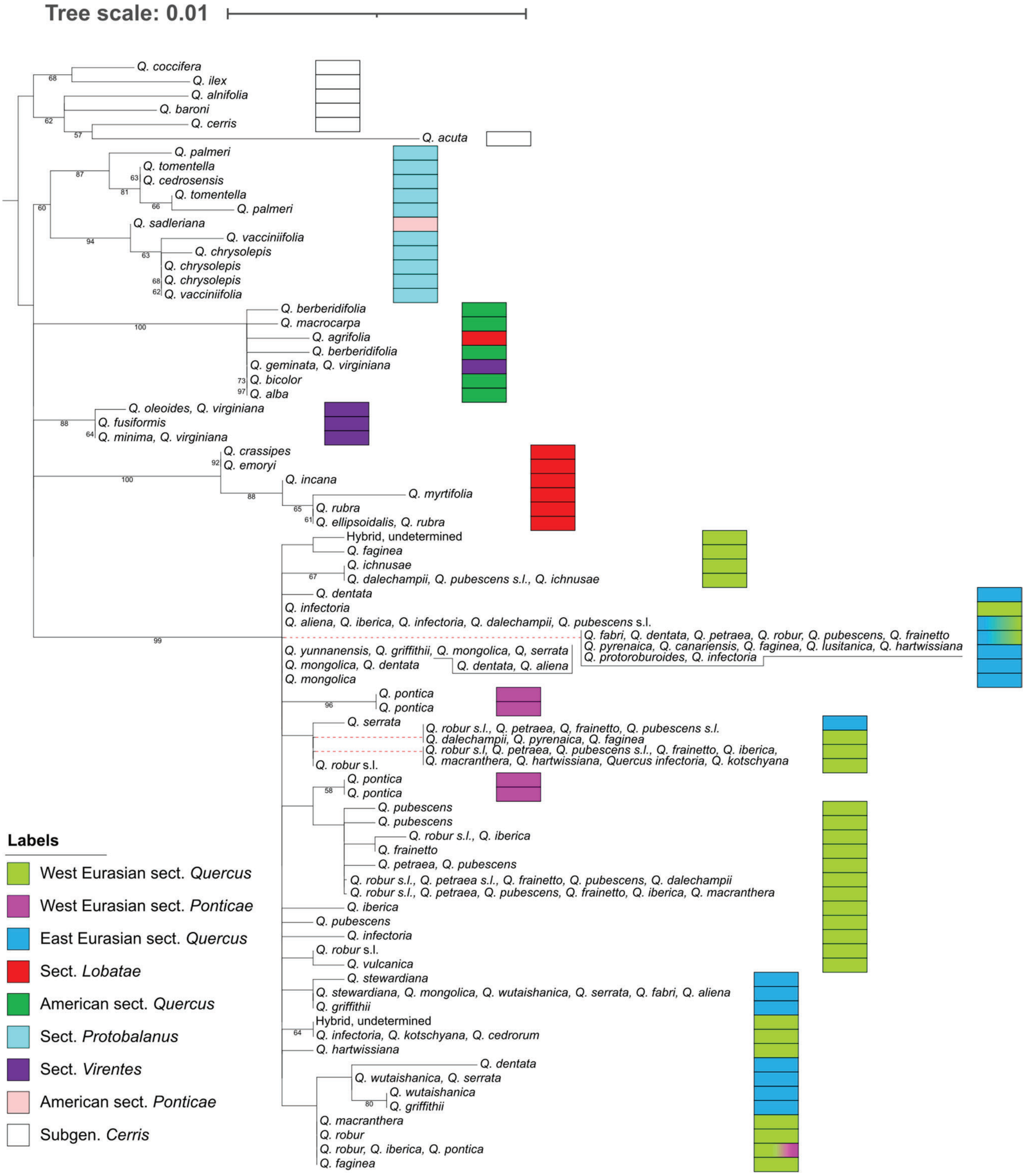


Fig. 2 Maximum likelihood phylogram of the trnH-psbA + trnK-matK concatenated regions of the investigated West Eurasian sect. *Quercus* dataset, integrated with GenBank haplotypes of subgen. *Quercus* and representatives of West Eurasian subgen. *Cerris*. Bootstrap support (> 50) values are reported above branches. Colouration refers to the major taxonomic and geographic affiliations of specimens

south–central Italy (BS = 64–67), no consistent differentiation could be observed at the taxonomic or geographic level, even between Western and Eastern Eurasian samples. Differentiation of a clade-basal group of sequences collecting both single and shared West and East Eurasian haplotypes is possible but low-supported (BS < 50).

The obtained RAxML topology is clearly mirrored by the Neighbor net graph shown in Fig. 3. All members of subgen. *Cerris* are connected to the North American cluster, with the two diverging Euro-Med members of sect. *Ilex* showing more affinity with the Eurasian sect. *Quercus*. Except for a few divergent sequences, all the Eurasian white oak haplotypes are highly mixed and organized in reticulated and little diverging clusters, with a huge trunk confirming the main split between North American and Eurasian sections *Quercus* and *Ponticae.* A composite cluster, corresponding to the low-supported clade-basal group of sequences observed in the RAxML tree, acts as the most direct connection between the two split groups.

trnH-psbA and trnK-matK variation in Eurasian white oaks

A more detailed look into the plastid differentiation patterns of West Eurasian white oaks could be obtained with a closer inspection of the molecular diversity of the sequenced markers (Table 1) and the resulting haplotype network (Fig. 4). The two marker sequences produced in this study and retrieved from GenBank (Table 1) allow a good comparison among sections and subgenera, totalling nearly 1000 individuals belonging to 47 oak taxa. In particular, all the currently accepted species belonging to Eurasian section *Quercus* and West Eurasian sections *Cerris* and *Ilex* were included in our evaluation. The markers' variation was moderate-to-low, and trnH-psbA showed higher diversity than trnK-matK across all lineages, except sect. *Ilex*. Both markers displayed remarkably lower values of molecular diversity in sect. *Quercus* than in sect. *Ilex*, and comparable estimates with sect. *Cerris* (characterized by lower numbers of individuals included).

Within sect. *Quercus*, the here investigated Euro-Mediterranean dataset ('primary dataset') was the least variable; its variation was only partially increased after the combination with the West Eurasian sequences from GenBank,

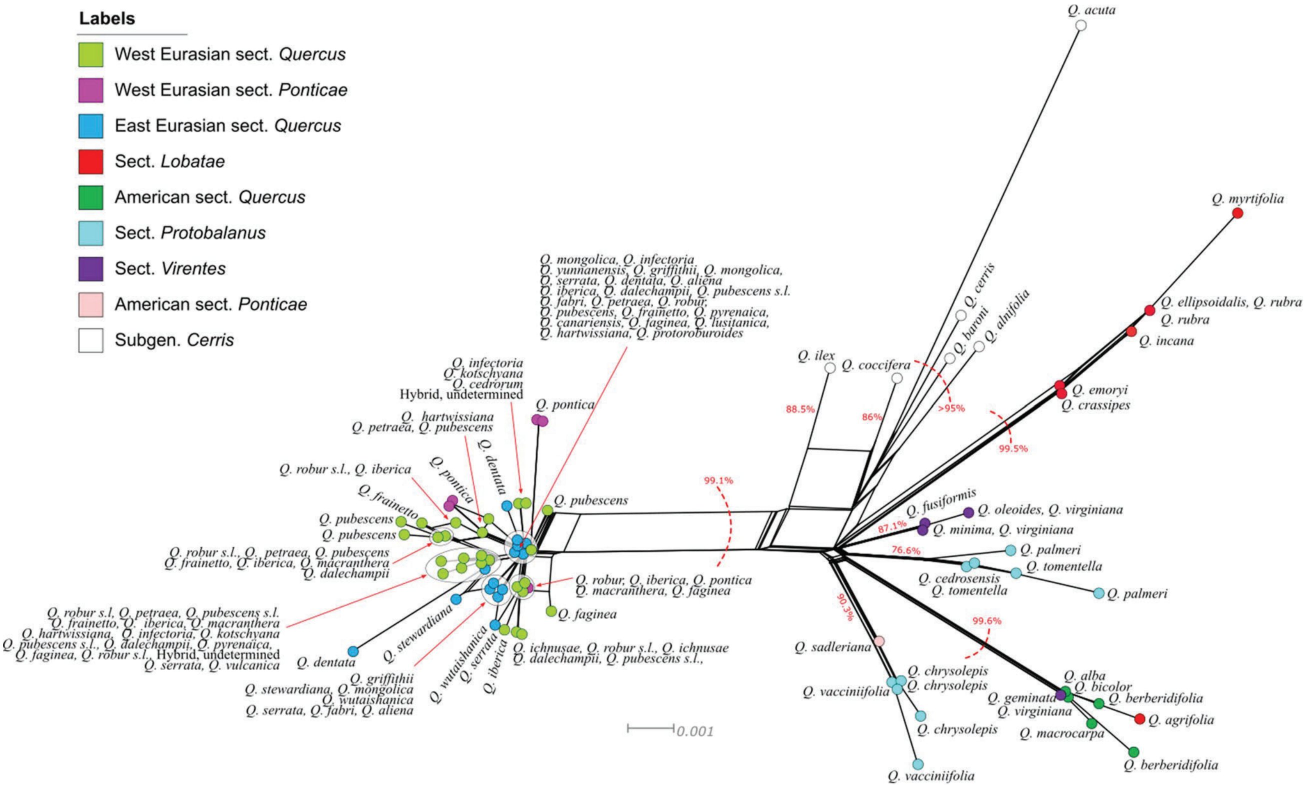


Fig. 3 Neighbor-Net splits graph of the trnH-psbA + trnK-matK concatenated regions of the investigated West Eurasian sect. *Quercus* dataset, integrated with GenBank haplotypes of subgen. *Quercus* and representatives of West Eurasian subgen. *Cerris*. Average Bootstrap support (> 50) percentages of main clusters are reported. Colouration as in Fig. 2

Table 1 Main diversity values of the trnH-psbA and trnK-matK marker regions in section *Quercus*, subdivided into the here investigated Euro-Mediterranean dataset (*Quercus*^{1}), the expanded GenBank dataset comprising all available West Eurasian white oaks (*Quercus*^{2}), and the East Eurasian members of sect. *Quercus* (*Quercus*^{3}), compared with GenBank data retrieved from West Eurasian members of subgen. *Cerris* (sects. *Cerris* and *Ilex*). T: number of species/taxa; N: number of individuals; *p*: uncorrected p-distance range (min. – max.); H: number of haplotypes (gaps included); Hd: haplotype diversity; PICs: Parsimony Informative Characters; T+K: combined markers; L: Major lineages identified (* = with only trnH-psbA considered; n.d.: not determined (marker sequences belonging to different samples)** = three of which are represented by highly-divergent, single haplotypes). Taxonomic and geographic details of sect. *Quercus* samples are provided in the Supplementary files S1, S2

		trnH-psbA						trnK-matK						T+K		
Subgen	Sect	T	N	*p*	H	Hd	PICs	T	N	*p*	H	Hd	PICs	H	Hd	L
Quercus	*Quercus*^{1}	12	270	0.0–0.0058	9	0.7713	4	7	270	0.0–0.0032	5	0.5557	2	14	0.8286	1
	Quercus^2	22	425	0.0–0.0089	19	0.8639	5	15	425	0.0–0.0051	8	0.5430	2	29	0.9271	1
	Quercus^3	9	81	0.0–0.0119	13	0.7568	5	9	81	0.0–0.0047	4	0.2080	3	14	0.848	5**
Cerris	*Ilex*	4	124	0.0–0.0128	29	0.8881	7	4	124	0.0–0.0116	23	0.9142	10	53	0.9561	3
	Cerris	12	207	0.0–0.0082	12	0.5148	5	10	47	0.0–0.0014	4	0.2007	1	n.d	n.d	2*

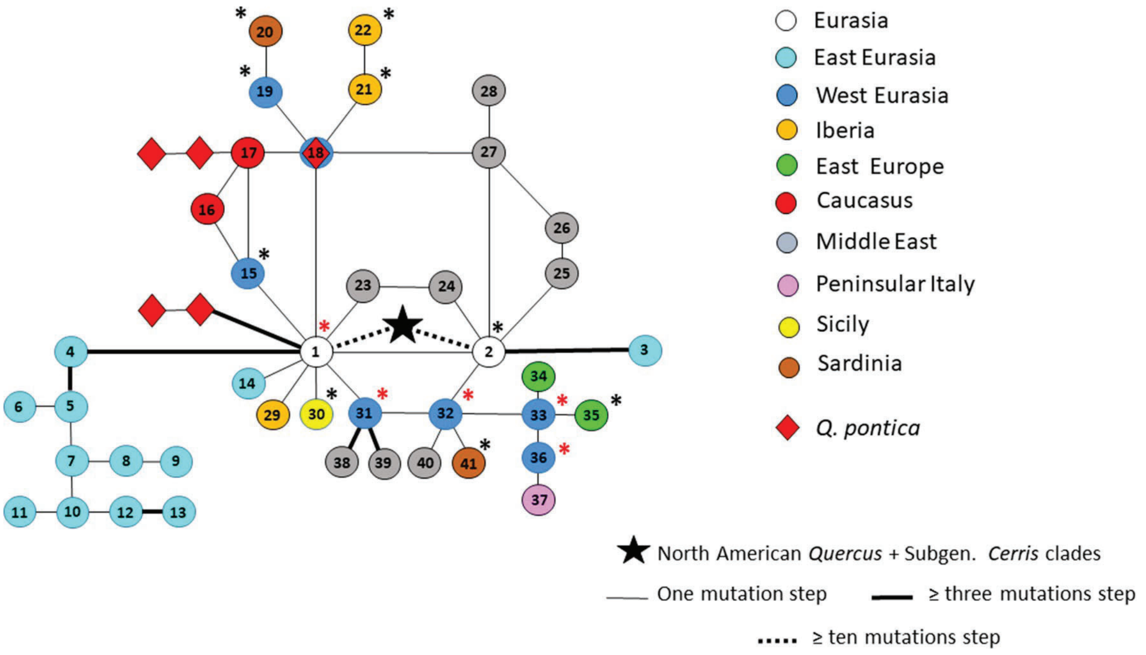


Fig. 4 Median Joining haplotype network of the trnH-psbA + trnK-matK concatenated regions, combining the investigated Euro-Mediterranean samples (primary dataset) and all available GenBank haplotypes of Eurasian subgen. *Quercus*. Colouration and symbols identify major lineages (sects. *Quercus* and *Ponticeae*) and the geographic distribution of the haplotypes (detailed in the Supplementary files S1, S2). Line thickness proportional to the number of mutations separating each haplotype (1, 3–4); dashed line corresponds to > 10 mutations. Asterisks indicate the haplotypes detected in the primary dataset (those with the highest frequency are in red)

that included samples from all over Europe, the Middle East, and the Caucasus region. In contrast, the East Eurasian dataset showed higher diversity, especially with trnH-psbA, and prefigures to host higher plastome diversity than the West Eurasian counterparts, due to the comparable values scored by a lower number of individuals included in the analysis. The relationships of the 41 Eurasian white oak haplotypes are reported in the haplotype network (Fig. 4). Only single mutations separate every West Eurasian sect. *Quercus* haplotype from the nearest, preventing the identification of any lineage, contrarily to the patterns detected in sects. *Ilex* and *Cerris* (Simeone et al. 2016, 2018). The network is rather intricate and unfolds around two major variants (labelled #1 and 2). Haplotype #1 is found in 46 individuals from 13 species and extends from Japan to Portugal, across China, Bulgaria, Greece, Cyprus, C and S Italy, France, England, Spain, Algeria and Morocco. Haplotype #2 has a lower frequency (13 individuals) and was detected in five species across Korea, Georgia (E Caucasus), Lebanon, S Italy and Sicily. Both haplotypes correspond to the basal group of sequences identified in the Eurasian white oak clade of Figs. 1 and 2. It is interesting to note that a BLAST search performed against all *Quercus* complete chloroplast genomes available on GenBank revealed 100% sequence identity of haplotype #1 with three accessions of *Q. robur* (OW028778, LT996900, England; MN562095, not specified) and one accession of *Q. fabri* (MK105456). Likewise, haplotype #2 matched one accession of *Q. fabri* (MK922346) and six accessions of *Q. mongolica* and *Q. dentata* (MK089571, MK105460, NC_043858, MK105453-105455). The closest connection with the North American sect. *Quercus Ilex* and *Cerris* clades (Figs. 1 and 2), the location at the core of the network and the wide taxonomic and geographic distribution of these two haplotypes suggest their possible ancestry (Posada and Crandall 2001). Several variants/haplotype groups directly depart from either one of these two haplotypes. Ten haplotypes from China and Korea (#4–13) form a discrete, divergent lineage, possibly comprising further sublineages (e.g. haplotypes #4, 5–12, 13); two other East Asian haplotypes are either very close (#14) or divergent (#3) from the putative ancestral variants.

The geographic distribution of shared or related West Eurasian haplotypes cover either very large or narrow regions and appear quite intermingled. For instance, haplotypes #23–28 extend from Lebanon to Turkey and Iran, and are only distantly related to the sympatric haplotypes #38–40. Haplotypes #15–19 cover a region spanning from S Italy to Georgia across Bulgaria, Turkey and Ukraine; interestingly, two haplotypes found in *Q. pontica* are directly connected to this cluster, whereas two, more divergent, depart from haplotype #1, and two further are included in haplotype # 18. However, other diverging Caucasian white oaks samples are placed in haplotypes #32, 33. The latter two haplotypes and #36 cover various territories in central, south, east Europe and the Near East, whereas haplotype #31 extends westwards from peninsular Italy, Sicily, and Sardinia to Spain. Together with #1, this highly related group of four haplotypes (#31, 32, 33, 36) collects the highest number of individuals across the entire dataset (up to 94 individuals each). Haplotype #33 also matched a complete chloroplast genome of *Q. petraea* (LT996899, England). Other haplotypes with remarkable frequencies, but distributed on narrower regions are #17, 18 and 24, respectively collecting 21, 26 and 17 individuals from the Caucasus, the Black Sea coasts and Lebanon, and #19 and 41, each one collecting 12 individuals from Sardinia. Some derivate haplotypes located at the tips of the network were scored from the Mediterranean major islands (e.g., #20, 30, 41), south central Spain (#21, 22) and central Anatolia (#38, 39); except for haplotype #41, they are all singlets. No further 100% identity scores with the GenBank complete *Quercus* genomes were identified.

Geographic structuring of the West Eurasian white oak plastid DNA variation

Dissecting the network into haplotype clusters based on molecular identity, affinity, scored frequency, and geographic positioning revealed some interesting geographic patterns (Fig. 5a–d). The two potentially ancestral variants (Fig. 5a; haplotypes #1, 2) are rather uniformly distributed across the Euro-Mediterranean region, all located within 35° and 44° Lat. North, except for the British (52° Lat. North) and one Caucasian sample. The four most frequent (and closely related) haplotypes (Fig. 5b; haplotypes #31, 32, 33, 36) identify a west central Mediterranean distribution (#31: Iberia and Italy, major islands included), contrasted to a central east European (#33: England, France, Italian Peninsula, Austria, Czech Republic, north east Croatia, Romania, Bulgaria, Georgia, Armenia), and two south eastern Mediterranean distributions (#32: Italian Peninsula and Sicily, west Croatia, Bulgaria, Georgia and Lebanon; #36: Italian Peninsula, south Croatia, Serbia, Bulgaria, Greece). Figure 5c shows the distribution of the less frequent, disjunct haplotypes: one (#15) derives from the ancestral variants (Fig. 4), is related with the Caucasian haplotypes (#16, 17) and connects southeast Italy and Bulgaria; likewise, one haplotype group (#23–28) departs from the ancestral variants and connects Lebanon to Iran across Turkey. Haplotypes #19 (connecting Sardinia and south Italy), and #18 (connecting Ukraine, Turkey and Georgia) identify well-known biogeographical links within each region; however, the two haplotypes result, unexpectedly, closely related. Finally, Fig. 5d shows the distribution of the single, narrowly distributed or highly divergent haplotypes: two haplotypes (#41 and 35) identified four populations in Sardinia (12 individuals) and one in Croatia (three individuals), all other were just singlets. As shown in Fig. 4, haplotypes #41, 35, 20, 30, 34, 37 and 40 are directly derived or closely related to co-occurring main (#1, 32, 33 and 36) or disjunct (#19) haplotypes. In contrast, haplotypes #21, 22 (South Spain) and #38, 39 (Turkey) are related to haplotypes from geographically very distant regions (#18 and 31, respectively).

Discussion

This work complements the current knowledge on plastid DNA phylogeography of West Eurasian oaks (sects. *Ilex* and *Cerris*, Simeone et al. 2016; Vitelli et al. 2017; Simeone et al. 2018), filling the large gap represented in particular by sect. *Quercus* and extending and improving upon the only available diversity studies that were conducted more than 20 years ago with dated molecular tools (Petit et al. 2002a and references therein). The two markers used herein acted synergistically, enabling identification of congruent

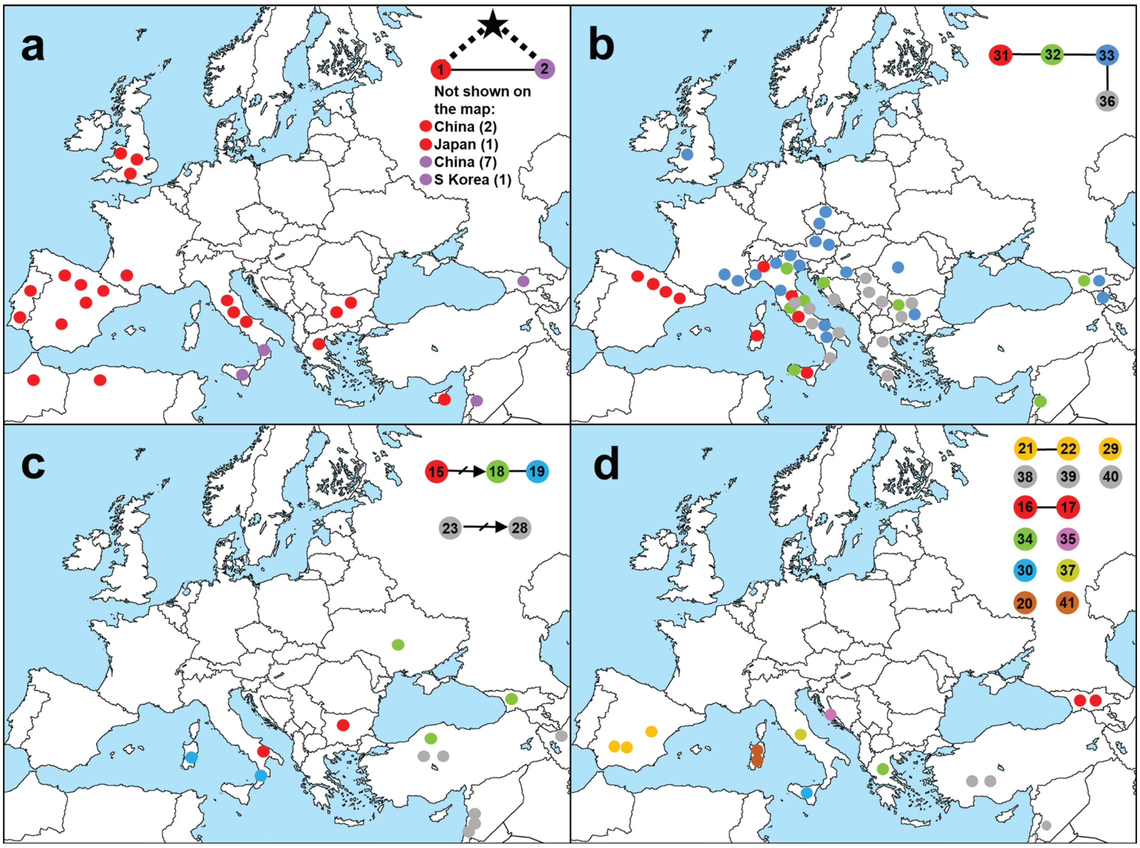


Fig. 5 (**a**–**d**) Geographic patterns of the plastid haplotype variation in West Eurasian sect. *Quercus*, (investigated and GenBank retrieved datasets), including 100% identical trnH-psbA + trnK-matK haplotypes detected in the complete chloroplast genomes of Eurasian subgen. *Quercus*. Connections between haplotypes are reported (see Fig. 4). **a**: Potential ancestral haplotypes; **b**: most frequent West Eurasian haplotypes; **c**: less frequent, disjunct haplotypes; **d**: local, unique haplotypes

widespread patterns and additional derived haplotypes with a narrower distribution (Supplementary file S3). Moreover, our analyses took advantage of the sequences available on GenBank to reveal molecular patterns consistent with major (genus level) and circumstantial (regional level) oak phylogenies, and outline a compelling framework of the white oaks evolution in Europe which will deserve new attention in future studies.

Phylogenetic patterns

The RAxML tree topology shown in Fig. 2 perfectly matches the most recent inter and intrasectional differentiation obtained with both more numerous and/or more powerful plastid markers (five DNA regions: Yang et al. 2020; over 200 coding and non-coding loci from RNA-seq data: Yang et al. 2021; whole genome sequencing: Zhou et al. 2022), and complements the evidence recently derived from nuclear data (RAD-sequencing; Hipp et al. 2020) of the complex genus evolution. As a side result, the peculiar relationships of the sect. *Ilex* Euro-Med lineage in relation to the Eurasian sect. *Quercus* is highlighted (see Fig. 3). As originally suggested in Simeone et al. (2016), the Euro-Med lineage may have been among the earliest diverging *Quercus* plastomes and represent the legacy of an ancient cross-sectional oak lineage (cf. Yang et al. 2021) established in the West Mediterranean area. Our phylogenetic reconstructions (Figs. 1 and 2) also separate New World and Old World oaks of the same evolutionary lineage, i.e. sect. *Quercus* and *Ponticeae*, complying with a geographic differentiation in their primordial members predating divergence and subsequent manifestation in modern taxa (Denk and Grimm 2010). In fact, the observed sectional plastome non-monophyly has already been documented in American and Eurasian oaks (Pham et al. 2017; Crowl et al. 2020; Manos and Hipp 2021; Zhou et al. 2022), and explained with chloroplast capture via hybridization in the early diversification of the genus. In sect. *Ponticae* (see Figs. 1 and 3) it has been postulated that the relict species *Q. sadleriana* survived by introgressing plastomes from sympatric *Protobalanus* members in the past (McVay et al. 2017; Hipp et al. 2020). Similarly, its Caucasian sister (*Q. pontica*) introgressed plastomes of sympatric

(Caucasian) members of sect. *Quercus*, likely from different sources and at different times in the past. On the other hand, the intra-sectional deep incongruence between plastid data and taxonomic identity is well-acknowledged across the entire genus (Yang et al. 2021). However, the lack of resolution we observed within the Eurasian white oaks, even between members from western and eastern Eurasia, was somewhat unexpected and is in sharp contrast with sects. *Cerris* and *Ilex* (c.f. Simeone et al. 2018; Yang et al. 2021).

The molecular diversity values reported in Table 1, collected from a vast and comprehensive Eurasian oak dataset, help to explain the low resolution of our phylogenetic reconstructions. In particular, we can see that despite the higher numbers of species and individuals investigated, the West Eurasian white oaks (both those investigated here and the GenBank expanded dataset) showed: (1) fewer, and (2) less variable haplotypes (in terms of sequence divergence) than West Eurasian members of sect. *Ilex*, and only slightly higher variation than West Eurasian members of sect. *Cerris*, of which there were far fewer individuals included in the comparison. Interestingly, Yang et al. (2020) found the same differences comparing five plastid markers across East Asian members of the three sections, and suggested different evolutionary dynamics associated with their distinct origins (New/Old World) as a possible explanation. In our work, the variation found suggests the identification of a single West Eurasian white oak haplotype lineage (largely unresolved in the RAxML tree and Neighbour-Net graph), in sharp contrast with the three lineages found in (sympatric) sect. *Ilex* (WAHEA, Cerris-Ilex, West-Med; Simeone et al. 2016) and the (at least) two in sect. *Cerris* (L1, L2; Simeone et al. 2018). In these cited studies, the different intra-sectional lineages were identified based on their relative positions in the phylogeographic reconstructions (e.g., high-supported clades and subclades in the RAxML tree, congruent geographic distribution) and the high number of mutations (up to five) separating the closest haplotypes of each lineage. Instead, the poorly resolved West Eurasian white oak haplotypes (Figs. 1 and 2) are each separated by single mutations (with the only exception of two singlets likely corresponding to geographically isolated samples; Fig. 4). The East Eurasian samples showed some divergent haplotype clusters and singlets that might suggest the occurrence of further lineages (c.f. Yan et al. 2019; Yang et al. 2020). However, differences in the representativeness of the sampling designs across West and East Eurasia may have contributed to revealing a sort of genetic continuum in the more densely sampled Western regions, in contrast to the more punctuated diversity in the less covered Asian region.

The Northeast Asian origins of sect. *Ilex* (first) and sect. *Cerris* (soon after) have been traced back to the Eocene (40–50 million years ago; Denk et al. 2023). Subsequent range expansions lead their progenitors to penetrate and colonize Western Eurasia along two different routes (*Ilex*: via the Tibet-Himalayan Corridor; *Cerris*: across Northern and Central Asia; Jiang et al. 2019; Denk et al. 2023) by > 20 million years ago (Early-Middle Miocene), causing major East–West Eurasian splits, and soon after differentiating their main intra-sectional lineages and species groups. All gathered data exhibited by the plastid markers in the Euro-Mediterranean members of these two sections (Vitelli et al. 2017; Simeone et al. 2018) show clear phylogeographical patterns that are indicative of reticulation, lineage sorting, diversification and quick dispersal not involving severe bottlenecks.

In contrast, colonization of Eurasia by a stock of North American sect. *Quercus* oaks can be dated considerably later (ca. 10 to 20 million years ago; Hipp et al. 2020), with the major intra-sectional split between the plastomes of the two white oak groups explained on the basis of their long-term geographic isolation due to submersion of the North Atlantic and Bering land bridges since the late Neogene (Denk et al. 2017). According to some authors, species differentiation and the division into eastern and western Eurasian lineages would have taken place in the late Miocene (ca. 10 Ma), as the likely result of decreasing temperatures and an intensification of the Asian monsoon system (Yan et al. 2019; Hipp et al. 2020). By the Late Miocene – Early Pliocene, the West Eurasian white oak progenitors were established in most European regions investigated in the present study (Jiménez-Moreno and Suc 2007; Kvaček et al. 2008, 2020; Velitzelos et al. 2014; Barrón et al. 2017; Niccolini et al. 2022; Vieira et al. 2023). During this period, these oak populations experienced multiple extirpation, biome shifts and complex repopulation phases over time (Kremer and Hipp 2020). Heavy vegetation changes with continuous remodelling in the structure and composition of both local and regional floras were caused by considerable geomorphological and climate changes along with the Messinian Salinity Crisis (Krijgsman et al. 1999; Krijgsman 2002), the late Pliocene development of the Mediterranean climate (Suc 1984), the Pleistocene Glacial/Interglacial cycles (Pons et al. 1995) and the concurrent uplift of major mountain systems associated with intense volcanic activity, and repeated, temporary land connections across sea straits (Blondel et al. 2010; Nieto Feliner 2014). As a result, a mosaic of local/regional conditions varying with latitude, longitude, altitude and sea proximity (Suc 1984; Suc and Popescu 2005) occurred especially in the Mediterranean regions, where temperate *Quercus* forests became progressively dominant around 1.4–1.3 Ma (Combourieu-Nebout et al. 2015; Magri et al. 2017) and found effective shelters from the widespread tree extinctions across much of Northern and Central Europe during the ice ages (Brewer et al. 2002).

The preliminary climatic deterioration of the late Pliocene, and especially the upheavals linked to the Pleistocene glacial and interglacial cycles, probably affected the newly arrived mesophylous white oaks more severely than the long-established, xero-thermic *Ilex* and pre-adapted *Cerris* oaks (Denk et al. 2023), further depleting their plastid diversity. The current geographical distribution of the West Eurasian species belonging to sect. *Quercus* and those of sect. *Cerris* and *Ilex,* as well as their ecological features, suggest some interpretative keys to explain the haplotype depletion characterizing white oaks. Sects. *Ilex* and *Cerris* oaks show a strictly steno-Mediterranean distribution (the former) and Euro-Mediterranean-Pontic (the latter). The European species of Sect. *Quercus* show a much wider northern distribution, seemingly related to their ability to cope with microthermic climates (e.g., *Q. petraea s.l.* and *Q. robur*, exhibiting their centre of distribution in Central Europe and extending westwards to the whole British Archipelago and northwards to southern Scandinavia and Siberia). It is therefore probable that sects. *Cerris* and *Ilex* species underwent only limited range contractions during the glaciations or were able to reach glacial refugia with relative ease by virtue of their privileged long-established southern distribution, thus conserving a huge portion of the populations and their genetic diversity. In contrast, a large part of white oaks populations (i.e., those of central and northern Europe) were constrained between the ice caps advancing from the north and the southern Pyrenees-Alps-Carpathians longitudinal mountainous alignment and were drastically reduced. This dramatic entrapment, which almost totally prevented oak forests migration southwards causing a virtual mass extinction (see Bennett et al. 1991), had the consequence that significant portions of the genetic diversity brought by the “northern” white oaks populations was irretrievably lost. The highly heterogeneous and changing landscapes of the post-glacial periods then allowed preservation of the genetic pools that survived in the earlier inhabited southern regions, and promoted diversification triggering adaptation, isolation and drift in the backup sources for subsequent recolonization (Hipp et al. 2018).

Phylogeographic patterns

The two haplotypes located at the centre of the Network (Fig. 4, haplotypes #1, 2), closer to the connection with the North American *Cerris* and *Ilex* sister lineages, can be considered the ancestral Eurasian white oak haplotypes. These haplotypes are exhibited by up to ten East samples and a large number of West Eurasian ones, with a fourth Asian haplotype separated by just one mutation. Yan et al. (2019) also found one shared haplotype between two East and three West Eurasian white oaks (far east Russia, North Europe) by using trnH-psbA and three other plastid marker sequences, strengthening the suggestion of extremely ancient genetic imprints still surviving in some Eurasian white oak plastomes. Given the geographical distribution of the samples included in our study, #1 appears to be dominant in the west-central European regions, whereas #2 is restricted to southern (Sicily), southeastern (Lebanon) and eastern (Georgia) latitudes, with a higher frequency in East Asia. Such a spatial separation could be explained by a different (western/eastern), possibly coeval, origin of the two ancestral haplotypes. Subsequent molecular differentiation (suggested by the relatively high number of derived lineages (Table 1) seems to have occurred with different efficacies on the two sides of the continent, probably reflecting the different ecological opportunities offered by the two regions or promoted by the different topography and survival rate of these oaks during the Pleistocene (c.f. Li et al. 2019). In West Eurasia, all the most frequent European haplotypes (# 31–33, 36) are directly linked to the ancestral variants; the white oaks pronounced ability for rapid migration (Kremer and Hipp 2020) likely worked in concert with the complex palaeogeology and palaeoclimatology of Europe and the Mediterranean basin to allow a highly efficient recolonization across Europe, but resulting at the same time in a phylogeographic structure which is difficult to dissect. Nevertheless, some basic patterns can be identified.

The ancestral haplotypes H01 and H02 are concentrated in southern Europe, whereas they are almost totally absent from central-northern Europe. We cannot exclude the possibility that the asymmetric North/South distribution may partially arise from the preponderance of southern European populations in our dataset. However, the two haplotypes occur in a wide number of Euro-Mediterranean territories (Fig. 5a), where regions able to host them are known to have existed for prolonged periods during both the Miocene and Pleistocene (Médail and Diadema 2009). It is therefore much more likely that this asymmetric distribution is to be linked to the survival of original gene pools and the role played by the Quaternary glaciations in repeatedly resetting the genetic memory of the European white oak populations. The most accredited theory is that the vegetation landscape of the Central and northern Europe, currently occupied by the deciduous temperate forests Biome, was almost completely covered by the *Artemisia* sp. steppe-like grasslands and the cushion-like Tundra during the Quaternary cold periods (Magri 2010; Tzedakis et al. 2013). Although it cannot be excluded that isolated stands of conifers could have survived in refugia in central Europe during the last glaciation (Fickert et al. 2007; Parducci et al. 2012a) a topic on which the debate is still open, (see Birks et al. 2012 and Parducci et al. 2012b),) the hypothesis of the occurrence of northern refugia for the significantly more thermophilous white oaks seems untenable. Accordingly, the absence of ancestral haplotypes of white oaks from the European territories north of the Alps is somewhat to be expected. In contrast, the absence of ancestral haplotypes within a large belt of central and southern Europe including Italian Po valley, northern Croatia and Serbia was not expected. It is probable that the cold and semi-arid conditions which characterized the Late Dryas (12,500 years BP) and that persisted in the first two millennia of the Holocene played a crucial role. Pollen data reports that in northern Italy such prolonged harsh climate events pushed the boreal coniferous forests to the foothills of the southern slopes of the Alps and the cold steppic grasslands to the Adriatic Sea coasts, causing complete extinction of deciduous forests or their confinement within a few small-size enclaves (Ravazzi et al. 2007; Kaltenrieder et al. 2009; Pini et al. 2022).

The presence of haplotype # H01 in Great Britain might seem out of context, considering that the ancestral haplotypes are absent from countries located much further south than Great Britain, such as France, Austria, and Serbia. However, this British occurrence is not surprising (see Cottrell et al. 2002; Nocchi et al. 2022), being explainable by an efficient migration of Iberian white oaks in the post-glacial period (even though the lack of basic information on the provenance of the British sequences downloaded from GenBank means that anthropic plantations of southern European germplasm cannot be excluded). As far as Southern Europe is concerned, in contrast to Sicily (see below), no ancestral haplotypes were found in Sardinia, despite our extensive sampling and the well-known richness in systematically isolated plant paleo-endemisms of this island (Mansion et al. 2009; Schmitt et al. 2021; Fois et al. 2022). In fact, the conservation and evolution of the genetic heritage of the white oaks in Sardinia and Sicily differed according to the differing paleogeographic histories of these two islands. The Sardinian block detached from the Catalan-Provençal plate (southern France and north-eastern Spain at present) in the early Miocene (ca. 30–15 Ma) and started a backward migration that brought it to the centre of the proto-Tyrrhenian Sea during the Messinian age. Since then, no further land connections occurred with the mainland, with possible exceptions during the Messinian salinity crisis, when it is assumed Sardinia was connected to the Apulian platform and to north Africa (Hsü et al. 1977), and during the LGM when a connection with the Tuscan Archipelago has been hypothesized (Médail and Diadema 2009; Schmitt et al. 2021).

Consequently, Sardinia displays four differently derived plastid variants which can be related to the paleogeographic events mentioned above. The lack of ancestral haplotypes may reflect the Early Miocene geological detachment of the Sardinian block from the Western European landmass (i.e., before the arrival of the original North American white oak plastomes); haplotype # 19 (*Q. congesta, Q. ichnusae, Q. virgiliana*) might be indicative of the ancient (Messinian) links with the Southern Italian Peninsula, coupled with more recent (Pleistocene glacial periods) immigrations from Northern Italy (haplotype # 31; *Q. congesta, Q. pubescens*), likely facilitated by land connection via the Tuscan Archipelago. Further recent diversification (derived haplotypes # 20, 41; *Q. congesta,* Q. *ichnusae, Q. virgiliana*) could have been triggered subsequently owing to isolation.

In contrast, Sicily (with an equally ancient history and a more complex orography) was less isolated. Both at the Miocene-Pliocene boundary and during the Pleistocene, there were no barriers to oak migration from northern Italian and Balkan territories via adjacent Calabria and Apulia (see alsoVitelli et al. 2017; Simeone et al. 2018). According to some authors (Brullo et al. 1999; Pignatti et al. 2017), in Sicily at present there are no *Q. petraea* subsp. *petraea* and *Q. pubescens s.s.*, but rather their southern xerothermic forms (*Q. petraea* subsp. *austrotyrrhenica, Q. amplifolia, Q. congesta, Q. dalechampii, Q. leptobalanos, Q. virgiliana*). Although the most recent bio-systematic revisions do not seem to confirm this taxonomic and nomenclatural variability (Di Pietro et al. 2020a, 2021), we can only hypothesize that the pronounced paleogeographic and paleoclimatic vicissitudes that Sicily experienced over geological history allowed an early island colonization, local preservation of ancient haplotypes, subsequent diversification and exchange with the mainland. Morphologically only slightly observable, this variability is expressed in a phylogeographic key by four different haplotypes, including the potentially ancestral #2 (at its westernmost occurrence; *Q. congesta*), the early derived #31, 32 (in all the taxa occurring in Sicily) and an isolated derivate from the ancestral haplotype #1 (#30; *Q. virgiliana*).

Finally, the geographical distribution of the most frequent haplotypes identified in our study (# 31- 33, 36) also seems to be well structured and consistent with the current biogeographical map of Europe (Rivas-Martínez et al. 2004). The major western/eastern separation is demonstrated by the geo-vicariance of haplotypes # 31 and # 36, while the overall distribution of haplotype # 36 perfectly matches the boundaries of the Apennine-Balkan Province. The wide distributions of haplotypes # 32 and 33 (extending from southern Italy to the Caucasus and Lebanon, and from central Europe to Great Britain) likely provide evidence of biogeographic connections prior to Quaternary glaciations and may reflect ancient East/West vicariance. The evolutionary interpretations of the haplotypes found in the most densely sampled area of our study (the Italian Peninsula and its major islands), are presented in Table 2.

Phylogeography of the European white oak plastome revisited

Despite the unbalanced sampling designs, we may sketch a comparison of our results with the distribution patterns proposed by (Petit et al. 2002a, b), representing the latest synthesis and the current state of the art for the European

Table 2 Haplotypes detected in the Italian Peninsula, distribution, frequency and evolutionary interpretation

#	N	Species	Occurrence	Distribution	Additional species	Haplotypes interpretation
1	16	*Q. petraea* *Q. pubescens* *Q. frainetto*	C Italy (Latium, Umbria, Molise)	Eurasia	*Q. robur*†* *Q. pyrenaica*†* *Q. faginea*†* *Q. canarensis** *Q. lusitanica** *Q. protoroburoides** *Q. hartwissiana** *Q. infectoria** *Q. fabri** *Q. dentata**	Ancestral type, reflecting America/Eurasia vicariance, and reticulation among the progenitors of modern Eurasian white oaks, also present in N Africa, common
2	7	*Q. congesta* *Q. dalechampii*	S Italy (Calabria), Sicily	Eurasia	*Q. iberica** *Q. infectoria** *Q. aliena** *Q. fabri** *Q. mongolica**	Ancestral type, reflecting America/Eurasia vicariance and reticulation among the progenitors of modern Eurasian white oaks, relatively more common in East Eurasia
31	38	*Q. robur* *Q. petraea* *Q. frainetto* *Q. pubescens* *Q. congesta* *Q. virgiliana* *Q. dalechampii*	N and C Italy (Lombardy, Emilia-Romagna, Latium), Sardinia, Sicily	SW and SC Europe	*Q. faginea*†* *Q. pyrenaica**	Early derived, reflecting ancient divergence between CW and CE Europe, common
32	24	*Q. robur* *Q. petraea subsp. austrotyrrhenica* *Q. frainetto* *Q. pubescens* *Q. virgiliana* *Q. congesta* *Q. leptobalana*	Italian peninsula (Lombardy, Latium, Abruzzo), Sicily	CE Europe, Caucasus, Near East	*Q. iberica** *Q. macranthera** *Q. hartwissiana** *Q. infectoria** *Q. kotschyana** *Q. robur subsp. imeretina** *Q. robur subsp. pedunculiflora**	Early derived, reflecting ancient divergence between CW and CE Europe, common
33	40	*Q. robur* *Q. petraea* *Q. frainetto* *Q. pubescens* *Q. dalechampii*	Italian Peninsula (Piedmont, Lombardy, Veneto, Friuli, Tuscany, Basilicata, Apulia)	C and E Europe, Caucasus	*Q. banatus* †* *Q. iberica** *Q. macranthera**	Early derived, reflecting ancient divergence between CW and CE Europe, common
15	1	*Q. pubescens*	SE Italy (Apulia)	SE Italy and Bulgaria	*Q. petraea**	Early derived, rare, disjunct, potentially reflecting an eroded link between Italy and the Balkans
30	1	*Q. virgiliana*	Sicily	Sicily	/	Early derived, rare, reflecting ancient genetic drift

Table 2 (continued)

#	N	Species	Occurrence	Distribution	Additional species	Haplotypes interpretation
36	35	*Q. petraea* *Q. petraea subsp. austrotyrrhenica* *Q. frainetto* *Q. pubescens* *Q. virgiliana* *Q. dalechampii* *Q. congesta* *Q. dalechampii*	CS Italian Peninsula (Latium, Umbria, Marche, Molise, Apulia, Calabria)	S and SE Europe	/	Derived, centred around the SE Mediterranean, potentially reflecting secondary trans-Adriatic contacts, common
19	13	*Q. dalechampii* *Q. congesta* *Q. virgiliana* *Q. ichnusae*	S Italy (Calabria), Sardinia	S Italy and Sardinia	/	Derived, relatively common, disjunct, potentially reflecting an ancient link between the two areas
41	12	*Q. congesta* *Q. virgiliana* *Q. ichnusae*	Sardinia	Sardinia	/	Derived, relatively common, reflecting local differentiation
20	1	*Q. ichnusae*	Sardinia	Sardinia	/	Derived, rare, reflecting local differentiation or genetic drift
37	1	*Q. pubescens**	C Italy (Latium)	C Italy	/	Derived, rare, reflecting local differentiation or genetic drift

#: haplotype number; N: number of individuals; †: haplotypes detected in the investigated dataset (outside Italy); *: haplotypes matched on GenBank white oak plastome diversity. In their seminal works conducted on over 2600 white oak populations across Europe (overlooking, however, the southeast Mediterranean and Near east areas), Petit et al. (2002a) detected 32 chloroplast PCR–RFLP haplotypes grouped in six major lineages (A–F). In contrast, our data include the overlooked regions but are somewhat deficient as regards the Northern European regions. However, we detected a comparable number of genetic variants (29) and we can expect the greater part of the molecular plastome diversity has been captured by our investigation. In fact, Petit et al. (2002a) also identified fewer chloroplast variants in Northern Europe and all present in the southern regions.

Our results match several of the previously assessed findings and provide additional insights. We confirm that many haplotypes have an extremely large distribution while others are more delimited, and that non-coeval molecular signatures coexist in the three Mediterranean peninsulas and major islands (Sicily and Sardinia). In addition, the same major areas of genetic similarity are found in our work too, confirming the previously acknowledged refugial role and the subsequent recolonization routes (Petit et al. 2002b). These certainly include the patterns of the most widespread haplotypes #31, 32, 33 and 36, identifying clear phylogeographic relationships between North Spain, Italy and major Tyrrhenian islands (#31), the Italian Peninsula and the southern Balkans (#36), Italy and Central-East Europe (#32, 33). Other congruent relationships are also evidenced by less widespread haplotypes such as #15 (South Italy and the Balkans), #18 (Black Sea coasts) and #19 (South Italy and Sardinia), whereas haplotypes #23–28 identify the previously overlooked Near East region. The occurrence of unique Iberian and Italian haplotypes (#20–22, 29, 30, 37) is also confirmed (cf. Olalde et al. 2002; Fineschi et al. 2002), together with the new evidence of further rare variants in the Southeast Mediterranean area (#16, 23, 25–28, 35, 38–40). Finally, not a single species showed an exclusive haplotype composition: the most densely sampled species (22 to 68 individuals each of *Q. robur, Q. petraea, Q. frainetto, Q. iberica*) displayed six to eight highly shared haplotypes, the only exception being a peculiar form of *Q. robur* from Anatolia (*Q. haas*).

However, we also document some significant novelties as compared to the generally accepted scenario. Our extensive dataset, combining new sampling points with a high number of GenBank sequences from the whole of Eurasia, enabled us to sketch out for the first time a suggestive phylogenetic backbone of the white oak plastome, to identify ancestral signatures and highlight a well-structured phylogeographic distribution. Evidence of ancestral haplotypes possibly dating back to the Miocene, i.e. prior to the white oaks species' differentiation and separation between the Eastern and Western Eurasian, opens up new interpretative scenarios on the evolution of white oaks and their spread across Europe. Furthermore, 17 unique variants were found, all exhibited by isolated (Sardinian, Sicilian) or disjunct (Anatolian, Lebanese, South Iberian) populations of species otherwise sharing the largest part of their plastome signatures with numerous congenerics, independently of geographic proximity or taxonomic affinity. The only exceptions are the Anatolian endemic *Q. vulcanica* and a couple of undefinable hybrids from Lebanon. In fact, the majority of the unique haplotypes (either ancient or derived) are displayed by xerophylous species, such as *Q. faginea, Q. ichnusae, Q. infectoria, Q. pubescens, Q. virgiliana*. This finding can be explained by means of a combination of ancestral legacies, ability to adapt to Mediterranean ecological extremes and isolation (i.e., 'phylogenetic conservatism and ecological opportunity' Cavender-Bares 2019; Hipp et al. 2020). As such, they may provide new input for future, more informative studies on the white oak phylogenomics.

Another newly emerging result is the multitasking phylogeographic role played by the Italian Peninsula, which hosted numerous glacial refugia, and did not simply act as a crossroad for widespread haplotypes but rather as a real biogeographical threshold. This is evident observing the structured distribution of the ancestral haplotypes, as well as the distribution of their derived variants that find their easternmost (#31) or westernmost (#32, 36 boundary precisely in the Italian Peninsula. Among these, and with a sharpness that has never emerged in previous works on European white oaks, a well-defined Amphi-Adriatic haplotype area has here been outlined. This creates interesting parallels with what has recently emerged from other fields of research, for example the new insight on the coenological and synchorological features of the *Quercus frainetto* and *Q. cerris* Apennine-Balkan forests (Di Pietro et al. 2020c), and the biosystematics studies regarding some other diagnostic (in phytogeographical terms) genera, such as *Campanula, Salvia, Sesleria* (Kuzmanović et al. 2017; Janković et al. 2019; Radosavljević et al. 2022). Obviously, we are aware of the paucity of data from Greece, Anatolia, and Eastern Europe from which it would be reasonable to expect a greater diversity than that detected by means of the relatively few samples included in our analyses. Greater availability of data may have enabled us to obtain results similar to those obtained for *Q. cerris* (Bagnoli et al. 2016), also considering the remarkable occurrence of ancestral (Bulgaria, Georgia), inter-sectionally shared (Georgia), disjunct (Ukraine), highly diverse (Bulgaria) and unique (Croatia, Georgia) haplotypes found in our study.

A final comment is also due regarding the genetic variation found in *Q. pubescens s.l.*, the most xero-thermic white oak in western Eurasia. This species revealed the highest number of haplotypes (18) in the entire dataset, including both ancient and derived variants. Five haplotypes, uniquely located in Spain, Italy (2), Croatia and Greece, were found solely in its typical form (*Q. pubescens* Willd.), whereas seven haplotypes can be assigned to its (largely Italian) taxonomically critical species (e.g. *Q. congesta*, *Q. virgiliana* etc.). The remaining were interspecifically and geographically highly shared. This high variability of *Q. pubescens s.l.* plastid DNA matches other findings highlighted in previous papers, such as this species' wide morphological variability (probably the widest in the whole genus) which was demonstrated as deriving from a genetic base (Viscosi et al. 2009; Curtu et al. 2011; Fortini et al. 2015b) and its pronounced tendency to introgressive hybridization which, according to some authors, represented one of the most effective mechanisms of its postglacial expansion (Lepais and Gerber 2011).

All this indicates the long, complex evolutionary history and the high tendency to local adaptation and differentiation of the downy oaks, which, although it is still far from being completely understood, bodes well for the adaptation of this xerothermic oak to the current ongoing climatic changes.

Conclusion

The rise of phylogenomic analysis over the last few years has provided superior tools for clarifying the evolutionary diversification of *Quercus*. However, the recent boost in the availability of genomic data deriving from one to few individuals per species may not provide reliable and complete information, especially if samples from biogeographically important areas are neglected and diagnostic natural populations are not considered. Findings resulting from our methodological trade-off (few markers, many samples, biogeographical diagnosis, GenBank exploitation, comprehensive comparative analyses) are complex or even puzzling, but at the same time confirm basic geographic patterns and highlight important variants bearing notable biogeographic or evolutionary signatures.

Our reconstructions are congruent with the general framework provided by Petit et al. (2002a, b). We gathered evidence that the West Eurasian white oaks, which originated from a limited genetic source and were only recently distributed across the continent after passing through a long series of impacting events in highly heterogeneous landscapes, have preserved a poorly differentiated plastid DNA, where signatures of a distant past abide with more recent, still unfixed genetic diversity. Finally, we provide a phylogenetic inference in which geographical areas (and their oak populations) preserving very ancient molecular signatures, overlapping combination of extensive Pliocene to Pleistocene migration waves, and local, more recent differentiation, are highlighted. Such a prolonged preservation, diversification and cohabitation dynamics of the white oak plastome certainly requires more in-depth multidisciplinary studies aimed at identifying the drivers of species evolution, identity and community assembly, in order to implement adequate conservation strategies. Some Eurasian regions have never been included in a thorough phylogenetic work and these were largely overlooked in recent phylogenomic studies; they include the focal area of our study (Central and South Italy, Sardinia, Sicily), together with central Iberia, Anatolia, the Middle East and the Caucasus. More specifically, the Italian Peninsula emerges as a clear phylogeographic threshold for lineages of different origins and provenance. Despite extensive blurring of specific molecular footprints via admixing over time and incomplete reproductive barriers, ancient and derived plastid variants co-occur in Italy, intriguingly encompassing some "major" species (*Q. frainetto, Q. petraea s.s.*, *Q. pubescens s.s.*, *Q. robur s.s.*) and peculiar, endemic, and still enigmatic phenotypes that urge a better assessment. Beyond the taxonomic and nomenclatural issues on their specific binomials, our results stress the potential biological and conservation significance of *Q. petraea* subsp. *austrothyrrenica*, *Q. congesta*, *Q. virgiliana* and *Q. ichnusae* in Italy, Sardinia and Sicily (this work), together with *Q. faginea* in Andalucia, *Q. vulcanica* and *Q. macranthera* subsp. *syspirensis* in Anatolia, *Q. kotschyana* and *Q. cedrorum* in the Middle East, *Q. hartwissiana*, *Q. iberica* and *Q. robur* subsp. *imeretina* in the Caucasian region (Ekhvaia et al. 2018; Hipp et al. 2020; Douaihy et al. 2020; Piredda et al. 2021). Future research on genomic variants with potential phylogenetic importance or exploitable (adaptive) traits capable of increasing the European forest cover, withstand the ongoing climate change and reverse the biodiversity crisis, should be focused more on oaks from Southern latitudes and should be inclusive of all peculiar xero-types having still unresolved taxonomy.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10342-023-01610-8.

Acknowledgements The authors wish to acknowledge two anonymous referees and the Editor for valuable feedbacks on an earlier version of the manuscript. Special thanks go to the colleagues who have helped in collecting specimens of white oaks in different places in Europe. They are J. Loidi (Spain), K. Chytrý (Czech Republic), B. Vincent (France), W. Willner (Austria), P. Dimopoulos (Greece), Ž. Škvorc and N. Jasprika (Croatia), R. Tzonev (Bulgaria); A.L. Conte, F. Conti, E. Farris, L. Filesi, L. Gianguzzi, D. Iamonico, G. Misano, L. Rosati, G. Spampinato (Italy) I. Jankovic, N. Kuzmanovic, D. Lakusic, (Serbia). For the same reason we thank the Park Rangers D. Di Fazio and colleagues (Ausoni Mountains and Fondi Lake Regional Park) and M. Patriarca, P. Perrella and F. Soscia (Aurunci Mountains Regional Park). We thank P. Di Marzio and C. Ravazzi for the useful suggestions for statistical analysis procedures and palaeobotanical information. Jim McManus kindly improved our English.

Author contributions R.D.P., P.F. and M.C.S. designed the study and wrote the first draft of the paper; E.P., P.F., R.D.P. and M.C.S. (together with colleagues mentioned in the acknowledgments) collected the plant material; E.P., S.C. and L.Q. generated and analysed the molecular data, R.D.P., P.F. and M.C.S. analysed the phylogeographic

comparative data, R.D.P., P.F., E.P., S.C., L.Q. and M.C.S. discussed the results and finalized the paper.

Funding Open access funding provided by Università degli Studi di Roma La Sapienza within the CRUI-CARE Agreement. The research was supported by the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2: Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research; Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, Project funded by the European Union—'NextGenerationEU NBFC'. R. Di Pietro: CUP B83C22002950007, Project title "National Biodiversity Future Center"; P. Fortini: Project: "Strengthening of research structures and creation of 'National R&S samples on some Key Enabling Technologies". CUP H73C22000300001: M.C. Simeone: Project ECS 0000024 Rome Technopole CUP B83C22002820006, Investment 1.5.

Data availability Used alignments and inference files are included in the Online Data Archive available at: https://10.0.23.196/m9.figshare.22787504. TrnH-psbA and trnK-matK sequences are deposited in the NCBI archive under accession numbers OQ990790–OQ990817. Other relevant data are contained within the manuscript and its supporting materials.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The Authors declare no potential sources of conflict of interest.

References

Amaral-Franco J (1990) Quercus. In: Castroviejo, S. et al. Flora Ibérica II CSIC Madrid

Antonecchia G, Fortini P, Lepais O, Gerber S, Legér P, Scippa, G S, Viscosi V (2015) Genetic structure of a natural oak community in central Italy: Evidence of gene flow between three sympatric white oak species (Quercus, Fagaceae). Ann for Res (2): 205–216

Backs JR, Ashley MV (2021) Quercus conservation genetics and genomics: past, present, and future. Forests 12:882. https://doi.org/10.3390/f12070882

Barrón E, Averyanova A, Kvaček Z et al (2017) The Fossil History of Quercus. In: Gil-Pelegrín E, Peguero-Pina JJ, Sancho-Knapik D (eds) Oaks Physiological Ecology. Springer International Publishing, Cham, Exploring the Functional Diversity of Genus Quercus L, pp 39–105

Bennett KD, Tzedakis PC, Willis KJ (1991) Quaternary refugia of north European trees. J Biogeogr 18:103–115

Birks HH, Giesecke T, Hewitt GM et al (2012) Comment on "glacial survival of boreal trees in Northern Scandinavia." Science 338:742–742. https://doi.org/10.1126/science.1225345

Blair C (2023) Organellar DNA continues to provide a rich source of information in the genomics era. Mol Ecol 32:2144–2150. https://doi.org/10.1111/mec.16872

Blasi C, Di Pietro R (1998) Two new phytosociological types of Quercus pubescens s.l. woodland communities in southern Latium. Plant Biosyst 132(3):207–223. https://doi.org/10.1080/11263504.1998.10654205

Blasi C, Di Pietro R, Filesi L (2004) Syntaxonomical revision of Quercetalia pubescenti-petraeae in the Italian Peninsula. Fitosociologia 41:87–164

Blondel J, Aronson J, Bodiou J-Y, Boeuf G (2010) The Mediterranean region: biological diversity in space and time. Oxford University Press Inc., New York

Bock B, Tison J-M (2012) Revisions nomenclaturales et taxonomiques (note n 2). Bull De La Societe Botanique Du Centre-Ouest (nouv Ser) 43:209–220

Brewer S, Cheddadi R, de Beaulieu JL, Reille M (2002) The spread of deciduous Quercus throughout Europe since the last glacial period. For Ecol Manage 156:27–48. https://doi.org/10.1016/S0378-1127(01)00646-6

Brullo S, Marcenò C (1985) Contributo alla conoscenza della classe Quercetea ilicis in Sicilia. Not Fitosoc 19(1):183–229

Brullo S, Guarino R, Siracusa G (1999) Taxonomical revision about the deciduous oaks of Sicily. Webbia 54:1–72. https://doi.org/10.1080/00837792.1999.10670670

Bryant D, Moulton V (2004) Neighbor-Net: an agglomerative method for the construction of phylogenetic networks. Mol Biol Evol 21:255–265. https://doi.org/10.1093/molbev/msh018

Burger WC (1975) The species concept in Quercus. Taxon 24:45–50. https://doi.org/10.2307/1218998

Cavender-Bares J (2019) Diversification, adaptation, and community assembly of the American oaks (Quercus), a model clade for integrating ecology and evolution. New Phytol 221:669–692. https://doi.org/10.1111/nph.15450

Cavender-Bares J, Ackerly DD, Hobbie SE, Townsend PA (2016) Evolutionary legacy effects on ecosystems: biogeographic origins, plant traits, and implications for management in the era of global change. Annu Rev Ecol Evol Syst 47:433–462. https://doi.org/10.1146/annurev-ecolsys-121415-032229

Combourieu-Nebout N, Bertini A, Russo-Ermolli E et al (2015) Climate changes in the central Mediterranean and Italian vegetation dynamics since the Pliocene. Rev Palaeobot Palynol 218:127–147. https://doi.org/10.1016/j.revpalbo.2015.03.001

Cottrell JE, Munro RC, Tabbener HE et al (2002) Distribution of chloroplast DNA variation in British oaks (Quercus robur and Q. petraea): the influence of postglacial colonisation and human management. For Ecol Manage 156:181–195

Crowl AA, Manos PS, McVay JD et al (2020) Uncovering the genomic signature of ancient introgression between white oak lineages (Quercus). New Phytol 226:1158–1170. https://doi.org/10.1111/nph.15842

Csaikl UM, Burg K, Fineschi S et al (2002) Chloroplast DNA variation of white oaks in the alpine region. For Ecol Manage 156:131–145

Curtu AL, Moldovan IC, Enescu CM, Craciunesc I, Sofletea N (2011) Genetic differentiation between Quercus frainetto Ten. and Q. pubescens Willd. in Romania. Not Bot Horti Agrobo 39:275–282

Denk T, Grimm GW (2010) The oaks of western Eurasia: Traditional classifications and evidence from two nuclear markers. Taxon 59:351–366

Denk T, Grimm GW, Manos PS et al (2017) An updated infrageneric classification of the oaks: review of previous taxonomic schemes and synthesis of evolutionary patterns. In: Gil-Pelegrín E, Peguero-Pina JJ, Sancho-Knapik D (eds) Oaks Physiological Ecology. Springer International Publishing, Cham, Exploring the Functional Diversity of Genus Quercus L, pp 13–38

Denk T, Grimm GW, Hipp AL et al (2023) Niche evolution in a northern temperate tree lineage: biogeographical legacies in cork oaks (Quercus section Cerris). Annals of Botany 131 (5): 769–787 https://doi.org/10.1093/aob/mcad032

Di Pietro R, Viscosi V, Peruzzi L, Fortini P (2012) A review of the application of the name Quercus dalechampii. Taxon 61:1311–1316. https://doi.org/10.1002/tax.616012

Di Pietro R, Di Marzio P, Medagli P et al (2016) Evidence from multivariate morphometric study of the Quercus pubescens complex in southeast Italy. Botanica Serbica 40:83–100

Di Pietro R, Conte AL, Di Marzio P et al (2020a) A multivariate morphometric analysis of diagnostic traits in southern Italy and Sicily pubescent oaks. Folia Geobot 55:163–183. https://doi.org/10.1007/s12224-020-09378-0

Di Pietro R, Di Marzio P, Antonecchia G et al (2020b) Preliminary characterization of the Quercus pubescens complex in southern Italy using molecular markers. Acta Bot Croat 79(1):15–25

Di Pietro R, Fortini P, Ciaschetti G et al (2020c) A revision of the syntaxonomy of the Apennine-Balkan Quercus cerris and Q. frainetto forests and correct application of the name Melittio-Quercion frainetto. Plant Biosyst 154:887–909. https://doi.org/10.1080/11263504.2019.1701127

Di Pietro R, Conte AL, Di Marzio P et al (2021) Does the genetic diversity among pubescent white oaks in southern Italy, Sicily and Sardinia islands support the current taxonomic classification? Eur J Forest Res 140:355–371. https://doi.org/10.1007/s10342-020-01334-z

Douaihy B, Saliba C, Stephan J et al (2020) Tracking diversity and evolutionary pathways of Lebanese oak taxa through plastome analyses. Botany Letters 167:315–330. https://doi.org/10.1080/23818107.2020.1765197

Dumolin S, Demesure B, Petit RJ (1995) Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. Theoret Appl Genetics 91:1253–1256. https://doi.org/10.1007/BF00220937

Ekhvaia J, Simeone MC, Silakadze N, Abdaladze O (2018) Morphological diversity and phylogeography of the Georgian durmast oak (Q. petraea subsp. iberica) and related Caucasian oak species in Georgia (South Caucasus). Tree Genetics & Genomes 14:17. https://doi.org/10.1007/s11295-018-1232-6

Fickert T, Friend D, Grüninger F, Molnia B, Richter M (2007) Did debris-covered glaciers serve as pleistocene refugia for plants? a new hypothesis derived from observations of recent plant growth on glacier surfaces. Arct Antarct Alp Res 39(2):245–257. https://doi.org/10.1657/1523-0430(2007)39[245:DDGSAP]2.0.CO;2

Fineschi S, Taurchini D, Grossoni P et al (2002) Chloroplast DNA variation of white oaks in Italy. For Ecol Manage 156:103–114. https://doi.org/10.1016/S0378-1127(01)00637-5

Fois M, Farris E, Calvia G et al (2022) The endemic vascular flora of sardinia: a dynamic checklist with an overview of biogeography and conservation status. Plants 11:601. https://doi.org/10.3390/plants11050601

Fortini P, Viscosi V, Maiuro L et al (2009) Comparative leaf surface morphology and molecular data of five oaks of the subgenus Quercus Oerst (Fagaceae). Plant Biosyst 143:543–554. https://doi.org/10.1080/11263500902722980

Fortini P, Antonecchia G, Di Marzio P et al (2015a) Role of micromorphological leaf traits and molecular data in taxonomy of three sympatric white oak species and their hybrids (Quercus L.). Plant Biosyst 149:546–558. https://doi.org/10.1080/11263504.2013.868374

Fortini P, Di Marzio P, Di Pietro R (2015b) Differentiation and hybridization of Quercus frainetto, Q. petraea, and Q. pubescens (Fagaceae): insights from macro-morphological leaf traits and molecular data. Plant Syst Evol 301:375–385. https://doi.org/10.1007/s00606-014-1080-2

Fortini P, Di Marzio P, Conte AL et al (2022) Morphological and molecular results from a geographical transect focusing on Quercus pubescens/Q. virgiliana ecological-altitudinal vicariance in peninsular Italy. Plant Biosyst 156:1498–1511. https://doi.org/10.1080/11263504.2022.2131923

Grossoni P, Bruschi P, Bussotti F et al (2021) The taxonomic interpretation of Mediterranean oaks of Quercus sect. Quercus (Fagaceae): uncertainties and diverging concepts. Flora Mediterranea 31:271–278. https://doi.org/10.7320/FlMedit31SI.271

Guarino R, Bazan G, Paura B (2015) Downy-oak woods of italy: phytogeographical remarks on a controversial taxonomic and ecologic issue. In: Box EO, Fujiwara K (eds) Warm-Temperate Deciduous Forests around the Northern Hemisphere. Springer International Publishing, Cham, pp 139–151

Hipp AL (2015) Should hybridization make us skeptical of the oak phylogeny. Int Oaks 26:9–17

Hipp AL, Manos PS, González-Rodríguez A et al (2018) Sympatric parallel diversification of major oak clades in the Americas and the origins of Mexican species diversity. New Phytol 217:439–452. https://doi.org/10.1111/nph.14773

Hipp AL, Manos PS, Hahn M et al (2020) Genomic landscape of the global oak phylogeny. New Phytol 226:1198–1212. https://doi.org/10.1111/nph.16162

Hsü KJ, Montadert L, Bernoulli D et al (1977) History of the Mediterranean salinity crisis. Nature 267:399–403. https://doi.org/10.1038/267399a0

Hubert F, Grimm GW, Jousselin E et al (2014) Multiple nuclear genes stabilize the phylogenetic backbone of the genus Quercus. Syst Biodivers 12:405–423. https://doi.org/10.1080/14772000.2014.941037

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267. https://doi.org/10.1093/molbev/msj030

Janković I, Satovic Z, Liber Z et al (2019) Genetic and morphological data reveal new insights into the taxonomy of Campanula versicolor s.l. (Campanulaceae). Taxon 68:340–369. https://doi.org/10.1002/tax.12050

Jiang X-L, Hipp AL, Deng M et al (2019) East Asian origins of European holly oaks (Quercus section Ilex Loudon) via the Tibet-Himalaya. J Biogeogr 46:2203–2214. https://doi.org/10.1111/jbi.13654

Jiménez-Moreno G, Suc J-P (2007) Middle Miocene latitudinal climatic gradient in Western Europe: evidence from pollen records. Palaeogeogr Palaeoclimatol Palaeoecol 253:208–225. https://doi.org/10.1016/j.palaeo.2007.03.040

Kaltenrieder P, Belis CA, Hofstetter S et al (2009) Environmental and climatic conditions at a potential Glacial refugial site of tree species near the Southern Alpine glaciers. New insights from multiproxy sedimentary studies at Lago della Costa (Euganean Hills, Northeastern Italy). Quatern Sci Rev 28:2647–2662. https://doi.org/10.1016/j.quascirev.2009.05.025

Kaplan Z, Danihelka J, Chrtek J et al (2022) Distributions of vascular plants in the Czech Republic Part 11. Preslia 94:335–427

Kremer A, Hipp AL (2020) Oaks: an evolutionary success story. New Phytol 226:987–1011. https://doi.org/10.1111/nph.16274

Krijgsman W (2002) The mediterranean: mare nostrum of earth sciences. Earth Planet Sci Lett 205:1–12. https://doi.org/10.1016/S0012-821X(02)01008-7

Krijgsman W, Hilgen FJ, Raffi I et al (1999) Chronology, causes and progression of the Messinian salinity crisis. Nature 400:652–655. https://doi.org/10.1038/23231

Kubitzki K (1993) Fagaceae. In: Kubitzki K, Rohwer JG, Bittrich V (eds) Flowering plants dicotyledons: magnoliid, hamamelid and caryophyllid families. Springer, Berlin, Heidelberg, pp 301–309

Kučera P (2018) New name for Central Europaean oak formerly labelled as Quercus dalechampii. Biologia 73:313–317. https://doi.org/10.2478/s11756-018-0048-z

Kumar S, Stecher G, Li M et al (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096

Kuzmanović N, Lakušić D, Frajman B et al (2017) Phylogenetic relationships in Seslerieae (Poaceae) including resurrection of Psilathera and Sesleriella, two monotypic genera endemic to the Alps. Taxon 66:1349–1370

Kvaček Z, Teodoridis V, Gregor H-J (2008) The Pliocene leaf flora of Auenheim, Northern Alsace (France). Documenta Naturae 155:1–108

Kvaček Z, Teodoridis V, Denk T (2020) The Pliocene flora of Frankfurt am Main, Germany: taxonomy, palaeoenvironments and biogeographic affinities. Palaeobio Palaeoenv 100:647–703. https://doi.org/10.1007/s12549-019-00391-6

Lepais O, Gerber S (2011) Reproductive patterns shape introgression dynamics and species succession within the European white oak species complex. Evolution 65:156–170

Li Y, Zhang X, Fang Y (2019) Landscape features and climatic forces shape the genetic structure and evolutionary history of an oak species (Quercus chenii) in East China. Front Plant Sci 10:1060. https://doi.org/10.3389/fpls.2019.01060

Li Y, Wang T-R, Kozlowski G et al (2022) Complete chloroplast genome of an endangered species quercus litseoides, and its comparative, evolutionary, and phylogenetic study with other quercus section cyclobalanopsis species. Genes 13:1184. https://doi.org/10.3390/genes13071184

Liu X, Chang E, Liu J, Jiang Z (2021) Comparative analysis of the complete chloroplast genomes of six white oaks with high ecological amplitude in China. J for Res 32:2203–2218. https://doi.org/10.1007/s11676-020-01288-3

Magri D (2010) Persistence of tree taxa in Europe and Quaternary climate changes. Quatern Int 219:145–151. https://doi.org/10.1016/j.quaint.2009.10.032

Magri D, Di Rita F, Aranbarri J et al (2017) Quaternary disappearance of tree taxa from Southern Europe: timing and trends. Quatern Sci Rev 163:23–55. https://doi.org/10.1016/j.quascirev.2017.02.014

Manos PS, Hipp AL (2021) An updated infrageneric classification of the North American Oaks (Quercus Subgenus Quercus): review of the contribution of phylogenomic data to biogeography and species diversity. Forests 12:786. https://doi.org/10.3390/f12060786

Manos PS, Cannon CH, Oh S-H (2008) Phylogenetic relationships and taxonomic status of the paleoendemic fagaceae of western North America: recognition of a new genus. Notholithocarpus Madr 55:181–190. https://doi.org/10.3120/0024-9637-55.3.181

Mansion G, Selvi F, Guggisberg A, Conti E (2009) Origin of Mediterranean insular endemics in the Boraginales: integrative evidence from molecular dating and ancestral area reconstruction. J Biogeogr 36:1282–1296. https://doi.org/10.1111/j.1365-2699.2009.02082.x

McVay JD, Hipp AL, Manos PS (2017) A genetic legacy of introgression confounds phylogeny and biogeography in oaks. Proc R Soc Lond B 284:20170300. https://doi.org/10.1098/rspb.2017.0300

Médail F, Diadema K (2009) Glacial refugia influence plant diversity patterns in the Mediterranean Basin. J Biogeogr 36:1333–1345. https://doi.org/10.1111/j.1365-2699.2008.02051.x

Menitsky YL (2005) Oaks of Asia (Translated from Russian). Science Publishes, Inc, Enfield, New Hampshire, USA, p 549

Mucina L, Bültmann H, Dierßen K et al (2016) Vegetation of Europe: hierarchical floristic classification system of vascular plant, bryophyte, lichen, and algal communities. Appl Veg Sci 19:3–264

Niccolini G, Martinetto E, Lanini B et al (2022) Late Messinian flora from the post-evaporitic deposits of the Piedmont Basin (Northwest Italy). Fossil Imprint 78:189–216

Nieto Feliner G (2014) Patterns and processes in plant phylogeography in the mediterranean basin. a review. Perspect Plant Ecol Evolut Syst 16:265–278. https://doi.org/10.1016/j.ppees.2014.07.002

Nocchi G, Brown N, Coker TLR et al (2022) Genomic structure and diversity of oak populations in British parklands. Plants, People, Planet 4:167–181. https://doi.org/10.1002/ppp3.10229

Okaura T, Quang ND, Ubukata M, Harada K (2007) Phylogeographic structure and late Quaternary population history of the Japanese oak Quercus mongolica var. crispula and related species revealed by chloroplast DNA variation. Genes Genet Syst 82:465–477. https://doi.org/10.1266/ggs.82.465

Olalde M, Herrán A, Espinel S, Goicoechea PG (2002) White oaks phylogeography in the Iberian Peninsula. For Ecol Manage 156:89–102. https://doi.org/10.1016/S0378-1127(01)00636-3

Pang X, Liu H, Wu S et al (2019) Species identification of oaks (Quercus L., Fagaceae) from gene to genome. Int J Mol Sci 20:5940. https://doi.org/10.3390/ijms20235940

Parducci L, Jørgensen T, Tollefsrud MM et al (2012a) Glacial survival of boreal trees in northern Scandinavia. Science 335:1083–1086. https://doi.org/10.1126/science.1216043

Parducci L, Edwards M, Bennett K, Alm T, Elverland E et al (2012b) Response to comment on "glacial survival of boreal trees in Northern Scandinavia." Science 338(6108):742–2012

Pasta S, De Rigo D, Caudullo G (2016) Quercus pubescens in Europe: distribution, habitat, usage and threats. In: San Miguel Ayanz J, de Rigo D, Caudullo G, Houston Durrant T, Mauri A (eds) European atlas of forest tree species. Publ. Off. EU, Luxembourg, e019e5c+European Atlas of forest tree species:156–157

Pattengale ND, Alipour M, Bininda-Emonds ORP et al (2009) How Many Bootstrap Replicates Are Necessary? In: Batzoglou S (ed) Research in Computational Molecular Biology. Springer, Berlin, Heidelberg, pp 184–200

Petit RJ, Csaikl UM, Bordács S et al (2002a) Chloroplast DNA variation in European white oaks: Phylogeography and patterns of diversity based on data from over 2600 populations. For Ecol Manage 156:5–26. https://doi.org/10.1016/S0378-1127(01)00645-4

Petit RJ, Brewer S, Bordács S et al (2002b) Identification of refugia and post-glacial colonisation routes of European white oaks based on chloroplast DNA and fossil pollen evidence. For Ecol Manage 156:49–74. https://doi.org/10.1016/S0378-1127(01)00634-X

Pham KK, Hipp AL, Manos PS, Cronn RC (2017) A time and a place for everything: phylogenetic history and geography as joint predictors of oak plastome phylogeny. Genome 60:720–732. https://doi.org/10.1139/gen-2016-0191

Pignatti S, Guarino R, La Rosa M (2017) Flora d'Italia, 2nd edn. Edagricole, Bologna

Pini R, Furlanetto G, Vallé F et al (2022) Linking North Atlantic and Alpine Last Glacial Maximum climates via a high-resolution pollen-based subarctic forest steppe record. Quatern Sci Rev 294:107759. https://doi.org/10.1016/j.quascirev.2022.107759

Piredda R, Simeone MC, Attimonelli M et al (2011) Prospects of barcoding the Italian wild dendroflora: oaks reveal severe limitations to tracking species identity. Mol Ecol Resour 11:72–83. https://doi.org/10.1111/j.1755-0998.2010.02900.x

Piredda R, Grimm GW, Schulze E-D et al (2021) High-throughput sequencing of 5S-IGS in oaks: Exploring intragenomic variation and algorithms to recognize target species in pure and mixed samples. Mol Ecol Resour 21:495–510. https://doi.org/10.1111/1755-0998.13264

Pons A, Suc J-P, Reille M, Combourieu-Nebout N (1995) The history of dryness in regions with a mediterranean climate. In: Roy J, Aronson J, di Castri F (eds) Time Scales of Biological Responses to Water Constraints. Publishing, Amsterdam, Acad, pp 169–188

Posada D, Crandall KA (2001) Intraspecific gene genealogies: trees grafting into networks. Trends Ecol Evol 16:37–45. https://doi.org/10.1016/S0169-5347(00)02026-7

Von Raab-Straube E, Raus T (2013) Euro+ Med-Checklist Notulae, 1. Willdenowia 43:

Radosavljević I, Satovic Z, Di Pietro R et al (2022) Phylogeographic structure of common sage (Salvia officinalis L.) reveals microrefugia throughout the Balkans and colonizations of the Apennines. Sci Rep 12:15726. https://doi.org/10.1038/s41598-022-20055-4

Ravazzi C, Peresani M, Pini R, Vescovi E (2007) Il Tardoglaciale nelle Alpi italiane e in Pianura Padana. Evoluzione stratigrafica, storia della vegetazione e del popolamento antropico [The Late Glacial in the Italian Alps and in the Po Valley. Stratigraphic evolution, history of vegetation and anthropic population]. Alpine Med Quat 20:163–184

Rivas-Martínez S, Penas A, Díaz TE (2004) Bioclimatic and biogeographic maps of Europe. University of León URL: www.globalbioclimatics.org/form/maps.htm

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC et al (2017) DnaSP 6: DNA sequence polymorphism analysis of large data sets. Mol Biol Evol 34:3299–3302. https://doi.org/10.1093/molbev/msx248

Schmitt T, Fritz U, Delfino M et al (2021) Biogeography of Italy revisited: genetic lineages confirm major phylogeographic patterns and a pre-Pleistocene origin of its biota. Front Zool 18:34. https://doi.org/10.1186/s12983-021-00418-9

Simeone MC, Piredda R, Papini A et al (2013) Application of plastid and nuclear markers to DNA barcoding of Euro-Mediterranean oaks (Quercus, Fagaceae): problems, prospects and phylogenetic implications. Bot J Linn Soc 172:478–499. https://doi.org/10.1111/boj.12059

Simeone MC, Grimm GW, Papini A et al (2016) Plastome data reveal multiple geographic origins of Quercus Group Ilex. PeerJ 4:e1897. https://doi.org/10.7717/peerj.1897

Simeone MC, Cardoni S, Piredda R et al (2018) Comparative systematics and phylogeography of Quercus Section Cerris in western Eurasia: inferences from plastid and nuclear DNA variation. PeerJ 6:e5793. https://doi.org/10.7717/peerj.5793

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Stamatakis A (2006) Phylogenetic models of rate heterogeneity: a high performance computing perspective. In: Proceedings 20th IEEE International Parallel & Distributed Processing Symposium. 1639535

Suc J-P (1984) Origin and evolution of the Mediterranean vegetation and climate in Europe. Nature 307:429–432. https://doi.org/10.1038/307429a0

Suc J-P, Popescu S-M (2005) Pollen records and climatic cycles in the North Mediterranean region since 2.7 Ma. Geol Soc, London, Special Pub 247:147–158

Thiers B (2016) Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. https://sweetgum.nybg.org/science/ih/

Trinajstić I (2007) About the problem of differentiation between the oaks quercus pubescens willd. and Quercus virgiliana. (Ten.) Ten. Šumarski List 131:57–60

Tzedakis PC, Emerson BC, Hewitt GM (2013) Cryptic or mystic? Glacial tree refugia in northern Europe. Trends Ecol Evol 28:696–704. https://doi.org/10.1016/j.tree.2013.09.001

Velitzelos D, Bouchal JM, Denk T (2014) Review of the Cenozoic floras and vegetation of Greece. Rev Palaeobot Palynol 204:56–117. https://doi.org/10.1016/j.revpalbo.2014.02.006

Vieira M, Zetter R, Grímsson F, Denk T (2023) Niche evolution versus niche conservatism and habitat loss determine persistence and extirpation in late Neogene European Fagaceae. Quatern Sci Rev 300:107896. https://doi.org/10.1016/j.quascirev.2022.107896

Vitelli M, Vessella F, Cardoni S et al (2017) Phylogeographic structuring of plastome diversity in Mediterranean oaks (Quercus Group Ilex, Fagaceae). Tree Genet Genomes 13:3. https://doi.org/10.1007/s11295-016-1086-8

Wellstein C, Spada F (2015) The Status of Quercus pubescens Willd. in Europe. In: Box EO, Fujiwara K (eds) Warm-Temperate Deciduous Forests around the Northern Hemisphere. Springer International Publishing, Cham, pp 153–163

Yan M, Liu R, Li Y et al (2019) Ancient events and climate adaptive capacity shaped distinct chloroplast genetic structure in the oak lineages. BMC Evol Biol 19:202. https://doi.org/10.1186/s12862-019-1523-z

Yang J, Guo Y-F, Chen X-D et al (2020) Framework phylogeny, evolution and complex diversification of Chinese oaks. Plants 9:1024. https://doi.org/10.3390/plants9081024

Yang Y-Y, Qu X-J, Zhang R et al (2021) Plastid phylogenomic analyses of Fagales reveal signatures of conflict and ancient chloroplast capture. Mol Phylogenet Evol 163:107232. https://doi.org/10.1016/j.ympev.2021.107232

Zhou B-F, Yuan S, Crowl AA et al (2022) Phylogenomic analyses highlight innovation and introgression in the continental radiations of Fagaceae across the Northern Hemisphere. Nat Commun 13:1320. https://doi.org/10.1038/s41467-022-28917-1

forests

MDPI

Article

Chloroplast Haplotype Diversity in the White Oak Populations of the Italian Peninsula, Sicily, and Sardinia

Romeo Di Pietro [1,2], Luca Quaranta [3], Claudia Mattioni [2,4], Marco Cosimo Simeone [5], Piera Di Marzio [2,3], Elisa Proietti [1] and Paola Fortini [2,3,*]

[1] Department of Planning, Design, and Architecture Technology (PDTA), Sapienza University of Rome, Via Flaminia 70, 00196 Roma, Italy; romeo.dipietro@uniroma1.it (R.D.P.); elisa.proietti@uniroma1.it (E.P.)
[2] National Biodiversity Future Center (NBFC), 90133 Palermo, Italy; claudia.mattioni@cnr.it (C.M.); piera.dimarzio@unimol.it (P.D.M.)
[3] Department of Biosciences and Territory, University of Molise, Via Hertz s.n.c., 86090 Pesche, Italy; l.quaranta1@studenti.unimol.it
[4] CNR—Research Institute on Terrestrial Ecosystems (IRET), Viale Guglielmo Marconi 2, 05010 Porano, Italy
[5] Department of Agricultural and Forestry Science (DAFNE), University of Tuscia, Via S. Camillo de' Lellis, 01100 Viterbo, Italy; mcsimeone@unitus.it
* Correspondence: fortini@unimol.it

Abstract: A phylogeographic study on the chloroplast DNA of natural white oak forests (*Quercus* subgen. *Quercus*, sect. *Quercus*) was carried out to identify possible haplotype-structured distribution within the Italian Peninsula, Sicily, and Sardinia. Sixty white oak populations belonging to *Q. frainetto*, *Q. robur* and the collective groups *Q. petraea* and *Q. pubescens* were considered and analyzed by combining five Chloroplast Simple Sequence Repeat (cpSSR) markers. A total of 28 haplotypes were detected. Central and southern Italy displayed the highest variability (14 and 10 haplotypes, respectively), followed by northern Italy (7), Sardinia (7), and Sicily (5). A complex geographical structure of the haplotype distribution emerged, highlighting (i) a high number of low-frequency haplotypes; (ii) the marked isolation of Sardinia; (iii) the occurrence of haplotypes widely distributed throughout the Italian Peninsula; (iv) the idiosyncrasy of Sicily, which exhibits exclusive haplotypes, and haplotypes shared with Sardinia and the rest of the Italian Peninsula. The haplotype distribution was also found to be partially related to the taxonomic identity of the specimens, with the following features emerging: a geographic separation between the central Italy and southern Italy *Q. frainetto* populations, an unexpected discontinuity between the Calabrian and Sicilian *Q. petraea* subsp. *austrotyrrhenica* populations, and the absence of the most common haplotype among the *Q. pubescens* populations of central and southern Italy.

Keywords: cpDNA; genetic structure; geographical isolation; paleogeography; phylogeography; *Quercus*

check for updates

Citation: Di Pietro, R.; Quaranta, L.; Mattioni, C.; Simeone, M.C.; Di Marzio, P.; Proietti, E.; Fortini, P. Chloroplast Haplotype Diversity in the White Oak Populations of the Italian Peninsula, Sicily, and Sardinia. *Forests* **2024**, *15*, 864. https://doi.org/10.3390/f15050864

Academic Editors: Nadezhda Tchebakova and Sergey V. Verkhovets

Received: 12 April 2024
Revised: 7 May 2024
Accepted: 13 May 2024
Published: 15 May 2024

1. Introduction

Progressing toward a comprehensive assessment of forest biodiversity is crucial to achieving modern and effective governance of territories and landscapes, especially in Southern Europe, which is a too often overlooked long-term repository of molecular, taxonomical, and ecological diversity [1]. The genus *Quercus* is divided into two subgenera, *Cerris* and *Quercus*, and includes more than 400 species [2,3]. It has a mainly Northern Hemisphere distribution, denoting great adaptive plasticity within a wide range of habitats from the desert and Mediterranean maquis to the subtropical rainforest. At the European level, *Quercus* is a dominant genus and constitutes a great part of the forests of Central and Southern Europe from sea level to the montane belt [4]. The white oaks (*Quercus* subgenus *Quercus* sect. *Quercus*) comprise the most important species characterizing the Temperate deciduous forests' biome in Central and Southern Europe, representing a very important natural, scientific, economic, and social resource [5]. The actual number of species belonging to white oaks in Europe is a source of great debate among European taxonomists, especially as regards the species composition of the collective groups of *Q. pubescens* and *Q. petraea* [6–11]. Italy hosts about 1.2 M ha of wooded land dominated by white oaks [12], and consequently, it plays a leading role in this debate. Moreover, some white oak taxa currently considered valid species in the floras and checklists of several S European countries (i.e., *Quercus congesta*, *Q. dalechampii*, and *Q. virgiliana*), together with other critical oaks endemic to southern Italy, Sicily, and Sardinia (i.e., *Q. amplifolia*, *Q. ichnusae*, and *Q. leptobalana*), were described for the first time in Italy [13–20]. Although a clear correlation between the high level of taxonomical–nomenclatural splitting and the nuclear DNA variability among S European white oaks did not emerge from recent studies [21–27], there can be no denying that the eco-morphological and coenological variability of white oaks observable in nature is extraordinarily high. As far as Italy is concerned, it is probable that the explanation for a great part of this variability is to be found in the significant role played by the Italian Peninsula as a glacial refuge for the thermophilous forests during the end of the Tertiary and the whole of the Quaternary [28]. Chloroplast DNA (cpDNA) variability can be particularly useful for studying natural processes, such as species evolution, reticulation, migration routes, isolation, and drift, and to dissect the geographical patterns of lineage distribution subtending community assembly and diversification, local ecological peculiarities, and regional history. The first investigations on European white oaks' plastid genome were carried out ca. 30 years ago [29,30] using Restriction Fragment Length Polymorphisms of some PCR-amplified intergenic regions of the cpDNA (PCR-RFLP). Subsequently, Petit et al. [31,32] expanded the analyses to over 2600 European populations and found 39 main variants (haplotypes), grouped into 6 lineages. Three lineages were preserved in southern Italy and major islands during the Quaternary glaciations [33]. Deepening some partial results of this fundamental work, Fineschi et al. [34] provided a more detailed picture of the chloroplast PCR-RFLP variability of the Italian populations, confirming the occurrence of three main plastid lineages and cataloguing six widely distributed and four rare haplotypes. However, neither the sampling strategy nor the interpretation of the results were carried out on the basis of detailed taxonomic and/or biogeographical information. Since then, the entire line of research on the plastid DNA of the Italian white oaks has been considered sufficiently analyzed, with the exception of a few regional contributions limited to restricted areas (see Lupini et al. [35]). Indeed, the PCR-RFLP procedure proved useful in uncovering the complex background of oak cpDNA variability and building initial spatial models on a wide geographical scale. However, the low mutation rate obtained through PCR-RFLP analyses generally does not allow high levels of information at finer spatial scales to be retrieved [36–39]. Consequently, chloroplast microsatellites (or Simple Sequence Repeats, SSRs) have progressively replaced PCR-RFLPs over the years, becoming the most used markers due to their higher efficacy, low cost, and ease of analyses. Exemplary utilizations of cpSSRs include the assessment of geographic patterns of diversity in other widespread Italian oaks such as *Quercus cerris* and *Quercus suber* [40,41] and clear genealogical reconstructions of several white oaks in Western Europe [42], Central Europe [38,43], the Balkans [39,44–46], and Northeastern Europe [47–50]. The emerging scenario unambiguously indicates Southern Europe to have hosted a mosaic of refugia for the persistence of tree species during the Quaternary [28,51], with white oak populations exhibiting complex patterns of genetic variation. A more recent study further highlighted the Italian Peninsula and major islands as a repository of chloroplast DNA diversity and bio-ecological distinctiveness [52]. On these bases, we argue that the long-lasting PCR-RFLP framework of the white oaks in Italy [34] needs a critical re-assessment. Our study carries out a new and updated analysis of the cpDNA variability of white oak populations of the Italian Peninsula, Sicily, and Sardinia using cpSSRs in order to fill the existing gap and upgrade the dated diversity structure currently available. The data obtained will contribute to the creation of more informed sampling designs for future genomic studies and to a better interpretation of recent [26,27,53] and ongoing works [54] on white oaks' diversity. We will address the following questions: (i) Is it possible to correlate the distribution of haplotypes in natural white oak forests in Italy with biogeographical patterns? (ii) Are there hotspots of genetic diversity and rare or divergent genetic variants worthy of special management and conservation measures? (iii) Is it possible to identify a haplotype structure linked to the taxonomical identity of the Italian white oak populations?

2. Materials and Methods

2.1. Field Sampling and Plant Material

Sixty natural oak populations belonging to subgen. *Quercus* sect. *Quercus* were collected in the Italian Peninsula, Sicily, and Sardinia (Figure 1, Table A1). The study area was divided into five geographical sectors, i.e., northern Italy, central Italy, southern Italy, Sicily, and Sardinia, with this phytogeographical regionalization being the most widely accepted for the Italian territory at present [55,56]. The plant material was collected during autumn between 2016 and 2018. A total of 3 individuals per population, for a total of 180 individuals, were sampled at a distance of at least 50 m to each other in order to reduce the likelihood of sampling sibling trees.

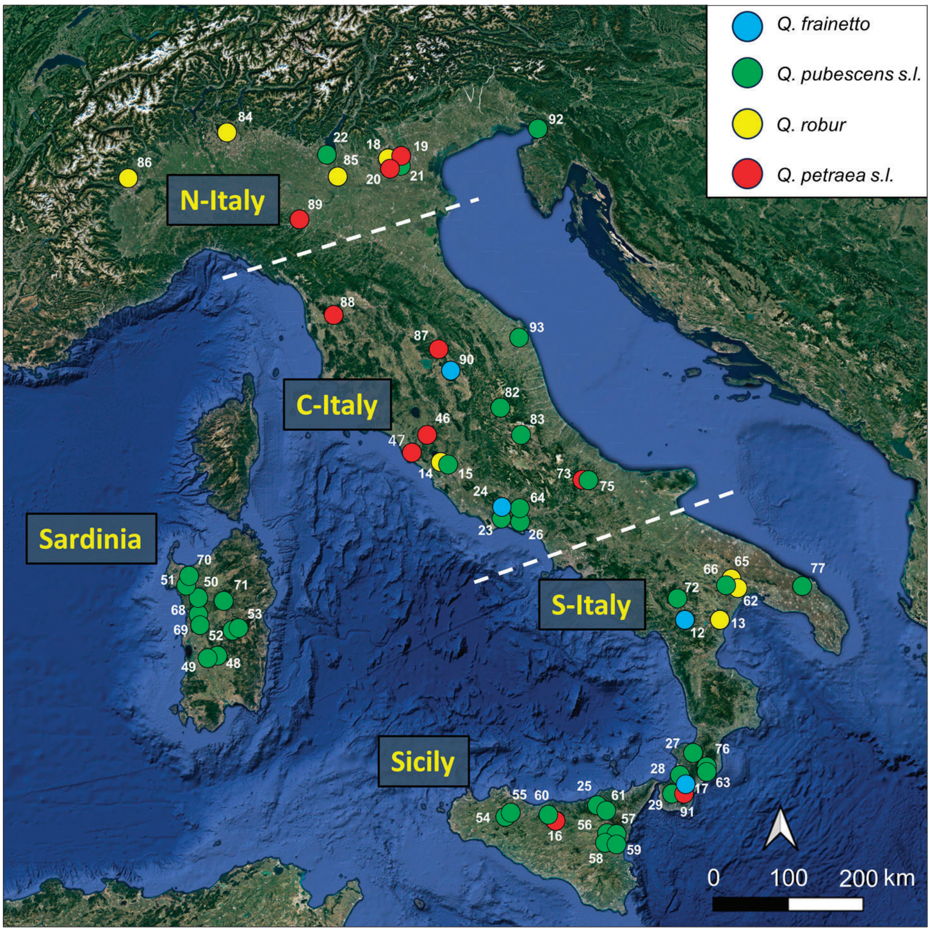


Figure 1. A distribution map of the white oak populations analyzed in Italy and the geographic sectors considered. The numbers correspond to the population codes as reported in Table A1.

Where possible, the white oak populations were sampled in areas where phytosociological tables or ecological descriptions were already published. As regards the name of the phytosociological associations related to the sampled populations, reference was made to the nomenclatural epithet originally assigned by the authors who described them (Table A2). For example, for southern Italy and the islands, reference was made to associations such as *Oleo-Quercetum virgilianae*, *Lonicero-Quercetum virgilianae*, *Ornithogalo-*

Quercetum ichnusae, *Glechomo-Quercetum congestae*, *Arabido-Quercetum congestae*, *Quercetum leptobalanae*, etc. [57–61], although *Q. congesta*, *Q. ichnusae*, *Q. leptobalana*, and *Q. virgiliana*, despite being accepted as valid species in Pignatti et al. [16], were considered synonyms of *Q. pubescens* Willd. in Bartolucci et al. [17]. This choice was made to retain available taxonomic, biogeographic, and ecological information that would have been lost by merging under the same name taxa whose taxonomic position is not yet definitively established at national and European levels. However, to ease the interpretation of the results, a re-arrangement (grouping) of single species into the following species complexes was carried out: (i) *Quercus petraea* complex (including *Q. petraea* (Matt.) Liebl. subsp. *petraea* and *Q. petraea* subsp. *austrotyrrhenica* Brullo, Guarino & Siracusa), (ii) *Q. pubescens* complex (including *Q. congesta* C. Presl., *Q. dalechampii* Ten., *Q. ichnusae* Mossa, Bacch. et Brullo, *Q. leptobalana* Guss. *Q. pubescens* Willd, and *Q. virgiliana* Ten. (Ten.)). Instead, both *Q. robur* L. and *Q. frainetto* Ten. were considered only single taxa. Indeed, regarding *Q. frainetto,* neither have infraspecific taxa been recorded in Italy, nor are similar taxa that could somehow be confused with it known at present. As far as *Q. robur* is concerned, no specimens were collected in the administrative regions (Campania and Calabria) where the subspecies *Q. robur* L. subsp. *brutia* (Ten.) *O. Schwarz* was considered to occur [17]. Plant material (including twigs, leaves, and fruits) and voucher specimens were deposited and preserved in the herbarium of the University of Molise (IS) [62].

2.2. DNA Extraction and cpSSR Amplification

Five polymorphic chloroplast SSR loci were used (Table 1); the primers were designed by Deguilloux et al. [37] and Sebastiani et al. [63].

Table 1. Details of the cpSSRs used in the present study.

Locus	**Location**	**Repeat Motif**	**Primers Sequence (5′–3′) Sense and Antisense**	**T_m (°C)**	**PCR Product Size (bp)**
μdt1	trnE-trnT intergenic	(A)11	ATCTTACACTAAGCTCGGAA TTCAATAACTTGTTGATCCC	48	81–83
μdt3	trnD-trnY intergenic	(A)11	TGTTAGTAATCCTTTCGTTT AGGTATAAAGTCTAAGGTAA	46	125–128
μcd4	ycf6-psbM intergenic	(T)12	TTATTTGTTTTTGGTTTCACC TTTCCCATAGAGAGTCTGTAT	45	94–99
μcd5	ycf6-psbM intergenic	(A)8	CCCCCGGATCTCTGTCAACTG TAATAAACGAGAATCACATAA	45	74–77
Cmcs6	ndhG-ndhI intergenic	(T)10	GAAAAAGGACCCTTCCTAAT CTTATGATCGTCACGAATTG	55	200–203

DNAs were extracted from silica gel-dried leaves (about 50 mg per tree) of 180 samples, using the NucleoSpin™ Plant II Ks (Macherey-Nagel, Oensingen - Switzerland), following the manufacturer's instructions. Polymerase chain reactions were carried out using the software GeneAmp® 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The reaction was performed in a 20 μL total volume containing 20 ng of genomic DNA following the Qiagen multiplex k protocol. The cycling parameters were as follows: 15 min at 95 °C; 30 cycles for 30 s at 95 °C, 44 s at 57 °C, and 30 s at 72 °C; and a final step of 30 min at 60 °C. Amplification products (1 μL) were added to 20 μL formamide and 0.2 μL GeneScan™ 500 LIZ™ and denatured at 95 °C for 5 min. The samples were run on ABI PRISM 3100 DNA-sequencer. The resulting raw data were collected by using GeneMapper 6 software (Applied Biosystems).

2.3. Data Analysis

Fragment-sized polymorphisms were identified as different length variants that were combined to define haplotypes by using GenAlEx 6.5 [64]. The same software was used to compute the main genetic statistic parameters, i.e., the number of observed alleles (*Na*), number of effective alleles (*Ne*), number of private alleles (*Np*), Shannon's information index (*i*), haploid genetic diversity (*h*), and unbiased genetic diversity (*uh*). An assessment of allele frequency was carried out using FSTAT version 2.9.4 [65]. Within- (H_S) and among (H_T)-population genetic diversity and the two main coefficients of gene differentiation (G_{ST} and R_{ST}) were assessed by using the software program PermutCpSSR v2.0 [66] with 1000 permutations. These parameters were computed on the entire dataset and according to the following grouping criteria: "geographical sector", "single species", and "species complex".

The Bayesian Analysis of Population Structure (BAPS, version 6.0; [67]) was used to investigate the geographical patterns underlying the genetic diversity of the white oak dataset and to confirm the division into geographical sectors, for mixture and admixture analysis (LocPrior option). The genetic structure of the investigated populations was also tested using STRUCTURE software v.2.3.4 [68], setting 20 runs with K from 1 to 7. Each run had a burn-in period of 100,000 iterations and 500,000 Monte Carlo Markov iterations, assuming an admixture model (with LocPrior) with correlated allele frequencies.

Structure HARVESTER [69] was used to obtain ΔK and mean L(K) values. Post-processing was carried out using CLUMPAK [70]. To define the genealogical relations among the haplotypes, a minimum spanning network (MSN) based on allele length differences was created using the software Arlequin 3.5.1.3 [71] and an MSN diagram was produced. Finally, a principal coordinate analysis (PCoA) was performed using the Nei's genetic distance matrix generated with GeneAlEx to evaluate dissimilarity among individuals.

3. Results

The five cpSSRs identified eighteen alleles in total (Table A3). The observed number of alleles per locus ranged from 3 (cmcs6, udt1, and ucd5) to 5 (ucd4), with a mean of 3.6 (Table 2). The number of effective alleles (*Ne*) ranged from 1.798 to 3.744, averaging 2.546. Shannon's diversity index ranged from 0.677 to 1.441, indicating medium to high polymorphism at the investigated loci. The values of genetic diversity (*h* and *uh*) denoted adequate investigative potential for the markers used across the dataset.

Table 2. Descriptive polymorphism parameters of the five cpSSR loci in the oak dataset investigated (60 populations; 180 individuals). *Na*: number of alleles; *Ne*: number of effective alleles; *i*: Shannon's diversity index; *h*: haploid genetic diversity; *uh*: unbiased genetic diversity; *se*: standard error.

Locus	*Na*	*Ne*	*i*	*h*	*Uh*
udt1	3	1.798	0.677	0.444	0.446
ucd5	3	1.980	0.852	0.495	0.498
ucd4	5	3.744	1.441	0.733	0.737
udt3	4	2.775	1.137	0.640	0.643
CmCs6	3	2.432	0.975	0.589	0.592
Mean (*se*)	3.600 (0.400)	2.546 (0.345)	1.000 (0.130)	0.580 (0.051)	0.583 (0.052)

3.1. Overall Haplotype Distribution

Polymorphisms detected in the 5 cpSSR loci resulted in a combined total of 28 haplotypes across the dataset investigated. The overall haplotype distribution, according to individual occurrence, geographical sector, and taxonomy, is shown in Table 3. In total, 6 haplotypes were found to be shared by 10–30 individuals, while 22 were restricted to a fewer number of individuals. A total of 9 of the 28 haplotypes identified were detected in single individuals (singletons). The most frequent haplotype (#V) was found to be distributed throughout the three sectors of the Italian Peninsula and within all of the taxa investigated. The second most frequent haplotype (#E) was found also in Sardinia and Sicily, but missing in southern Italy, and was detected in all of the species investigated except for *Q. frainetto*. Haplotypes #C and #X were found to be exclusive to central and southern Italy, respectively, with the latter exhibited only by the *Q. pubescens* complex. Haplotype #U was found to be distributed throughout the Italian Peninsula and in all of the oak species except for *Q. robur*. Haplotype #B was found in just a few individuals of *Q. petraea* and *Q. pubescens* complexes from central Italy, Sardinia, and Sicily. With the exception of haplotype #W, which was detected only in five individuals of *Q. petraea* and *Q. robur* from southern and northern Italy, the less frequent haplotypes (including singletons) were found to be concentrated in central Italy (7), Sardinia (5), southern Italy (4), Sicily (3), and northern Italy (2). Finally, three haplotypes, each identified only in two to four individuals, were found to be exclusive to a single species (#BB: *Q. frainetto*; #GG: *Q. petraea*; #Y: *Q. robur*).

Table 3. The distribution of the 28 cpSSR haplotypes according to haplotype code (*H*); the number of individuals involved (*N*); the percentage of individuals with the same haplotype (*Freq.*); the number of populations involved (*Pops*); the geographical sector (*Gs*); and taxonomy (*species/species complex*). Alphabetical and numerical (for singletons) codes of the haplotypes were automatically assigned using GenAlEx 6.5.

H	*N*	*Freq.*	*Pops*	*Gs*	*Species*	*Species Complex*
6	1	0.56	1	Sardinia	*Q. ichnusae*	*Q. pubescens*
7	1	0.56	1	South	*Q. dalechampii*	*Q. pubescens*
8	1	0.56	1	South	*Q. congesta*	*Q. pubescens*
9	1	0.56	1	Central	*Q. petraea*	*Q. petraea*
10	1	0.56	1	South	*Q. congesta*	*Q. pubescens*
11	1	0.56	1	Central	*Q. petraea*	*Q. petraea*
12	1	0.56	1	Central	*Q. petraea*	*Q. petraea*
13	1	0.56	1	Central	*Q. pubescens*	*Q. pubescens*
14	1	0.56	1	Sicily	*Q. congesta*	*Q. pubescens*
A	3	1.67	2	Sicily	*Q. leptobalana*, *Q. virgiliana*	*Q. pubescens*
B	10	5.56	8	North, Sicily, and Sardinia	*Q. congesta*, *Q. dalechampii*, *Q. petraea*, *Q. pubescens*, *Q. virgiliana*	*Q. petraea*, *Q. pubescens*
BB	2	1.11	1	Central	*Q. frainetto*	*Q. frainetto*
C	13	7.22	5	Central	*Q. frainetto*, *Q. pubescens*, *Q. robur*	*Q. frainetto*, *Q. pubescens*, *Q. robur*
CC	9	5	5	Central	*Q. frainetto*, *Q. petraea*, *Q. pubescens*	*Q. frainetto*, *Q. petraea*, *Q. pubescens*
D	7	3.89	4	Sicily	*Q. congesta*, *Q. leptobalana*, *Q. petraea* subsp. *austrotyrrhenica*, *Q. virgiliana*	*Q. petraea*, *Q. pubescens*
DD	8	4.44	3	Central and South	*Q. congesta*, *Q. dalechampii*, *Q. pubescens*	*Q. pubescens*
E	21	11.67	11	North, Central, Sicily, and Sardinia	*Q. congesta*, *Q. dalechampii*, *Q. petraea*, *Q. pubescens*, *Q. robur*, *Q. virgiliana*	*Q. petraea*, *Q. pubescens*, *Q. robur*
GG	2	1.11	1	Central	*Q. petraea*	*Q. petraea*
J	5	2.78	3	North	*Q. petraea*, *Q. robur*	*Q. petraea*, *Q. robur*
O	6	3.33	4	Sardinia	*Q. congesta*, *Q. ichnusae*, *Q. virgiliana*	*Q. pubescens*
R	3	1.67	2	Sardinia	*Q. ichnusae*, *Q. virgiliana*	*Q. pubescens*
S	6	3.33	3	Sardinia	*Q. congesta*, *Q. ichnusae*, *Q. virgiliana*	*Q. pubescens*
T	8	4.44	4	Sardinia	*Q. congesta*, *Q. ichnusae*, *Q. virgiliana*	*Q. pubescens*
U	14	7.78	5	North, Central, and South	*Q. frainetto*, *Q. petraea*, *Q. pubescens*	*Q. frainetto*, *Q. petraea*, *Q. pubescens*
V	30	16.67	13	North, Central, and South	*Q. frainetto*, *Q. petraea*, *Q. petraea* subsp. *austrotyrrhenica*, *Q. pubescens*, *Q. robur*	*Q. frainetto*, *Q. petraea*, *Q. pubescens*, *Q. robur*
W	5	2.78	3	North and South	*Q. petraea*, *Q. robur*	*Q. petraea*, *Q. robur*
X	15	8.33	6	South	*Q. congesta*, *Q. dalechampii*, *Q. pubescens*, *Q. virgiliana*	*Q. pubescens*
Y	4	2.22	2	North	*Q. robur*	*Q. robur*

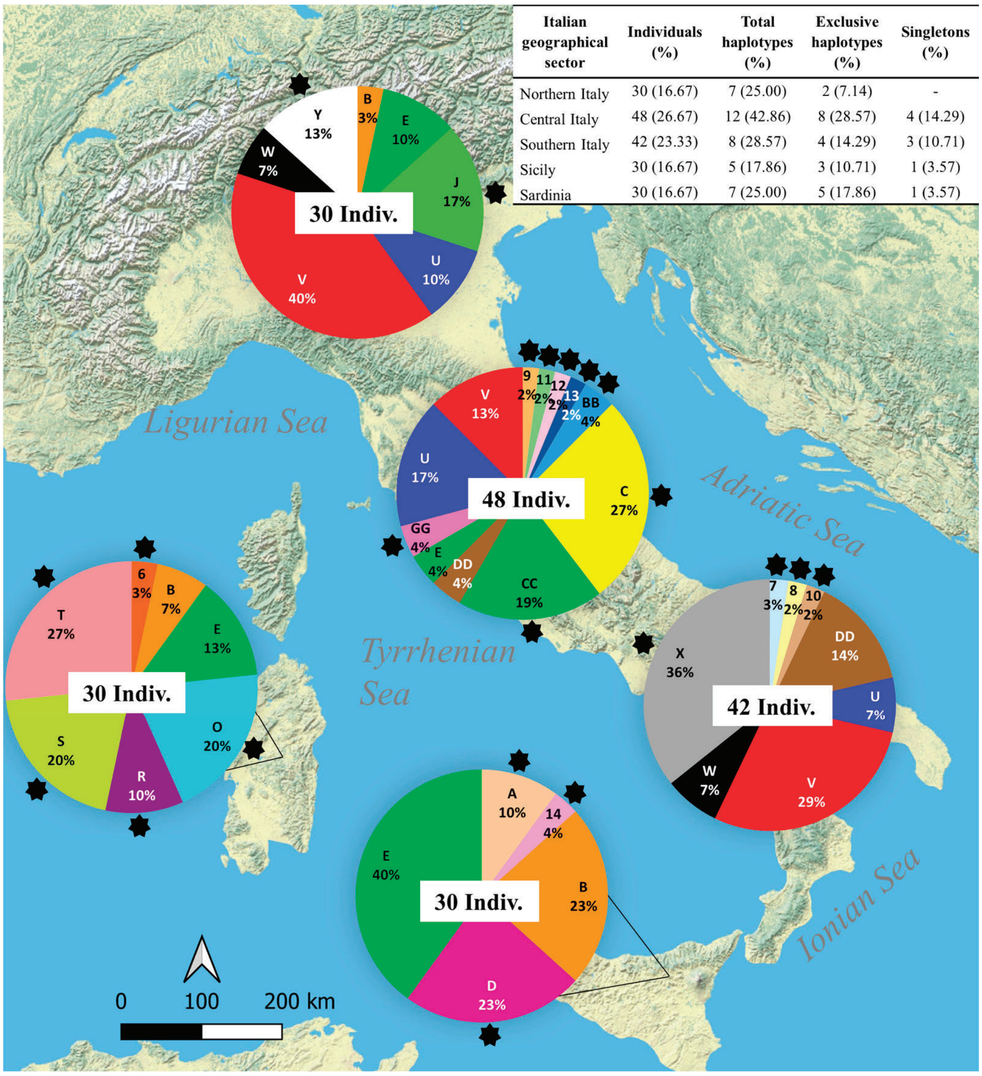
3.2. Haplotype Diversity and Geographical Distribution

Central Italy, southern Italy, and Sardinia displayed the highest number of alleles (*Na*) and the highest level of total genetic diversity (H_T) (Table 4). The number of effective alleles (*Ne*) were found to be low in all of the geographical sectors considered. Central Italy was the only sector displaying private alleles (*Np*). Relatively low values of genetic diversity within populations (H_S) were detected across all of the geographical sectors. The genetic differentiation index (G_{ST}) was moderately low (mean = 0.555), ranging from 0.345 in Sicily to 0.839 in southern Italy. In contrast, the coefficient R_{ST} was found to be rather high (mean = 0.745). A statistically significant geographical structure across populations (R_{ST} vs. G_{ST}) was detected for northern Italy, central Italy, and Sardinia, whereas in southern Italy and Sicily, the correlation was not significant.

Table 4. The parameters of genetic diversity per geographical sector. *N*: the number of individuals; *Na*: the number of different alleles; *Ne*: the number of effective alleles; *Np*: the number of private alleles; *h*: total genetic diversity; *uh*: unbiased genetic diversity; H_S: the diversity within populations; H_T: the diversity among populations; G_{ST}: genetic differentiation index; R_{ST}: genetic differentiation index considering similarities among haplotypes. The statistical significance of the $R_{ST} > G_{ST}$ ratio was calculated according to the PERMUT cpSSR manual; ^{ns}: non-significant; $p > 5\%$; * $p < 5\%$; ** $p = 0\%$ (to be significant, the 1000 permutations must not exceed the observed R_{ST} value of 5%). The standard error is reported in parentheses.

Geographical Sector	*N*	*Na*	*Ne*	*Np*	*h*	*uh*	H_S	H_T	G_{ST}	R_{ST}
Northern Italy	30	2.200 (0.200)	1.698 (0.115)	0.000 (0.000)	0.399 (0.045)	0.413 (0.047)	0.333 (0.136)	0.799 (0.084)	0.583 (0.190)	0.628 * (0.217)
Central Italy	48	3.200 (0.490)	2.503 (0.457)	0.200 (0.200)	0.558 (0.060)	0.570 (0.061)	0.381 (0.110)	0.908 (0.045)	0.581 (0.110)	0.861 ** (0.080)
Southern Italy	42	2.800 (0.374)	1.675 (0.253)	0.000 (0.000)	0.349 (0.093)	0.358 (0.095)	0.137 (0.070)	0.851 (0.047)	0.839 (0.090)	0.876 ^{ns} (0.090)
Sicily	30	2.000 (0.316)	1.377 (0.205)	0.000 (0.000)	0.212 (0.104)	0.219 (0.108)	0.500 (0.114)	0.763 (0.055)	0.345 (0.166)	0.398 ^{ns} (0.233)
Sardinia	30	2.400 (0.400)	1.863 (0.293)	0.000 (0.000)	0.421 (0.068)	0.435 (0.071)	0.500 (0.114)	0.869 (0.036)	0.425 (0.123)	0.961 ** (0.011)
Mean (among geogr. sectors)		2.520 (0.356)	1.823 (0.265)	0.040 (0.040)	0.388 (0.074)	0.399 (0.076)	0.370 (0.110)	0.838 (0.053)	0.555 (0.136)	0.745 (0.126)

The number of haplotypes and relative frequencies in each geographical sector are reported in Figure 2. The highest number of haplotypes (12) were found in central Italy, of which eight were exclusive (four singletons), whereas northern Italy exhibited the lowest number (seven haplotypes, of which two were exclusive). Southern Italy exhibited eight haplotypes (four exclusives and three singletons), Sardinia exhibited seven haplotypes (five exclusives, of which one was a singleton), and Sicily exhibited five haplotypes (three exclusives, of which one was a singleton).



Italian geographical sector	Individuals (%)	Total haplotypes (%)	Exclusive haplotypes (%)	Singletons (%)
Northern Italy	30 (16.67)	7 (25.00)	2 (7.14)	-
Central Italy	48 (26.67)	12 (42.86)	8 (28.57)	4 (14.29)
Southern Italy	42 (23.33)	8 (28.57)	4 (14.29)	3 (10.71)
Sicily	30 (16.67)	5 (17.86)	3 (10.71)	1 (3.57)
Sardinia	30 (16.67)	7 (25.00)	5 (17.86)	1 (3.57)

Figure 2. Distribution and frequency of cpSSR white oak haplotypes within five geographical sectors. Asterisks indicate haplotypes exclusive to geographical sector in which they occur.

3.3. Genetic Diversity and Haplotype Distribution According to Taxonomy

Table 5 reports the genetic diversity displayed by species complexes. The *Q. pubescens* complex exhibited the highest mean values for *Na* (3.200) and *Ne* (2.661), while *Q. robur* exhibited the lowest values for these parameters (2.200 and 1.747, respectively). Only *Q. petraea* and *Q. pubescens* complexes showed private alleles. The genetic diversity among populations (H_T) was found to be high in all of the species complexes considered (mean = 8.872), whereas the genetic diversity within populations (H_S) was found to be relatively low (0.133–0.351), except for *Q. petraea* (0.467). The occurrence of a taxonomical structure ($R_{ST} > G_{ST}$) has been highlighted for all of the species complexes (*Q. pubescens* exhibits the highest value), although statistical significance was achieved only for *Q. petraea* and *Q. pubescens*.

Table 5. The parameters of genetic diversity per species complexes. *N*: the number of individuals; *Na*: the number of different alleles; *Ne*: the number of effective alleles; *Np*: the number of private alleles; *h*: total genetic diversity; *uh*: unbiased genetic diversity; H_S: the diversity within populations; H_T: the diversity among populations; G_{ST}: genetic differentiation index; R_{ST}: the coefficient of genetic differentiation index; ^{ns}: non-significant; $p > 5\%$; ** $p = 0\%$. The statistical significance of the $R_{ST} > G_{ST}$ ratio is calculated according to the PERMUT cpSSR manual (to be significant, the 1000 permutations must not exceed the observed R_{ST} value of 5%). The standard error is reported in parentheses.

Species Complex	*Na*	*Ne*	*Np*	*h*	*uh*	H_S	H_T	G_{ST}	R_{ST}
Q. frainetto	2.600 (0.400)	2.002 (0.256)	0.000 (0.000)	0.468 (0.065)	0.501 (0.070)	0.133 (0.133)	0.900 (0.117)	0.852 (0.138)	0.988 ^{ns} (0.006)
Q. petraea	2.800 (0.200)	1.827 (0.175)	0.200 (0.200)	0.434 (0.050)	0.449 (0.052)	0.467 (0.133)	0.800 (0.116)	0.417 (0.126)	0.841 ** (0.111)
Q. pubescens	3.200 (0.490)	2.661 (0.349)	0.600 (0.245)	0.600 (0.047)	0.606 (0.048	0.351 (0.062)	0.940 (0.011)	0.626 (0.066)	0.880 ** (0.045)
Q. robur	2.200 (0.374)	1.747 (0.232)	0.000 (0.000)	0.377 (0.100)	0.393 (0.104)	0.292 (0.146)	0.849 (0.078)	0.657 (0.181)	0.721 ^{ns} (0.172)
Mean	2.700 (0.366)	2.059 (0.253)	0.200 (0.111)	0.470 (0.066)	0.487 (0.069)	0.311 (0.1189)	0.872 (0.080)	0.638 (0.128)	0.858 (0.084)

Table 6 and Figure 3 depict the haplotype distribution for the different oak species and species complexes considered. In total, the *Q. pubescens* species complex exhibited 20 haplotypes, of which 13 were found to be exclusive (6 singletons). Within the *Q. pubescens* species complex, *Q. congesta*, *Q. dalechampii*, *Q. ichnusae,* and *Q. pubescens* exhibited one to three exclusive haplotypes, all of which corresponded to singletons, whereas *Q. leptobalana* and *Q. virgiliana* exhibited none. The *Q. petraea* species complex exhibited twelve haplotypes (four exclusives, of which three were singletons); none of these exclusive haplotypes were found in *Q. petraea* susbsp. *austrotyrrhenica*. Both *Q. robur* and *Q. frainetto* displayed a lower number of haplotypes (six and five, respectively) compared to the *Q. petraea* and *Q. pubescens* species complexes. Moreover, both of these species were found to be characterized by one exclusive haplotype and no singletons. The ratio between the number of haplotypes and the number of individuals showed that the highest value (0.40) was found for *Q. petraea* and the lowest (0.17) for *Q. pubescens*; *Q. frainetto* and *Q. robur* exhibited values of 0.33 and 0.29, respectively.

Table 6. Haplotype distribution according to the oak species and species complexes. The percentage values related to the total number of individuals {180} and the total number of haplotypes (28) are reported in parentheses.

Taxa	Individuals	Haplotypes	Haplotypes/Individuals	Exclusive Haplotypes	Singletons
Q. congesta	27 {15.0}	11 (39.3)		3 (10.7)	3 (10.7)
Q. dalechampii	9 {5.0}	5 (17.9)		1 (3.6)	1 (3.6)
Q. ichnusae	9 {5.0}	5 (17.9)		1 (3.6)	1 (3.6)
Q. leptobalana	3 {1.7}	2 (7.1)		-	-
Q. pubescens	42 {23.3}	9 (32.1)		1 (3.6)	1 (3.6)
Q. virgiliana	21 {11.7}	9 (32.1)		-	-
***Q. pubescens* complex**	**111 {61.7}**	**20 (71.4)**	**0.18**	**13 (46.4)**	**6 (21.4)**
Q. petraea	24 {13.3}	11 (39.3)		4 (14.3)	3 (10.7)
Q. petraea subsp. *austrotyrrhenica*	6 {3.3}	2 (7.1)		-	-
***Q. petraea* complex**	**30 {16.7}**	**12 (42.9)**	**0.40**	**4 (14.3)**	**3 (10.7)**
Q. frainetto	**15 {8.3}**	**5 (17.9)**	**0.33**	**1 (3.6)**	-
Q. robur	**24 {13.3}**	**6 (21.4)**	**0.25**	**1 (3.6)**	-

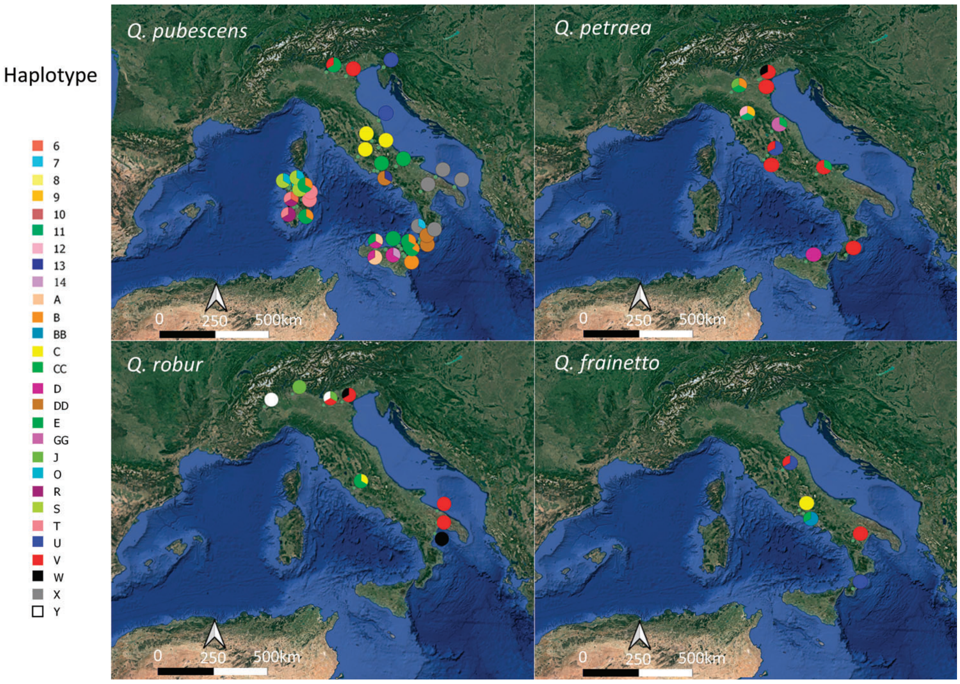


Figure 3. Haplotype distribution according to the four species complexes considered.

3.4. Genetic Structure

Figure 4 shows the results of BAPS and STRUCTURE clustering analyses. According to BAPS, the best partition was established at $K = 5$, which exactly corresponds to the number of geographical sectors into which the study area has been considered a priori to be divided. Instead, according to STRUCTURE, the number of genetic clusters (K) that best fit the data were found to be three, as also confirmed by the ΔK value obtained by using the Evanno method [72] (see Figure A1). The three clusters identified by STRUCTURE can be considered to geographically correspond to the Italian Peninsula, Sicily, and Sardinia. Owing to the fact that the best ΔK value suggested might not always represent perfectly the underlying structure of the data, and that additional information might emerge from interpretation of the K values close to the best ΔK value suggested by the Evanno method, the genetic structures for $K = 2$, $K = 4$, and $K = 5$ were analyzed and are discussed. In the specific case of our dataset, three further clusters were identifiable, passing from $K = 3$ to $K = 5$, in addition to the two main genetic clusters identified for $K = 2$ (Figure 4).

According to the minimum spanning network (MSN), seven main clusters of haplotypes were identified. These clusters were distinguished on the basis of the highest values of the genetic distance between adjacent haplotypes readable within each branch of the tree network. The five haplotypes exclusive to Sardinia were found to be concentrated in the two extremes of the network (#T, #R, and singleton #6 on the left side of the network displayed in Figure 5, with #S and #O haplotypes arranged on the right side). The Sicilian haplotypes are almost completely included within a single branch of the network and divided into two groups (light blue and blue in Figure 5) associated with the high-frequency haplotypes #E, #C, and #D. The two haplotypes exclusive to the northern Italy sector (#J and #Y) are arranged within the first branches of high-frequency haplotypes #E and #V. The haplotypes exclusive to the central Italy sector broadly exhibit two main concentration areas: one characterized by a group of singletons surrounding the high-frequency haplotype #X, which includes only individuals from southern Italy, and the other linked to a singleton from Sicily and to haplotype #CC (the latter also includes populations from southern Italy). The geographical distribution of the seven groups of haplotypes identified by the MSN is displayed in Figure A2.

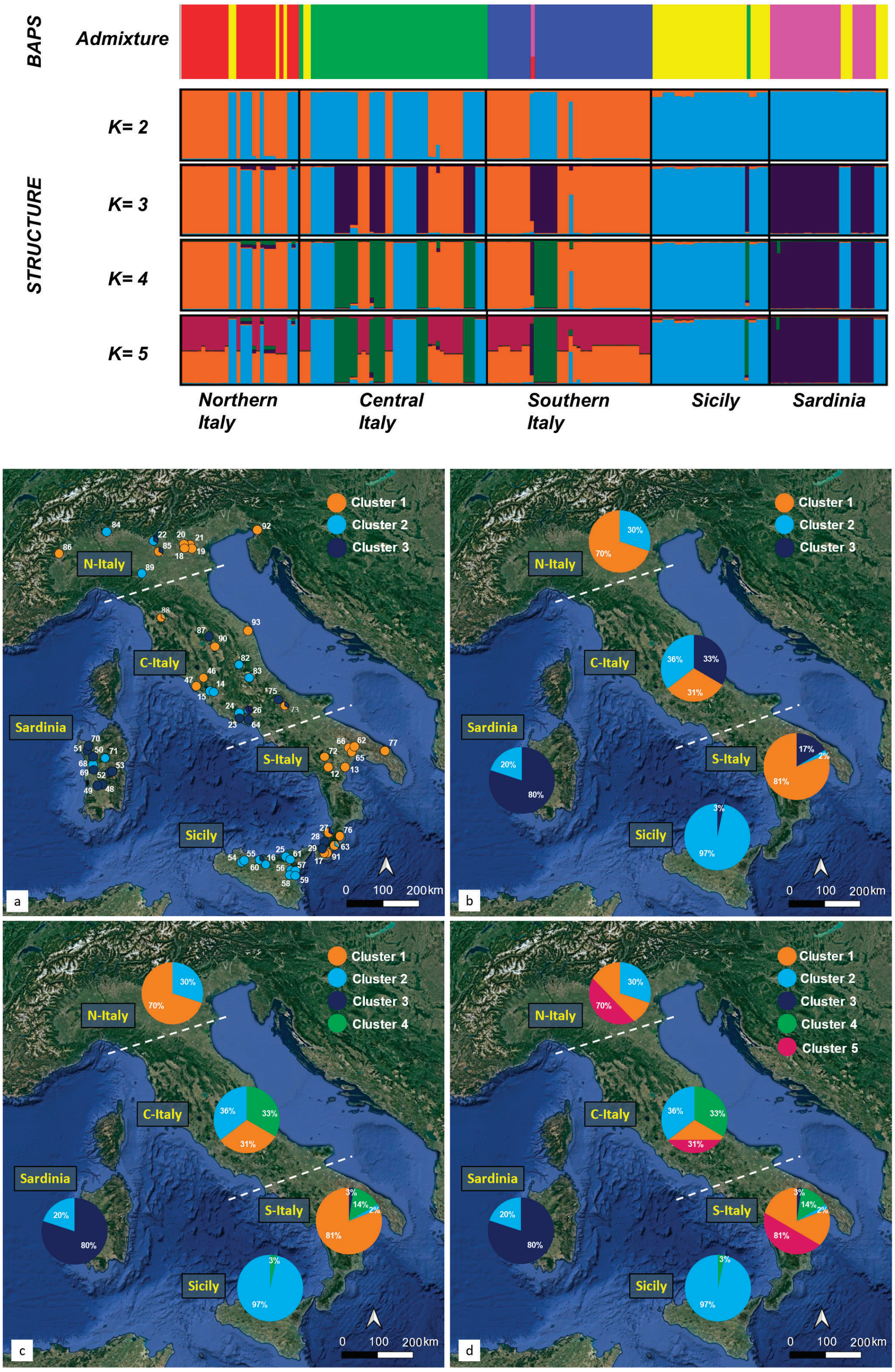


Figure 4. (**Top**): The results of BAPS and STRUCTURE clustering analyses. Each individual is represented by a vertical line. Individuals are arranged by geographical sector. In the STRUCTURE diagram, orange = 'Cluster 1', light blue = 'Cluster 2', dark violet = 'Cluster 3', green = 'Cluster 4', and red = 'Cluster 5'. (**Bottom**): (**a**) the spatial pattern and distribution within each individual population of the genetic clusters identified by STRUCTURE for $K = 3$ (The numbers correspond to the population codes as reported in Table A1); (**b**–**d**) the percentages of occurrence within each geographical sector of the genetic clusters identified by STRUCTURE with $K = 3$ (**b**), $K = 4$ (**c**), and $K = 5$ (**d**).

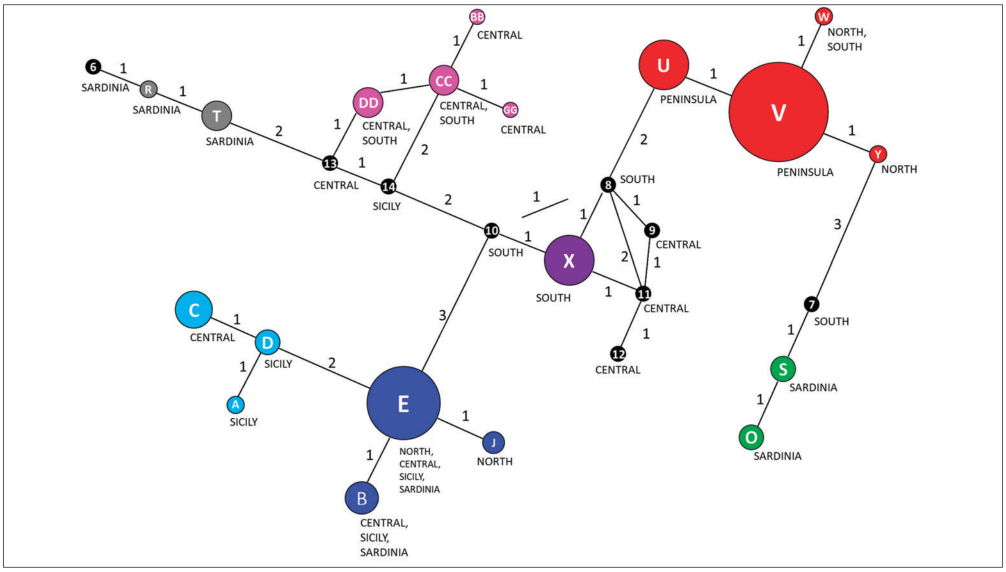


Figure 5. The minimum spanning network (MSN) of the 28 haplotypes, obtained using the pairwise distance. The size of the circles is proportional to the haplotype frequency, while the length of the branches is proportional to the distance between haplotypes. The different colors used identify different groups of haplotypes.

In the PCoA (Figure 6), the first two axes bear 79.94% of the total variance. The seven groups identified in the MSN are also distinguishable in the PCoA diagram, roughly exhibiting a U-shaped distribution. Along axis 2, a separation between haplotypes exclusive to the Italian Peninsula (#V, #U, #W, #Y, and #X) is evident, arranged in the lower part of the diagram, and haplotypes from both the peninsula and the two islands are arranged in the upper part of the diagram. Along axis 1, the two haplotypes exclusive to Sardinia (#T and #R) are found to be clearly identifiable and separated from the rest in the upper right side of the PCoA diagram; the other two Sardinian endemic haplotypes #O and #S are arranged on the other side of axis 1. The proximity of haplotypes #C, #A, and #D to the group of Sardinian endemic haplotypes #O and #S at the left end of the diagram does not seem to fully express the genetic distance between these two groups of haplotypes, which is indeed well observable in the MSN. In fact, these two groups are found to be clearly separated from each other when a third axis is considered (Figure A3). Finally, haplotypes #CC, #GG, and #DD are found to be clearly distinguishable in the lower right side of the diagram. Figure A4 highlights the relationships between the clusters identified in the structure analysis and those derived from the MSN classification through their spatial distribution in the PCoA diagram. In general, a significant correspondence emerges between these two types of classification, where the five clusters identified by STRUCTURE are distributed neatly in the graphic ellipses corresponding to the groups derived from the MSN. The only significant difference concerns the two groups of haplotypes exclusive to Sardinia for which both the MSN and the PCoA itself identify two distinct lineages (#O and #S vs. #R and #T), where, instead, the STRUCTURE analysis maintains these lineages associated within the same group (dark-blue color) for values of ΔK ranging between 3 and 5.

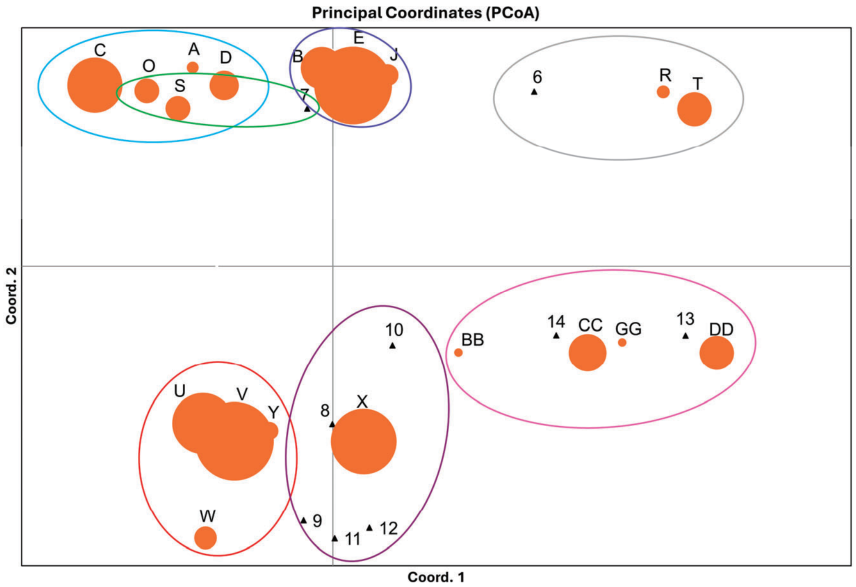


Figure 6. Principal coordinate analysis (PCoA) plot based on genetic distance and representing variability along the first two axes. Ellipses (with related colors) correspond to the seven groups of haplotypes displayed by the MSN of Figure 5.

4. Discussion

In this study, we used cpSSRs to investigate the genetic diversity and population genetic structure of 60 white oak populations of Italy. The overall dataset showed levels of total and within-population genetic diversity in line with the results obtained for 160 Italian white oak populations analyzed using chloroplast PCR-RFLP [34] and for 92 white oak populations from Western Europe investigated with 6 cpSSRs [42]. Our G_{ST} values also fall well within the ranges observed for 90 Central European white oak populations investigated at ten cpSSR loci [38] and, more generally, within the data compiled on 138 Angiosperm species investigated using different molecular techniques at the cpDNA [73]. Despite the generally lower number of individuals and populations considered, the number of cpSSR haplotypes detected in the present work are also comparatively higher than those found in Western, Central, and Eastern Europe, irrespective of the number of species investigated (two to five) [38,39,42,49].

4.1. Haplotype Distribution and Paleoecological and Paleogeographic Events

The haplotype distribution that we have found among Italian white oak populations follows a rather clear geographical structure which accords well with the current biogeographic features of the Italian Peninsula and with its paleoclimatic and paleogeographic history. Considering only the three geographical sectors into which the Italian Peninsula was divided for this study, (i.e., north, central, and south—leaving aside the islands of Sicily and Sardinia), the northern Italy sector stands out as the one showing the lowest number of haplotypes. This result was largely expected considering that northern Italy includes the southern slope of the Alps and the Po River plain whose deciduous forests were strongly affected by the Quaternary glaciations. The paleoclimatic conditions which prevailed along the entire southern slope of the Alps and the Po River plain after the Last Glacial Maximum (LGM) did not occur as a homogeneously increasing warming [74,75]. It was characterized by significant temperature oscillations, which strongly influenced the density and distribution of the deciduous oak forests and presumably had consequences for their haplotype richness and diversity. During the Bolling–Allerod stage (15 Ky BP), the white oak forests (e.g., the *Q. petraea-pubescens* group) expanded northwards, covering the Alps' foothills and partially replacing the boreal coniferous forests that had descended the mountain slopes up to the plain during the LGM. During the subsequent Younger Dryas

(YD) (12-10 ky BP), a cold and arid period was established which lasted for the first two millennia of the Holocene and led to a contraction of oak forests almost to the point of their complete disappearance.

The identification of two exclusive haplotypes (#J and #Y) for the white oak populations of northern Italy, alongside the occurrence in this sector of the most common haplotype in the whole dataset (#V), and the identification of a haplotype shared only with the southern Italy sector (#W) comply at least in part with the aforementioned chronological reconstruction. In fact, the foregoing scenario is explainable by hypothesizing the presence of isolated secondary glacial refugia located within the foothill areas of the Po valley characterized by mesoclimatic conditions significantly different from the typical regional macroclimate and thus able to host enclaves of deciduous forests [76,77]. Documented evidence of such glacial "micro-refuges" is available especially for geothermal areas (e.g., Arquà Petrarca thermal lake), where a continuous pollen record for deciduous oak forests across the whole LGM period was found [78]. It would be interesting to know whether the refuge areas of northern Italy represent a site of origin and genetic differentiation for the north Italy-exclusive haplotypes or, as is more probable, a conservative site for lineages shared with other South European refuge areas. Further research on a wider scale will be needed to provide additional information on this issue.

Moving southwards, we observed that both the central and southern Italy sectors exhibited a significant amount of signatures (14 and 10 haplotypes, respectively, with several singletons). Surprisingly, the central Italy white oak populations displayed a higher percentage of exclusive haplotypes compared to southern Italy ones (six vs. four), contradicting the generally held assumption that due to the effects of the glaciations being felt more intensively in Northern Europe and the Alps, the forest genetic diversity must necessarily become higher moving southwards. In general, this is indeed the case, and although the Italian Peninsula partly escapes this tendency (the Apennines are a mountain range oriented toward the meridians connecting the Alps to Sicily), it is true that the effects of the glaciation in the northern and central Apennines were much greater than in the southern Apennines [79–81]. However, this did not lead to a concentration of glacial refuges in southern Italy alone, but they were also found to be equally numerous in central Italy, especially along the Tyrrhenian side [28,33,82,83]. Instead, very few glacial refuges were found in the northern Apennines [84–86]. The Apennine range is very heterogeneous and extensive in latitude, meaning that it can be considered to be divided into two main blocks, the northern Apennines and the central–southern Apennines, having a different genesis and evolution. In biogeographic terms, the northern Apennines, made up mainly of siliceous rocks, show stronger links with the western Alps than with the rest of the Apennines; it is not by chance that many alpine species or species typical of conifer boreal forests or the alpine tundra find their southernmost limit in the northern Apennines (e.g., *Picea abies*, *Pinus sylvestris*, *Rhododendron ferrugineus*, *Vaccinium vitis-idaea*, *Empetrum ermaphroditum*, *Juncus trifidus*, etc.). In contrast, the central and southern Apennines (both mainly composed of limestone rocks) show many floristic and coenological similarities to each other, hosting many endemics and exhibiting a close link with the Balkan Peninsula, testified to by a very high number of amphi-Adriatic species with a high physiognomical role which do not occur in the northern Apennines and in the Alps (e.g., *Anemone apennina, Carpinus orientalis*, *Carex kitaibeliana*, *Cytisus spinescens, Edraianthus graminifolius*, *Festuca bosniaca*, *Hippocrepis glauca, Leontopodium nivale*, *Lomelosia crenata, Salvia officinalis*, *Sesleria juncifolia*, etc.). The numerous palynological studies carried out in central Italy [87–91] have shown that even during the YD, the percentage of deciduous tree species' pollen (with oaks constituting the predominant part) remained much higher (close to 40%) than the percentage recorded in the northern Apennines and Po Valley [92]. This allowed for a reduced depletion of the genetic variability within the oak populations of central Italy and its rapid and intense widespread redistribution during the post-glacial reforestation stage. In the case of our study, the greater number of haplotypes found in central Italy compared to southern Italy could merely be due, at least in part, to the higher number of populations that we collected in the former area. However, the reasons are likely to be deeper. Indeed, a molecular study on *Quercus robur* in the Italian Peninsula [54] has recently identified central Italy as the geographical sector showing the highest genetic diversity for this species, attributing this result to the occurrence of a possible mosaic of small refugia that allowed *Q. robur* to persist during the LGM. What is more, a high genetic variability (in terms of allelic variability) for *Castanea sativa* was found in central Italy too, and this was explained by considering this area a convergence node of migratory routes from glacial refuges scattered throughout the Italian Peninsula [93,94].

The two large islands of Sicily and (especially) Sardinia are only partially involved in the biogeographical context of the peninsula of Italy. The difference in the number of haplotypes that emerges when comparing these two islands with each other can be attributed to the degree of isolation that they experienced during the paleogeographic evolution of the central Mediterranean Basin (see also Fortini et al. [52]). The Corsican–Sardinian block detached from the present Catalan–Provençal coast during the lower Miocene and rotated toward the center of the Tyrrhenian Sea where it settled definitively in the early Miocene. Connections with the mainland, if any, were short-lived and occurred during the Messinian salinity crisis with Sicily and North Africa and during the LGM (Quaternary Age) with the Italian Peninsula via the Tuscan Archipelago. In line with this, we found that five haplotypes, out of the seven found in Sardinia, are exclusive to this island, while the other two are shared with the central sector of the Italian Peninsula and with Sicily, respectively. In contrast, Sicily had repeated land connections with Calabria (the southernmost spur of the Italian Peninsula), especially during the Quaternary cold periods, and for this reason, it exhibits a significantly greater number of haplotypes shared with the peninsula of Italy.

Genetic features of the Sardinian haplotypes appear to be linked to the geographical position of the collection sites, where specimens from geographical areas very close to each other exhibit genetically related haplotypes. For example, #R and #T haplotypes, both of which are found exclusively in populations coming from central Sardinia, are very similar to each other (they differ in only one marker, with length of fragments very similar to each other) and are therefore interpreted as close allelic variants. A similar situation was found for haplotypes #O and #S in the northern Sardinia populations. The taxonomic identity of the specimens appears to have no influence on this result. This statement, which is quite obvious if we take Bartolucci et al. [17] as nomenclatural–taxonomic reference (since [17] reports *Q. pubescens* to be the only white oak present in Sardinia), also remains valid when adopting more divisive taxonomic frameworks [16,61,95]. This is because we found the allelic variants coming from pairs of similar haplotypes to involve different putative species (*Q. congesta*/*Q. ichnusae*, *Q. virgiliana*/*Q. congesta*). It is therefore conceivable that the white oak populations showing the two haplotype pairs (R-T; O-S) of central and northern Sardinia, respectively, belong to different phylogenetic lineages as the result of an ancient splitting from a common haplotype stock or to signatures deriving from different events of colonization from the mainland. The MSN (Figure 6) seems to support this hypothesis by placing the two pairs of Sardinian haplotypes (#O-#S and #R-#T) at the two ends of the MSN tree, separated from each other by significant genetic distance. More than a hypothesis, instead (by virtue of the congruent geographical distribution), is that within each pair of similar Sardinian haplotypes, one of the two could be the natural derivation of the other. If this were the case, however, it would not be possible to establish, within each pair, which haplotype should be considered the older and which the derived one, as both are exclusive to Sardinia. This result is consistent with what has already been found by Fortini et al. [52] for the same Sardinian populations, where the individuals bearing #O-#S and #R-#T haplotypes fall into two different plastid haplotypes on the basis of the variability in the two combined plastid loci (*trn*H-*psb*A and *trn*K-*mat*K).

Pairs of genetically similar haplotypes occupying the same geographical area are identified for Sicily too, although in this case, the ecological factor also seems to play a role. In fact, haplotypes #B and #E are found, respectively, in populations from Etna volcano slopes and W-Nebrodi mountains, both developed on acidic substrates. Instead, haplotypes #D and #A come from the Madonie and Sicani mountains and therefore from oak populations developed on limestone substrates. Unlike Sardinia, the position of the exclusive Sicilian haplotypes (#D and #A) is more or less central in the MSN tree network (Figure 5), displaying closer connections with haplotypes occurring in the peninsula of Italy (e.g., #C, #U, and #B), and is therefore perfectly in agreement with Sicily's lower degree of geographic (and genetic) isolation. Again, this interpretation appears to be consistent with the cpDNA results published in Fortini et al. [52].

One piece of datum that seems interesting concerns the degree of haplotype variability observed within single populations (Figure 3). It emerges that southern Italy is characterized by a higher number of homogeneous haplotype populations compared to central and northern Italy, whilst Sicily and Sardinia exhibit the highest degree of intra-population variability. This result could be useful in providing preliminary information on the propensity of the different Italian geographical sectors to promote intra-population diversity. This will only be verifiable by carrying out more exhaustive and homogeneous spatial sampling.

4.2. Haplotype Distribution vs. Taxonomical Identity

The calculation of the haplotype variability expressed by the oak macro-species (i.e., *Q. frainetto*, *Q. robur*, *Q. petraea* s.l., and *Q. pubescens* s.l.) shows that this variability is not shared equally among the different macro-species, since each individual macro-species exhibits haplotypes which do not occur in the other ones. These results are not completely in agreement with what was recently published for the Crimean Peninsula (southern Ukraine) by Semerikova et al. [50]. In this latter study, where the cpDNA of the same group of white oak species (except for *Q. frainetto*) was investigated, no significant differences in haplotype composition were highlighted when comparing the different species. Instead, we found that *Q. frainetto* exhibits a gene diversity value (0.468) higher than that exhibited by both *Q. petraea* and *Q. robur* complexes, although the number of haplotypes (5) identified within the *Q. frainetto* populations are lower than those identified within *Q. petraea* (11) and *Q. robur* (7) populations. Moreover, *Q. frainetto* exhibits a dominance of the #U haplotype in two populations located at the two boundaries of the species' Italian distribution range (S-Umbria in the north and S-Calabria in the south), while the #V haplotype occurs in another population from southern Italy and central Italy, too. On the other hand, the populations of *Q. frainetto* occurring within the Tyrrhenian side of central Italy show completely different plastid genetic features which involve other haplotypes (#BB, #C, and #CC). Owing to the fact that using microsatellites in the study of plastid DNA does not allow the events to be dated chronologically, the reasons for the aforementioned haplotype distribution can only be hypothesized. One possible explanation might be that as *Q. frainetto* is an amphi-Adriatic species with a range centered in the Balkan Peninsula, the observed haplotype distribution could be the result of different migratory waves that took place from the Balkan to the Italian Peninsula, taking advantage of the land bridges that periodically connected the two sides of the Adriatic Sea. The first appearance of *Q. frainetto* and other SE European oaks (e.g., *Q. trojana* and *Q. ithaburensis* subsp. *macrolepis*, both restricted to SE Italy) in the proto-Italian Peninsula is thought to date back to the late Miocene, when land bridges temporarily existed between the SW Balkans and the Apulian shelf [96]. The current wider occurrence and ecological amplitude of *Q. frainetto* in southern Italy compared to central Italy [97] could have resulted precisely from this tertiary migration on the S Balkans–S Italy route (together with the greater survival rate of *Q. frainetto* in S Italy during the Quaternary due to the low impact that glaciations had in this area). These southern populations may have later reached central Italy and undergone subsequent genetic differentiation. However, it cannot be excluded that further *Q. frainetto* waves of migration from the Balkans may have occurred further north during the cold periods of the Quaternary along the CW Balkans–C Apennines route, taking advantage of the drying up of the northern and central Adriatic Sea. These Quaternary migrations could have raised the chloroplast genetic variability in central Italy *Q. frainetto* populations while maintaining qualitative differences with the populations of southern Italy.

Somewhat unexpected are certain aspects of the haplotype diversity observable inside the collective group of *Quercus petraea*. In particular, the separation between the populations of the Aspromonte massif (S-Calabria) from those of the Madonie mountains (Sicily) strikes us as a little peculiar. Indeed, both of these populations are currently assigned to the southern Italy endemic taxon *Q. petraea* subsp. *austrotyrrhenica* and are considered taxonomically separated from the populations of the rest of the Italian Peninsula generally ascribed to *Q. petraea* subsp. *petraea* (see [6,16,17]). The fact that the presumed *Q. petraea* subsp. *austrotyrrhenica* populations from the southern end of Calabria show stronger phylogeographic links with the populations of the rest of the Italian Peninsula currently assigned to subsp. petraea rather than with the populations of *Q. petraea* subsp. *austrotyrrhenica* occurring in Sicily is in partial disagreement with the aforementioned taxonomical framework. This haplotype distinction between oak populations belonging to two virtually bordering areas (Calabria and Sicily), separated from each other by only a 3 km wide stretch of sea (Strait of Messina), lends support to the call to reconsider the effectiveness of the Strait as a barrier to the migration of white oaks, as already hypothesized in previous papers (see [26,98]).

The geographical haplotype distribution observed within the *Q. pubescens* collective group (the one including the largest number of populations in our dataset) is characterized by the complete absence of haplotype #V (the haplotype displaying the widest distribution in our database and involving the highest number of individuals) in all of the populations of *Q. pubescens* s.l. occurring in central and southern Italy, whereas this haplotype (#V) acts as the most common in the *Q. pubescens* populations of northern Italy. The *Q. pubescens* collective group has also been found to be the one displaying the highest number of exclusive haplotypes (seven plus six singletons), showing a wide gap when compared to the other white oak species (*Q. petraea,* one plus three singletons; *Q. frainetto,* one; *Q. robur,* one). Obviously, this higher haplotype diversity found in the *Q. pubescens* complex cannot fail to be related, at least in part, to the preponderance of populations belonging to this complex in our sampling. However, the figures tell us that the diversity would still be higher than in the other species complexes even considering the percentage ratios excluding singletons. This high diversity rate of *Q. pubescens* at the cpDNA level confirms what already emerged from nuclear DNA analyses carried out on white oak populations in various SE European countries [8,24,99]. Analyzing it from an interdisciplinary perspective, this result is also consistent with the well-known high morphological variability in *Q. pubescens* normally observed within both individuals and populations in Italy [16,23,100] and with the wide ecological amplitude of the Italian *Q. pubescens* communities [101–103].

4.3. Haplotype Distribution vs. Geographical Structure

The genetic features of the populations reflect a clear geographical structure. The five optimal clusters identified by BAPS (Figure 4; upper part) are clearly related to the five geographical sectors into which the Italian territory has been divided (N Italy, C Italy, S Italy, Sicily, and Sardinia). The BAPS analysis, therefore, considers the five sectors as belonging to the same hierarchical level and having roughly the same importance in a phylogeographic key. More detailed information was obtained from the STRUCTURE analysis, which instead identifies the optimal number of clusters as three. These three clusters correspond to the Italian Peninsula (taken as a whole), Sicily, and Sardinia. Accordingly, the three sectors (northern, central, and southern) of the Italian Peninsula should more correctly be considered at the rank of "sub-clusters", thus bearing a "phylogeographic weight" that is lower than the weights of Sicily and Sardinia. Compared to the BAPS analysis, a higher degree of admixture within the single geographical sector and subsectors emerges from the STRUCTURE results. Starting from the two main genetic macro-clusters identified by $K = 2$ (orange and light-blue groups), we witness a splitting into new groupings of the genetic structure of the populations by increasing delta K until it equals what the Baps analysis considers the optimal number ($K = 5$). For $K = 3$, a genetic subdivision of the light-blue group (identifiable in the dark-blue group of Figure 4) is observable. This new dark-blue group occurs mainly in Sardinia, but also in Sicily (minimally) and in central and southern Italy, and it was found to be characterized by several haplotypes exclusive to the aforementioned three sectors (#C and #CC for central Italy; #X for southern Italy; #T, #S, #R, and #O for Sardinia). The occurrence of the dark-blue group could be hypothesized to be the result of a trans-Tyrrhenian W-E migration (from Sardinia to the Italian Peninsula) and subsequent colonization. The geographical isolation of Sardinia allowed this island to preserve its haplotype structure characterized by two main genetic lineages, as displayed by the simulation with $K = 4$, where, instead, both Sicily (minimally) and the peninsula of Italy exhibit a further "green" subgroup including the oak individuals previously included in the dark-blue group. The simulation with $K = 5$ shows a clear development of the other main genetic lineage (orange group) in the three subsectors of the peninsula of Italy, while Sicily and Sardinia remain stable, reflecting the consequences of the geographical isolation. However, considering the presence of such a high number of haplotypes restricted to Sardinia, some populations of the island would be expected to contain signs of ancestral cpDNA. Instead, this expectation was not confirmed in Fortini et al. [52], where the two most ancestral haplotypes identified on the basis of gene sequences were found to be missing in Sardinia, whereas they were found in central and southern Italy and in Sicily.

4.4. What Has Changed Compared to Previous Investigations?

Comparing the results of our study with those published twenty years ago by Fineschi et al. [34,98], we can make the following observations. Central Italy is confirmed to be the geographical sector, out of the three constituting the Italian Peninsula, displaying the highest haplotype diversity within populations (H_S in Table 4). However, in our study, Sicily and Sardinia exhibit values of H_S significantly higher than those found in the three geographical sectors of the Italian Peninsula and significantly higher than the H_S values found in Fineschi et al. [34] (2002) for these two islands. Furthermore, Sicily has here been confirmed as the geographical sector displaying the lowest total and among-population haplotype diversity (Table 4). However, in our study, it is central Italy and not Sardinia (as instead found in Fineschi et al. [34]) that is the sector displaying the highest value for this parameter. The genetic differentiation among populations (G_{ST}) calculated for the whole Italian Peninsula was found to be significantly lower (0.554) than that found in Fineschi et al. [34] (0.828), and this is probably due to the higher number of populations investigated and the different molecular approach used in this latter study [73]. The highest G_{ST} value per geographic sector was, in our study, found for southern Italy (0.839), with relatively low values for Sicily (0.345) and Sardinia (0.425), whereas the latter island exhibited by far the highest value (1.000) in Fineschi et al. [34]. To summarize, the results of the two cpDNA-based studies both carried out on the whole Italian Peninsula and its major islands twenty years apart from each other displayed some confirmations and several unexpected differences. It is probable that some of these differences could be related to the different number of populations sampled (161 in Fineschi et al. [34] and 60 in our study). Despite the significantly higher number of populations and individuals analyzed by Fineschi et al. [34], however, the final number of haplotypes found twenty years ago (10) were much lower than the number of haplotypes found in our study (28). The difference in markers used certainly influenced this result, testifying to a greater efficiency of cpSSRs for this type of analysis. Nonetheless, we believe that the nature of the differences found goes far beyond a mere (quantitative) numerical issue. In fact, they also appertained to the qualitative aspect of the data and led us to propose new or partially different interpretations to those advanced in previous papers by other authors.

5. Conclusions

Our study, based on the application of cpSSR markers, allowed us to improve the only available, partial picture of the white oaks' genetic diversity in the Italian Peninsula and its major islands, detailing the extent and organization of the chloroplast DNA diversity, the geographical distribution of lineages, and the spatio-temporal dynamics in an unprecedented taxonomic investigation. The cpSSR variation produced a substantial level of polymorphism, allowing for the detection of about three times the number of haplotypes currently catalogued in Italy. The results obtained and the new interpretations that we advanced turned out to be only partially in agreement with those argued previously by other authors. The cpDNA diversity was found to be higher in central Italy than in southern Italy, whereas northern Italy (expected) and Sicily (surprisingly) displayed the lowest degree of haplotype diversity. The cpDNA features of the Sardinian populations turned out to be significantly different from those of the rest of the Italian Peninsula and Sicily, confirming geographical isolation to have played a major role in producing long-term differentiation and genetic segregation. On the other hand, the occurrence of haplotypes displaying a wide distribution within the whole study area, such as "E" and "#V", might well indicate a distant common ancestry (see [52,104]). The observation of the haplotype variability for taxonomic macro-species (*Q. frainetto*, *Q. petraea*, *Q. pubescens*, and *Q. robur*) highlighted some unexpected discontinuities in their haplotype distribution (see *Q. petraea* subsp. *austrotyrrhenica* and *Q. frainetto*) which allowed us to hypothesize relationships between these species' current distribution and precise palaeoecological or paleogeographic events. All of these findings underline the importance of the peninsula of Italy and its major islands as a mosaic of multiple refugia for the persistence of tree species and their genetic variation during the Quaternary [51], and throw into relief the regrettable lack of similar studies in the other macro-refugial areas of Southeastern Europe. It is desirable, in view of the predicted future environmental changes, that the improved genealogical patterns and biogeographical legacies that have emerged in our study might provide useful pointers for developing adequate management and conservation strategies to preserve biodiversity, especially, as is the case with white oaks, when taxonomy is still uncertain [105]. Accordingly, special measures (e.g., seed banking for artificial plantations and the implementation of bio-ecologically representative conservation networks) will certainly be needed for the individuals presenting rare genetic variants. In our study, as well as in previous ones [31], it has emerged that a high number of trees exhibit singletons or very rare haplotypes. This testifies to progressive genetic erosion among white oaks, the causes of which may reasonably be assumed to have a long-lasting human impact (see Fady et al. [106] and Médail [107]), and it is possible that the frequent and increasingly intense (especially in the Mediterranean area) episodes of oak decline are linked to global warming [108–110]. From this perspective, scientists, policy makers, and forest stakeholders still need to develop an effective set of options in order to harmonize landscape planning, management, and preservation of the immeasurable value of forest genetic resources.

Author Contributions: R.D.P., P.F., L.Q., C.M. and M.C.S. designed the study and wrote the first draft of the paper; R.D.P., P.F., E.P. and M.C.S. (together with the colleagues mentioned in the acknowledgments) collected the plant material; L.Q., C.M. and P.D.M. generated and analyzed the molecular data; R.D.P., P.F. and M.C.S. analyzed and interpreted the phylogeographic issues; R.D.P., P.F., E.P., P.D.M., C.M., L.Q. and M.C.S. discussed the results and finalized the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by (a) MUR PRIN 2022, project code: 2022B3HA72, project title: "Xerothermic deciduous oaks, a still-unsolved genetic, taxonomic and coenological issue of a valuable forest resource in Mediterranean countries. From the safeguard of landscape identity to the planning of Urban forests" (PI. R. Di Pietro, UO P. Fortini) CUP B53D23011890006; (b) the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2: Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of the Italian Ministry of University and Research; Award Number: project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, project funded by the European Union—"NextGenerationEU NBFC"; (A) R. Di Pietro: CUP B83C22002950007, project title "National Biodiversity Future Center"; (B) P. Fortini and P. Di Marzio Project: Strengthening of research structures and creation of "National R&S samples on some Key Enabling Technologies": CUP

H73C22000300001; (C) MC Simeone: Project ECS 0000024 Rome Technopole CUP B83C22002820006, Investment 1.5; (c) PROGET_20232024_Wikiplantbase_START_UP_DIMARZIO.

Data Availability Statement: The genetic analysis and inference file outputs are openly available at https://figshare.com/articles/dataset/_/25375858 (accessed on 11 April 2024). Other relevant data are contained within the manuscript.

Acknowledgments: The authors wish to thank their colleagues who have helped in collecting the specimens of white oaks in different places of Italy. They are A.L. Conte, F. Conti, E. Farris, L. Filesi, L. Gianguzzi, D. Iamonico, G. Misano, L. Rosati, and G. Spampinato (Italy). For the same reason, we thank the Park Directors L. De Filippis, G. De Marchis, and G. Marzano, the Park Rangers D. Di Fazio and colleagues (Ausoni Mountains and Fondi Lake Regional Park) and M. Patriarca, P. Perrella, and F. Soscia (Aurunci Mountains Regional Park). We also thank Marcello Cherubini from the IRET-CNR Institute for their help with the laboratory activity. Jim McManus kindly improved our English.

Conflicts of Interest: The authors declare no potential sources of conflicts of interest.

Appendix A

Table A1. Taxonomic, bioclimatic, lithological, and geographic features of the sampling stands. Pop: population identification code; BR: bioclimatic region; Lith: lithology; Alt: altitude (m a.s.l.).

Pop	Species Complex	Taxon	BR [1]	Lith. [2]	Alt.	Latitude (N); Longitude (E) (WGS84) Adm. Region—Sector [3]
IT12	-	*Q. frainetto*	To	Tes	725	40.2422; 16.0551 Basilicata—S-IT
IT13	-	*Q. robur*	Mo	Cls	5	40.1741; 16.6997 Basilicata—S-IT
IT14	-	*Q. robur*	tMo	Tcs	112	41.5515; 12.2112 Lazio—C-IT
IT15	Q. pub.	*Q. pubescens*	tMo	Ei	105	41.9546; 12.4195 Lazio—C-IT
IT16	Q. petr.	*Q. petraea* subsp. *austrotyrrhenica*	To	Cas	1408	37.8583; 14.0580 Sicily—SIC
IT17	Q. petr.	*Q. petraea* subsp. *austrotyrrhenica*	Mo	M	195	38.14604; 16.0599 Calabria—S-IT
IT18	Q. pub.	*Q. pubescens*	Ts	Cas	42	45.2745; 11.7416 Veneto—N-IT
IT19	Q. petr.	*Q. dalechampii*	Ts	Ei	10	45.3430; 11.7617 Veneto—N-IT
IT20	Q. petr.	*Q. petraea*	Ts	Ei	242	45.3173; 11.6877 Veneto—N-IT
IT21	-	*Q. robur*	Ts	Ei	56	45.3227; 11.7408 Veneto—N-IT
IT22	Q. pub.	*Q. pubescens*	Ts	Cls	76	45.4319; 10.6256 Lombardia- N-IT
IT23	Q. pub.	*Q. pubescens*	Mo	Tcs	16	41.3640; 13.3391 Lazio—C-IT
IT24	-	*Q. frainetto*	tMo	Ei	89	41.4862; 13.3105 Lazio—C-IT
IT25	Q. pub.	*Q. virgiliana*	tMo	Cas	669	38.0689; 14.7373 Sicily—SIC
IT26	-	*Q. frainetto*	tTo	Cas	576	41.3588; 13.5265 Lazio—C-IT
IT27	Q. pub.	*Q. dalechampii*	Mo	Tcs	260	38.6265; 16.1628 Calabria—S-IT
IT28	Q. pub.	*Q. dalechampii*	Mo	Tcs	70	38.3778; 15.9396 Calabria—S-IT

Table A1. *Cont.*

Pop	Species Complex	Taxon	BR [1]	Lith. [2]	Alt.	Latitude (N); Longitude (E) (WGS84) Adm. Region—Sector [3]
IT29	Q. pub.	*Q. congesta*	Mo	Ii	980	38.2107; 15.9163 Calabria—S-IT
IT46	Q. petr.	*Q. petraea*	To	Ei	609	42.3236; 12.1302 Lazio—C-IT
IT47	Q. petr.	*Q. petraea*	tMo	Ei	358	42.1469; 11.9407 Lazio—C-IT
IT48	Q. pub.	*Q. ichnusae*	tMo	Cas/Ei	283	39.8158; 8.9486 Sardinia—SAR
IT49	Q. pub.	*Q. virgiliana*	tMo	Cas	567	39.7871; 8.7892 Sardinia—SAR
IT50	Q. pub.	*Q. ichnusae*	Mo	M	529	40.4685; 8.6721 Sardinia—SAR
IT51	Q. pub.	*Q. virgiliana*	Mo	Ei	246	40.6136; 8.5034 Sardinia—SAR
IT52	Q. pub.	*Q. congesta*	tMo	Ii	831	40.1096; 9.2489 Sardinia—SAR
IT53	Q. pub.	*Q. congesta*	Mo	Ii	921	40.1289; 9.3027 Sardinia—SAR
IT54	Q. pub.	*Q. leptobalana*	Mo	Cas	919	37.8841; 13.3831 Sicily—SIC
IT55	Q. pub.	*Q. virgiliana*	tMo	Cas	538	37.9563; 13.4239 Sicily—SIC
IT56	Q. pub.	*Q. congesta*	tMo	Ei	1298	37.7095; 14.9621 Sicily—SIC
IT57	Q. pub.	*Q. dalechampii*	To	Ei	1400	37.7062; 14.9672 Sicily—SIC
IT58	Q. pub.	*Q. virgiliana*	tMo	M	544	37.5917; 15.0447 Sicily—SIC
IT59	Q. pub.	*Q. virgiliana*	tMo	M	540	37.6086; 15.0723 Sicily—SIC
IT60	Q. pub.	*Q. congesta*	tMo	Cas	1179	37.9054; 13.9936 Sicily—SIC
IT61	Q. pub.	*Q. congesta*	tMo	Cas	1327	37.9415- 14.8762 Sicily—SIC
IT62	-	*Q. robur*	Mo	Tcs	342	40.6259; 16.8121 Puglia—S-IT
IT63	Q. pub.	*Q. congesta*	tMo	Ii	1190	38.4813; 16.3411 Calabria—S-IT
IT64	Q. pub.	*Q. pubescens*	tTo	Cas	576	41.3588; 13.5266 Lazio—C-IT
IT65	-	*Q. robur*	Mo	Cas	39	41.0925; 16.8223 Puglia—S-IT
IT66	Q. pub.	*Q. virgiliana*	Mo	Cas	39	41.0925; 16.8223 Puglia—S-IT
IT68	Q. pub.	*Q. pubescens*	Mo	Cls	91	38.0689; 9.0594 Sardinia—SAR
IT69	Q. pub.	*Q. ichnusae*	Mo	M	780	40.2382; 8.6978 Sardinia—SAR
IT70	Q. pub.	*Q. congesta*	Mo	Ei	147	40.7461; 8.5361 Sardinia—SAR
IT71	Q. pub.	*Q. congesta*	Mo	Ii	916	40.4277; 9.0069 Sardinia—SAR

Table A1. *Cont.*

Pop	Species Complex	Taxon	BR [1]	Lith. [2]	Alt.	Latitude (N); Longitude (E) (WGS84) Adm. Region—Sector [3]
IT72	Q. pub.	*Q. pubescens*	To	Tes	796	40.4600; 15.9745 Basilicata—S-IT
IT73	Q. petr.	*Q. petraea*	To	C	610	41.3316; 14.3650 Molise—C-IT
IT75	Q. pub.	*Q. pubescens*	To	Cas	610	41.3302; 14.3701 Molise—C-IT
IT76	Q. pub.	*Q. pubescens*	To	Ii	1091	38.4813;16.3411 Calabria—S-IT
IT77	Q. pub.	*Q. pubescens*	Mo	Tcs	23	40.6584;17.887 Puglia—S-IT
IT82	Q. pub.	*Q. pubescens*	To	Cas	1014	42.6205; 13.2173 Lazio—C-IT
IT83	Q. pub.	*Q. pubescens*	To	Cas	1303	42.3362; 13.5901 Abruzzo—C-IT
IT84	-	*Q. robur*	Ts	Cls	202	45.6213; 9.0943 Lombardia—N-IT
IT85	-	*Q. robur*	Ts	Tcs	21	45.2045; 10.7534 Lombardia—N-IT
IT86	-	*Q. robur*	Ts	Cls	276	45.1485; 7.5962 Piemonte—N-IT
IT87	Q. petr.	*Q. petraea*	To	Tes	346	43.2837; 12.3420 Umbria—C-IT
IT88	Q. petr.	*Q. petraea*	To-s	Cls	67	43.7353; 10.7334 Toscana—C-IT
IT90	-	*Q. frainetto*	tTo-s	Tcs	204	43.0946;12.4672 Umbria—C-IT
IT91	-	*Q. frainetto*	Mo	Tcs	347	38.22470- 16.0453 Calabria—S-IT
IT93	Q. pub.	*Q. pubescens*	Ts	Cas	458	45.6282;13.8784 Friuli—N-IT
IT93	Q. pub.	*Q. pubescens*	tTo-s	Cls	10	43.4676; 13.5917 Marche—C-IT

[1] Bioclimatic regions (according to Blasi et al. [111]): Mo = Mediterranean oceanic, tMo = trans Mediterranean oceanic, To = Temperate oceanic, To-s = Temperate oceanic–semicontinental, Ts = Temperate semicontinental, and tTo = trans Temperate oceanic. [2] Lithology (according to Blasi et al. [111]): Cas = carbonate sedimentary, C = clastic, Cls = clastic sedimentary, Cs/Ei = carbonate sedimentary/effusive igneous, Ei = effusive igneous, Ii = intrusive igneous, Me = metamorphic, Tcs = terrace clastic sedimentary, and Tes = terrigenous sedimentary. [3] Geographic sector (according to Pignatti [55] and Blasi et al. [56]): C-IT = central Italy, N-IT = northern Italy, S-IT = southern Italy, SAR = Sardinia, and SIC = Sicily.

Table A2. Loci, alleles, and allele frequency (total number of individuals: 180).

udt1	udt1_81	0.678
	udt1_82	0.311
	udt1_83	0.011
ucd5	ucd5_74	0.217
	ucd5_75	0.667
	ucd5_77	0.117
ucd4	ucd4_94	0.139
	ucd4_95	0.356
	ucd4_97	0.322
	ucd4_98	0.072
	ucd4_99	0.111

Table A2. *Cont.*

udt3	udt3_125	0.139
	udt3_126	0.461
	udt3_127	0.356
	udt3_128	0.044
Cmcs6	CmCs6_200	0.328
	CmCs6_202	0.139
	CmCs6_203	0.533
Mean		**0.278**

Table A3. Syntaxonomic classification of the forest communities within each site of collection.

Pop.	**Species Complex**	**Taxon**	**Geograph. Sector**	**Site of Collection (Adm. Province)**	**Syntaxonomy**
IT12	-	*Q. frainetto*	S Italy	Rustico, San Mart. d'Agri (PZ)	*Melittio albidae-Quercion frainetto* Quezel et al. in Bonin & Gamisans 1976
IT13	-	*Q. robur*	S Italy	Oasi Pantano di Policoro (MT)	*Rubio peregrinae-Fraxinetum oxycarpae* Biondi & Allegrezza 2004 var with *Q. robur*
IT14	-	*Q. robur*	C Italy	Insugherata (RM)	*Rubio peregrinae-Quercetum cerris* Di Pietro et al. 2010 var. with *Q. robur*
IT15	Q. pub.	*Q. pubescens*	C Italy	Insugherata (RM)	*Roso sempervirentis-Quercetum pubescentis* Biondi 1986
IT16	Q. petr.	*Q. petraea* subsp. *austrothyrr.*	S Italy	Bosco Pomieri, Geraci Siculo (PA)	*Ilici-Quercetum austrotyrrhenicae* Brullo & Marcenò in Brullo 1984 corr. Brullo 2002
IT17	Q. petr.	*Q. petraea* subsp. *austrothyrr.*	S Italy	Pollia (Asprom.). San Luca	*Aristolochio luteae-Quercetum austrotyrrhenicae* Brullo et al. 1999
IT18	Q. pub.	*Q. pubescens*	N Italy	Colli Euganei (PD)	*Fraxino orni-Ostryon carpinifoliae* Tomazic 1940 (*Quercetalia pubescenti-petraeae*)
IT19	Q. petr.	*Q. petraea*	N Italy	Colle S.Daniele (PD)	*Physospermo-Quercion petraeae* A.O. Horvat 1976
IT20	Q. petr.	*Q. petraea*	N Italy	M. Venda (PD)	*Melampyro vulgati-Quercetum petraeae* Puncer et Zupančić 1979
IT21	Q. robur	*Q. robur*	N Italy	Torreglia; M.Rua; Roccapendice (PD)	*Asparago tenuifolii-Quercetum roboris* (Lausi 1967). Marincek 1994
IT22	Q. pub.	*Q. pubescens*	N Italy	Ome, San Martino (BS)	*Quercetalia pubescenti-petraeae* Klika 1933
IT23	Q. pub.	*Q. pubescens*	C Italy	Monte San Biagio (LT)	*Pistacio terebinthi-Quercetum pubescentis* (Blasi et Di Pietro 1998) Allegrezza et al. 2003
IT24	-	*Q. frainetto*	C Italy	Selvapiana Amaseno (FR)	*Quercus frainetto* community (*Crataego-Quercion cerridis* Arrigoni 1998)
IT25	Q. pub.	*Q. virgiliana*	S Italy	Valle del Fitalia (Frazzanò, ME)	*Erico arborae-Quercetum virgilianae* Brullo et Marcenò 1985
IT26	-	*Q. frainetto*	C Italy	Parco Monti Aurunci (LT)	*Quercus frainetto* community (*Crataego-Quercion cerridis* Arrigoni 1998)
IT27	Q. pub.	*Q. dalechampii*	S Italy	Serre, Sant'Angelo-Pizzoni (VV)	indivisuals of *Q. dalechampii* in the *Erico arborae-Quercetum virgilianae* Brullo e Marcenò 1985
IT28	Q. pub.	*Q. dalechampii*	S Italy	Aspromonte, Croce Mammone (RC)	indivisuals of *Q. dalechampii* in the *Oleo oleaster-Quercetum virgilianae* Brullo 1984
IT29	Q. pub.	*Q. congesta*	S Italy	Aspromonte, Piani di Carmelia (RC)	*Erico-Quercetum congestae* Brullo et al. 2001
IT46	Q. petr.	*Q. petraea*	C Italy	Monti Cimini (VT)	individuals of *Q. petraea* in *Coronillo emeri-Quercetum cerridis* Blasi 1984 var. with Q. petraea
IT47	Q. petr.	*Q. petraea*	C Italy	Tolfa mountains (Roma)	*Carici olbiensis-Quercetum petraeae* Di Pietro et al. 2010

Table A3. *Cont.*

Pop.	Species Complex	Taxon	Geograph. Sector	Site of Collection (Adm. Province)	Syntaxonomy
IT48	Q. pub.	*Q. ichnusae*	Sardinia	Senis (OR)	*Ornithogalo pyrenaici-Quercetum ichnusae* Bacchetta et al. 2004
IT49	Q. pub.	*Q. virgiliana*	Sardinia	Pau (OR)	*Lonicero implexae-Quercetum virgilianae* Bacchetta et al. 2004
IT50	Q. pub.	*Q. ichnusae*	Sardinia	Monte Traessu (SS)	*Ornithogalo pyrenaici-Quercetum ichnusae* Bacchetta et al. 2004
IT51	Q. pub.	*Q. virgiliana*	Sardinia	Ittiri SS)	*Lonicero implexae-Quercetum virgilianae* Bacchetta et al. 2004
IT52	Q. pub.	*Q. congesta*	Sardinia	Fonni (Muggiana) (NU)	*Glechomo sardoae-Quercetum congestae* Bacchetta et al. 2004
IT53	Q. pub.	*Q. congesta*	Sardinia	Fonni (Govossai) (NU)	*Glechomo sardoae-Quercetum congestae* Bacchetta et al. 2004
IT54	Q. pub.	*Q. leptobalana*	Sicily	Bosco Ficuzza (PA)	*Quercetum leptobalani* Brullo 1984
IT55	Q. pub.	*Q. virgiliana*	Sicily	Marineo (PA)	*Oleo-Quercetum virgilianae* Brullo 1984
IT56	Q. pub.	*Q. congesta*	Sicily	Etna Volcano (CT)	*Arabido turritae-Quercetum congestae* Brullo e Marcenò 1985
IT57	Q. pub.	*Q. dalechampii*	Sicily	Etna Volcano (CT)	*Quercenion dalechampii* Brullo 1984
IT58	Q. pub.	*Q. virgiliana*	Sicily	Etna. M. Ceraulo, Mascalucia (CT)	*Oleo-Quercetum virgilianae* Brullo 1984
IT59	Q. pub.	*Q. virgiliana*	Sicily	Etna. Tre castagni, (CT)	*Celtido aetnensis-Quercetum virgilianae* Brullo e Marcenò 1985
IT60	Q. pub.	*Q. congesta*	Sicily	Madonie, Piani Torre-Zucchi (PA)	*Conopodio capillifolii-Quercetum congestae* Maniscalco & Raimondo 2009
IT61	Q. pub.	*Q. congesta*	Sicily	Valle del Flascio. Nebrodi (ME)	*Festuco heterophyllae-Quercetum congestae* Brullo & Marcenò 1986
IT62	Q. robur	*Q. robur*	S Italy	Tafuri (BA)	*Quercus robur* isolated individuals in *Stipo-Quercetum dalechampii* Biondi et al. 2004
IT63	Q. pub.	*Q. congesta*	S Italy	Serre, Sant'Angelo Nardodipace (VV)	*Festuco heterophyllae-Quercetum congestae* Brullo & Marcenò 1986
IT64	Q. pub.	*Q. pubescens*	C Italy	Parco Monti Aurunci (LT)	*Pistacio terebinthi-Quercetum pubescentis* (Blasi & Di Pietro 1998) Allegrezza et al. 2002
IT65	Q. robur	*Q. robur*	S Italy	Tafuri, Murgean Plateau (BA)	*Quercus robur* isolated individuals in *Stipo-Quercetum dalechampii* Biondi et al. 2004
IT66	Q. pub.	*Q. virgiliana*	S Italy	Selva S. Vito Gravina Laterza (TA)	individuals of *Q. virgiliana* in the *Teucrio siculi-Quercetum trojanae* Biondi et al. 2004
IT68	Q. pub.	*Q. pubescens*	Sardinia	M.te Zara. Monastir (CA)	*Lonicero implexae-Quercetum virgilianae* Bacchetta et al. 2004
IT69	Q. pub.	*Q. ichnusae*	Sardinia	Bosco S. Antonio. Macomer (NU)	*Ornithogalo pyrenaici-Quercetum ichnusae* Bacchetta et al. 2004
IT70	Q. pub.	*Q. congesta*	Sardinia	Sant'Orsola (SS)	*Glechomo sardoae-Quercetum congestae* Bacchetta et al. 2004
IT71	Q. pub.	*Q. congesta*	Sardinia	M. Rasu, Catena del Marghine (SS)	*Glechomo sardoae-Quercetum congestae* Bacchetta et al. 2004
IT72	Q. pub.	*Q. pubescens*	S Italy	Laurenzana (PZ)	*Centaureo centaurii-Quercetum pubescentis* Ubaldi & Zanotti 1995
IT73	Q. petr.	*Q. petraea*	C Italy	Monte Vairano (CB)	individuals of *Q. petraea* in *Aremonio-Quercetum cerridis* Blasi et al. ex Terzi et al. 2022
IT75	Q. pub.	*Q. pubescens*	C Italy	Monte Vairano (CB)	*Cytiso sessilifolii-Quercetum pubescentis* Blasi et al. 1982

Table A3. *Cont.*

Pop.	Species Complex	Taxon	Geograph. Sector	Site of Collection (Adm. Province)	Syntaxonomy
IT76	Q. pub.	*Q. pubescens*	S Italy	Mar di Pace—Nardodipace (VV)	*Erico-Quercetum congestae* Brullo et al. 2001
IT77	Q. pub.	*Q. pubescens*	S Italy	Bosco del Compare (BR)	Individuals of *Q. pubescens* in *Fraxino orni-Quercetum ilicis* Horvatic (1956) 1958
IT82	Q. pub.	*Q. pubescens*	C Italy	Torrita, Monti della Laga (RI)	*Cytiso sessilifolii-Quercetum pubescentis* Blasi et al. 1982
IT83	Q. pub.	*Q. pubescens*	C Italy	Barisciano, San Colombo (AQ)	*Cytiso sessilifolii-Quercetum pubescentis* Blasi et al. 1982
IT84	-	*Q. robur*	N Italy	Parco delle Groane—Solaro (MI)	*Polygonato multiflori—Quercetum roboris* Sartori 1984
IT85	-	*Q. robur*	N Italy	Bosco Fontana—Marmirolo (MN)	*Polygonato multiflori—Quercetum roboris* Sartori 1984
IT86	-	*Q. robur*	N Italy	Parco La Mandria Ven. Reale (TO)	*Quercus robur* community belonging to *Carpinion betuli* Issler 1931
IT87	Q. petr.	*Q. petraea*	C Italy	Montecorona Umbertide (PG)	*Hieracio racemosi-Quercetum petraeae* Pedrotti et al. 1982
IT88	Q. petr.	*Q. petraea*	C Italy	Parco Castelfranco di Sotto (PI)	individuals of *Q. petraea* in *Melico-Quercetum cerridis* Arrigoni, in Arrigoni et al. 1990
IT89	Q. petr.	*Q. petraea*	N Italy	Parco dei boschi di Carrega (PR)	*Physospermo cornubiensis-Quercetum petraeae* Oberdorfer & Hofmann 1967
IT90	-	*Q. frainetto*	C Italy	Collestrada (PG)	*Malo florentinae-Quercetum frainetto* Biondi et al. 2001 ex Terzi et al. 2022
IT91	-	*Q. frainetto*	S Italy	Parco Naz. Aspromonte—Platì (RC)	*Cytiso-Quercetum frainetto* Scelsi & Spampinato 1996
IT92	Q. pub.	*Q. pubescens*	N Italy	Basovizza (TS)	*Ostryo-Quercetum pubescentis* (Horvat 1959). Trinajstić 1974.
IT93	Q. pub.	*Q. pubescens*	C Italy	Selva di Castelfidardo (AN)	*Roso sempervirentis-Quercetum pubescentis* Biondi 1986

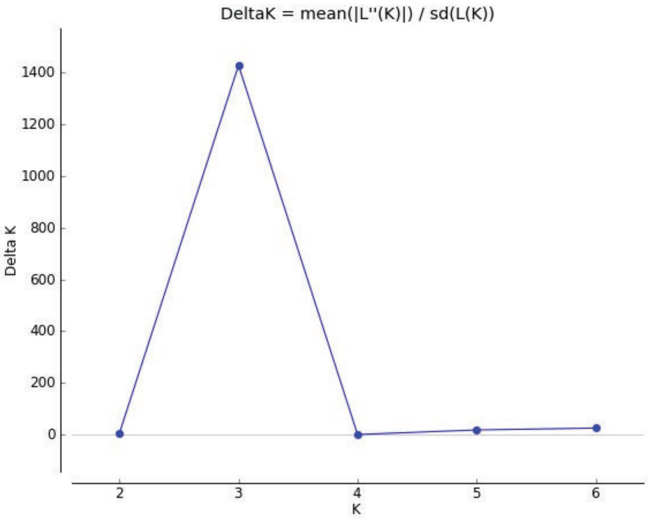


Figure A1. The optimal number of genetic groups in the studied oak populations determined by the ΔK Evanno model.

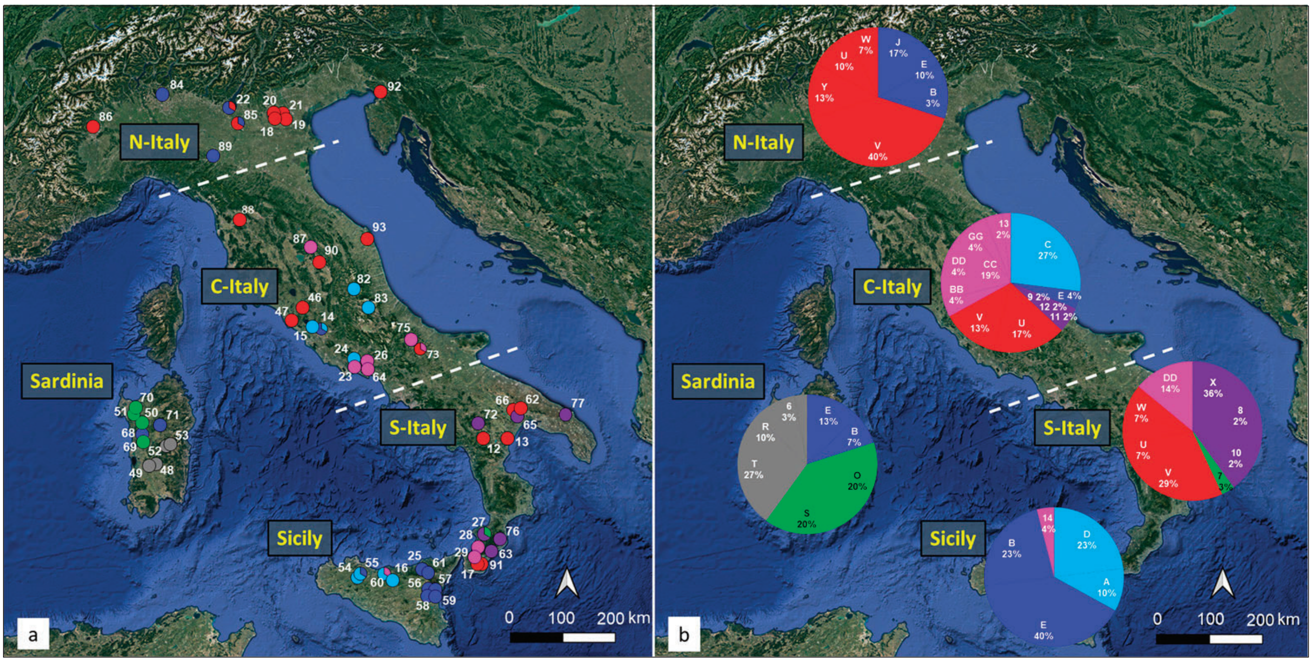


Figure A2. The geographical distribution of the seven groups of haplotypes identified by the MSN diagram displayed in Figure 5, according to (**a**) single populations (The numbers correspond to the population codes as reported in Table A1); (**b**) the percentage value per geographical sector. The colors used for filling both single-population circles and pie-chart sectors correspond to those used for circumscribing the different groups of haplotypes in Figure 5.

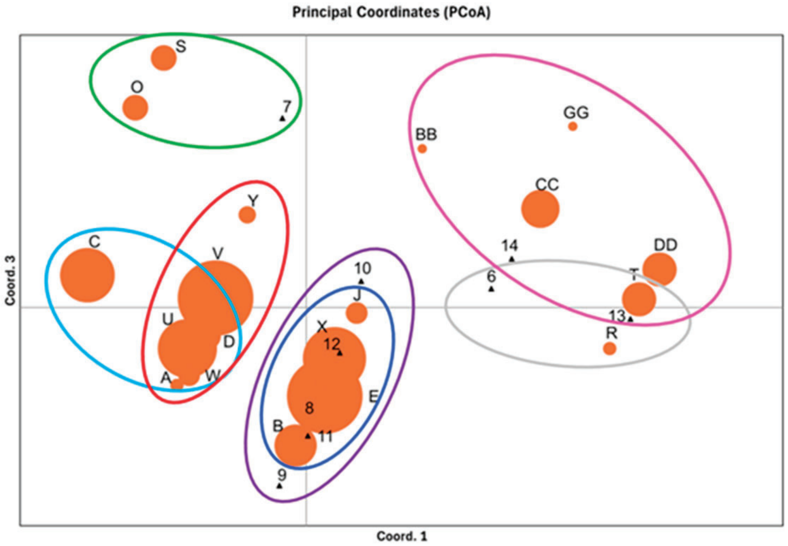


Figure A3. Principal coordinate analysis (PCoA) plot based on genetic distance representing the haplotype distribution observable along the first and third axes. The colors of the ellipsis contours circumscribing the different groups of haplotypes are the same as those used in the PCoA diagram of Figure 6 (axes 1 and 2).

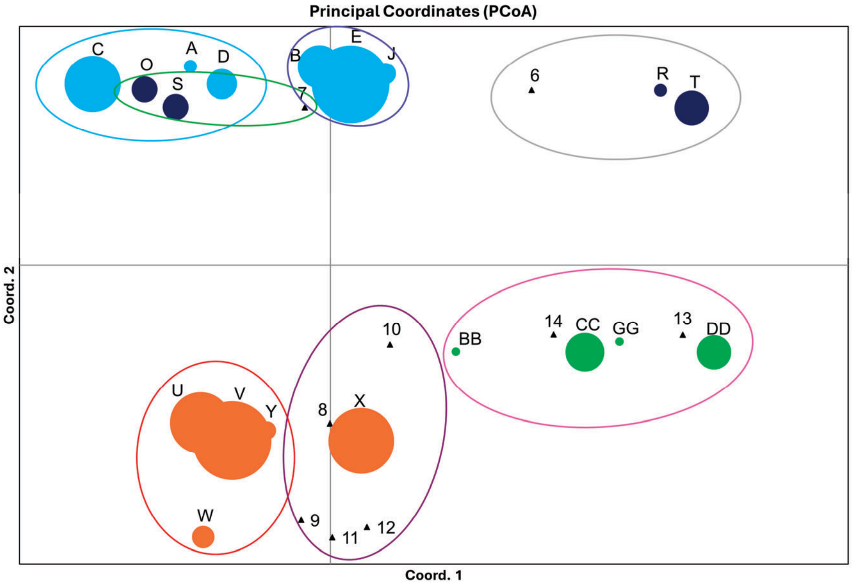


Figure A4. Principal coordinate analysis (PCoA) displaying the ellipses that include the groups of haplotypes derived from the MSN classification (Figure 5). Ellipsis contours are colored with the colors already used in the MSN diagram (Figure 5). Haplotypes are filled with the colors derived from the STRUCTURE analysis for $K = 4$ (see Figure 4).

References

1. Hampe, A.; Petit, R.J. Conserving biodiversity under climate change: The rear edge matters. *Ecol. Lett.* **2005**, *8*, 461–467. [CrossRef] [PubMed]
2. Denk, T.; Grimm, G.W.; Manos, P.S.; Deng, M.; Hipp, A.L. An updated infrageneric classification of the oaks: Review of previous taxonomic schemes and synthesis of evolutionary patterns. In *Oaks Physiological Ecology. Exploring the Functional Diversity of Genus Quercus L.*; Gil-Pelegrín, E., Peguero-Pina, J.J., Sancho-Knapik, D., Eds.; Tree Physiology; Springer International Publishing: Cham, Switzerland, 2017; pp. 13–38, ISBN 978-3-319-69099-5.
3. Govaerts, R.; Nic Lughadha, E.; Black, N.; Turner, R.; Paton, A. The world checklist of vascular plants, a continuously updated resource for exploring global plant diversity. *Sci. Data* **2021**, *8*, 215. [CrossRef] [PubMed]
4. Mucina, L.; Bültmann, H.; Dierßen, K.; Theurillat, J.-P.; Raus, T.; Čarni, A.; Šumberová, K.; Willner, W.; Dengler, J.; García, R.G.; et al. vegetation of Europe: Hierarchical floristic classification system of vascular plant, bryophyte, lichen, and algal communities. *Appl. Veg. Sci.* **2016**, *19*, 3–264. [CrossRef]
5. Plomion, C.; Martin, F. Oak genomics is proving its worth. *New Phytol.* **2020**, *226*, 943–946. [CrossRef] [PubMed]
6. Brullo, S.; Guarino, R.; Siracusa, G. Taxonomical revision about the deciduous oaks of Sicily. *Webbia* **1999**, *54*, 1–72. [CrossRef]
7. Di Pietro, R.; Viscosi, V.; Peruzzi, L.; Fortini, P. A Review of the application of the name *Quercus dalechampii*. *Taxon* **2012**, *61*, 1311–1316. [CrossRef]
8. Fortini, P.; Di Marzio, P.; Di Pietro, R. Differentiation and hybridization of *Quercus frainetto*, *Q. petraea*, and *Q. pubescens* (Fagaceae): Insights from macro-morphological leaf traits and molecular data. *Plant Syst. Evol.* **2015**, *301*, 375–385. [CrossRef]
9. Wellstein, C.; Spada, F. The status of *Quercus pubescens* Willd. in Europe. In *Warm-Temperate Deciduous Forests around the Northern Hemisphere*; Box, E.O., Fujiwara, K., Eds.; Geobotany Studies; Springer International Publishing: Cham, Switzerland, 2015; pp. 153–163, ISBN 978-3-319-01261-2.
10. Kučera, P. New name for central europaean oak formerly labelled as *Quercus dalechampii*. *Biologia* **2018**, *73*, 313–317. [CrossRef]
11. Grossoni, P.; Bruschi, P.; Bussotti, F.; Pollastrini, M.; Selvi, F. The taxonomic interpretation of Mediterranean oaks of *Quercus* Sect. *Quercus* (Fagaceae): Uncertainties and diverging concepts. *Flora Mediterr.* **2021**, *31*, 271–278. [CrossRef]
12. Gasparini, P.; Papitto, G. The italian forest inventory in brief. In *Italian National Forest Inventory—Methods and Results of the Third Survey: Inventario Nazionale delle Foreste e dei Serbatoi Forestali di Carbonio—Metodi e Risultati della Terza Indagine*; Gasparini, P., Di Cosmo, L., Floris, A., De Laurentis, D., Eds.; Springer International Publishing: Cham, Switzerland, 2022; pp. 1–15, ISBN 978-3-030-98678-0.
13. Presl, C.B. Quercus. In *Deliciae Pragensis Historiam Naturalem Spectantes*; Presl, J.S., Presl, C.B., Eds.; Sumtibus Calve: Pragae, Czech Republic, 1822; Volume 1, p. 32.
14. Gussone, G. *Florae Siculae Synopsis*; Ex Typis Tramater: Naples, Italy, 1842; Volume 2.
15. Dimopoulos, P.; Raus, T.; Bergmeier, E.; Constantinidis, T.; Iatrou, G.; Kokkini, S.; Strid, A.; Tzanoudakis, D. *Vascular Plants of Greece: An Annotated Checklist*; Botanic Garden and Botanical Museum Berlin-Dahlem: Berlin, Germany; Hellenic Botanical Society: Athens, Greece, 2013.

16. Pignatti, S.; Guarino, R.; La Rosa, M. *Flora d'Italia*, 2nd ed.; Edagricole: Bologna, Italy, 2017; Volume 4, ISBN 88-506-5242-9.
17. Bartolucci, F.; Peruzzi, L.; Galasso, G.; Albano, A.; Alessandrini, A.; Ardenghi, N.M.G.; Astuti, G.; Bacchetta, G.; Ballelli, S.; Banfi, E.; et al. An updated checklist of the vascular flora native to Italy. *Plant Biosyst.* **2018**, *152*, 179–303. [CrossRef]
18. Kaplan, Z.; Danihelka, J.; Chrtek, J.; Prančl, J.; Grulich, V.; Jelinek, B.; Úradníček, L.; Řepka, R.; Smarda, P.; Vašut, R.; et al. Distributions of vascular plants in the Czech Republic. Part 11. *Preslia* **2022**, *94*, 335–427. [CrossRef]
19. Tenore, M. *Semina Anno 1830 Collecta, Quae in Horto Botanico Neapolitano pro Mutua Commutatione Offeruntur*; Fibreni: Naples, Italy, 1830.
20. Tenore, M. *Florae Neapolitanae Sylloge*; Fibreni: Naples, Italy, 1831.
21. Franjić, J.; Liber, Z.; Škvorc, Ž.; Idžojtić, M.; Šoštarić, R.; Stančić, Z. Morphological and molecular differentiation of the croatian populations of *Quercus pubescens* Willd. (Fagaceae). *Acta Soc. Bot. Pol.* **2006**, *75*, 123–130. [CrossRef]
22. Enescu, C.; Curtu, A.; Șofletea, N. Is *Quercus virgiliana* a distinct morphological and genetic entity among European white oaks? *Turk. J. Agric. For.* **2013**, *37*, 632–641. [CrossRef]
23. Di Pietro, R.; Di Marzio, P.; Medagli, P.; Misano, G.; Silletti, G.N.; Wagensommer, R.P.; Fortini, P. Evidence from multivariate morphometric study of the *Quercus pubescens* complex in southeast Italy. *Bot. Serbica* **2016**, *40*, 83–100.
24. Di Pietro, R.; Di Marzio, P.; Antonecchia, G.; Conte, A.; Fortini, P. Preliminary characterization of the *Quercus pubescens* complex in southern italy using molecular markers. *Acta Bot. Croat.* **2020**, *79*, 15–25. [CrossRef]
25. Di Pietro, R.; Conte, A.L.; Di Marzio, P.; Gianguzzi, L.; Spampinato, G.; Caldarella, O.; Fortini, P. A multivariate morphometric analysis of diagnostic traits in southern Italy and Sicily pubescent oaks. *Folia Geobot.* **2020**, *55*, 163–183. [CrossRef]
26. Di Pietro, R.; Conte, A.L.; Di Marzio, P.; Fortini, P.; Farris, E.; Gianguzzi, L.; Müller, M.; Rosati, L.; Spampinato, G.; Gailing, O. Does the genetic diversity among pubescent white oaks in southern Italy, Sicily and Sardinia islands support the current taxonomic classification? *Eur. J. For. Res.* **2021**, *140*, 355–371. [CrossRef]
27. Fortini, P.; Di Marzio, P.; Conte, A.L.; Antonecchia, G.; Proietti, E.; Di Pietro, R. Morphological and molecular results from a geographical transect focusing on *Quercus pubescens*/*Q. virgiliana* ecological-altitudinal vicariance in peninsular Italy. *Plant Biosyst.* **2022**, *156*, 1498–1511. [CrossRef]
28. Brewer, S.; Cheddadi, R.; de Beaulieu, J.L.; Reille, M. The spread of deciduous *Quercus* throughout Europe since the last glacial period. *For. Ecol. Manag.* **2002**, *156*, 27–48. [CrossRef]
29. Dumolin, S.; Demesure, B.; Petit, R.J. Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theoret. Appl. Genet.* **1995**, *91*, 1253–1256. [CrossRef] [PubMed]
30. Dumolin-Lapègue, S.; Demesure, B.; Fineschi, S.; Le Come, V.; Petit, R.J. Phylogeographic structure of white oaks throughout the european continent. *Genetics* **1997**, *146*, 1475–1487. [CrossRef] [PubMed]
31. Petit, R.J.; Csaikl, U.M.; Bordács, S.; Burg, K.; Coart, E.; Cottrell, J.; van Dam, B.; Deans, J.D.; Dumolin-Lapègue, S.; Fineschi, S.; et al. Chloroplast DNA variation in european white oaks: Phylogeography and patterns of diversity based on data from over 2600 populations. *For. Ecol. Manag.* **2002**, *156*, 5–26. [CrossRef]
32. Petit, R.J.; Brewer, S.; Bordács, S.; Burg, K.; Cheddadi, R.; Coart, E.; Cottrell, J.; Csaikl, U.M.; van Dam, B.; Deans, J.D.; et al. Identification of refugia and post-glacial colonisation routes of european white oaks based on chloroplast DNA and fossil pollen evidence. *For. Ecol. Manag.* **2002**, *156*, 49–74. [CrossRef]
33. Bennett, K.D.; Tzedakis, P.C.; Willis, K.J. Quaternary refugia of north european trees. *J. Biogeogr.* **1991**, *18*, 103–115. [CrossRef]
34. Fineschi, S.; Taurchini, D.; Grossoni, P.; Petit, R.J.; Vendramin, G.G. Chloroplast DNA variation of white oaks in Italy. *For. Ecol. Manag.* **2002**, *156*, 103–114. [CrossRef]
35. Lupini, A.; Aci, M.M.; Mauceri, A.; Luzzi, G.; Bagnato, S.; Menguzzato, G.; Mercati, F.; Sunseri, F. Genetic diversity in old populations of sessile oak from Calabria assessed by nuclear and chloroplast SSR. *J. Mt. Sci.* **2019**, *16*, 1111–1120. [CrossRef]
36. Provan, J.; Powell, W.; Hollingsworth, P.M. Chloroplast microsatellites: New tools for studies in plant ecology and evolution. *Trends Ecol. Evol.* **2001**, *16*, 142–147. [CrossRef] [PubMed]
37. Deguilloux, M.-F.; Dumolin-Lapègue, S.; Gielly, L.; Grivet, D.; Petit, R.J. A set of primers for the amplification of chloroplast microsatellites in *Quercus*. *Mol. Ecol. Notes* **2003**, *3*, 24–27. [CrossRef]
38. Neophytou, C.; Michiels, H.-G. Upper rhine valley: A migration crossroads of middle european oaks. *For. Ecol. Manag.* **2013**, *304*, 89–98. [CrossRef]
39. Chmielewski, M.; Meyza, K.; Chybicki, I.J.; Dzialuk, A.; Litkowiec, M.; Burczyk, J. Chloroplast microsatellites as a tool for phylogeographic studies: The case of white oaks in Poland. *iForest* **2015**, *8*, 765. [CrossRef]
40. Magri, D.; Fineschi, S.; Bellarosa, R.; Buonamici, A.; Sebastiani, F.; Schirone, B.; Simeone, M.C.; Vendramin, G.G. The distribution of *Quercus suber* chloroplast haplotypes matches the palaeogeographical history of the western Mediterranean. *Mol. Ecol.* **2007**, *16*, 5259–5266. [CrossRef] [PubMed]
41. Bagnoli, F.; Tsuda, Y.; Fineschi, S.; Bruschi, P.; Magri, D.; Zhelev, P.; Paule, L.; Simeone, M.C.; González-Martínez, S.C.; Vendramin, G.G. Combining molecular and fossil data to infer demographic history of *Quercus cerris*: Insights on european eastern glacial refugia. *J. Biogeogr.* **2016**, *43*, 679–690. [CrossRef]
42. Grivet, D.; Deguilloux, M.-F.; Petit, R.J.; Sork, V.L. Contrasting patterns of historical colonization in white oaks (*Quercus* spp.) in California and Europe. *Mol. Ecol.* **2006**, *15*, 4085–4093. [CrossRef]
43. Neophytou, C.; Semizer-Cuming, D.; Michiels, H.-G.; Kremer, A.; Jansen, S.; Fussi, B. Relict stands of central european oaks: Unravelling autochthony and genetic structure based on a multi-population study. *For. Ecol. Manag.* **2024**, *551*, 121554. [CrossRef]

44. Curtu, A.L.; Gailing, O.; Leinemann, L.; Finkeldey, R. Genetic variation and differentiation within a natural community of five oak species (*Quercus* spp.). *Plant Biol.* **2007**, *9*, 116–126. [CrossRef] [PubMed]
45. Danusevičius, D.; Baliuckas, V.; Buchovska, J.; Kembrytė, R. Geographical Structuring of *Quercus robur* L. Chloroplast DNA haplotypes in Lithuania: Recolonization, adaptation, or overexploitation effects? *Forests* **2021**, *12*, 831. [CrossRef]
46. Popović, M.; Katičić Bogdan, I.; Varga, F.; Šatović, Z.; Bogdan, S.; Ivanković, M. Genetic diversity in peripheral pedunculate oak (*Quercus robur* L.) provenances—Potential climate change mitigators in the center of distribution despite challenges in natural populations. *Forests* **2023**, *14*, 2290. [CrossRef]
47. Gailing, O.; Wachter, H.; Schmitt, H.-P.; Curtu, A.-L.; Finkeldey, R. Characterization of different provenances of slavonian pedunculate oaks (*Quercus robur* L.) in Münsterland (Germany) with chloroplast DNA markers: PCR-RFLPs and chloroplast microsatellites. *Allg. Forst. Jagdztg.* **2007**, *178*, 85–90.
48. Burger, K.; Gailing, O. Genetic Variability of indigenous (*Quercus robur* L.) and late flushing oak (*Quercus robur* L. subsp. *slavonica* (Gáyer) Mátyás) in adult stands compared with their natural regeneration. *Eur. J. For. Res.* **2022**, *141*, 1073–1088. [CrossRef]
49. Semerikova, S.A.; Isakov, I.Y.; Semerikov, V.L. Chloroplast DNA variation and phylogeography of pedunculate oak *Quercus robur* L. in the eastern part of the range. *Russ. J. Genet.* **2021**, *57*, 47–60. [CrossRef]
50. Semerikova, S.A.; Podergina, S.M.; Tashev, A.N.; Semerikov, V.L. Phylogeography of oaks in the crimea reveals pleistocene refugia and migration routes. *Russ. J. Ecol.* **2023**, *54*, 197–212. [CrossRef]
51. Médail, F.; Diadema, K. Glacial refugia influence plant diversity patterns in the Mediterranean basin. *J. Biogeogr.* **2009**, *36*, 1333–1345. [CrossRef]
52. Fortini, P.; Di Pietro, R.; Proietti, E.; Cardoni, S.; Quaranta, L.; Simeone, M.C. Dissecting the continuum and unravelling the phylogeographic knot of plastid DNA in european white oaks (*Quercus* sect. *Quercus*): Ancient signatures and multiple diversity reservoirs. *Eur. J. For. Res.* **2024**, *143*, 107–127. [CrossRef]
53. Proietti, E.; Filesi, L.; Di Marzio, P.; Di Pietro, R.; Masin, R.; Conte, A.L.; Fortini, P. Morphology, geometric morphometrics, and taxonomy in relict deciduous oaks woods in northern Italy. *Rend. Fis. Acc. Lincei* **2021**, *32*, 549–564. [CrossRef]
54. Avanzi, C.; Bagnoli, F.; Romiti, E.; Spanu, I.; Tsuda, Y.; Vajana, E.; Vendramin, G.G.; Piotti, A. The latitudinal trend in genetic diversity and distinctiveness of *Quercus robur* rear edge forest remnants calls for new conservation priorities. *bioRxiv*, 2023; *advance online publication*. [CrossRef]
55. Pignatti, S. La flora dell'Appennino meridionale: Distribuzione attuale ed ipotesi sull'origine. *Biogeographia* **1986**, *10*, 89–100. [CrossRef]
56. Blasi, C.; Filibek, G.; Burrascano, S.; Copiz, R.; Di Pietro, R.; Ercole, S.; Lattanzi, E.; Rosati, L.; Tilia, A. Primi risultati per una nuova regionalizzazione fitogeografia del territorio italiano. *Biogeographia* **2007**, *28*, 9–23. [CrossRef]
57. Brullo, S. Contributo alla conoscenza della vegetazione delle Madonie (Sicilia settentrionale). *Boll. Acc. Gioenia Sci. Nat.* **1984**, *16*, 351–420.
58. Brullo, S.; Marcenò, C. Contributo alla conoscenza della classe *Quercetea ilicis* in Sicilia. *Not. Fitosoc.* **1985**, *19*, 183–229.
59. Brullo, S.; Scelsi, F.; Spampinato, G. *La Vegetazione dell'Aspromonte*; Laruffa: Reggio Calabria, Italy, 2001; ISBN 88-7221-160-3.
60. Brullo, S.; Gianguzzi, L.; La Mantia, A.; Siracusa, G. La classe *Quercetea ilicis* in Sicilia. *Boll. Acc. Gioenia Sci. Nat* **2008**, *41*, 1–24.
61. Bacchetta, G.; Biondi, E.; Farris, E.; Filigheddu, R.; Mossa, L. A phytosociological study of the deciduous oak woods of Sardinia (Italy). *Fitosociologia* **2004**, *41*, 53–64.
62. Thiers, B. Index Herbariorum: A Global Directory of Public Herbaria and Associated Staff. New York Botanical Garden's Virtual Herbarium. 2015. Available online: http://sweetgum.nybg.org/ih (accessed on 20 March 2024).
63. Sebastiani, F.; Carnevale, S.; Vendramin, G.G. A new set of mono- and dinucleotide chloroplast microsatellites in Fagaceae. *Mol. Ecol. Notes* **2004**, *4*, 259–261. [CrossRef]
64. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* **2012**, *28*, 2537–2539. [CrossRef] [PubMed]
65. Goudet, J. *FSTAT (Version 2.9.4), a Program (for Windows 95 and Above) to Estimate and Test Population Genetics Parameters*; Department of Ecology & Evolution, Lausanne University: Vaud, Switzerland, 2003; Volume 53.
66. Pons, O.; Petit, R. Measuring and testing genetic differentiation with ordered vs. unordered alleles. *Genetics* **1996**, *144*, 1237–1245. [CrossRef] [PubMed]
67. Corander, J.; Cheng, L.; Marttinen, P.; Sirén, J.; Tang, J. BAPS: Bayesian Analysis of Population Structure, Manual v. 6.0, 14 February 2013. Available online: https://github.com/ocbe-uio/BAPS/blob/develop/BAPS6manual.pdf (accessed on 20 October 2023).
68. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959. [CrossRef] [PubMed]
69. Earl, D.A.; vonHoldt, B.M. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **2012**, *4*, 359–361. [CrossRef]
70. Kopelman, N.M.; Mayzel, J.; Jakobsson, M.; Rosenberg, N.A.; Mayrose, I. Clumpak: A program for identifying clustering modes and packaging population structure inferences across K. *Mol. Ecol. Resour.* **2015**, *15*, 1179–1191. [CrossRef] [PubMed]
71. Excoffier, L.; Lischer, H.E.L. Arlequin Suite Ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* **2010**, *10*, 564–567. [CrossRef]
72. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef] [PubMed]

73. Petit, R.J.; Duminil, J.; Fineschi, S.; Hampe, A.; Salvini, D.; Vendramin, G.G. Invited review: Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Mol. Ecol.* **2005**, *14*, 689–701. [CrossRef] [PubMed]
74. Walker, M.J.C. Climatic changes in Europe during the last glacial/interglacial transition. *Quatern. Int.* **1995**, *28*, 63–76. [CrossRef]
75. Schenk, F.; Väliranta, M.; Muschitiello, F.; Tarasov, L.; Heikkilä, M.; Björck, S.; Brandefelt, J.; Johansson, A.V.; Näslund, J.-O.; Wohlfarth, B. Warm summers during the younger dryas cold reversal. *Nat. Commun.* **2018**, *9*, 1634. [CrossRef] [PubMed]
76. Ravazzi, C.; Peresani, M.; Pini, R.; Vescovi, E. Il tardoglaciale nelle Alpi Italiane e in Pianura Padana. Evoluzione stratigrafica, storia della vegetazione e del popolamento antropico. *Alp. Mediterr. Quat.* **2007**, *20*, 163–184.
77. Vescovi, E. Long-Term Population Dynamics of Major Forest Trees under Strongly Changing Climatic Conditions. Ph.D. Thesis, University of Bern, Bern, Switzerland, 2007.
78. Kaltenrieder, P.; Belis, C.A.; Hofstetter, S.; Ammann, B.; Ravazzi, C.; Tinner, W. Environmental and climatic conditions at a potential glacial refugial site of tree species near the southern alpine glaciers. new insights from multiproxy sedimentary studies at Lago Della Costa (Euganean hills, northeastern Italy). *Quat. Sci. Rev.* **2009**, *28*, 2647–2662. [CrossRef]
79. Acquafredda, P.; Palmentola, G. Il glacialismo quaternario nell'Italia meridionale dal massiccio del Matese all'Aspromonte. *Biogeographia* **1986**, *10*, 13–18. [CrossRef]
80. Dramis, F.; Kotarba, A. Southern Limit of Relict Rock Glaciers, Central Apennines, Italy. *Permafr. Periglac.* **1992**, *3*, 257–260. [CrossRef]
81. Di Pietro, R.; Terzi, M.; Fortini, P. A revision of the high-altitude acidophilous and chionophilous grasslands of the Apennines (peninsular Italy), a long-lasting intricate syntaxonomic issue. *Phytocoenologia* **2017**, *47*, 261–304. [CrossRef]
82. Follieri, M.; Magri, D.; Sadori, L. 250,000-year pollen record from Valle Di Castiglione (Roma). *Pollen Spores* **1988**, *30*, 329–356.
83. Di Pasquale, G.; Saracino, A.; Bosso, L.; Russo, D.; Moroni, A.; Bonanomi, G.; Allevato, E. Coastal pine-oak glacial refugia in the Mediterranean basin: A biogeographic approach based on charcoal analysis and spatial modelling. *Forests* **2020**, *11*, 673. [CrossRef]
84. Lowe, J.J. Lateglacial and early holocene lake sediments from the northern Apennines, Italy—Pollen stratigraphy and radiocarbon dating. *Boreas* **1992**, *21*, 193–208. [CrossRef]
85. Bertoldi, R.; Chelli, A.; Roma, R.; Tellini, C. New data from northern Apennines (Italy) pollen sequences spanning the last 30,000 yrs. *Il Quat.* **2007**, *20*, 3–20.
86. Vescovi, E.; Ammann, B.; Ravazzi, C.; Tinner, W. A new late-glacial and holocene record of vegetation and fire history from Lago Del Greppo, northern Apennines, Italy. *Veget. Hist. Archaeobot.* **2010**, *19*, 219–233. [CrossRef]
87. Follieri, M.; Giardini, M.; Magri, D.; Sadori, L. Palynostratigraphy of the last glacial period in the volcanic region of central Italy. *Quatern. Int.* **1998**, *47*, 3–20. [CrossRef]
88. Magri, D. Late quaternary vegetation history at Lagaccione near Lago Di Bolsena (Central Italy). *Rev. Palaeobot. Palyno.* **1999**, *106*, 171–208. [CrossRef]
89. Magri, D.; Sadori, L. Late Pleistocene and Holocene pollen stratigraphy at Lago Di Vico, Central Italy. *Veg. Hist. Archaeobot.* **1999**, *8*, 247–260. [CrossRef]
90. Sadori, L. The lateglacial and holocene vegetation and climate history of Lago Di Mezzano (central Italy). *Quat. Sci. Rev.* **2018**, *202*, 30–44. [CrossRef]
91. Di Rita, F.; Anzidei, A.P.; Magri, D. A lateglacial and early holocene pollen record from Valle Di Castiglione (Rome): Vegetation dynamics and climate implications. *Quatern. Int.* **2013**, *288*, 73–80. [CrossRef]
92. Magri, D. Lineamenti della vegetazione tardoglaciale in Italia peninsulare e in Sicilia. In *Il Tardiglaciale in Italia–Lavori in Corso*; British Archaeological Reports, International Series 1859; Mussi, M., Ed.; Archaeopress: Oxford, UK, 2008; pp. 1–8, ISBN 1-4073-0342-2.
93. Mattioni, C.; Martin, M.A.; Chiocchini, F.; Cherubini, M.; Gaudet, M.; Pollegioni, P.; Velichkov, I.; Jarman, R.; Chambers, F.M.; Paule, L.; et al. Landscape genetics structure of european sweet chestnut (*Castanea Sativa* Mill): Indications for conservation priorities. *Tree Genet. Genomes* **2017**, *13*, 39. [CrossRef]
94. Mattioni, C.; Ranzino, L.; Cherubini, M.; Leonardi, L.; La Mantia, T.; Castellana, S.; Villani, F.; Simeone, M.C. Monuments unveiled: Genetic characterization of large old chestnut (*Castanea sativa* Mill.) trees using comparative nuclear and chloroplast DNA analysis. *Forests* **2020**, *11*, 1118. [CrossRef]
95. Mossa, L.; Bacchetta, G.; Brullo, S. *Quercus ichnusae* (Fagaceae), a new species from Sardinia. *Isr. J. Plant Sci.* **1999**, *47*, 199–207. [CrossRef]
96. Giuli, C.D.; Masini, F.; Valleri, G. Paleogeographic evolution of the adriatic area since oligocene to pleistocene. *Riv. Ital. Paleontol. Stratigr.* **1987**, *93*, 109–126. [CrossRef] [PubMed]
97. Di Pietro, R.; Fortini, P.; Ciaschetti, G.; Rosati, L.; Viciani, D.; Terzi, M. A revision of the syntaxonomy of the Apennine-Balkan *Quercus cerris* and *Q. frainetto* forests and correct application of the name *Melittio-Quercion frainetto*. *Plant Biosyst.* **2020**, *154*, 887–909. [CrossRef]
98. Fineschi, S.; Vendramin, G. Choroplastic diversity in italian oaks: Evidence of a higher genetic richness in southern and insular populations. *Forests* **2004**, *1*, 82. [CrossRef]
99. Kerkez Janković, I.; Jovanović, M.; Milovanović, J.; Aleksić, J.; Šijačić-Nikolić, M. Genetic diversity and genetic structure of three sympatric oak species in serbian landscape of outstanding features "Kosmaj" assessed by nuclear microsatellites. *Seefor* **2023**, *14*, 117–127. [CrossRef]

100. Fortini, P.; Viscosi, V.; Maiuro, L.; Fineschi, S.; Vendramin, G.G. Comparative leaf surface morphology and molecular data of five oaks of the subgenus *Quercus* Oerst (Fagaceae). *Plant Biosyst.* **2009**, *143*, 543–554. [CrossRef]
101. Blasi, C.; Di Pietro, R. Two New Phytosociological Types of *Quercus pubescens* s.l. woodland communities in southern Latium. *Plant Biosyst.* **1998**, *132*, 207–223. [CrossRef]
102. Allegrezza, M.; Baldoni, M.; Biondi, E.; Taffetani, F.; Zuccarello, V. Studio fitosociologico dei boschi a *Quercus pubescens* s.l. delle Marche e delle zone contigue dell'Appennino centro-settentrionale (Italia centrale). *Fitosociologia* **2002**, *39*, 161–171.
103. Blasi, C.; Di Pietro, R.; Filesi, L. Syntaxonomical revision of *Quercetalia pubescenti-Petraeae* in the italian peninsula. *Fitosociologia* **2004**, *41*, 87–164.
104. Schaal, B.A.; Hayworth, D.A.; Olsen, K.M.; Rauscher, J.T.; Smith, W.A. Phylogeographic studies in plants: Problems and prospects. *Mol. Ecol.* **1998**, *7*, 465–474. [CrossRef]
105. Andrello, M.; D'Aloia, C.; Dalongeville, A.; Escalante, M.A.; Guerrero, J.; Perrier, C.; Torres-Florez, J.P.; Xuereb, A.; Manel, S. Evolving spatial conservation prioritization with intraspecific genetic data. *Trends Ecol. Evol.* **2022**, *37*, 553–564. [CrossRef] [PubMed]
106. Fady, B.; Esposito, E.; Abulaila, K.; Aleksic, J.M.; Alia, R.; Alizoti, P.; Apostol, E.-N.; Aravanopoulos, P.; Ballian, D.; Kharrat, M.B.D.; et al. Forest Genetics Research in the Mediterranean Basin: Bibliometric Analysis, Knowledge Gaps, and Perspectives. *Curr. For. Rep.* **2022**, *8*, 277–298. [CrossRef]
107. Médail, F. Plant biogeography and vegetation patterns of the Mediterranean islands. *Bot. Rev.* **2022**, *88*, 63–129. [CrossRef]
108. Allen, C.D.; Macalady, A.K.; Chenchouni, H.; Bachelet, D.; McDowell, N.; Vennetier, M.; Kitzberger, T.; Rigling, A.; Breshears, D.D.; Hogg, E.H. (Ted); et al. A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *For. Ecol. Manag.* **2010**, *259*, 660–684. [CrossRef]
109. Natalini, F.; Alejano, R.; Vázquez-Piqué, J.; Cañellas, I.; Gea-Izquierdo, G. The role of climate change in the widespread mortality of holm oak in open woodlands of southwestern Spain. *Dendrochronologia* **2016**, *38*, 51–60. [CrossRef]
110. Conte, A.L.; Di Pietro, R.; Iamonico, D.; Di Marzio, P.; Cillis, G.; Lucia, D.; Fortini, P. Oak decline in the Mediterranean basin: A study case from the southern Apennines (Italy). *Plant Sociol.* **2019**, *56*, 69–80.
111. Blasi, C.; Capotorti, G.; Copiz, R.; Guida, D.; Barbara, M.; Smiraglia, D.; Zavattero, L. Terrestrial Ecoregions of Italy Explanatory Notes. Global Map S.r.l.: Firenze, Italy, 2019; ISBN 978-88-97091-04-2.

Historically, the taxonomy of the genus Quercus has undergone significant revisions, reflecting the complexity of this genus but also the advances in morphological analysis, molecular phylogenetics, and biogeographical studies. From the Nixon (1993) revision, that proposed a classification based primarily on morphological traits, grouping oaks into two subgenera, *Cyclobalanopsis* and *Quercus* (containing only the section Quercus that included white oaks, evergreen oaks and cerris oaks), the last phylogenetic analyses, have separated the genus *Quercus* into two principal clades: the white oaks (subgenus *Quercus*) and the cerris oaks (subgenus *Cerris*) (Denk et al., 2017). These phylogenetic studies incorporating molecular data, proposed by Hipp (2015) and Denk et al. (2017), have led to a revised framework that better aligns with the evolutionary history of the genus. These studies revealed that Nixon's system, while useful, did not fully capture the genetic divergence among oak lineages, necessitating a reassessment of subgeneric relationships. One of the major insights from Denk et al. (2017) was the confirmation that the subgenus *Cerris* forms a monophyletic clade, distinct from the white oaks (*Quercus* subgenus). This revision was driven by the discovery of deep genetic splits between these groups, likely dating back to the Miocene (~23–5 million years ago), a period of pronounced climatic fluctuations that shaped Mediterranean ecosystems. By integrating molecular markers from nuclear and plastid genomes, researchers demonstrated that *Cerris* species share unique genetic signatures, reproductive traits, and ecological adaptations that distinguish them from other oaks. Unlike white oaks, which exhibit rapid acorn germination and less protective cupules, *Cerris* oaks develop acorns over two growing seasons, with heavily scaled or spiny cupules that likely evolved as a defence mechanism against seed predation (Hipp et al., 2015; Denk et al., 2017).

Insight the Cerris clade, in terms of ecological relevance, one of the most important species for the Italian and Mediterranean basin is *Quercus cerris* L. (Turkey oak).

Turkey oak is a deciduous tree species, native of southeastern Europe and western Asia, that create the physiognomy of Mediterranean and temperate forest ecosystems (Figure 4). Its range extends in the Mediterranean basin from the Anatolian Peninsula in Turkey and Balkans to the Italian Peninsula, that represent the western limit of its natural distribution. It is commonly found at altitudes ranging from sea level to approximately 1,400 meters, where it grows in a variety of landscapes, including coastal, hill, and mountain regions (De Rigo et al., 2016). Turkey oak occupies a large ecological niche in these areas, thriving in temperate and sub-Mediterranean climates characterized by hot, dry summers and mild, wet winters (Blasi et al., 2004).

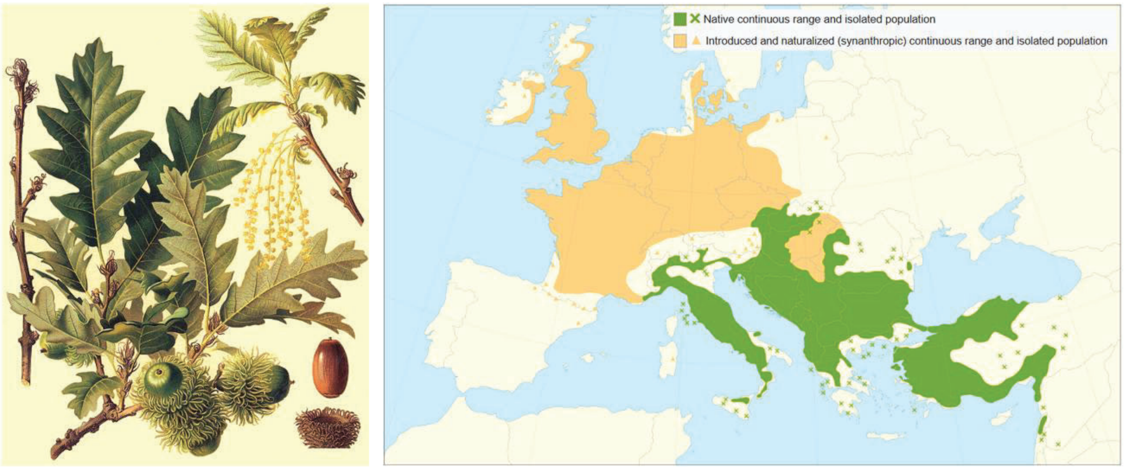


Figure 4 Distribution and diagnostics characteristics of Quercus cerris

In Asia, the species is primarily distributed in Turkey, where it forms extensive populations, particularly in the central and western regions of the country, and extends through the Caucasus, Armenia, and parts of Iran. In these areas, it grows alongside other Mediterranean and temperate species, establishing mixed forests that are rich in biodiversity. Its ability to adapt to diverse soil types and environmental conditions has facilitated its spread, though its range in Asia is limited compared to its European distribution.

In Europe, *Quercus cerris* thrives in a range of climatic conditions, where it contributes significantly to forest composition and structure. Its distribution spans from the Iberian Peninsula to the Balkans, including Greece and parts of southern Romania. In these regions, it is found in both coastal lowlands and higher elevations, frequently forming mixed oak forests with other species such as *Q. ilex* (holm oak), *Q. frainetto* (Italian oak), and *Q. pubescens* (pubescent oak). The presence of *Q. cerris* is also noted in various forest ecosystems, including those influenced by seasonal droughts and temperate rainfall, demonstrating its adaptability to both xeric and mesic environments (Guarino et al., 2015).

The species is widespread across the Italian Peninsula, predominantly in central and southern Italy, and is frequently found in association with other oaks such as *Q. pubescens* and *Q. frainetto* in mixed forest stands (Blasi et al., 2004). It is particularly abundant in the hilly and mountainous areas of the Apennine chain. The ecological role of *Q. cerris* in Italy is particularly pronounced in its adaptability to diverse soil types, ranging from sandy substrates to clay-rich soils, along with its resilience to seasonal droughts, highlights the remarkable plasticity and adaptability of *Q. cerris*. In addition, its capacity to thrive in dry, rocky soils and its resistance to drought stress, which is increasingly important in the context of climate change (Ameztegui et al., 2024). This broad ecological amplitude underscores its importance in maintaining biodiversity and ecological connectivity across heterogeneous landscapes playing a pivotal role in maintaining biodiversity in these areas, where it contributes to forest structure and ecological processes. (Blasi et al., 2004).

Climate change and global warming put at risk the conservation of this species and the entire Mediterranean biodiversity, threatening ecosystems and habitats. The

Mediterranean region is recognized as a biodiversity hotspot but is increasingly vulnerable to the climate change impacts, including rising temperatures, altered precipitation patterns, and extreme weather events (IPCC; 2019; Ameztegui et al. 2024). This vulnerability is enhanced by the built-up areas expansion, increasing the impact on the environmental and ecosystem functions. Large cities are the first to be affected; still, urbanization processes gradually affect smaller settlements and even remote rural villages (Antrop 2006). The expansion of small cities generally proceeds slowly and determines a hybrid urban landscape, where portions of natural forests or abandoned lands are often incorporated into urban tissue. In this context, urban and peri-urban forests are increasingly recognized as critical components of the landscape, playing a pivotal role in nature-based solutions (NBSs), providing a wide range of ecosystem services, regulating infiltration and stormwater runoff; mitigating microclimate extremes; reducing the urban heat island effect; combating soil, air, and water pollution; and limiting the spread of invasive species (Konijnendijk et al. 2006; De Groot et al. 2010; Dobbs et al. 2014; Livesley et al. 2016; Watts et al. 2017; Morabito et al. 2015).

In this context, *Quercus cerris* stands out as a species of strategic importance due to its ecological versatility and ability to tolerate environmental stressors. The species' adaptability, combined with its potential to thrive in urban settings, has led to its inclusion in reforestation initiatives under Italy’s National Recovery and Resilience Plan (PNRR). Furthermore, given that Italian populations are located at the westernmost limit of the species' natural range, the significant ecological pressures, environmental fluctuations and increasingly severe climatic stressors to which they are subjected, continuously tests their adaptive capacity (Pignatti et al., 2017).

My thesis focuses on the importance of monitoring the health status of this species across the Italian territory to provide insights into its response to climate change in both natural and urban environments. For these reasons during my PhD thesis I have investigated *Q. cerris* in different conditions. I have analysed the *Quercus cerris* seedling fitness in wood stands, developed in different lithological and physiographic conditions and subjected to different forestry structures and practices to understand the different adaptation strategies implemented by the seedlings. Moreover, I have evaluated the impact of environmental conditions on this species in urban, peri-urban and natural forests dominated by *Q. cerris*.

This thesis is focused on developing methods for assessing the health of *Quercus cerris* populations in urban and natural contexts through the analysis of leaf functional traits. By integrating traits widely described in the literature and combining it with pigment analysis, whose physiological role is known but whose approach as traits has not yet been investigated.

Plant functional traits are measurable morphological, physiological, or phenological features that influence a species’ ecological performance and responses to environmental conditions (Kraft et al. 2008; Pérez-Harguindeguy et al., 2013); Valladares et al. 2014; Li et al. 2015; Choat et al. 2018). Among these, leaf traits such as specific leaf area (SLA), leaf dry matter content (LDMC), and chlorophyll content, are particularly valuable indicators of tree health and stress tolerance (Castro-Díez et al. 2000; Garnier et al. 2001; Cornellisen et al. 2003; Pérez-Harguindeguy et al., 2013; Díaz et al. 2022).

Finally, the understanding of these species can be useful in order to protecting and creating green areas in cities, having native species that can better adapt to different environmental conditions, achieving a result that contemplates biodiversity at the service of citizens through ecosystem services (Bolund et al. 1999; Savard et al. 2000; Alvey 2006; Bailey et al. 2007; Capotorti et al. 2019; Lombardía et al. 2023; Gentili et al. 2024).

References

1. Nixon K.C Infrageneric classification of *Quercus* (Fagaceae) and typification of sectional names. *Ann Sci For* **1993** 50:25s–34s
2. Denk, T.; Grimm, G.W.; Manos, P.S.; Deng, M.; Hipp, A.L. An Updated Infrageneric Classification of the Oaks: Review of Previous Taxonomic Schemes and Synthesis of Evolutionary Patterns. In Oaks Physiological Ecology. Exploring the Functional Diversity of Genus Quercus L.; Gil-Pelegrín, E., Peguero-Pina, J.J., Sancho-Knapik, D., Eds.; Tree Physiology; Springer International Publishing: Cham, **2017**; pp. 13–38 ISBN 978-3-319-69099-5.
3. Hipp, A.L. Should hybridization make us skeptical of the oak phylogeny. *Int Oaks* **2015**, 26:9–17
4. De Rigo, D.; Enescu, C.M.; Houston Durrant, T.; Caudullo, G. Quercus Cerris in Europe: Distribution, Habitat, Usage and Threats. *Eur. Atlas For. Tree Species San-Miguel-Ayanz J Rigo Caudullo G Houst. Durrant T Mauri Eds* **2016**, 148–149.
5. Blasi, C., Marignani, M., & Rosati, L. Mediterranean vegetation types and Quaternary refuges in the Italian Peninsula. *J Biogeogr*. **2004** 31(3), 423–436.
6. Guarino, R.; Bazan, G.; Paura, B. Downy-Oak Woods of Italy: Phytogeographical Remarks on a Controversial Taxonomic and Ecologic Issue. In *Warm-Temperate Deciduous Forests around the Northern Hemisphere*; Box, E.O., Fujiwara, K., Eds.; Geobotany Studies; Springer International Publishing: Cham, 2015; pp. 139–151 ISBN 978-3-319-01261-2.
7. 2019 Refinement to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories — IPCC.
8. Ameztegui, A.; Coll, L.; Cáceres, M.D.; Morán-Ordóñez, A. Disturbance Impacts on Mediterranean Forests across Climate and Management Scenarios. *J. Environ. Manage.* **2024**, *371*, 123193, doi:10.1016/j.jenvman.2024.123193.
9. Antrop, M. Landscape Change and the Urbanization Process in Europe. *Landsc. Urban Plan.* **2004**, *67*, 9–26.
10. Konijnendijk, C.C.; Ricard, R.M.; Kenney, A.; Randrup, T.B. Defining Urban Forestry – A Comparative Perspective of North America and Europe. *Urban For. Urban Green.* **2006**, *4*, 93–103, doi:10.1016/j.ufug.2005.11.003.
11. De Groot, R.S.; Alkemade, R.; Braat, L.; Hein, L.; Willemen, L. Challenges in Integrating the Concept of Ecosystem Services and Values in Landscape Planning, Management and Decision Making. *Ecol. Complex.* **2010**, *7*, 260–272, doi:10.1016/j.ecocom.2009.10.006.
12. Dobbs, C.; Kendal, D.; Nitschke, C.R. Multiple Ecosystem Services and Disservices of the Urban Forest Establishing Their Connections with Landscape Structure and Sociodemographics. *Ecol. Indic.* **2014**, *43*, 44–55, doi:10.1016/j.ecolind.2014.02.007.
13. Livesley, S.J.; McPherson, E.G.; Calfapietra, C. The Urban Forest and Ecosystem Services: Impacts on Urban Water, Heat, and Pollution Cycles at the Tree, Street, and City Scale. *J. Environ. Qual.* **2016**, *45*, 119–124, doi:10.2134/jeq2015.11.0567.

14. Watts, N.; Adger, W.N.; Ayeb-Karlsson, S.; Bai, Y.; Byass, P.; Campbell-Lendrum, D.; Colbourn, T.; Cox, P.; Davies, M.; Depledge, M.; et al. The Lancet Countdown: Tracking Progress on Health and Climate Change. *Lancet Lond. Engl.* **2017**, *389*, 1151–1164, doi:10.1016/S0140-6736(16)32124-9.

15. Morabito, M.; Crisci, A.; Gioli, B.; Gualtieri, G.; Toscano, P.; Stefano, V.D.; Orlandini, S.; Gensini, G.F. Urban-Hazard Risk Analysis: Mapping of Heat-Related Risks in the Elderly in Major Italian Cities. *PLOS ONE* **2015**, *10*, e0127277, doi:10.1371/journal.pone.0127277.

16. Pignatti, S.; Guarino, R.; La Rosa, M. *Flora d'italia*; 2nd ed.; Edagricole: Bologna, 2017; Vol. 2; ISBN 88-506-5242-9.

17. Kraft, N.J.B.; Valencia, R.; Ackerly, D.D. Functional Traits and Niche-Based Tree Community Assembly in an Amazonian Forest. *Science* **2008**, *322*, 580–582, doi:10.1126/science.1160662.

18. Pérez-Harguindeguy, N., Díaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., Bret-Harte, M. S., Cornwell, W. K., Craine, J. M., Gurvich, D. E., Urcelay, C., Veneklaas, E. J., Reich, P. B., Poorter, L., Wright, I. J., Ray, P., Enrico, L., Pausas, J. G., de Vos, A. C., Buchmann, N., Funes, G., Quétier, F., Hodgson, J. G., Thompson, K., Morgan, H. D., ter Steege, H., van der Heijden, M. G. A., Sack, L., Blonder, B., Poschlod, P., Vaieretti, M. V., Conti, G., Staver, A. C., Aquino, S., Cornelissen, J. H. C. New handbook for standardised measurement of plant functional traits worldwide. *Australian Journal of Botany* **2013**, 61(3), 167–234. https://doi.org/10.1071/BT12225

19. Valladares, F.; Matesanz, S.; Guilhaumon, F.; Araújo, M.B.; Balaguer, L.; Benito-Garzón, M.; Cornwell, W.; Gianoli, E.; van Kleunen, M.; Naya, D.E.; et al. The Effects of Phenotypic Plasticity and Local Adaptation on Forecasts of Species Range Shifts under Climate Change. *Ecol. Lett.* **2014**, *17*, 1351–1364, doi:10.1111/ele.12348.

20. Li, R.; Zhu, S.; Chen, H.Y.H.; John, R.; Zhou, G.; Zhang, D.; Zhang, Q.; Ye, Q. Are Functional Traits a Good Predictor of Global Change Impacts on Tree Species Abundance Dynamics in a Subtropical Forest? *Ecol. Lett.* **2015**, *18*, 1181–1189, doi:10.1111/ele.12497.

21. Choat, B.; Brodribb, T.J.; Brodersen, C.R.; Duursma, R.A.; López, R.; Medlyn, B.E. Triggers of Tree Mortality under Drought. *Nature* **2018**, *558*, 531–539, doi:10.1038/s41586-018-0240-x.

22. Castro-Díez, P.; Puyravaud, J.P.; Cornelissen, J.H.C. Leaf Structure and Anatomy as Related to Leaf Mass per Area Variation in Seedlings of a Wide Range of Woody Plant Species and Types. *Oecologia* **2000**, *124*, 476–486, doi:10.1007/PL00008873.

23. Garnier, E.; Shipley, B.; Roumet, C.; Laurent, G. A Standardized Protocol for the Determination of Specific Leaf Area and Leaf Dry Matter Content. *Funct. Ecol.* **2001**, *15*, 688–695, doi:10.1046/j.0269-8463.2001.00563.x.

24. Cornelissen, J. h. c.; Cerabolini, B.; Castro-Díez, P.; Villar-Salvador, P.; Montserrat-Martí, G.; Puyravaud, J. p.; Maestro, M.; Werger, M. j. a.; Aerts, R. Functional Traits of Woody Plants: Correspondence of Species Rankings between Field Adults and Laboratory-Grown Seedlings? *J. Veg. Sci.* **2003**, *14*, 311–322, doi:10.1111/j.1654-1103.2003.tb02157.x.

25. Díaz, S.; Kattge, J.; Cornelissen, J.H.C.; Wright, I.J.; Lavorel, S.; Dray, S.; Reu, B.; Kleyer, M.; Wirth, C.; Prentice, I.C.; et al. The Global Spectrum of Plant Form and Function: Enhanced Species-Level Trait Dataset. *Sci. Data* **2022**, *9*, 755, doi:10.1038/s41597-022-01774-9.

26. Bolund, P.; Hunhammar, S. Ecosystem Services in Urban Areas. *Ecol. Econ.* **1999**, *29*, 293–301, doi:10.1016/S0921-8009(99)00013-0.

27. Savard, J.-P.L.; Clergeau, P.; Mennechez, G. Biodiversity Concepts and Urban Ecosystems. *Landsc. Urban Plan.* **2000**, *48*, 131–142, doi:10.1016/S0169-2046(00)00037-2.

28. Alvey, A.A. Promoting and Preserving Biodiversity in the Urban Forest. *Urban For. Urban Green.* **2006**, *5*, 195–201, doi:10.1016/j.ufug.2006.09.003.

29. Bailey, S. Increasing Connectivity in Fragmented Landscapes: An Investigation of Evidence for Biodiversity Gain in Woodlands. *For. Ecol. Manag.* **2007**, *238*, 7–23, doi:10.1016/j.foreco.2006.09.049.

30. Capotorti, G.; Mollo, B.; Zavattero, L.; Anzellotti, I.; Celesti-Grapow, L. Setting Priorities for Urban Forest Planning. A Comprehensive Response to Ecological and Social Needs for the Metropolitan Area of Rome (Italy). *Sustainability* **2015**, *7*, 3958–3976, doi:10.3390/su7043958.

31. Gentili, R.; Quaglini, L.A.; Galasso, G.; Montagnani, C.; Caronni, S.; Cardarelli, E.; Citterio, S. Urban Refugia Sheltering Biodiversity across World Cities. *Urban Ecosyst.* **2024**, *27*, 219–230, doi:10.1007/s11252-023-01432-x.

Plant Sociology 59(1) 2022, 11–24 | DOI 10.3897/pls2022591/02

Topical Collection: "Species and Community Variability"

PENSOFT.

Società Italiana di Scienza
della Vegetazione (SISV)

Analysis of the functional traits of *Quercus cerris* L. seedlings in the Molise region (southern Italy)

Luca Quaranta[1], Piera Di Marzio[1], Romeo Di Pietro[2], Fabrizio Ferretti[3], Umberto Di Salvatore[4], Paola Fortini[1]

1 *Museo Erbario, Department of Bioscience and Territory, University of Molise, Pesche, Italy*
2 *Department of Planning, Design and Architecture Technology, Sapienza Università di Roma, Roma, Italy*
3 *CREA Research Centre for Forestry and Wood Azienda "Vivaio Feudozzo" – "Tenuta San Pietro Avellana, Isernia (IS), Italy*
4 *CREA Research Centre for Politics and Bioeconomy, Via Lombardia 7, I-65012 Cepagatti, PE, Italy*

Corresponding author: Piera Di Marzio (piera.dimarzio@unimol.it)

Subject editor: Fotios Xystrakis ♦ **Received** 29 November 2021 ♦ **Accepted** 25 January 2022 ♦ **Published** 16 May 2022

Abstract

This study deals with the analysis of seedling fitness in three *Quercus cerris* wood stands, namely Selva di Castiglione (SC), Bosco della Ficora (BF) and Bosco di San Leo (BSL), developed in different lithological and physiographic conditions and subjected to different forestry practices. A phytosociological study was carried out for analysing the coenological features of the forest stands and to classify these latter from a syntaxonomic point of view. The Pignatti-Ellenberg index (PEi) was calculated on the matrix composed of the phytosociological relevés in order to highlight possible ecological differences or gradients among stands. The phenotypic parameters considered were the stem and root length and the leaf area, whereas the plant functional traits (PFTs) were specific leaf area (SLA), leaf dry matter content (LDMC), leaf thickness (Lth) and chlorophyll content (CHL). The results showed that seedlings coming from different sampling sites exhibited similar values in all the phenotypic parameters. Instead, statistically significant differences were observed in the PFTs. The results suggested that the different adaptation strategies implemented by the seedlings are to be related to the physical environment of the sampling sites and to the different forest structures. The Selva di Castiglione forest stand (SC) exhibited better growth conditions for seedlings testified by higher values of SLA and CHL and lower values of LDMC and Lth. These were interpreted as greater investment in carbon production aimed at rapid development and renewal of the seedling rather than carbon storage aimed at ensuring leaf longevity.

Keywords

functional trait, high forest, oak, phenotypic response, phytosociology, southern Apennines

Introduction

Quercus cerris L. (Turkey oak, Fagaceae) acts as a dominant species in a large part of the oak forests of central and southern Italy (Blasi et al. 2004; Di Pietro et al. 2020b; Terzi et al. 2021). In the Molise region, the *Q. cerris* forests are widely distributed within the hilly, submontane and lower montane belt, ranging in altitude between 300 and 1200 m a.s.l. (Blasi and Paura 1995; Blasi et al. 2004, 2005). In particular, a wide *Q. cerris* belt separates thermophilous mixed oak woods with *Q. cerris*, *Q. pubescens* Willd. and *Q. frainetto* Ten. of the hilly belt from the beech woods of the montane belt. The *Q. cerris* belt is linked to Flysch substrates or the occurrence of large karst basins on limestone bordered by mild slopes covered by leached rich in clay soils. However, the abundance of *Q. cerris* woods in the Molise region is also due to the application of forestry management (e.g., different forest systems and silvicultural systems) that have advantaged this species over other tree species (e.g., *Q. petraea* (Matt.) Liebl. and *Q. frainetto*). The high-forest system of the Turkey oak woods, which is typically adopted in many parts of Italy and especially in the Molise region, finds its historical reason in the production of railway sleepers and, only marginally, of firewood (Di Martino 1996). Owing to the importance of Turkey oak for the regional economy, and paying attention to the ongoing climate change, we carried out an in-situ study aimed at identifying possible phenotypic response and plant functional traits (PFTs) variations from *Q. cerris* seedlings coming from wood stands characterised by different environmental conditions and forestry treatment. PFT variations have proved to be useful for establishing ecological and evolutionary trends for plant species and communities. They have often been used to interpret plant-environment relationships, to make previsions on possible consequences and to quantify a wide range of natural and/or human-driven processes in terms of species or community ecology (Pèrez-Harguindeguy et al. 2013; Adler et al. 2014; Pérez-Ramos et al. 2019). The measurements of the functionality patterns of seedlings of *Q. cerris* growing under different forest management, could provide useful information on the health state of the woods as well as address forest management policies in the next future. Surprisingly the available literature on such studies (i.e., observations in situ on *Q. cerris* seedlings) is relatively scarce in Italy. Most of the ecological studies performed on *Q. cerris* dealt with adult trees (e.g., Gratani and Foti 1998) or analysed, in greenhouses or under controlled conditions, the response of woody species seedlings to various types of induced stress (Otieno et al. 2005; Aref et al. 2013; Karavin et al. 2013; Chiatante et al. 2015; Abdala-Roberts et al. 2018; Torres-Ruis et al. 2019). Other studies have focused on the phenotypic plasticity of a species as a measure of its adaptability and fitness to environmental and climatic changes (Valladares et al. 2000, 2014; Pérez-Ramos et al. 2019). Field studies on the vitality of plants using PFTs, however, are more frequently applied to grassland communities (Bolzan et al. 2007; Catorci et al. 2013; Chelli et al. 2019; Baltieri et al. 2020) than to forest ones.

This study is part of an interdisciplinary and broader line of research that has been undertaken by the Laboratory of Systematic Botany and Floristics of the University of Molise in the last decade, which aims to shed light on the morphological, ecological, and bio-molecular features of *Quercus* species (Antonecchia et al. 2015; Fortini et al. 2015a, 2015b; Di Pietro et al. 2016; Conte et al. 2019; Di Pietro et al. 2020a, 2020c; 2021). Specifically, the present paper aims to evaluate the response of *Quercus cerris* seedlings to different ecological and forest management conditions, using PFTs and the analysis of morphological trait variations.

Materials and methods

Study area

The study was carried out in three *Quercus cerris* woods located in the submontane-lower montane belt of the Molise Region (Italy), namely Selva di Castiglione (SC), Bosco di San Leo (BSL), Bosco della Ficora (BF) (Fig. 1). According to Blasi et al. (2010) and Rivas-Martínez et al. (2011), the macro-bioclimate of the study area can be defined as temperate oceanic/sub-Mediterranean while based on the classifications of the Ecoregions of Italy (Blasi et al. 2018), the study area falls under code 1C3a1. The code identifies the territory according to the following characteristics: 1 = Temperate Division, C = Apennine Province, 3 = Southern Apennine Section, a = Campanian Apennine Subsection. The first wood stand (Selva di Castiglione–SC) is developed within the bottom of a plain on clayey substrates, while the other two stands (Bosco di San Leo–BSL and Bosco della Ficora–BF) are located within slopes with declivity on arenaceous-pelithic substrate. Physiographic information on the sites of collection is summarised in Table 1.

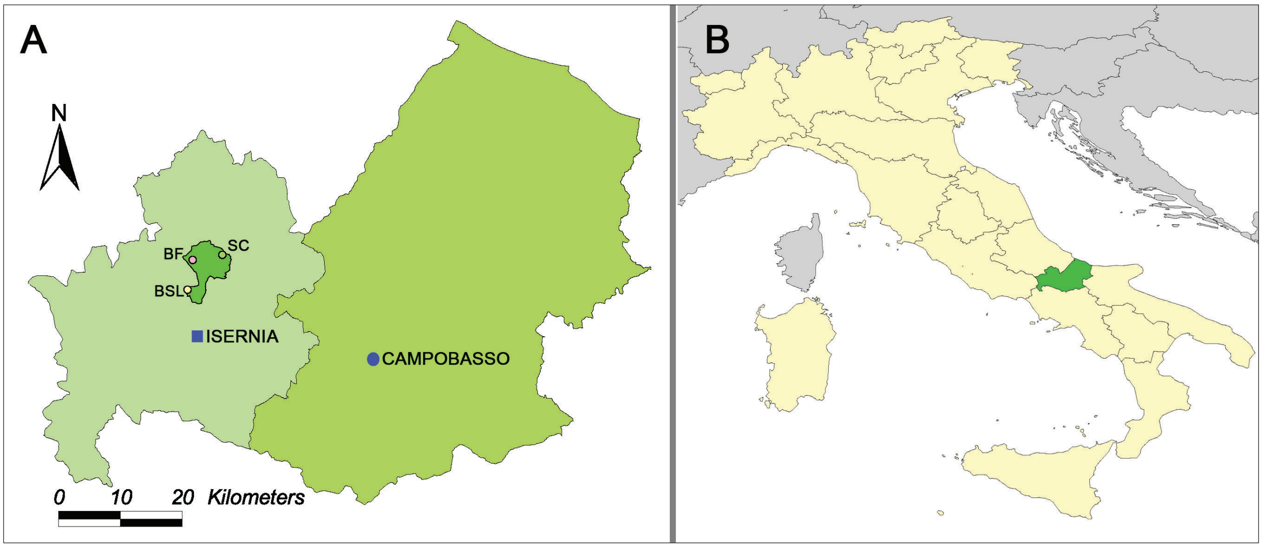


Figure 1. A) Location of the three sampled sites (Municipality of Carovilli) in the Province of Isernia in the Molise Region. BF = Bosco della Ficora, BSL = Bosco di San Leo, SC = Selva di Castiglione. B) Location of the Molise Region in Italy.

Table1. Characteristics of the sampled sites (Cantiani et al. 2010).

	Coordinate (DMS)	Stand development	Age	Altitude (m a.s.l.)	Aspect	Slope (%)	Substrate
Bosco di San Leo (BSL)	41°40’33”N 14°15’18”E	Mature high forest	135	810	NW	15	arenaceous-pelithic
Bosco della Ficora (BF)	41°42’55”N 14°18’55”E	Young high forest	55	920	N-NW	15	arenaceous-pelithic
Selva di Castiglione (SC)	41°44’12”N 14°18’50”E	Adult high forest	90	1000	-	0	varicoloured clay

Silvicultural description

The sampled stands have already been the subject of experimentation on the *Q. cerris* forests by the Council for Research and Analysis of Agricultural Economics (CREA). From a forestry point of view, the three stands have different histories and are currently passing through different silvicultural stages. The BSL stand is an old forest (135 years) and is a result of a natural regeneration stage starting from a senescent high forest subjected to thinning interventions. The average diameter of Turkey oak individuals ranges between 30 and 35 cm. The BF stand is a young high forest (average age 55 years), which having never been subjected to silvicultural interventions, is currently characterised by an excessive density of tree individuals. The SC stand is an adult forest (average age ranging between 60 and 100 years). In 2006 and 2007, the following silvicultural treatments were applied to the three forest stands (Cantiani et al. 2010): BSL, shelterwood cutting (seed-tree); SC, high thinning (grade: heavy) and BF, selective thinning.

Data and methods

To obtain coenological information on the three *Q. cerris* stands a field-sampling campaign was carried out during the 2019 summer period using the phytosociological approach (Braun-Blanquet 1964). Species and syntaxa nomenclature in the phytosociological table followed Bartolucci et al. (2018) and Biondi et al. (2014), respectively. The following Ellenberg indicator values (L - light availability; T - temperature) and edaphic factors (U - soil moisture; N - soil nutrient) as reported in Pignatti (2005) were also considered to infer possible ecological differences between the three *Q. cerris* stands investigated. For the indicator values that were missing for some species in Pignatti (2005), reference was made to Sürmen et al. (2014). Subsequently, Pignatti-Ellenberg index was applied to the matrix of phytosociological relevés by calculating the weighted averaging using species abundances to evaluate the average response of each forest community to the abiotic factors associated with Ellenberg indicator values, following what is reported in Gristina and Marcenò (2008) from the unpublished data of Pignatti and Bona (2007).

The in-field collection of *Quercus cerris* seedlings followed mainly the protocol reported in Gottardini et al. (2016) according to the following steps: 1) identification of a sampling stand, with a random collection of thirty 3 to 4-year-old seedlings, starting from the centre of the site and maintaining a distance of 6–7 m between each seedling collected, choosing seedlings with fully expanded leaves free from damage from herbivores or pathogens (Garnier et al. 2001); 2) eradication of the seedlings by taking an entire clod of soil with a spade up to 30 cm deep and subsequent removing of the soil from the root through water; 3) measurements of the soil pH (classes of pH soil according to National Soil Survey Center 1998) precisely where the individual seedlings were eradicated; 4) measurement of the bulk density of the soil through a metal cylinder (classes of bulk density soil according to National Soil Survey Center 1998); 5) application of a damp cotton cover to each seedling and store it in dark in the cooler and 6) measurements of the chlorophyll leaf content by the at LEAF CHL PLUS instrument (FT Green LLC, Wilmington, US), whose function is comparable to the SPAD 502 (Minolta) (Zhu et al. 2012; Novichonok et al. 2016). The measurements were repeated three times in the central part of the lamina of three leaves directly in the field.

The first stage of field sampling was carried out during three consecutive days (24, 25 and 26 June 2019) under the same climatic conditions (no wind and no clouds). A total of 90 seedlings were collected and further analysed. The chlorophyll contents of the leaves of the seedlings were determined in August 2019 for 30 different seedlings in each stand due to difficulties in obtaining the instrumentation for measuring the chlorophyll content during the June 2019 field campaign. Laboratory measurements followed Pérez-Harguindeguy et al. (2013). Stem length, root length and leaf area of each seedling were measured on fresh materials. Three leaves for each seedling were fixed on a sheet and scanned using the Epson GT15000 scanner, and the leaf area (LA, cm^2) was calculated using the ImageJ software (Rasband 1997–2018). Fresh leaf mass saturated (LFMsat.) was calculated (after seven hours from the collection) and dry leaf mass (LDM), was calculated after drying leaves for 72 h at 60 °C until a stable weight was achieved. The leaf measures were used to evaluate the following plant functional traits: specific leaf area (SLA), leaf

Table 2. Description of the functional traits analysed (Pérez-Harguindeguy et al. 2013). §: the dimensionless value is converted into the total chlorophyll value (mg/cm2) using the conversion table https://www.atleaf.com/SPAD.

PFTs	Unit	Formula
Specific leaf area (SLA): is the area of a fresh leaf, divided by its oven-dry mass	cm^2/g	LA/LDM
Leaf dry matter content (LDMC): is the oven-dry mass of a leaf, divided by its water-saturated fresh mass	mg/g	LDM/LFMsat.
Leaf thickness (Lth)	-	1/SLA*LDMC
Leaf water content (LWC)	-	1000-LDMC
Leaf mass per area (LMA) is the oven-dry mass of a leaf, divided by its leaf area	g/cm^2	1/SLA
Leaf chlorophyll content (CHL)	mg/cm^2	§

dry matter content (LDMC), leaf thickness (Lth), leaf water content (LWC) and leaf mass per area (LMA) (Table 2).

It should be noted (Table 2) that some of the PFTs investigated express approximately the same concept providing redundant information (e.g., SLA/LMA or LDMC /LWC). Accordingly, we have decided to make a further selection considering three PFTs only, namely SLA, LDMC and Lth. A spreadsheet (Excel 2016) containing all the field and laboratory data was arranged and processed with XLSTAT 2020 3.1.3 software (Addinsoft 2005–2020). The data underpinning the analysis reported in this paper are deposited at "Mendeley Data" at https://doi.org/10.17632/6m2d4rxc7n.1. All the data were previously subjected to a normality check and then to a non-parametric comparison analysis, according to the Kruskal-Wallis method, with a paired comparison according to the Dunn method (with Bonferroni correction for significance, set at alpha = 0.05).

Results

Phytosociological survey

No published phytosociological relevés or previous syntaxonomic interpretation were available for the study area. All the three wood stands were assigned to a single association, *Roso arvensis-Quercetum cerridis* Ubaldi 2003 (syn: *Aremonio agrimonioidis-Quercetum cerridis* Blasi et al. 2005 ex Terzi et al. 2021). The phytosociological relevés (Table 3) showed some differences among the three sampled sites. Bosco della Ficora (BF) and Selva di Castiglione (SC) woods are characterised by a tree layer composed of *Quercus cerris* and *Carpinus betulus*. In central Europe, the abundance of *Carpinus betulus* is considered as favoured by clumsy cutting forest practices that lead the climax *Quercus petraea* and *Q. robur* forest to be fragmented and subsequently invaded by the hornbeam (Oberdorfer, 1992). In the case of our study area, the occurrence of *Carpinus betulus* in the dominant tree layer is a sign of moist and eutrophic soil conditions and a low degree of disturbance (Blasi et al. 2005). Indeed, the abundance of *Carpinus betulus* sharply decreases passing from northern Italy to southern Italy and this species normally does not participate in recolonisation stages after oak forest coppicing where it is substituted by *Fraxinus ornus, Acer opalus* subsp. *obtusatum*, *Corylus avellana* and *Ostrya carpinifolia* (the latter especially on limestone or calcareous marls). The BF and the SC woods show a mesophilous-microthermic aspect due to the presence of several *Fagetalia sylvaticae* species such as *Asperula taurina*, *Euphorbia amygdaloides*, *Crataegus laevigata*, *Cardamine bulbifera*, *Neottia nidus-avis*, *Euphorbia dulcis*, *Viola reichenbachiana* (Di Pietro et al. 2004). In one relevé from the BF wood, *Abies alba* also occurs. The SC stand exhibits abundance of species typical of the *Quercus cerris* undergrowth such as *Fragaria vesca*, *Festuca heterophylla*, *Allium pendulinum* and *Luzula forsteri*. Bosco di San Leo wood (BSL) acts as a more thermophilous *Q. cerris* wood, characterised by a thick secondary tree layer dominated by *Carpinus orientalis*, *Fraxinus ornus* and *Acer campestre* and a shrub layer dominated by *Cornus sanguinea*, *Ligustrum vulgare*, *Hedera helix* and *Rubus ulmifolius* (Taffetani et al. 2012). In this paper, we have classified the *Roso arvensis-Quercetum cerridis* in the alliance *Erythronio-Carpinion betuli* (*Fagetalia sylvaticae*) due to the occurrence of several *Fagetalia* species. However, this classification should be considered preliminary. A still unresolved debate is open on the classification of the *Quercus cerris* woods of the montane belt of central-southern Italy both at the class level (*Carpino-Fagetea* vs. *Quercetea pubescentis*) and at the alliance level (*Erythronio-Carpinion* vs. *Physospermo-Quercion cerridis* vs. *Melittio-Quercion frainetto* vs *Geranio striati-Fagion*). As this is not the paper in which to discuss this issue, we refer to the following bibliography: Biondi et al. 2002; Ubaldi 2003; Blasi et al. 2005; Di Pietro and Fascetti 2005; Rosati et al. 2005; Biondi et al. 2008, 2014; Mucina et al. 2017 and Di Pietro et al. 2020.

Syntaxonomic framework

QUERCO ROBORIS-FAGETEA SYLVATICAE Br.-Bl. et Vlieger in Vlieger 1937
FAGETALIA SYLVATICAE Pawlowski in Pawlowski, Sokolowski et Wallish 1928
Erythronio-Carpinion betuli (Horvat, 1958) Marincek in Wallnofer, Mucina et Grass 1993
Roso arvensis-Quercetum cerridis Ubaldi 2003

Table 3. *Roso arvensis-Quercetum cerridis* Ubaldi 2003.

Date	6/6/2019	6/6/2019	6/6/2019	6/6/2019	7/22/2010	7/22/2010	6/25/2019	
Forest Stand acronym	SC	SC	BF	BF	BSL	BSL	BSL	
Relevé number	1	2	3	4	5	6	7	
Aspect	-	-	NNW	E	N	NNE	NNE	
Elevation (m a.s.l.)	980	980	920	890	810	810	810	
Slope (%)	-	-	10	5	5	10	5	
Soil surface aspect, rocky outcrops (%)	-	-	-	-	-	-	-	
Soil surface aspect, stoniness (%)	-	-	-	-	1	-	-	
Upper tree layer cover (%)	65	65	55	70	65	70	50	
Upper tree layer height (m)	20	20	22	17	18	20	28	
Lower tree layer cover (%)	80	50	40	30	30	20	70	
Lower tree layer height (m)	2.5	2.5	3.4	5.6	4,5	6	4	
Shrubs layer cover (%)	-	-	-	10	50	40	90	
Shrubs layer height (%)	-	-	-	2	1.5	2	2	
Herbaceous layer cover (%)	60	40	95	75	30	40	10	
Area (m^2)	200	200	200	200	200	200	300	
Coverage (%)	100	80	90	90	100	100	100	
Species per relevé	26	26	35	26	22	22	24	frq.
***Roso arvensis-Quercetum cerridis* Ubaldi 2003**								
Rosa arvensis	1	+	1	1	2	+	1	V
Ligustrum vulgare	1	2	1	2	2	2	2	V
Lonicera caprifolium	2	2	2	2	+	+	.	V
Crataegus laevigata	+	.	.	.	.	.	1	II
***Erythronio-Carpinion betuli* (Horvat 1958) Marincek in Wallnofer, Mucina & Grass 1993**								
Carpinus betulus	4	3	2	2	.	+	.	IV
Primula vulgaris	+	1	+	.	1	+	.	IV
Cardamine bulbifera	2	2	+	+	.	.	.	III
Pulmonaria apennina	+	.	+	.	.	+	.	III
Viola odorata	.	.	.	.	+	.	.	I
Carex sylvatica	.	.	.	.	.	.	1	I
***Geranio versicoloris-Fagion* Gentile 1970**								
Cyclamen hederifolium	+	+	.	+	.	.	.	III
Allium pendulinum	2	1	+	.	.	.	.	III
Anemone apennina	+	+	+	+	.	.	.	III
Ranunculus lanuginosus	.	.	+	.	1	.	.	II
Geranium versicolor	.	.	+	.	.	.	.	I
Lathyrus venetus	.	.	.	+	.	.	.	I
***Fagetalia sylvaticae* Pawłowski in Pawłowski, Sokołowski & Wallisch 1928**								
Euphorbia amygdaloides	.	.	2	.	.	.	1	II
Euphorbia dulcis	.	.	+	+	.	.	.	II
Fraxinus excelsior	+							
Asperula taurina	.	.	1	.	.	.	.	I
Neottia nidus-avis	.	.	+	.	.	.	.	I
Mycelis muralis	.	+	.	.	.	.	.	I
Galium odoratum	.	.	.	.	.	+	.	I
Abies alba	.	.	.	1	.	.	.	I
***Quercetalia pubescenti-petraeae* Klika 1933**								
Brachypodium rupestre	1	1	2	1	.	.	+	IV
Sorbus torminalis	.	+	.	.	.	1	+	III
Cornus mas	.	.	1	+	.	.	1	III
Fraxinus ornus	.	.	.	+	.	1	1	III
Carpinus orientalis	.	.	.	.	1	2	2	III
Veronica chamaedrys	+	+	.	.	.	.	.	II
Aegonychon purpurocaeruleum	.	.	2	.	.	.	.	I
Helleborus foetidus	.	.	.	.	.	+	.	I
Scutellaria columnae	.	.	.	.	.	.	1	I
***Querco-Fagetea* Br.-Bl. & Vlieger in Vlieger 1937**								
Quercus cerris	4	3	3	4	4	4	4	V
Hedera helix	1	1	1	1	2	3	1	V
Rubus hirtus	+	1	2	3	+	+	.	V
Daphne laureola	1	+	+	.	+	+	.	IV
Acer campestre	.	.	2	2	2	1	3	IV
Ajuga reptans	+	1	.	.	+	.	1	III
Festuca heterophylla	2	2	1	+	.	.	.	III
Crataegus monogyna	.	.	1	2	+	.	+	III

Table 3. Continuation.

Date	6/6/2019	6/6/2019	6/6/2019	6/6/2019	7/22/2010	7/22/2010	6/25/2019	
Forest Stand acronym	SC	SC	BF	BF	BSL	BSL	BSL	
Relevé number	1	2	3	4	5	6	7	
Aspect	-	-	NNW	E	N	NNE	NNE	
Elevation (m a.s.l.)	980	980	920	890	810	810	810	
Slope (%)	-	-	10	5	5	10	5	
Soil surface aspect, rocky outcrops (%)	-	-	-	-	-	-	-	
Soil surface aspect, stoniness (%)	-	-	-	-	1	-	-	
Upper tree layer cover (%)	65	65	55	70	65	70	50	
Upper tree layer height (m)	20	20	22	17	18	20	28	
Lower tree layer cover (%)	80	50	40	30	30	20	70	
Lower tree layer height (m)	2.5	2.5	3.4	5.6	4,5	6	4	
Shrubs layer cover (%)	-	-	-	10	50	40	90	
Shrubs layer height (%)	-	-	-	2	1.5	2	2	
Herbaceous layer cover (%)	60	40	95	75	30	40	10	
Area (m^2)	200	200	200	200	200	200	300	
Coverage (%)	100	80	90	90	100	100	100	
Species per relevé	26	26	35	26	22	22	24	frq.
Fragaria vesca	2	2	+	.	.	.	.	III
Cruciata glabra	+	+	+	+	.	.	.	III
Luzula forsteri	1	1	.	.	+	.	.	III
Dioscorea communis	.	+	+	.	.	.	.	II
Digitalis micrantha	+	.	.	.	.	.	.	I
Lilium bulbiferum	.	1	.	.	.	.	.	I
Melica uniflora	.	.	.	+	.	.	.	I
Bromopsis ramosa	.	.	.	.	+	.	.	I
Campanula trachelium	.	.	.	.	.	.	1	I
Epipactis helleborine	.	.	.	.	.	.	+	I
***Rhamno-Prunetea* Rivas Goday & Borja ex Tuxen 1962**								
Euonymus europaeus	.	.	1	1	+	1	.	III
Cornus sanguinea	.	.	.	.	3	2	1	III
Pyrus communis	.	.	+	+	2	.	.	III
Clematis vitalba	.	.	1	.	.	.	1	II
Prunus spinosa	.	.	.	.	1	+	.	II
Rubus umifolius	.	.	.	.	.	1	3	II
Ulmus minor	.	.	.	.	.	.	1	I
Other species								
Agrimonia eupatoria	.	.	.	+	+	+	.	III
Carex remota	.	.	.	.	1	.	1	II
Hieracium gr. murorum	+	.	.	.	.	.	.	I
Malva thuringiaca	.	.	+	.	.	.	.	I
Campanula glomerata	.	.	.	.	.	.	+	I
Dactylis glomerata	.	+	.	.	.	.	.	I
Astragalus glyciphyllos	.	.	+	.	.	.	.	I
Carex sylvatica	.	.	1	.	.	.	.	I
Arum italicum	.	.	.	+	.	.	.	I
Orobanche hederae	.	.	.	.	.	+	.	I

Pignatti-Ellenberg's indices

Pignatti-Ellenberg's indices calculated for each forest stand were found to be the following: the SC stand: L= 5.179, T= 5.885, U= 5.408 and N= 5.600. For the BF stand, they were L= 5.932, T= 6.590, U= 4.644 and N= 4.962. For the BSL stand, they were L= 5.627, T= 6.742, U= 4.185 and N=3.341. The comparison among stands showed for the SC stand, higher values for soil moisture (U) and nutrients (N) compared to those of the BF and the BSL stands. In contrast, the BF and the BSL stands exhibited higher values for temperature and light (T and L) (Fig. 2).

Bulk density and pH of soil

The bulk density is an indicator of soil compaction. The three sampled stands exhibit similar mean values of bulk density (p-value is not significant), although the BSL and the BF stands showed a wide range of values, whereas the SC stand showed a narrow range of values together with the presence of some outliers (Fig. 3). According to the Natural Resources Conservation Service (USDA-NRCS 2021), the values we have found are considered "ideal conditions for plant growth". Based on the classes of the pH soil described by the National Soil Survey Center

(1998), the SC stand is classified as "extremely acid", with an average pH value of 4.5, the BF stand is classified "very strongly acid", with an average value of 4.7 and the BSL stand is classified as "strongly acid", with an average value of 5.6 (Fig. 3).

Morphological measurements

Seedling root lengths were found to be slightly shorter in the SC stand compared to the other two stands, whereas stem lengths were found to be slightly shorter for the

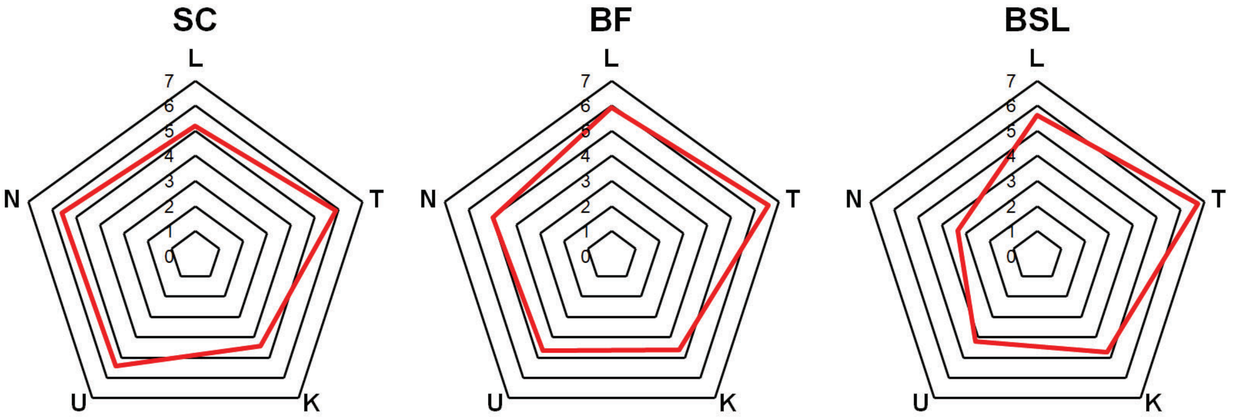


Figure 2. Ecograms for the three forest stands based on the average values for Pignatti-Ellenberg indices: L – light; T – temperature; K – continentality; U – soil moisture; N – soil nutrients.

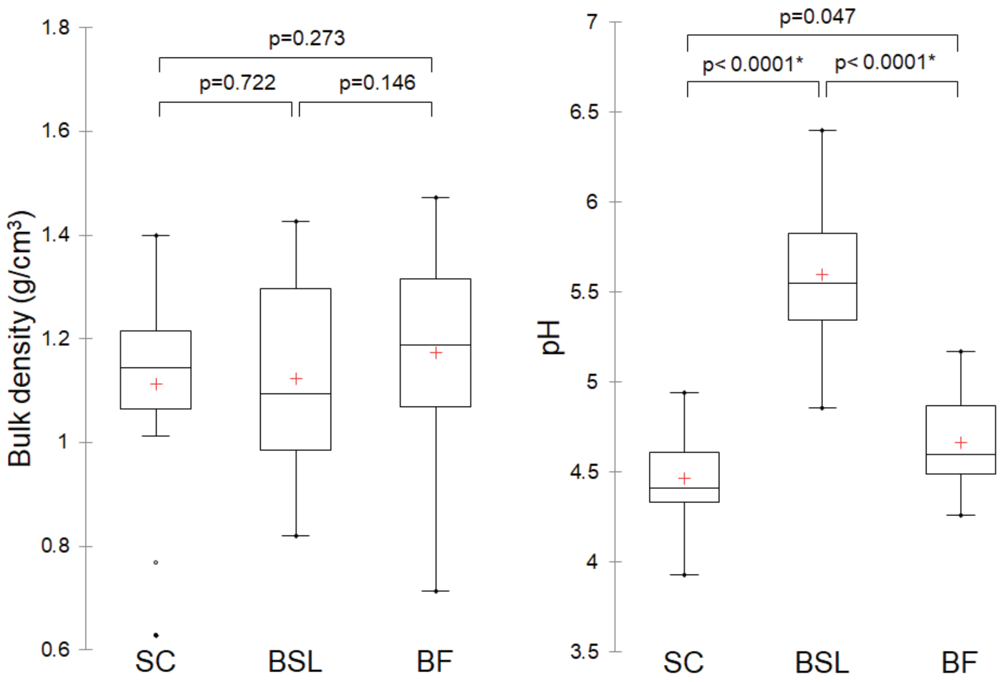


Figure 3. Bulk density and pH of soil in the three wood stands. Non-parametric comparison analysis (Kruskal-Wallis test) and pair comparison according to the Dunn method with Bonferroni correction for significance. Number of replicates for each stand: 30. Legend: red cross = mean; horizontal bar in the box = median; lower limit of the box = first quartile; upper limit of the box = third quartile; empty dots and stars outer the whiskers' bounds = outliers; filled diamonds = minimum and maximum values. Empty dots: values found in the [Q1 – 3 (Q3 - Q1); Q1 - 1.5 (Q3 - Q1)] interval or in the [Q3 + 1.5 (Q3 - Q1); Q3 + 3 (Q3 - Q1)] interval. See Appendix 1, Table A1 for the summary statistics.

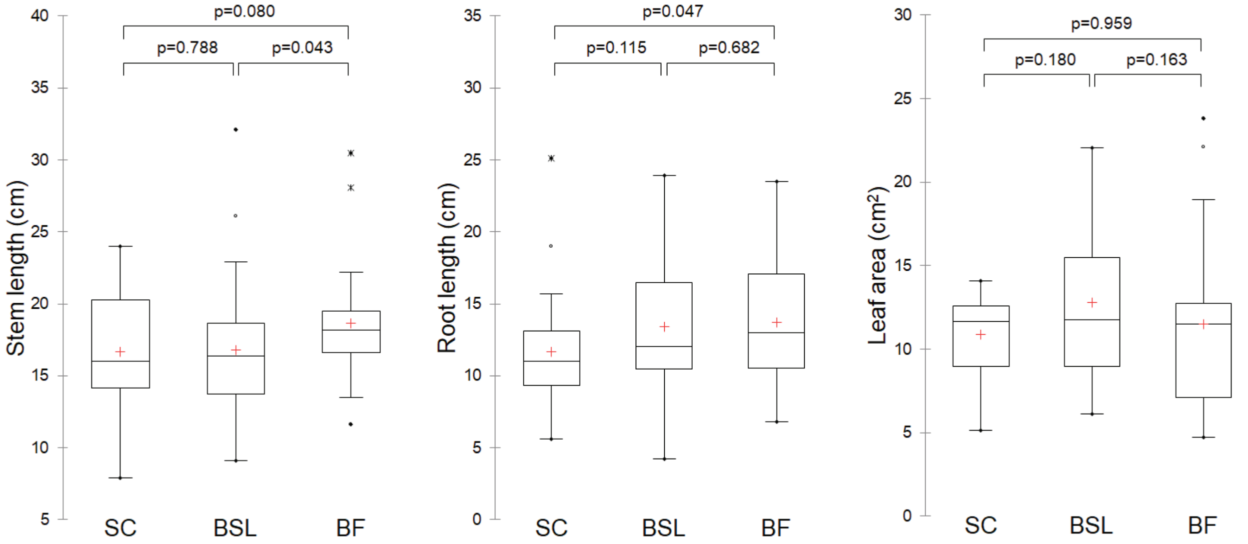


Figure 4. Stem length, root length and leaf area values in the three wood stands. Non-parametric comparison analysis (Kruskal-Wallis test) and pair comparison according to the Dunn method with Bonferroni correction for significance. Number of replicates for each stand: 30. Legend: red cross = mean; horizontal bar in the box = median; lower limit of the box = first quartile; upper limit of the box = third quartile; empty dots and stars outer the whiskers' bounds = outliers; filled diamonds = minimum and maximum values. Asterisks: values found outside the [Q1 - 3 (Q3- Q1); Q3 + 3 (Q3 - Q1)] interval. Empty dots: values found in the [Q1 – 3 (Q3 - Q1); Q1 - 1.5 (Q3 - Q1)] interval or in the [Q3 + 1.5 (Q3 - Q1); Q3 + 3 (Q3 - Q1)] interval. See Appendix 1, Table A2 for the summary statistics.

BSL stand. However, these differences in the mean values of the stem and root lengths turned out not to be statistically significant, with the p-value very close to the threshold (alpha=0.05, Fig. 4). Differences in the values of leaf area among the three stands investigated turned out to also be statistically insignificant (Fig. 4).

Plant functional traits

Specific leaf area (SLA) measurements showed the highest mean value in the SC stand (265.551), which differed significantly from the BF stand (215.078) and the BSL stand (205.224) (p=0.000 and p=0.0001 respectively), the values of the latter being very similar (p=0.508) (Fig. 5). Leaf dry matter content (LDMC) measurements showed that the mean value of the SC stand (339.652) differs significantly from those of the BF stand (407.723) and the BSL stand (416.036), with the SC displaying the lowest mean value (p=0.001) (Fig. 5). For Lth, the low values obtained for the SC stand (1.362), differed significantly from those obtained for the BF stand (1.983) and the BSL stand (2.301) (p=0.001) (Fig. 5).

Leaf chlorophyll content

The SC stand exhibited the highest average value for CHL (0.022 mg/cm^2). This value differs significantly from those displayed by the BSL (0.016 mg/cm^2) and BF stands (0.014 mg/cm^2) (Fig. 6).

Discussion

All three wood stands investigated were assigned to the same association, which is *Roso arvensis-Quercetum cerridis* Ubaldi 2003. Nevertheless, owing to their different silvicultural treatment and ecological features, the three forest stands exhibited clear floristic-coenological differences. The BSL stand is dominated by *Q. cerris* in the upper tree layer and *Carpinus orientalis* in the lower tree layer, and the coverage of the shrub layer was found to be very high (40–90%). The PEi values were high for T and L. The BF stand is characterised by a dominance of *Quercus cerris*, with *Carpinus betulus* having a subordinate role, and by a high coverage by the herb layer (75–90%). The PEi values were found to be high for T and L. The SC stand is characterised by the co-dominance of *Quercus cerris* and *Carpinus betulus* in the upper three layer and by an almost totally missing shrub layer. The PEi values were high for U and N and low for T and L.

The stem and root lengths and the leaf areas of the seedlings were found to be rather similar in the three wood stands. Even if the *Q. cerris* seedlings of the three wood stands investigated are phenotypically very close each other, they showed a different response to environmental conditions if the PFTs values are considered. In

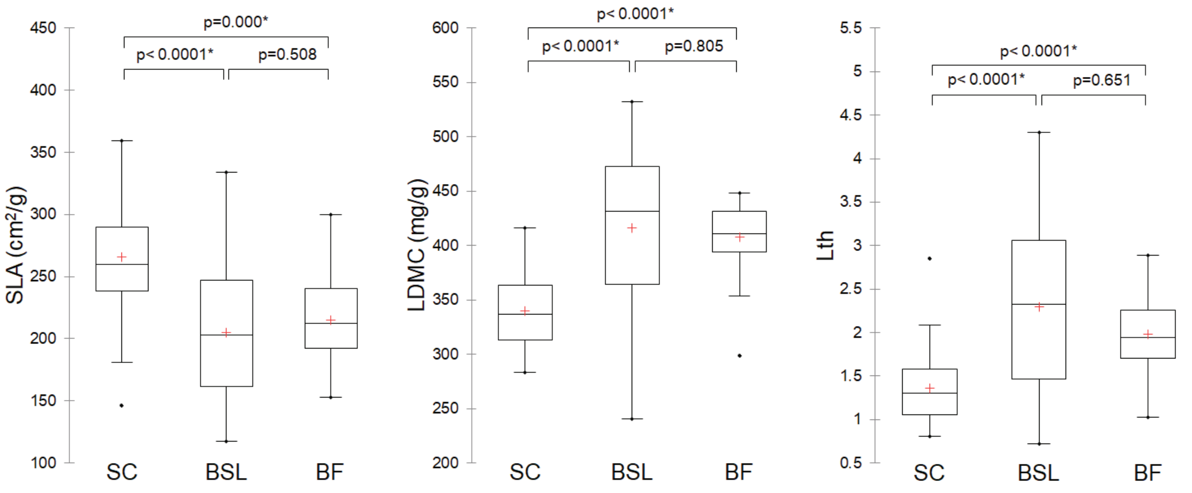


Figure 5. SLA, LDMC and Lth values in the three wood stands. Non-parametric comparison analysis (Kruskal-Wallis test) and pair comparison according to the Dunn method with Bonferroni correction for significance. Number of replicates for each stand: 30. Legend: red cross = mean; horizontal bar in the box = median; lower limit of the box = first quartile; upper limit of the box = third quartile; empty dots and stars outer the whiskers' bounds = outliers; filled diamonds = minimum and maximum values. Empty dots: values found in the [Q1 – 3 (Q3 - Q1); Q1 - 1.5 (Q3 - Q1)] interval or in the [Q3 + 1.5 (Q3 - Q1); Q3 + 3 (Q3 - Q1)] interval. See Appendix 1, Table A3 for the summary statistics.

the ecological literature, high values of SLA are normally related to environments characterised by being rich in nutrients and/or by shaded conditions. The higher values of SLA showed by the saplings of the SC stand compared to those of the BF and the BSL testify to the propensity of the SC seedlings for an investment in carbon oriented to a rapid growth of the saplings rather than to store material for longevity. High SLA values are related to a lower occurrence of dense tissues, which, as a negative counterpart, makes these seedlings more palatable to grazing herbivores. This negative effect is balanced by a more rapid production of new leaves, linked to a higher availability in nutrients, water, as suggested by the PEi values (see Fig. 3) (Wilson et al. 1999; Shipley et al. 2002; Wright et al. 2004; Pontes et al. 2007).

In both the BSL and the BF stands, the values of LDMC are higher than those measured in the SC, showing, therefore, for the seedlings of the BSL and the BF a greater propensity to longevity (Dijkstra and Lambers 1989; Niemann et al. 1992; Garnier and Laurent 1994; Kazakou et al. 2006). The low SLA and the high LDMC values in the BSL and the BF highlight different strategies compared to the SC stand. We could indirectly address these differences to a lower presence of resources for leaf development. The low Lth values of the SC stand are related to seedlings with thinner leaves. This is another PFT that identifies leaves with a greater propensity to grow. The Lth values are normally negatively correlated with photosynthesis and growth, while being positively correlated with leaf longevity and construction costs (Vile et al. 2005).

Considering that PFT values, such as SLA, LDMC and Lth, are in many cases used to estimate the growth rate of seedlings (Poorter and Garnier 1996; Wright and Westoby 2001), it can be assumed that the SC seedlings grow faster than those of the BF and the BSL. In broad terms, high values in SLA and low values in LDMC correspond to a rapid production of biomass, whereas the low values in SLA and high values in LDMC indicate the conservation of nutrients through long-lived leaves (Reich 1998; Garnier et al. 2001). Assuming that the SC stand seedlings benefit from a greater number of resources that lead these seedlings to exhibit higher SLA values, the next step could be making hypothesis on which kinds of resources are important. In this case, the response derives directly from the PEi, which indicates, for the SC stand, a higher availability of nutrients (N) and soil moisture (U). The flat geomorphological profile of the SC stand and the clayey substrate may play a crucial role in leading to a high degree of soil water retention. While refraining from drawing up a ranking of PEi by importance, we underline the effect that soil moisture can have for the deciduous forest communities occurring in Mediterranean or sub-Mediterranean environments, these latter being usually characterised by more or less prolonged dry periods during late spring and summer.

The pH of the soil, on the other hand, would seem to provide values in contrast, as it goes from 4.5 (SC) to 4.7

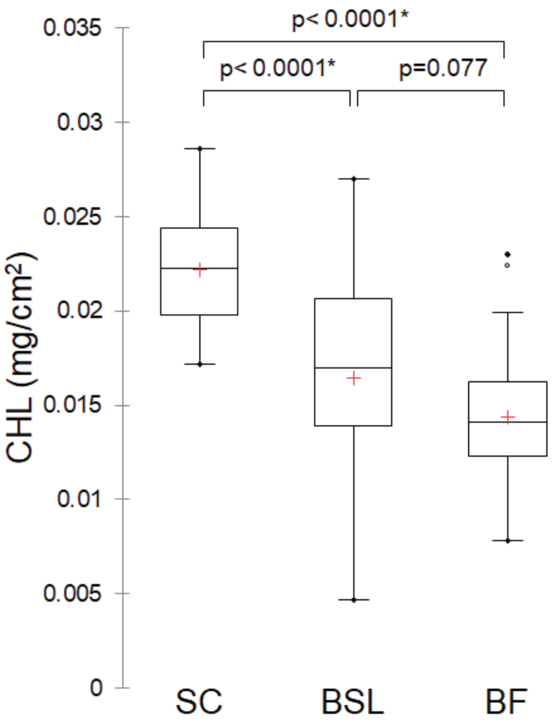


Figure 6. CHL values in the three wood stands. Non-parametric comparison analysis (Kruskal-Wallis test) and pair comparison according to the Dunn method with Bonferroni correction for significance. Number of replicates for each stand: 30. Legend: red cross = mean; horizontal bar in the box = median; lower limit of the box = first quartile; upper limit of the box = third quartile; empty dots and stars outer the whiskers' bounds = outliers; filled diamonds = minimum and maximum values. Empty dots: values found in the [Q1 – 3 (Q3 - Q1); Q1 - 1.5 (Q3 - Q1)] interval or in the [Q3 + 1.5 (Q3 - Q1); Q3 + 3 (Q3 - Q1)] interval. See Appendix 1, Table A4 for the summary statistics.

(BF) and 5.6 (BSL). These values are more consistent with a typical oligotrophic Quercetalia robori-petraea wood than with eutrophic *Quercus cerris* woods of *Erythronio-Carpinion*. Instead, the pH values of the BSL site were perfectly in line with this latter alliance. It would be interesting to understand if these low pH values of the SC and the BF could at least partially explain why the montane-belt *Quercus cerris* forests often give rise to almost monophytic communities, or in any case, communities lacking other species of oaks (see for example Di Pietro and Tondi 2005). It is possible that in addition to the altitude, the low soil pH values lead *Quercus cerris* to be less subjected to the competition from other oak species that are common in this area (e.g. *Q. pubescens* and *Q. frainetto*), whereas acidophilic oaks (e.g., *Q. petraea* and *Q. robur*) that potentially could take advantage from such low pH values are missing due to their rareness in southern Italy.

Another resource that could explain the high SLA values observed for the SC stand could be the light radiation. The PEi values showed a significant difference between the low value (5.19) of the SC stand and the values of the BF and the BSL stands (5.932 and 5.627, respectively).

Observing the headline of the phytosociological table, it emerges that the SC wood stand, while showing a high coverage index at the upper tree layer is characterised by an almost total lack of shrub layer. On the contrary, the BSL wood stand shows the highest cover indices for the shrub layer. It is possible, therefore, that in the SC population, the high coverage of the tree layer was much more effective in reducing the amount of light available for the herb layer than the dense shrub layer occurring in the BSL stand. Accordingly, the high Lth values observed in the BSL stand could be due to the low coverage value of the upper tree layer. In this case, a shrub layer, composed of prostrate species, such as *Hedera helix* and *Rubus ulmifolius* occurring in the BSL would not seem to represent an effective impediment to light in reaching the herb layer.

As far as values of CHL are concerned, these were found to be higher in the SC stand than in the other two stands. A greater quantity of chlorophyll favours a greater absorption capacity of solar radiation. Based on published studies (e.g., Curran et al. 1990; Filella et al. 1995; Gitelson et al. 2003) higher chlorophyll content suggests also a higher photosynthetic potential. This is in accordance with other PFT values observed in this study, such as the lower Lth values and with the higher soil moisture, which would turn out to be higher in the SC stand than in the other two stands (see Fig. 2). The soil moisture is one of the factors that is normally considered as influencing the photosynthetic potential (Zhang et al. 2011; Aref et al. 2013). All these results seem to agree that the seedlings of the SC stand, due to the lower light irradiance and higher soil moisture, are more prone to a rapid development than the seedlings of the BF and the BSL stands. The latter (BLS), growing on slightly drier substrates and greater light intensity, exhibits seedlings developing thicker leaves to cope with lower water availability and higher irradiance (Wright et al. 2004; Poorter et al. 2009).

Conclusions

In this study, phenotypic parameters, and some plant functional traits of *Quercus cerris* seedlings were tested for the first time in natural forest communities to highlight possible adaptation strategies to environmental conditions. The results do not show a significant link between phenotypic plasticity and environmental parameters. It emerged (at least in our case), that the phenotypic expression is not related with the growth strategy of the seedlings. Accordingly, the phenotypic plasticity of the *Quercus cerris* seedlings should not be considered a target element to detect the fitness to ensure the growth of the tree. On the contrary, PFT values allowed for highlighting significant differences between the three wood stands considered and for hypothesising possible strategies adopted by *Quercus cerris* in the first years after germination, depending on the environmental characteristics of the sites. Apparently, the PFT values evidenced that the seedlings of Selva di Castiglione (SC) were following a different development strategy than those of the Bosco della Ficora (BF) and Bosco di San Leo (BSL). Thus, the SC seedlings strategy, expressed by higher values of SLA and CHL and lower values of LDMC and Lth leads to the production of thinner leaves and testifies a greater investment in carbon aimed to a rapid growth of the seedling. The presence of more favorable conditions in the SC stand for the development of the seedlings is supported by the Pignatti-Ellenberg index (PEi) values, which confirm for this site a greater availability of nutrients and soil humidity.

Beyond contributing to a relatively still poor national PFTs database with the first data of in situ PFTs from southern Italy regarding a species of enormous territorial value such as *Quercus cerris*, this study emphasises the importance of investigating on the relationships between young plant development and environmental conditions. The in situ studies on plant functional traits of seedlings of forest species, providing crucial information on their ecology, may assume a great importance to address forest management practices. This information is likely to prove useful in the near future, precisely in the light of recent Italian and European policies linked to the containment of global warming. The guidelines for the “urban and extra-urban forest plan” of the National Recovery and Resilience Plan (Mission 2; Component 4; Investment 3.1) recently published by the Italian Ecological transition Ministry require specific references to natural forest communities and native woody flora to propose models of new urban forests that are consistent with the environmental characteristics of the sites and with the natural potential vegetation types. Yet, the new EU forestry strategy, which plans to plant 3 billion more trees in the European territory by 2030, can become an opportunity to apply the principles of ecology and vegetation science to sustainable land management.

Bibliography

Abdala-Roberts L, Galmán A, Petry WK, Covelo F, de la Fuente M, Glauser G, Moreira X (2018) Interspecific variation in leaf functional and defensive traits in oak species and its underlying climatic drivers. PLOS ONE 13: e0202548. https://doi.org/10.1371/journal.pone.0202548

Adler PB, Salguero-Gómez R, Compagnoni A, Hsu JS, Ray-Mukherjee J, Mbeau-Ache C, Franco M (2014) Functional traits explain variation in plant life history strategies. Proceedings of the National Academy of Sciences of the United States of America 111: 740–745. https://doi.org/10.1073/pnas.1315179111

Antonecchia G, Fortini P, Lepais O, Gerber S, Legér P, Scippa GS, Viscosi V (2015) Genetic structure of a natural oak community in central Italy: evidence of gene flow between three sympatric white oak species (*Quercus*, *Fagaceae*). Annals of Forest Research 58: 205–216. https://doi.org/10.15287/afr.2015.415

Aref I, El Atta H, El Obeid M, Ahmed A, Khan P, Iqbal M (2013) Effect of water stress on relative water and chlorophyll contents of Juniperus procera Hochst. ex Endlicher in Saudi Arabia. Life Science Journal 10(4): 681–685.

Baltieri M, Fantinato E, Vecchio SD, Buffa G (2020) Intraspecific variability of leaf traits and functional strategy of Himantoglossum adriaticum H. Baumann. Plant Sociology 57: 105–112. https://doi.org/10.3897/pls2020572/03

Bartolucci F, Peruzzi L, Galasso G, Albano A, Alessandrini A, Ardenghi NMG, et al. (2018) An updated checklist of the vascular flora native to Italy. Plant Biosystems 152(2): 79–303. https://doi.org/10.1080/11263504.2017.1419996

Biondi E, Blasi C, Allegrezza M, Anzellotti I, Azzella MM, Carli E, et al. (2014) Plant communities of Italy: The Vegetation Prodrome. Plant Biosystems 148: 728–814. https://doi.org/10.1080/11263504.2014.948527

Biondi, E, Casavecchia S, Biscotti N (2008) Forest biodiversity of the Gargano Peninsula and a critical revision of the syntaxonomy of the mesophilous woods of southern Italy. Fitosociologia 45: 93–127.

Biondi, E, Casavecchia, S, Pinzi, M, Allegrezza, M, Baldoni, M (2002) The syntaxonomy of the mesophilous woods of the Central and Northern Apennines (Italy). Fitosociologia 39: 71–93.

Blasi C, Capotorti G, Copiz R, Guida D, Mollo B, Smiraglia D, Zavattero L (2018) Terrestrial Ecoregions of Italy. Map and Explanatory notes. Global Map s.r.l., Firenze, Italy.

Blasi C, Di Pietro R, Filesi L (2004) Syntaxonomical revision of *Quercetalia pubescenti-petraeae* in the Italian Peninsula. Fitosociologia 41: 87–164.

Blasi C, Fortini P, Grossi G, Presti G (2005) Faggete e cerrete mesofile nell’Alto Molise. Fitosociologia 42(2): 67–81.

Blasi C, Paura B (1995) Su alcune stazioni a *Quercus frainetto* Ten. in Campania ed in Molise: analisi fitosociologica e fitogeografica. Annali Botanica (Roma), Studi sul Territorio, 51, Suppl. 10 (2) (1993): 353–366.

Bolzan A, Ferroni L, Vecchiettini M, Speranza M (2007) *Brachypodium pinnatum*, *Bromus erectus* and *Dactylis glomerata* leaf functional traits in grassland communities with different environmental conditions. Journal of Mountain Agriculture in the Balkans 10: 8–15.

Braun-Blanquet J (1964) Pflanzensoziologie. Grundzüge der Vegetationskunde. 3 Auflage, Springer Verlag, Wien and New York, 330 pp. https://doi.org/10.1007/978-3-7091-8110-2

Cantiani P, Ferretti F, Pelleri F, Sansone D, Tagliente G (2010) Le fustaie di cerro del Molise. Analisi del trattamento del passato per le attuali scelte selvicolturali. Annals of Silvicultural Research 36: 25–36.

Catorci A, Tardella FM, Cutini M, Luchetti L, Paura B, Vitanzi A (2013) Reproductive traits variation in the herb layer of a submediterranean deciduous forest landscape. Plant Ecology 214: 737–749. https://doi.org/10.1007/s11258-013-0203-0

Chelli S, Ottaviani G, Simonetti E, Wellstein C, Canullo R, Carnicelli S, Andreetta A, Puletti N, Bartha S, Cervellini M, Campetella G (2019) Climate is the main driver of clonal and bud bank traits in Italian forest understories. Perspectives in Plant Ecology, Evolution and Systematics 40: 125478. https://doi.org/10.1016/j.ppees.2019.125478

Chiatante D, Tognetti R, Scippa GS, Congiu T, Baesso B, Terzaghi M, Montagnoli A (2015) Interspecific variation in functional traits of oak seedlings (*Quercus ilex*, *Quercus trojana*, *Quercus virgiliana*) grown under artificial drought and fire conditions. Journal of Plant Research 128: 595–611. https://doi.org/10.1007/s10265-015-0729-4

Conte AL, Di Pietro R, Iamonico D, Di Marzio P, Cillis G, Lucia D, Fortini P (2019) Oak decline in the Mediterranean basin: a study case from the southern Apennines (Italy). Plant Sociology 56 (2): 69–80. https://doi.org/10.7338/pls2019562/051

Curran PJ, Dungan JL, Gholz HL (1990) Exploring the relationship between reflectance red edge and chlorophyll content in slash pine. Tree Physiology 7: 33–48. https://doi.org/10.1093/treephys/7.1-2-3-4.33

Di Martino P (1996) Storia del Paesaggio Forestale del Molise (Sec. XIX-XX). Istituto Regionale per gli Studi storici del Molise "V. Cuoco", Campobasso, 171pp.

Di Pietro R, Fascetti S (2005) A contribution to the knowledge of *Abies alba* woodlands in the Campania and Basilicata regions (southern Italy). Fitosociologia 42: 71–95.

Di Pietro R, Tondi GC (2005) A new mesophilous turkey oak woodland association from Laga Mts. (Central Italy). Haquetia 4 (2): 5–25. https://ojs.zrc-sazu.si/hacquetia/article/view/2964/2707

Di Pietro R, Izco J, Blasi C (2004) Contribute to the nomenclatural knowledge of the *Fagus sylvatica* beech-woodlands communities of southern Italy. Plant Biosystems 138 (1): 27–36-52. https://doi.org/10.1080/11263500410001684099

Di Pietro R, Di Marzio P, Antonecchia G, Conte AL, Fortini P (2020a) Preliminary characterization of the *Quercus pubescens* complex in southern Italy using molecular markers. Acta Botanica Croatica 79 (1): 15–25. https://doi.org /10.2478/botcro-2019-0026

Di Pietro R, Di Marzio P, Misano G, Silletti G, Wagensommer RP, Fortini, P (2016) Evidence from multivariate morphometric study of the *Quercus pubescens* complex in southeast Italy. Botanica Serbica 40 (1): 83–100. https:/doi.org/ 10.5281/zenodo.48865

Di Pietro R, Fortini P, Ciaschetti G, Rosati L, Viciani D, Terzi M (2020b) A revision of the syntaxonomy of the Apennine-Balkan *Quercus cerris* and *Q. frainetto* forests and correct application of the name *Melittio-Quercion frainetto*. Plant Biosystems 154: 887–909. https://doi.org/10.1080/11263504.2019.1701127

Di Pietro R, Conte AL, Di Marzio P, Gianguzzi L, Spampinato G, Caldarella O, Fortini P (2020c) A multivariate morphometric analysis of diagnostic traits in southern Italy and Sicily pubescent oaks. Folia Geobotanica 55: 163–183. https://doi.org/10.1007/s12224-020-09378-0

Di Pietro R, Conte AL, Di Marzio P, Fortini P, Farris E, Gianguzzi L, Müller M, Rosati L, Spampinato G, Gailing O (2021) Does the genetic diversity among pubescent white oaks in southern Italy, Sicily and Sardinia islands support the current taxonomic classification? European Journal of Forest Research 140, 355–371. https://doi.org/10.1007/s10342-020-01334-z

Dijkstra P, Lambers H (1989) Analysis of specific leaf area and photosynthesis of two inbred lines of *Plantago major* differing in relative growth rate. New Phytologist 113: 283–290. https://doi.org/10.1111/j.1469-8137.1989.tb02405.x

Filella I, Serrano L, Serra J, Peñuelas J (1995) Evaluating Wheat Nitrogen Status with Canopy Reflectance Indices and Discriminant Analysis. Crop Science 35: 1400–1405. https://doi.org/10.2135/cropsci1995.0011183X003500050023x

Fortini P, Antonecchia G, Di Marzio P, Maiuro L, Viscosi V (2015a) Role of micromorphological leaf traits and molecular data in taxonomy of three sympatric white oak species and their hybrids (*Quercus* L.). Plant Biosystems 149: 546–558. https://doi.org/10.1080/11263504.2013.868374

Fortini P, Di Marzio P, Di Pietro R, (2015b) Differentiation and hybridization of *Quercus frainetto*, *Q. petraea*, and *Q. pubescens* (*Fagaceae*): insights from macro-morphological leaf traits and molecular data. Plant Systematics and Evolution 301 (1): 375–385. https://doi.org/10.1007/s00606-014-1080-2

Garnier E, Laurent G (1994) Leaf anatomy, specific mass and water content in congeneric annual and perennial grass species. New Phytologist 128: 725–736. https://doi.org/10.1111/j.1469-8137.1994.tb04036.x

Garnier E, Shipley B, Roumet C, Laurent G (2001) A standardized protocol for the determination of specific leaf area and leaf dry matter content. Functional Ecology 15: 688–695. https://doi.org/10.1046/j.0269-8463.2001.00563.x

Gitelson AA, Gritz † Y, Merzlyak MN (2003) Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. Journal of Plant Physiology 160: 271–282. https://doi.org/10.1078/0176-1617-00887

Gottardini E, Cristofolini F, Cristofori A, Pollastrini M, Ferretti M (2016) Misura della clorofilla a, contenuto di clorofilla e tratti fogliari: campionamento, raccolta e misurazioni. Guida per studi in campo. Documento del progetto LIFE Future For CoppiceS, Azione B.2, pp 34. https://doi.org/10.13140/RG.2.2.19909.14562

Gratani L, Foti I (1998) Estimating forest structure and shade tolerance of the species in a mixed deciduous broad-leaved forest in Abruzzo, Italy. Annales Botanici Fennici 35: 75–83.

Gristina AS, Marcenò C. (2008) Gli indici di bioindicazione di Pignatti-Ellenberg nello studio floristico-vegetazionale del promontorio di Capo Zafferano (Sicilia nord-occidentale). Naturalista siciliano, 32(1-2): 61–96.

Karavin N (2013) Effects of leaf and plant age on specific leaf area in deciduous tree species *Quercus cerris* L. var. cerris. Bangladesh Journal of Botany 42: 301–306. https://doi.org/10.3329/bjb.v42i2.18034

Kazakou E, Vile D, Shipley B, Gallet C, Garnier E (2006) Co-variations in litter decomposition, leaf traits and plant growth in species from a Mediterranean old-field succession. Functional Ecology 20: 21–30. https://doi.org/10.1111/j.1365-2435.2006.01080.x

Mucina L, Bültmann H, Dierßen K, Theurillat JP, Raus T, Čarni A, et al. (2016) Vegetation of Europe: hierarchical floristic classification system of vascular plant, bryophyte, lichen, and algal communities. Applied Vegetation Science 19: 3–264. https://doi.org/10.1111/avsc.12257

National Soil Survey Center, 1998. Soil Quality Indicators: pH. Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Available online at the following link: https://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_052208.pdf

Niemann GJ, Pureveen JBM, Eijkel GB, Poorter H, Boon JJ (1992) Differences in relative growth rate in 11 grasses correlate with differences in chemical composition as determined by pyrolysis mass spectrometry. Oecologia 89: 567–573. https://doi.org/10.1007/BF00317165

Novichonok EV, Novichonok AO, Kurbatova JA, Markovskaya EF (2016) Use of the atLEAF+ chlorophyll meter for a non destructive estimate of chlorophyll content. Photosynthetica 54: 130–137. https://doi.org/10.1007/s11099-015-0172-8

Oberdofer E (1992) Süddentsche Pflanzengesellschaften. Teil IV: 157–159. G. Fisher. Jena.

Otieno DO, Schmidt MWT, Adiku S, Tenhunen J (2005) Physiological and morphological responses to water stress in two Acacia species from contrasting habitats. Tree Physiology 25: 361–371. https://doi.org/10.1093/treephys/25.3.361

Pérez-Harguindeguy N, Díaz S, Garnier E, Lavorel S, Poorter H, Jaureguiberry P, et al. (2013) New handbook for standardised measurement of plant functional traits worldwide. Australian Journal of Botany 61: 167–234. https://doi.org/10.1071/BT12225

Pérez-Ramos IM, Matías L, Gómez-Aparicio L, Godoy Ó (2019) Functional traits and phenotypic plasticity modulate species coexistence across contrasting climatic conditions. Nature Communications 10: 2555. https://doi.org/10.1038/s41467-019-10453-0

Pignatti S (2005) Valori di bioindicazione delle piante vascolari della flora d'Italia. Braun-Blanquetia 39: 3–97.

Pignatti S, Bona E (2007) Ellenberg 2000. Ecological bioindication engine. EVS, Rome (unpublished)

Pontes LDS, Soussana J-F, Louault F, Andueza D, Carrère P (2007) Leaf traits affect the above-ground productivity and quality of pasture grasses. Functional Ecology 21: 844–853. https://doi.org/10.1111/j.1365-2435.2007.01316.x

Poorter H, Garnier E (1996) Plant growth analysis: an evaluation of experimental design and computational methods. Journal of Experimental Botany 47: 1343–1351. https://doi.org/10.1093/jxb/47.9.1343

Poorter H, Niinemets Ü, Poorte L, Wright, IJ, Villar R (2009). Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. New Phytologist 182, 565–588. https://doi.org/10.1111/j.1469-8137.2009.02830

Rasband WS (1997-2018) ImageJ. U.S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/

Reich PB, Walters MB, Tjoelker MG, Vanderklein D, Buschena C (1998) Photosynthesis and respiration rates depend on leaf and root morphology and nitrogen concentration in nine boreal tree species differing in relative growth rate. Functional Ecology 12: 395–405. https://doi.org/10.1046/j.1365-2435.1998.00209.x

Rivas-Martínez S, Sáenz SR, Penas A (2011) Worldwide bioclimatic classification system. Global Geobotany 1: 1–634. https://doi.org/10.5616/gg110001

Rosati L, Di Pietro R, Blasi C (2005) La vegetazione forestale della Regione Temperata del Flysch del Cilento (Italia meridionale). Fitosociologia 42 (2): 33–65.

Sürmen B, Kutbay HG, Kiliç DD, Hüseyinova R, Kilinç M (2014) Ellenberg's indicator values for soil nitrogen concentration and pH in selected swamp forests in the Central Black Sea region of Turkey. Turkish Journal of Botany 38: 883–895. https://doi.org/10.3906/bot-1311-43

Shipley B, Vu T-T (2002) Dry matter content as a measure of dry matter concentration in plants and their parts. New Phytologist 153: 359–364. https://doi.org/10.1046/j.0028-646X.2001.00320.x

Taffetani F, Catorci A, Ciaschetti G, Cutini M, Di Martino L, Frattaroli AR, Paura B, Pirone G, Rismondo M, Zitti S (2012) The *Quercus cerris* woods of the alliance *Carpinion orientalis* Horvat 1958 in Italy. Plant Biosystems 146: 918–953. https://doi.org/10.1080/11263504.2012.682613

Terzi M, Ciaschetti G, Fortini P, Rosati L, Viciani D, Di Pietro R (2021) New syntaxon names for the Italian *Quercus cerris* woods. Mediterranean Botany 42: e75592. https://doi.org/10.5209/mbot.75592

Torres-Ruiz JM, Kremer A, Carins-Murphy MR, Brodribb T, Lamarque LJ, Truffaut L, Bonne F, Ducousso A, Delzon S (2019) Genetic differentiation in functional traits among European sessile oak populations. Tree physiology 39: 1736–1749. https://doi.org/10.1093/treephys/tpz090

Ubaldi D (2003) La vegetazione boschiva d'Italia: manuale di Fitosociologia forestale. Clueb, Bologna.

Valladares F, Martinez-Ferri E, Balaguer L, Pérez-Corona ME, Manrique E (2000) Low leaf-level response to light and nutrients in Mediterranean evergreen oaks: A conservative resource-use strategy? New Phytologist 148: 79–91. https://doi.org/10.1046/j.1469-8137.2000.00737.x

Valladares F, Matesanz S, Guilhaumon F, Araújo MB, Balaguer L, Benito-Garzón M, Cornwell W, Gianoli E, van Kleunen M, Naya DE, Nicotra AB, Poorter H, Zavala MA (2014) The effects of phenotypic plasticity and local adaptation on forecasts of species range shifts under climate change. Ecology Letters 17: 1351–1364. https://doi.org/10.1111/ele.12348

Vile D, Garnier É, Shipley B, Laurent G, Navas ML, Roumet C, et al. (2005) Specific Leaf Area and Dry Matter Content Estimate Thickness in Laminar Leaves. Annals of Botany 96: 1129–1136. https://doi.org/10.1093/aob/mci264

Wilson PJ, Thompson K, Hodgson JG (1999) Specific leaf area and leaf dry matter content as alternative predictors of plant strategies. New Phytologist 143: 155–162. https://doi.org/10.1046/j.1469-8137.1999.00427.x

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, et al. (2004) The worldwide leaf economics spectrum. Nature 428: 821–827. https://doi.org/10.1038/nature02403

Wright IJ, Westoby M (2001) Understanding seedling growth relationships through specific leaf area and leaf nitrogen concentration: generalisations across growth forms and growth irradiance. Oecologia 127: 21–29. https://doi.org/10.1007/s004420000554

Zhang YJ, Xie ZK, Wang YJ, Su PX, An LP, Gao H (2011) Effect of water stress on leaf photosynthesis, chlorophyll content, and growth of oriental lily. Russian journal of plant physiology 58: 844–850. https://doi.org/10.1134/S1021443711050268

Zhu J, Tremblay N, Liang Y (2012) Comparing SPAD and atLEAF values for chlorophyll assessment in crop species. Canadian Journal of Soil Science 92: 645–648. https://doi.org/10.4141/cjss2011-100

Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Bulk density/Moisture/Aeration. Available online at the following link: https://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_053260.pdf (Accessed on 28/11/2021)

Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Soil Quality Indicators: pH. Available online at the following link: https://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_052208.pdf (Accessed on 28/11/2021)

USA-NRCS, w/data. Bulk density/Moisture/Aeration. Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Available online at the following link: https://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_053260.pdf (Accessed on 28/11/2021)

Appendix - Summary statistics

Table A1. Summary statistics for Bulk density and pH.

	Bulk density (g/cm^3)				**pH**			
Stand	*Minimum*	*Maximum*	*Average*	*Standard deviation*	*Minimum*	*Maximum*	*Average*	*Standard deviation*
SC	0.627	1.399	1.113	0.181	3.93	4.94	4.469	0.267
BSL	0.820	1.425	1.123	0.180	4.86	6.40	5.599	0.360
BF	0.713	1.472	1.174	0.181	4.26	5.17	4.666	0.233

Table A2. Summary statistics for Stem length, Root length and Leaf area.

	Stem length (cm)				**Root length (cm)**				**Leaf area (cm^2)**			
Stand	*Minimum*	*Maximum*	*Average*	*Standard deviation*	*Minimum*	*Maximum*	*Average*	*Standard deviation*	*Minimum*	*Maximum*	*Average*	*Standard deviation*
SC	7.900	24.000	16.697	4.257	5.600	25.100	11.650	3.918	5.114	14.092	10.859	2.448
BSL	9.100	32.100	16.780	4.825	4.200	23.900	13.397	5.102	6.124	22.033	12.799	4.272
BF	11.600	30.500	18.633	3.698	6.800	23.500	13.707	4.512	4.710	23.806	11.495	4.742

Table A3. Summary statistics for the PFTs Specific Leaf Area (SLA), Leaf Dry Matter Content (LDMC) and Leaf thickness (Lth).

	SLA (cm^2/g)				**LDMC (mg/g)**				**Lth**			
Stand	*Minimum*	*Maximum*	*Average*	*Standard deviation*	*Minimum*	*Maximum*	*Average*	*Standard deviation*	*Minimum*	*Maximum*	*Average*	*Standard deviation*
SC	146.135	359.631	265.551	51.847	283.037	416.391	339.652	37.281	0.806	2.854	1.362	0.431
BSL	117.310	334.237	205.224	55.258	240.648	532.341	416.036	74.523	0.720	4.306	2.301	0.939
BF	152.591	299.809	215.078	34.135	298.322	448.317	407.723	31.926	1.022	2.887	1.983	0.419

Table A4. Summary statistics for the chlorophyll content (CHL).

	CHL (mg/cm^2)			
Stand	*Minimum*	*Maximum*	*Average*	*Standard deviation*
SC	0.017	0.029	0.022	0.003
BSL	0.005	0.027	0.016	0.006
BF	0.008	0.023	0.014	0.004

plants

Article

Quercus cerris Leaf Functional Traits to Assess Urban Forest Health Status for Expeditious Analysis in a Mediterranean European Context

Luca Quaranta [1,*], Piera Di Marzio [1,2] and Paola Fortini [1,2]

[1] Department Biosciences and Territory, University of Molise, 86090 Pesche, Italy; piera.dimarzio@unimol.it (P.D.M.); fortini@unimol.it (P.F.)
[2] National Biodiversity Future Center (NBFC), 90133 Palermo, Italy
* Correspondence: luca.quaranta@unimol.it

Abstract: In the Mediterranean basin, urban forests are widely recognized as essential landscape components, playing a key role in nature-based solutions by enhancing environmental quality and providing a range of ecosystem services. The selection of woody plant species for afforestation and reforestation should prioritize native species that align with the biogeographical and ecological characteristics of the planting sites. Among these, *Quercus cerris* L. (Turkey oak) is considered a promising candidate for urban reforestation. However, its fitness within urban forest environments remains poorly understood. This study aimed to identify suitable leaf functional traits for assessing the response of *Q. cerris* in urban forests and to analyze the main climatic variables influencing its performance in urban contexts. We also proposed practical, rapid monitoring tools to compare urban and natural forests across different seasons. The results demonstrated that *Q. cerris* experiences significant water stress in urban forests due to the combined effects of drought and high temperatures. To find the tools to mitigate this stress, the differences between leaf traits such as specific leaf area, thickness, and the contents of chlorophyll, anthocyanins, and flavonols in urban and natural forests were analyzed. Our findings underscore the high adaptability of *Q. cerris* to varied climatic and environmental conditions. This study provides a practical method for rapidly assessing the responses of tree species to climate change. In the future, this approach will be tested on other native species that are characteristic of Mediterranean forest ecosystems to help with choosing afforestation and reforestation strategies.

Keywords: Campobasso municipality; environmental data; phytosociological survey; plant functional traits; statistical analysis; plant stress; urbanization

Academic Editor: Giuseppe Fenu

Received: 17 December 2024
Revised: 16 January 2025
Accepted: 17 January 2025
Published: 20 January 2025

Citation: Quaranta, L.; Di Marzio, P.; Fortini, P. *Quercus cerris* Leaf Functional Traits to Assess Urban Forest Health Status for Expeditious Analysis in a Mediterranean European Context. *Plants* **2025**, *14*, 285. https://doi.org/10.3390/plants14020285

1. Introduction

Over the past fifty years, the gradual abandonment of farmland and grazing areas has facilitated the spontaneous regrowth of forests, particularly in Mediterranean Europe [1,2]. Despite these encouraging trends, the Mediterranean basin remains one of the most vulnerable regions globally to the impacts of climate change. Additionally, Mediterranean forests are significantly affected by high levels of degradation, which is primarily driven by anthropogenic activities [3]. These include the conversion of natural vegetation into extensive agricultural land, industrial development, and urban expansion [4]. The expansion of built-up areas replaces and transforms agricultural lands or natural forests, intensively impacting the environment and ecosystem functions. Large cities are the first to be affected; still, urbanization processes gradually affect smaller settlements and even remote rural villages [5]. The expansion of small cities generally proceeds slowly and determines a hybrid urban landscape, where portions of natural forests or abandoned lands are often incorporated into urban tissue. In this context, urban and peri-urban forests are increasingly recognized as critical components of the landscape, playing a pivotal role in nature-based solutions (NBSs). They contribute to environmental quality and multifunctionality by providing a wide range of ecosystem services, including regulating infiltration and stormwater runoff; mitigating microclimate extremes; reducing the urban heat island effect; combating soil, air, and water pollution; and limiting the spread of invasive species [6–11]. From the ecological perspective, urban and peri-urban forests are essential for maintaining the functions of the urban ecosystem [12–16].

Over the past fifty years, as most of the world's population has transitioned to urban living, many researchers and international organizations have focused on understanding urban forests. They have examined important factors such as knowledge, management, socio-economic impact, and future scenarios, recognizing the importance of ecosystem services in urban environments and the vital role that cities play in the lives of people worldwide [17–20]. Less investigated, however, are the functional responses of tree species and the state of health of the entire urban forest ecosystem in the local context in Mediterranean cities, where plants may be affected by environmental stress as a consequence of climatic features such as air pollutants, altered solar radiation, lower wind speed, lower relative humidity, and higher rainfall. The last factor, however, does not translate into a higher soil water content due to the surface runoff caused by the high waterproofing of urban soils. The direct aspect of these effects is the increase in air temperature compared to the surrounding non-urbanized areas [21–23].

In Italy, to increase the resilience of urban areas, the national strategic plan for urban forestry compiled a list of woody plant species recommended for afforestation and reforestation (NRRP; Protection and enhancement of urban and extra-urban green—M2C4 3.1) [24] (https://www.nbfc.it (accessed on 30 November 2024)) [25]. This list encompasses the principal species constituting the flora of the Mediterranean biogeographic region within temperate forest ecosystems [26–28], and it includes both shrubs and tree species, with several oak species represented among the latter. Notably, *Quercus cerris* L. (Turkey oak) is identified as a key species for urban reforestation efforts. *Quercus cerris* (Sect. Cerris) is an autochthonous deciduous tree native to southern Europe and Asia Minor (chorotype: Euri-Mediterranean); at the European level, the species is mainly present in the Balkan and Italian peninsulas. This is a dominant species in the mixed forests of the Mediterranean basin covering the entire Italian territory (except Sardinia) for a total of around 280.000 ha of natural woods and those frequently occurring together with other oaks (*Quercus frainetto* Ten., *Q. petraea* (Matt.) Liebl., *Q. pubescens* Willd., *Q. robur* L.). The Turkey oak is found in urban areas at elevations from sea level up to the Apennines (1000–1200 m a.s.l.). It is highly adaptable to urban environments, making it an excellent choice for urban reforestation. As indicated in many papers, this species is particularly drought-tolerant and is suitable for planting as a single plant or in groups, in parks, gardens, squares, and tree-lined avenues, as well as in large and medium spaces [29]. Despite the growing attention to the role of trees in urban vegetation patches, the assessment of the ecological response of *Q. cerris* in urban forests at the local scale is still lacking.

One of the concepts that has been more utilized for identifying species' environmental tolerance range values is the Ecological Indicator Value (EIV) system [30–33]. To date, the Ecological Indicator Values for Europe 1.0 (EIVE) [34] is the largest system database in Europe that can be used to retrieve information about specific species preferences in terms of light, temperature, soil moisture, soil nitrogen, and soil reaction, and it can be used to indirectly evaluate the ecological features of forests. Despite the robustness of the toll found in large applications in natural contexts [35–38], EIVE 1.0 is not suitable for indicating the real-time health status of trees in urban forests. This is particularly true because local conditions (reduced area, increased isolation, altered physical environment, etc.) can negatively impact the environmental tolerance of the species [4]. Indeed, the type of trees that can survive in a given location depends on their functional traits, which include all the physiological and morphological features that determine how they interact with and respond to their environment [39].

More suitable are the plant functional traits (PFTs), which are defined as the morphological, anatomical, physiological, biochemical, and phenological characteristics of plants that represent ecological strategies and determine how plants respond to environmental factors [40–45]. Numerous functional traits identified in the literature [40,46–49] can be utilized to evaluate the potential persistence and adaptive capacity of trees in urban forests facing various stressors. These stressors include climate change scenarios [50], heat islands, forest fragmentation, human impact, pollution, and competition from invasive species [51]. The functional traits documented to date are very numerous, encompassing various plant organs, including shoots, roots, and leaves. Since 2007, the TRY Plant Trait Database (https://www.try-db.org/ (accessed on 15 December 2024)) has aimed to collect a vast array of plant functional trait data from diverse sources, and it is highlighted as the most comprehensive archive of global plant data and is open access to the public [52]. Additionally, Pérez-Harguindeguy et al. [40] contributed to it by publishing a comprehensive framework for standardizing the measurement of plant functional traits worldwide. Among functional traits, those related to leaves are particularly advantageous due to their minimally invasive nature, suitability for studying plant communities, and repeatability over time, making them ideal for measuring characteristics of plants that influence their performance, survival, and ecological roles.

In this context, four leaf PFTs were selected for the purpose of the project: the specific leaf area (SLA), the leaf dry matter content (LDMC), the leaf thickness, and the chlorophyll content. This selection was based on their wide use in various studies and their ability to describe the strategies adopted by plants according to environmental conditions with non-invasive, repeatable, and expeditive analysis. In addition, two other pigments, i.e., anthocyanins and flavonols, were investigated on the basis of their relevant role in plant stress protection, but they have not been investigated thoroughly as traits [44,49,53–58].

The purposes of this paper are as follows:

1. To test and detect the selected leaf functional traits to study the response of tree species in urban forests;
2. To detect the main climatic variables that may affect urban forests;
3. To compare the health status of Quercus cerris in urban forests versus natural forests during different seasons;
4. To propose expedited and practical tools for monitoring the response of the trees in different urban forest conditions.

The results obtained in a Mediterranean urban context should help to monitor the health status of forest tree species in other climate regions, guiding the management of urban greenery, especially in large and medium-sized cities, which are experiencing increasingly rapid changes in environmental conditions.

2. Results

2.1. Community Description and Characterization

Based on the results of the coenological surveys (Table S1), it can be stated that the three stands were originally part of the natural forest, and these were separated over time due to the expansion of the city. Accordingly, these stands were classified within the association *Roso arvensis-Quercetum cerridis* Ubaldi 2003 (alliance *Crataego laevigatae-Quercion cerridis* Arrigoni 1997; order *Fagetalia sylvaticae* Pawlowski in Pawlowski, Sokolowski et Wallish 1928; class *Carpino-Fagetea* Jakucs ex Passarge 1968) [59].

Quercus cerris was found to be the dominant tree species across all of the sampled stands, alongside *Quercus frainetto*. Differences in the floristic composition were observed between natural forests and urban and peri-urban stands. The natural forest was characterized by high biodiversity, comprising 59 species, whereas the urban and peri-urban forests were deciduous mixed forests with a reduced floristic diversity of approximately 36 species, all of which were shared with the natural forest.

The tree layer in the natural forest predominantly consisted of *Quercus cerris*, *Q. petraea*, and *Q. frainetto*, while *Carpinus orientalis* dominated the shrub layer. Typical herbaceous forest species such as *Viola reichenbachiana*, *Fragaria vesca*, *Asperula taurina* subsp. *taurina*, and *Scutellaria columnae* were abundant. The tree layer in urban and peri-urban stands was primarily composed of *Quercus cerris*, *Q. frainetto*, and *Q. pubescens*, while the shrub layer included *Ligustrum vulgare*, *Prunus spinosa*, and *Ulmus minor*. The latter species was identified as the only indicator of anthropogenic influence within these forests.

A Kiviat diagram was used to illustrate the Ecological Indicator Values (EIVs) [34] for each forest stand, providing insight into their responses to abiotic factors (Figure 1).

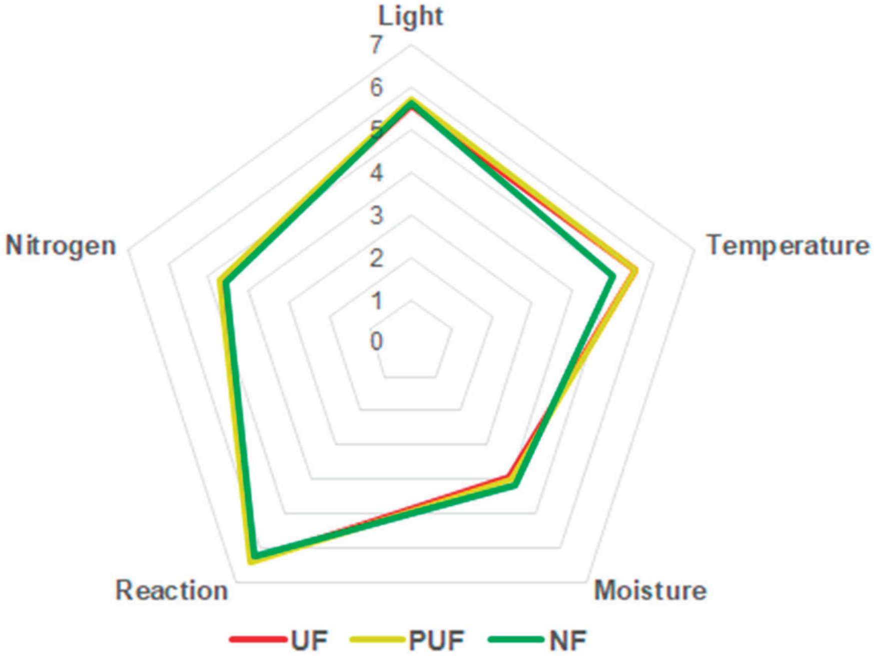


Figure 1. Kiviat diagram showing the weighted values of EIV 1.0 indexes (with an adimensional range value of 0 to 10) for the three stands (UF = urban forest; PUF = peri-urban forest; and NF = natural forest).

The weighted values were the following: L = 5.60, T = 5.53, M = 3.93, R = 6.36, and N = 4.67 for the UF stand; L = 5.74, T = 5.53, M = 3.97, R = 6.42, and N = 4.74 for the PUF stand; and L = 5.65, T = 5.00, M = 4.14, R = 6.26, and N = 4.58 for the NF stand. Comparing the EIVE indices of the three stands, no gradient was detected that passed from natural forest to urban forest, and the ecological requirements of the natural and urban forests appeared just marginally different.

2.2. Correlations Between Traits and Climatic Variables

The results of a Pearson's correlation analysis, which was conducted on the entire dataset, are shown in Figure 2.

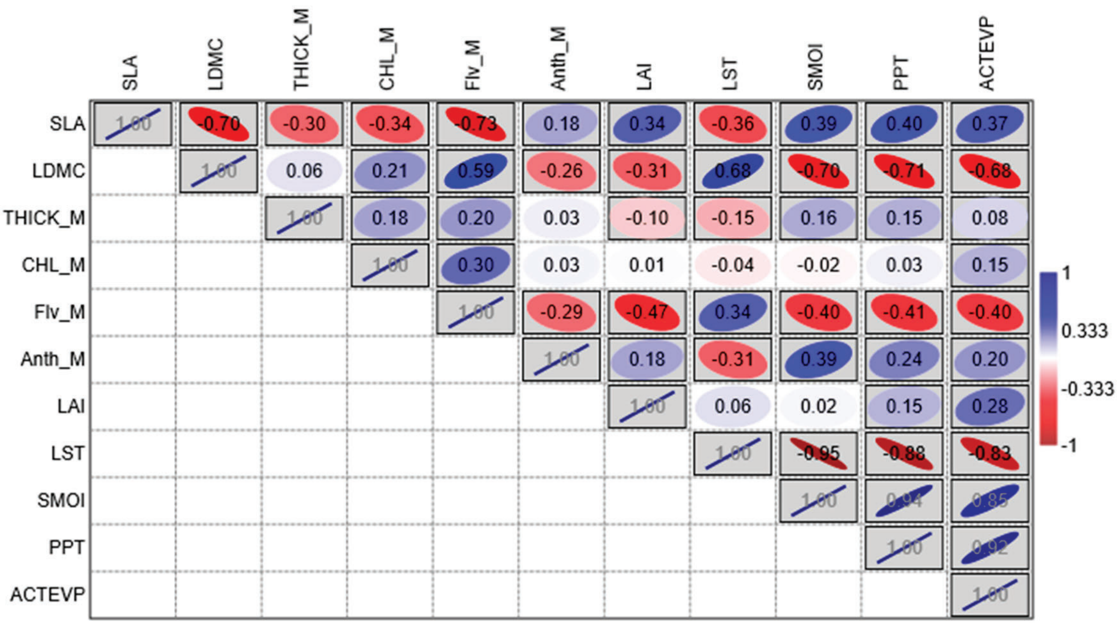


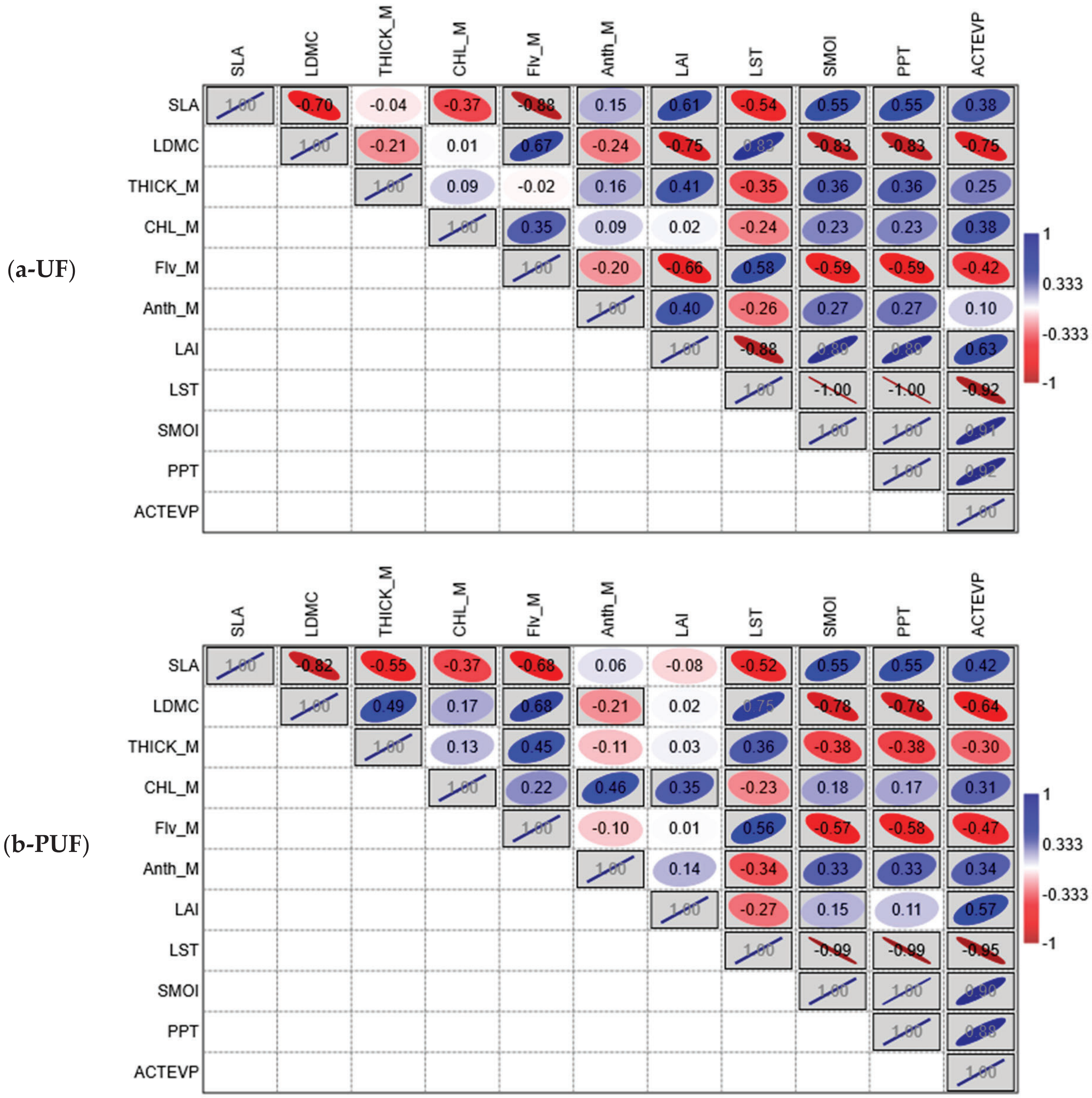
Figure 2. Correlation matrix showing the results of Pearson's correlation analysis, which was performed on the entire dataset (11 variables; three stands; three time sampling). Pearson correlation coefficient values and directions are displayed with the following rule: positive correlation from white to blue and negative correlation from white to red on the color scale. Boxes are marked gray when $p < 0.05$. The inclination of the ellipse is representative of the positivity or negativity of the correlation, while its smaller or larger range indicates the intensity of the correlation. SLA = specific leaf area; LDMC = leaf dry matter content; THICK_M = leaf thickness; Flv_M = flavonol content; CHL_M = chlorophyll content; Anth_M = anthocyanin content; LAI = leaf area index; LST = land surface temperature; PPT = precipitation; SMOI = soil moisture; and ACTEVP = actual evapotranspiration.

The specific leaf area (SLA) and leaf dry matter content (LDMC) diverged oppositely in terms of the variables, and they exhibited significant correlations with all the variables investigated. In detail, the SLA showed a moderate positive correlation with the climatic variables related to the presence of water in the stands (soil moisture—SMOI, precipitation—PPT, and actual evapotranspiration—ACTEVP) and a moderate negative correlation with the variable related to temperature (land surface temperature—LST). The correlation with anthocyanin content (Anth_M) and leaf area index (LAI) was weakly positive. Conversely, the leaf thickness (THICK_M), chlorophyll (CHL_M), and flavonol (Flv_M) content were moderately negatively correlated with the SLA. The relationships between LDMC and all the variables commented on above were opposite to those illustrated for SLA.

Interestingly, THICK_M showed a slightly positive correlation with all the pigments, and the correlation with Anth_M was not found to be statistically significant. Among the three pigments analyzed, CHL_M correlated the lowest with all the variables. Anth_M and Flv_M were negatively correlated with each other. They exhibited opposite correlations with SLA, LDMC, LST, and LAI variables. In contrast, the correlation with the variables related to the presence of water in the stands (PPT, ACTEVP, and SMOI) was negative for Flv_M and positive for Anth_M.

Pearson's correlation was performed on the three stands separately (Figure 3), and it highlighted a similar correlation, except for chlorophyll content, which presented a different correlation between the three sites. CHL_M in the urban forest negatively correlated with increasing temperature (LST), while the chlorophyll content in the natural forest positively correlated with temperature. On the other hand, variables related to soil water availability (PPT, SMOI, and ACTEVP) in urban and natural forests were oppositely correlated with chlorophyll.

Furthermore, principal component analysis (PCA) was undertaken to evaluate the variation between the three forest stands in the three time-sampling periods (June, July, and September) and their relationships with leaf traits and climatic variables. The value of the Kaiser–Meyer–Olkin (KMO) measure was 0.76393, which is reported by PAST 4.17 instruction [60] as "good", and the "scree plot" of the eigenvalues (Figure 4) showed that the higher part of the variability can be explained with the first axis.



(a-UF)

	SLA	LDMC	THICK_M	CHL_M	Flv_M	Anth_M	LAI	LST	SMOI	PPT	ACTEVP
SLA	1.00	-0.70	-0.04	-0.37	-0.88	0.15	0.61	-0.54	0.55	0.55	0.38
LDMC		1.00	-0.21	0.01	0.67	-0.24	-0.75	0.83	-0.83	-0.83	-0.75
THICK_M			1.00	0.09	-0.02	0.16	0.41	-0.35	0.36	0.36	0.25
CHL_M				1.00	0.35	0.09	0.02	-0.24	0.23	0.23	0.38
Flv_M					1.00	-0.20	-0.66	0.58	-0.59	-0.59	-0.42
Anth_M						1.00	0.40	-0.26	0.27	0.27	0.10
LAI							1.00	-0.88	0.80	0.80	0.63
LST								1.00	-1.00	-1.00	-0.92
SMOI									1.00	1.00	0.91
PPT										1.00	0.92
ACTEVP											1.00

(b-PUF)

	SLA	LDMC	THICK_M	CHL_M	Flv_M	Anth_M	LAI	LST	SMOI	PPT	ACTEVP
SLA	1.00	-0.82	-0.55	-0.37	-0.68	0.06	-0.08	-0.52	0.55	0.55	0.42
LDMC		1.00	0.49	0.17	0.68	-0.21	0.02	0.75	-0.78	-0.78	-0.64
THICK_M			1.00	0.13	0.45	-0.11	0.03	0.36	-0.38	-0.38	-0.30
CHL_M				1.00	0.22	0.46	0.35	-0.23	0.18	0.17	0.31
Flv_M					1.00	-0.10	0.01	0.56	-0.57	-0.58	-0.47
Anth_M						1.00	0.14	-0.34	0.33	0.33	0.34
LAI							1.00	-0.27	0.15	0.11	0.57
LST								1.00	-0.99	-0.99	-0.95
SMOI									1.00	1.00	0.90
PPT										1.00	0.89
ACTEVP											1.00

Figure 3. *Cont.*

(**c-NF**)

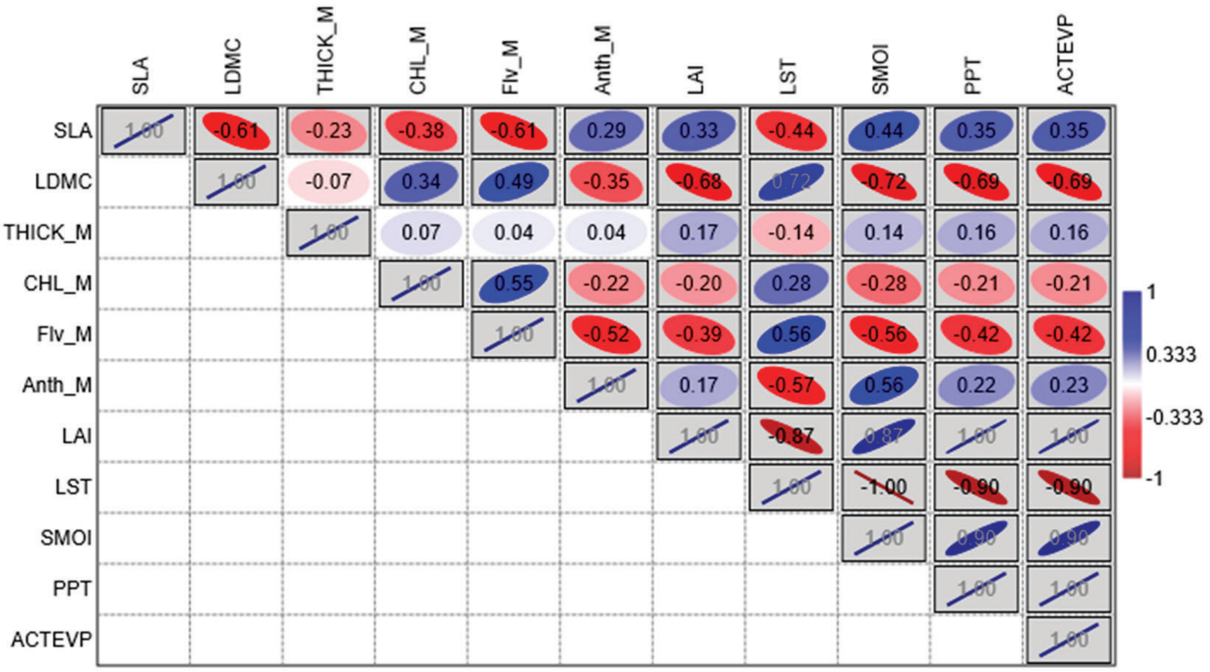


Figure 3. The correlation matrix shows the results of Pearson's correlation analysis, which was performed separately on (**a**) UF—urban forest, (**b**) PUF—peri-urban forest, and (**c**) NF—natural forest stands. Pearson's correlation coefficient values and directions are displayed with the following rule: positive correlation from white to blue and negative correlation from white to red on the color scale. Boxes are marked gray when $p < 0.05$. The inclination of the ellipse is representative of the positivity or negativity of the correlation, while its smaller or larger range indicates the intensity of the correlation. SLA = specific leaf area; LDMC = leaf dry matter content; THICK_M = leaf thickness; Flv_M = flavonol content; CHL_M = chlorophyll content; Anth_M = anthocyanin content; LAI = leaf area index; LST = land surface temperature; PPT = precipitation; SMOI = soil moisture; and ACTEVP = actual evapotranspiration.

	SLA	LDMC	THICK_M	CHL_M	Flv_M	Anth_M	LAI	LST	SMOI	PPT	ACTEVP
SLA	1.00	-0.61	-0.23	-0.38	-0.61	0.29	0.33	-0.44	0.44	0.35	0.35
LDMC		1.00	-0.07	0.34	0.49	-0.35	-0.68	0.72	-0.72	-0.69	-0.69
THICK_M			1.00	0.07	0.04	0.04	0.17	-0.14	0.14	0.16	0.16
CHL_M				1.00	0.55	-0.22	-0.20	0.28	-0.28	-0.21	-0.21
Flv_M					1.00	-0.52	-0.39	0.56	-0.56	-0.42	-0.42
Anth_M						1.00	0.17	-0.57	0.56	0.22	0.23
LAI							1.00	-0.87	0.87	1.00	1.00
LST								1.00	-1.00	-0.90	-0.90
SMOI									1.00	0.90	0.90
PPT										1.00	1.00
ACTEVP											1.00

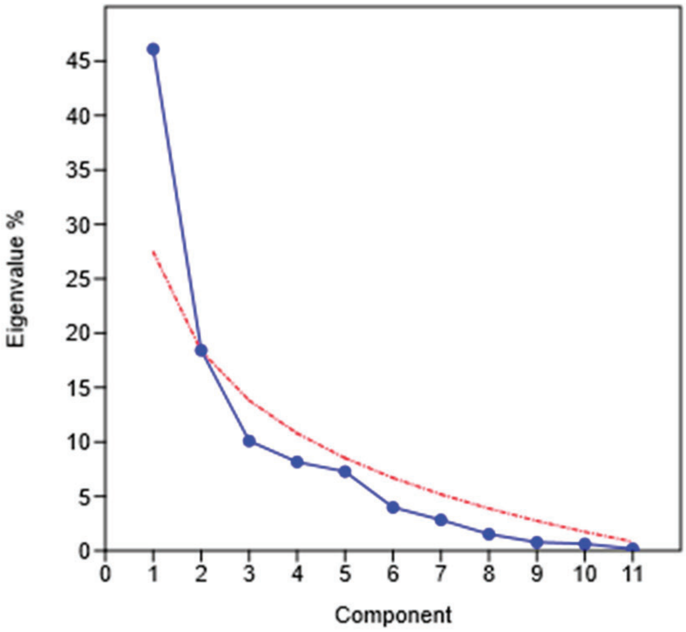


Figure 4. Principal component analysis scree plot of the eigenvalues (blue) with "broken stick" (red: eigenvalues expected under a random model), in which it is shown that the first two components explain more than 64% of the variance.

Figure 5 shows the scatterplot of the component 1 and 2 scores, accounting for around 64% of the total variance. Along component one (with a variance of 46.10%), the data collected in June and July formed a distinct group from those harvested in September. These latter were located in the left part of the graph, and they were mainly associated with the variables LST, LDMC, and Flv_M; furthermore, the data related to urban and peri-urban forests overlapped, while those related to natural forests were separated and very close to each other. In the right part of the graph, the data collected in June and July were mainly associated with SMOI, PPT, and ACTEVP variables. The data of June show an overlap for the urban and peri-urban forests, while the natural forest data were slightly separated. For the data of July, the separation of the natural forest data was more evident from the other two forest stands. It was also evident that the data of June and July of the natural forest overlapped entirely, while the clouds of urban and peri-urban forest data shifted for the two sampling times.

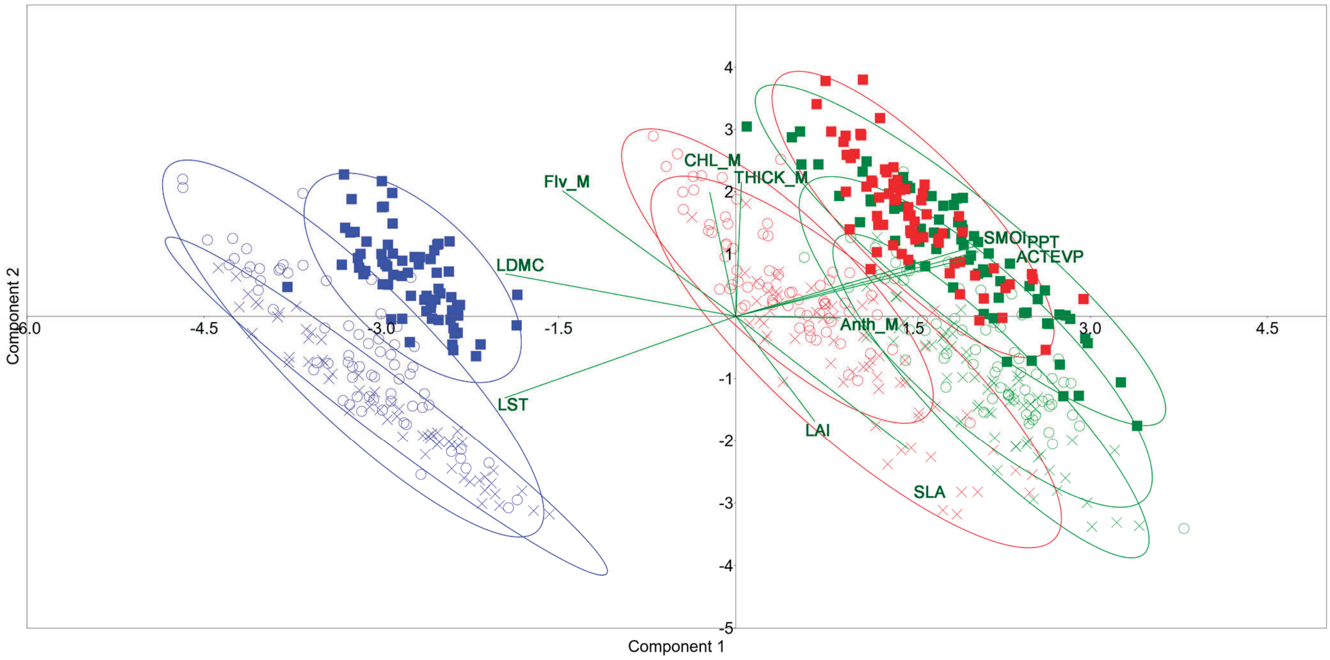


Figure 5. Scatter plot of the principal component analysis biplot depicting the relationship between the variables and the three stands. Urban forest (circle), peri-urban forest (X symbol), and natural forest (square) in three sampling times (June—green; July—red; September—blue). SLA = specific leaf area; LDMC = leaf dry matter content; THICK_M = leaf thickness; Flv_M = flavonol content; CHL_M = chlorophyll content; Anth_M = anthocyanin content; LAI = leaf area index; LST = land surface temperature; PPT = precipitation, SMOI = soil moisture; and ACTEVP = actual evapotranspiration.

2.3. Plant Functional Traits Variation in the Sampling Times

When considering the plant functional traits, the SLA values decreased in the three sampling times, with a higher statistically significant difference in the urban and peri-urban forests between June and July (Figure 6a). The LDMC values increased across all forest stands, though it was notable that the values observed in the natural forest did not reach a statistically significant difference when comparing June and July (Figure 6b).

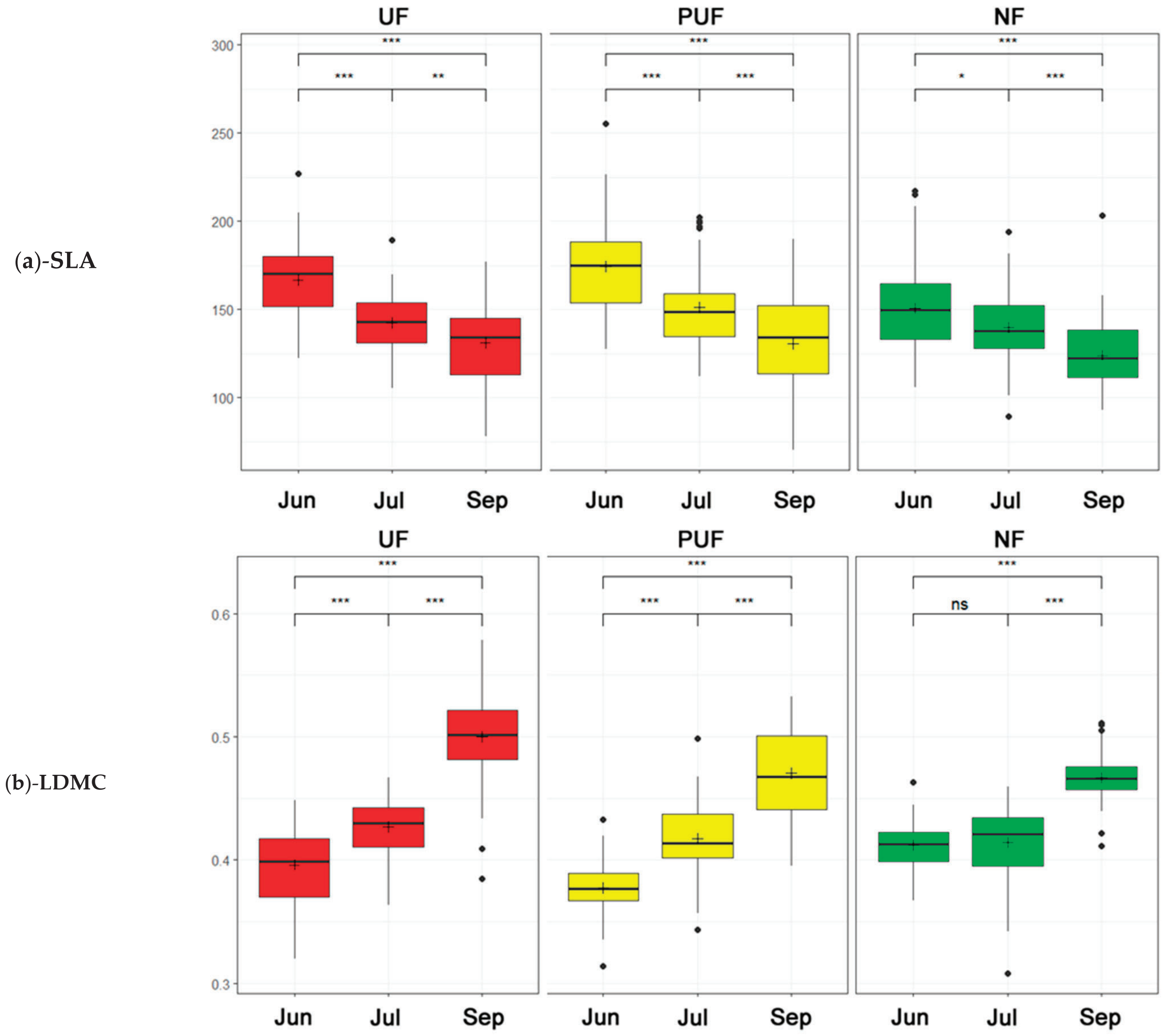


Figure 6. Two-way ANOVA box plots (a Tukey post hoc analysis was used to test statistical significance: ns = not significant, · = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) that describe the trends of the three stands (green—natural forest; yellow—peri-urban forest; and red—urban forest) in three periods (from left to right: Jun = June, Jul = July, and Sep = September). (**a**) Specific leaf area (SLA). (**b**) Leaf dry matter content (LDMC).

THICK_M values exhibited three distinct trends: a decline in urban forest between June and July, an uptick in peri-urban forest, and a lack of significant variation globally in natural forest (Figure 7a). The CHL_M values in the urban and peri-urban forests were higher in July and overall higher in the urban forest. In contrast, the natural forest had constant growth in the three periods that became significant between the first and third periods (Figure 7b).

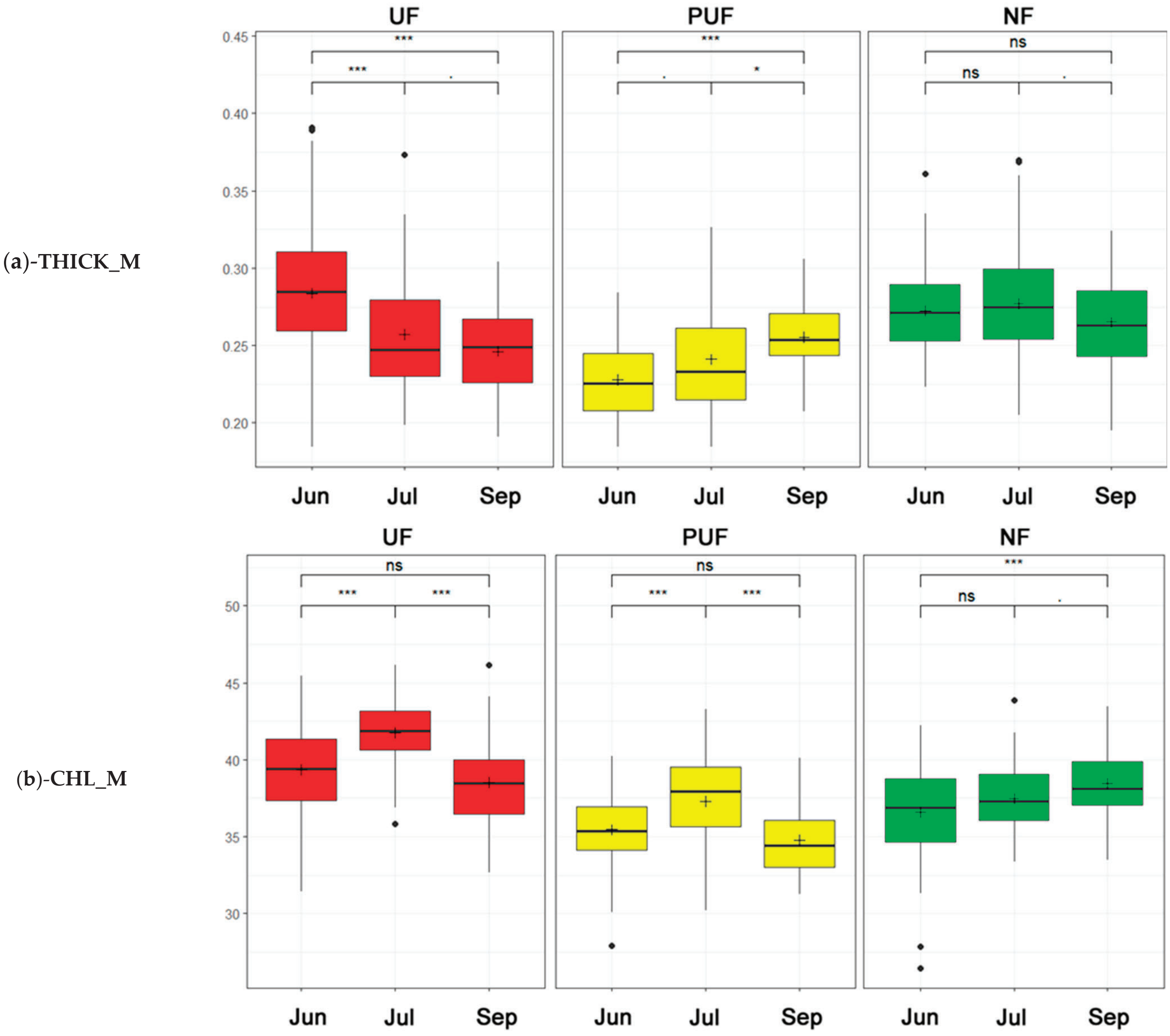


Figure 7. Two-way ANOVA box plots (a Tukey HSD post hoc analysis was used to test statistical significance: ns = not significant, · = $p < 0.1$, * = $p < 0.05$, *** = $p < 0.001$) that describe the trends of the three stands (green—natural forest; yellow—peri-urban forest; and red—urban forest) in three periods (from left to right: Jun = June, Jul = July, and Sep = September). (**a**) Leaf thickness (THICK_M). (**b**) Chlorophyll content (CHL_M).

Flv_M values demonstrated considerable growth in all three stands and during the three sampling periods, with elevated values observed in the natural forest compared to the other two forests (Figure 8a). Anth_M values exhibited a comparable trend in natural and urban forests, with a decline that reached a stabilization in September. In contrast, the peri-urban forest demonstrated consistent stability throughout June and July, followed by a decrease in September (Figure 8b).

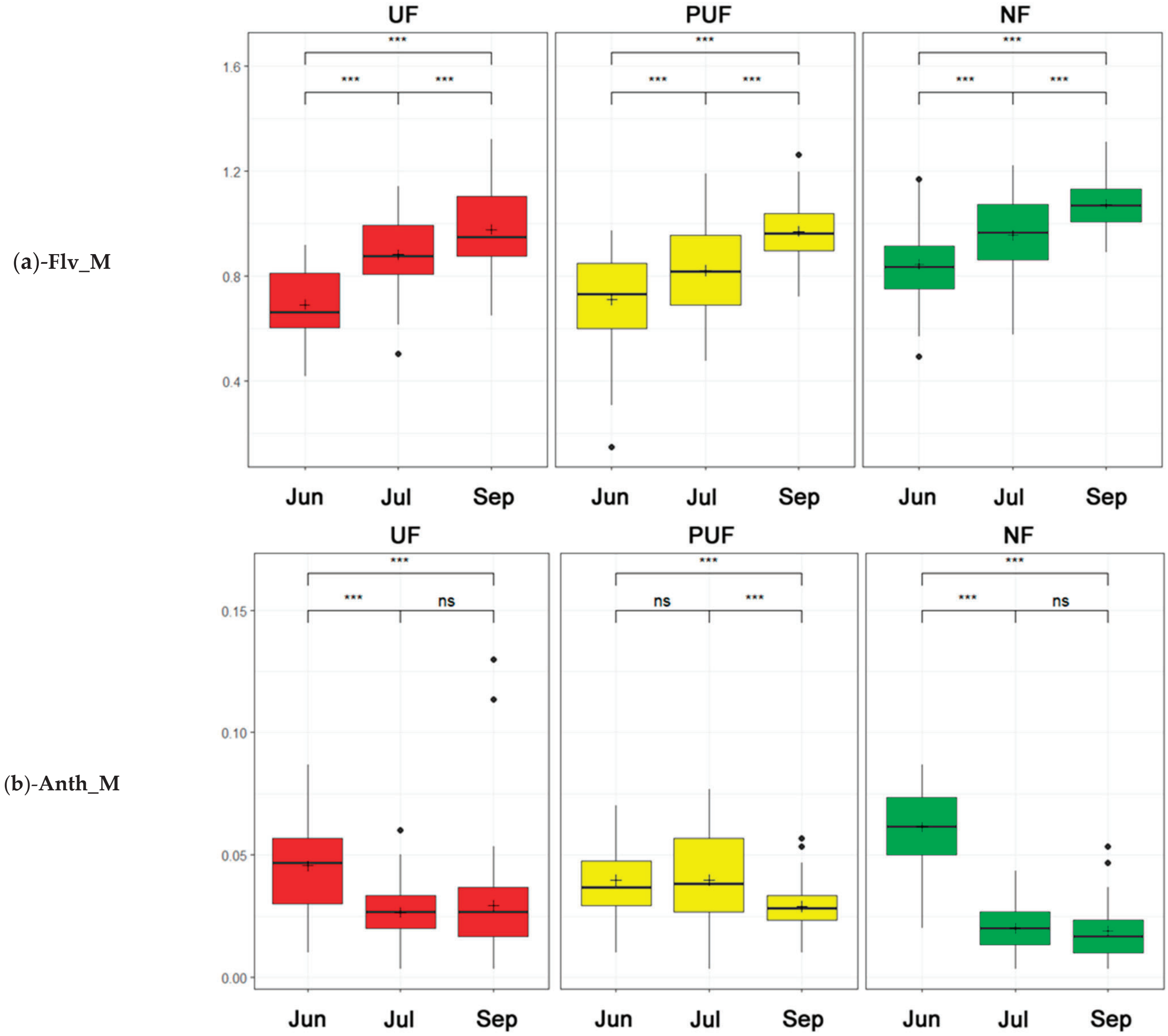


Figure 8. Two-way ANOVA box plots (a Tukey post hoc analysis was used to test statistical significance: ns = not significant, · = $p < 0.1$, *** = $p < 0.001$) that describe the trends of the three stands (green—natural forest; yellow—peri-urban forest; and red—urban forest) in three periods (from left to right: Jun = June, Jul = July, and Sep = September). (**a**) Flavonol content (Flv_M). (**b**) Anthocyanin content (Anth_M).

3. Discussion

The first finding from our phytosociological surveys testifies that the *Quercus cerris* urban and peri-urban forests were originally part of a continuum large natural forest, which underwent fragmentation due to the expansion of agricultural and built-up areas in the Campobasso municipality; furthermore, there was no evidence of planting or thinning in the three forests, and the impact of recreation in the urban forest was not significant. However, differences in the study area have inevitably occurred over time. The urban forest showed a poorer plant community composition, demonstrating the pressures of the city on nature. On the other side, the high presence of species shared with the natural forest (i.e., *Quercus cerris*, *Q. frainetto*, *Q. pubescens*, *Crataegus monogyna*, *Ligustrum vulgare*, *Prunus spinosa*, *Brachypodium sylvaticum*, and *Lathyrus venetus*) is a statement to nature's resilience amidst urban development. These differences did not emerge when comparing the EIVE values among the three stand species; in fact, no evident gradient passing from the urban to the natural forest was detected. It would, therefore, seem outwardly that the ecological requirements differed only marginally. The similarity in the values provides that the three stands are effectively part of the same ecological typology of vegetation and that, consequently, the behavioral responses of the turkey oak in the three stands are mainly influenced by the urbanization gradient.

The leaf traits investigated (SLA, LDMC, leaf thickness, and chlorophyll content) were selected based on their relevance in describing the adaptive strategies of plant species and their use in many of the studies in the last few decades [46–48,58,61–68]. In addition, the contents of anthocyanins and flavonols were investigated due to their significant physiological role (i.e., photo-oxidative stress protection and high- and low-temperature protection). The selected variables were also related to the leaf area index and climatic variables (PPT, SMOI, ACTEVP, and LST) that heavily affect plant species development and growth [66]. Finally, *Quercus cerris* was monitored in the three stands (UF, PUF, and NF) and in three different periods (June, July, and September) to evaluate the trends of the variables considered.

We observed that the variables showed different meanings when comparing Pearson's correlation results of the entire data set (three stands and three periods) with those obtained for the three stands separately (UF, PUF, and NF). The variables strongly correlated in the general analysis also had the same correlation in the analyses performed on the three subsets. In contrast, the ones that showed no significant correlation in the general analysis nullified each other with divergent correlations in the three stand analyses.

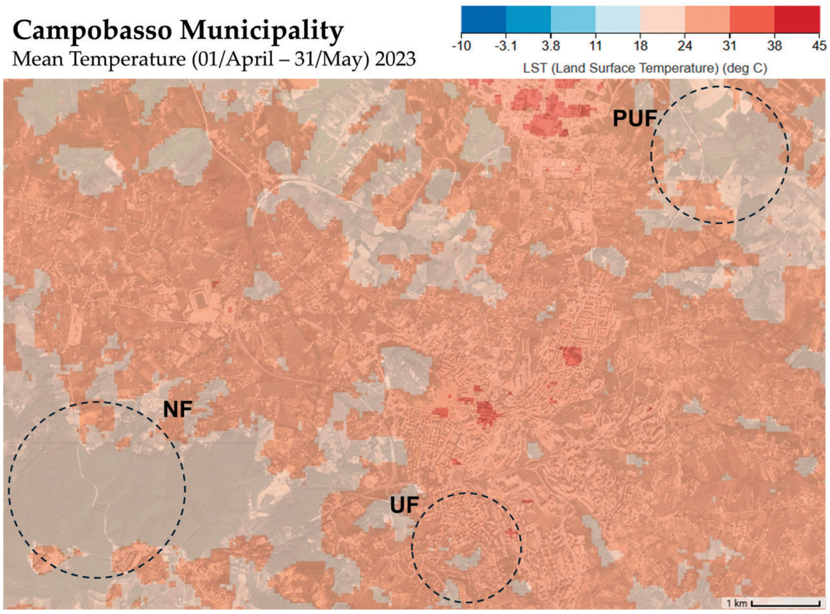
Amongst the correlations that showed the same trend in the three subsets, a strong negative correlation between SLA and LDMC (−0.70, see Figure 2) can be observed, which was quite predictable since these two indices described, on the one hand, opposite strategies for plants under different environmental conditions [46] and, on the other hand, they underwent variations according to leaf development (SLA decreasing and LDMC increasing as leaf age increases) [62,69–71]. These results were confirmed by the two-way ANOVA box plots calculated for each stand. *Q. cerris*, across the three forests, showed a decrease in specific leaf area (SLA) over the three periods, contrasting with an increase in LDMC (Figure 6). However, the variation of these trends differed between urban and natural forests. Specifically, the urban forest's SLA values decreased more significantly than those in the natural forest. SLA is a critical trait that reflects a plant's strategy for resource acquisition and utilization [72–74]. A less pronounced decrease in SLA suggests that NF plants maintain a relatively stable photosynthetic capacity under stress conditions [58]. Conversely, the significant increase in LDMC observed in the UF across all three periods suggested a strong adaptation to resource conservation and drought conditions [72,75,76]. This adaptive response, however, was absent in the natural forest during the first two periods (June and July) and only became evident in the last period (September).

The most interesting correlations were among SLA, LDMC, Flv_M, and Anth_M with the climatic variables. The results showed that an increase in temperature (LST) and a decrease in PPT, SMOI, and ACTEVP corresponded with a decrease in SLA values (generally increasing in developing leaves, in spring, and decreasing during leaf maturation), an increase in LDMC [62,69–71] (Figure 6), as well as an increase in temperature that corresponds with an increase in Flv_M and a decrease in Anth_M (Figures 2 and 8).

The combination of these results showed that increasing temperatures and leaf development play a role in the regulation of the two pigments: Anth_M was present more in juvenile or senescent leaves and under low-temperature conditions [53,77–80], while Flv_M increased to enhance plant's resilience to abiotic stressors such as high temperatures and drought [54]. The flavonol content in NF was significantly higher. Flavonols are crucial in protecting plants from environmental stress by acting as antioxidants and UV protectants.

The traits that showed few and weak correlations in the overall analysis were CHL_M and THICK_M. However, it is interesting to note that CHL_M showed a moderate negative correlation with SLA and a low positive correlation with LDMC (Figure 2). This result may be related to leaf development, which has a lower CHL_M content in young leaves (higher SLA) and increases as the leaf tissue matures (higher LDMC). Examining the results of the analyses performed on the three subsets, we observed that the divergent relationships of these two variables in the three stands may contribute to the weak correlation observed in the overall analysis. Specifically, CHL_M in urban and peri-urban forests was negatively correlated with LST and positively correlated with PPT, SMOI, and ACTEVP (Figure 3), whereas, in the natural forest, the correlation was the opposite. This discrepancy could be due to the significantly higher temperatures recorded, especially for the last sampling in September (mean temperature 1 July–31 August 2023) (Figure 9 and Table S2). Specifically, the UF (33.5 °C) and PUF (35.2 °C), compared to the NF (26.6 °C), exceeded the optimal threshold for chlorophyll synthesis (30 °C) [81,82].

(**a**)-**April–May**



(**b**)-**May–June**

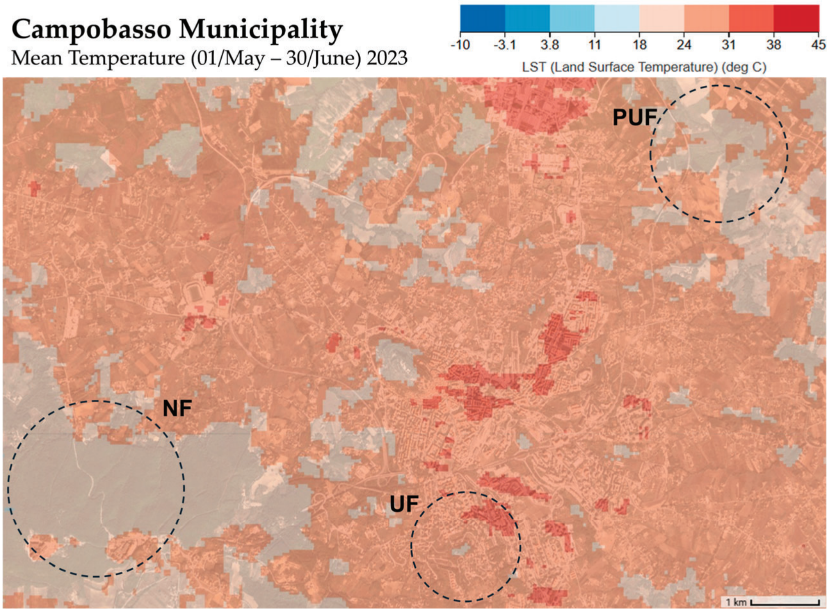


Figure 9. *Cont.*

(**c**)-**July–August**

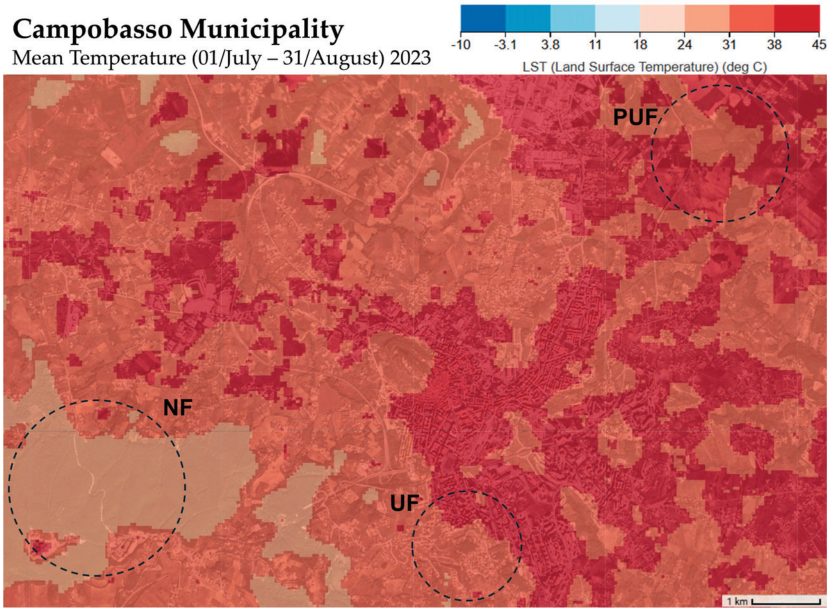


Figure 9. Land surface temperature (LST) maps of the Campobasso municipality (https://app.climateengine.org/climateEngine (accessed on 1 August 2024)), representing the mean temperature of the two months before each sampling date: (**a**) April–May for the June sampling; (**b**) May–June for the July sampling; and (**c**) July–August for the September sampling. NF—natural forest; PUF—peri-urban forest; and UF—urban forests.

In contrast, the positive correlation of CHL_M with climatic variables related to water availability emphasizes, in the urban and peri-urban forests, the water stress to which the two forests were subjected under synergetic conditions of drought and high temperatures. This correlation was expressed in the opposite way in the natural forest where, under near-optimal temperature conditions, *Q. cerris*, a species adapted to drought [83], did not appear to have any particular water requirements.

PCA confirmed the results obtained from the previous analysis. The environmental variables clearly drove the separation of the data collected in the three sampling times. LST was the most influencing climatic factor of the *Quercus cerris* responses registered in September, especially for urban and peri-urban stands; conversely, the variables related to water availability (PPT, ACTEVP, and SMOI) influenced the responses of the Turkey oak in June and July. This result is in accordance with those found by Salamanca-Fonseca et al. [84] around Bogotá city (Colombia), where they studied the effects of urban, peri-urban, and rural land covers on plant functional traits, confirming that urbanization locally increased temperatures and led to a reduction in evapotranspiration [85].

4. Materials and Methods

4.1. Study Area and Stands Selection

The study area is located in the Campobasso municipality (Molise region, Southern Italy) (Figure 10), a small Mediterranean city that is 80 km from the Adriatic Sea; it is one of the eight Italian municipalities included in the studies of the National Biodiversity Future Centre (NBFC). The stands were chosen within a grid measuring cells of 1 km × 1 km developed by an interdisciplinary group (Spoke 5: Urban Biodiversity), who aimed to improve our knowledge of biodiversity in Italian cities and to provide new insights to protect and enhance nature in built-up areas (https://www.nbfc.it/en/environments (accessed on 1 August 2024)). The three cells were selected along a gradient of fragmentation and green cover. They corresponded to an urban forest (UF), a peri-urban forest (PUF), and a natural forest (NF) [50]. The natural forest is included in the Special Area of Conservation (SAC) IT7222295 "Monte Vairano" (Directive Habitat 92/43 EEC).

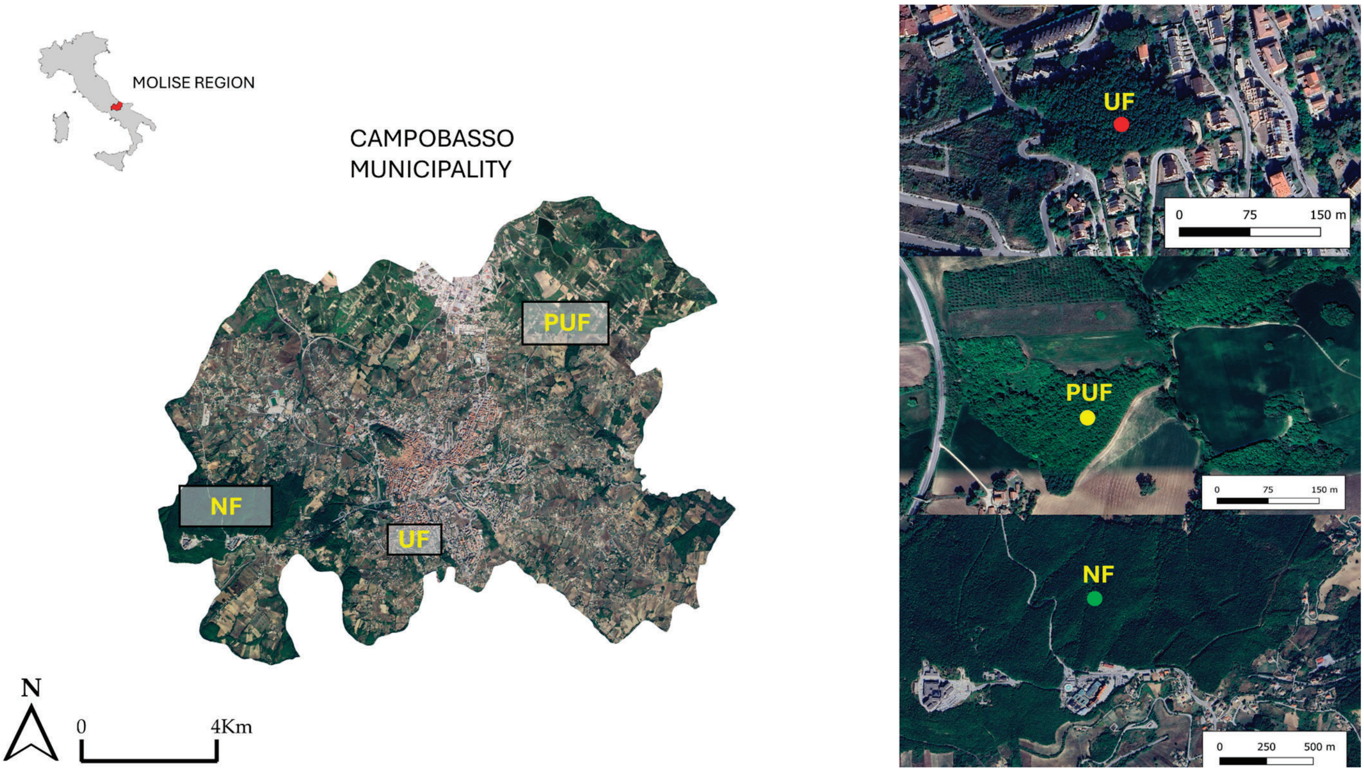


Figure 10. Location of the three stands, urban forest (UF), peri-urban forest (PUF), natural forest (NF), in the Campobasso municipality, and the location of the Molise region in Italy (red color).

The macro-bioclimate of the three stands, according to Blasi et al. [86] and Rivas-Martínez et al. [26], can be defined as temperate oceanic/sub-Mediterranean when based on the classifications of the Ecoregions of Italy [28], and the study area falls under code 1C3a1. This code identifies the territory using the following characteristics: 1 = Temperate Division, C = Apennine Province, 3 = Southern Apennine Section, a = Campanian Apennine Subsection. The urban and natural forest stands are characterized by sandstones and conglomerates, and the peri-urban forest stands are characterized by feldspathic quartz sandstones. The altitudes vary from 587 to 875 m a.s.l. The average slope for the three stands is slight and around 10–15° (Table 1). Soil pH was measured with a portable field pH meter (YY-1033 soil pH meter, YIYEGO).

Table 1. Description of the three stands studied.

Stands	Altitude (m a.s.l.)	Slope (°)	Lithology [1]	Area (ha)	pH
Urban forest (UF)	756	10	Sandstones and conglomerates	1.49	5.6
Peri-urban forest (PUF)	587	15	Feldspathic quartz sandstones	2.09	5.8
Natural forest (NF)	875	10	Sandstones and conglomerates	15.58	5.6

[1] Geological map of Molise region, scale 1:50,000 (https://www.isprambiente.gov.it/Media/carg/405_CAMPOBASSO/Foglio.html) (accessed on 3 September 2024).

4.2. Sampling Protocol

The three studied stands were coppice forests converted into high forests of Turkey oak and Hungarian oak with a dominant layer covering around 80%. Before proceeding with the leaf measurements, the phytosociological surveys were done to describe the

Quercus cerris community composition [87] and to obtain ecological information about the forests. The species and syntaxa nomenclature used followed Bartolucci et al. [88] and Biondi et al. [89], respectively.

The Ecological Indicator Values [34] (Table S3) were applied to the matrix of phytosociological relevés to calculate the weighted average response of each forest community to the abiotic factors, which express plant preferences for the following: temperatures, light, soil moisture, reaction, and nitrogen (using cover-abundance percentage values based on the Braun–Blanquet scale: r → 0.01%, + → 0.5%. 1 → 3%, 2 → 15%, 3 → 37.5%, 4 → 62.5%, 5 → 87.5%) [90].

Three sampling times were carried out in 2023 (June, July, and September), during three consecutive days under the same climatic conditions (no wind and no clouds), and in the 9:00–16:30 time frame—following Garnier et al. [64] and Vendramini et al. [61]—in order to monitor the trends of *Q. cerris* parameters as responses to environmental conditions. During the field sampling, the leaf area index (LAI) was measured using an AccuPAR LP-80 Ceptometer (METER group, Munich, Germany) (Table 2).

Table 2. The in-field and laboratory variables measured/calculated.

		Variables	Unit
Stand Structure	In-Field	Leaf area index (LAI: leaf area/ground area)	m^2/m^2
Leaf Functional Traits	In-Field	Fresh weight (FW) Leaf thickness (THICK) Chlorophyll content (CHL: chlorophyll fluorescence ratio) Anthocyanin content (Anth: anthocyanin fluorescence ratio) Flavonol content (Flv: flavonols fluorescence ratio)	g mm T940 nm/T660 mm F660 nm/F525 nm F660 nm/F325 nm
	Laboratory	Leaf area (LA) Dry weight (DW) Specific leaf area (SLA: leaf area/dry weight) Leaf dry matter content (LDMC: dry weight/fresh weight)	cm^2 g cm^2/g mg/g

In each stand, a circle plot of one hectare (ha) area was identified, maintaining a 25 m buffer zone from the boundary of the forest. From the dominant layer (average height around 20 m), seven individuals were randomly sampled (at least 20 m apart) and ten leaves were collected for each one. For the leaf collection, different branches were cut in several exposures of the tree crown to represent the individual's entire condition. The twigs were preserved in humidified air-tight bags for the subsequent steps [61].

The fresh weight (FW) was evaluated in the field with a portable balance (Kern-300 EMS) following the protocol proposed by Vendramini et al. [61]. The chlorophyll (CHL), anthocyanin (Anth), and flavonol (Flv) content were measured with the Opti-Sciences Inc. Multi-Pigment-Meter MPM-100. The thickness (THICK) was measured with a Neoteck Portable Digital Thickness Gauge. For better accuracy, these parameters were repeated in three different parts of the leaves: base, middle, and apex [91]. Subsequently, the three values were mediated (*_M). All the fresh leaves collected were scanned with an Epson GT15000 scanner (Epson Europe Electronics GmbH, Munich, Germany), and the images were analyzed with ImageJ 2.1.0/1.53c [92] to obtain the leaf area measurements. Finally, the leaves were placed in a dryer for 72 h at 70 degrees until there was complete water loss, and then the dry weight (DW) was measured.

The Plant Functional Traits, i.e., the specific leaf area (SLA) and leaf dry matter content (LDMC), were computed following Pérez-Harguindeguy et al. [40], Gottardini et al. [93], and Vendramini et al. [61] (Table 2).

4.3. Satellite Data

We used satellite data (Table 3) downloaded from the ClimateEngine.org website (https://www.climateengine.org/ (accessed on 1 August 2024)) to collect climatic data for the three different sampling periods: precipitation (PPT), soil moisture (SMOI), land surface temperature (LST), and actual evapotranspiration (ACTEVP). The data were averaged as the mean of the two months before each sampling period (April–May for the June sampling; May–June for the July sampling; and July–August for the September sampling).

Table 3. In the table, the following information are reported. *Satellite:* the name of the satellite used for the data collection. *Satellite data:* the parameter evaluated. Description: the satellite resolution and recording interval.

Satellite	Satellite Data	Description
Landsat 5/6/7/9	Land Surface Temperature (LST)	30 m of resolution—16 days
TerraClimate	Precipitation (PPT) [94] Soil Moisture (SMOI) [94] Actual Evapotranspiration (ACTEVP) [94]	4 km of resolution—monthly

4.4. Statistical Analysis

We collected field and laboratory data on *Quercus cerris* and organized all the information in a spreadsheet (Excel, 2016). The linear correlation between all the measured variables (traits and climatic variables) was calculated with Pearson's r correlation coefficient using PAST 4.17 software [60] by performing an analysis on the entire dataset (11 variables; three stands; and three time-sampling periods). Subsequently, the same analysis was performed on the three stands (UF, PUF, and NF) separately. With respect to the strength of the correlation, the following scale was used: $|r < 0.1|$ no correlation, $|0.1 < r < 0.3|$ low correlation, $|0.3 < r < 0.5|$ moderate correlation, $|0.5 < r < 0.7|$ high correlation, and $|0.7 < r < 0.1|$ very high correlation [95]. Subsequently, a principal component analysis (PCA) was performed using PAST 4.17 software. The data set was analyzed using the "correlation" option (which implies the normalization of the variables) because the variables were measured in different units. Finally, a two-way ANOVA (Rpackage stats version 4.2.1) [96] with a pairwise Tuckey HSD post hoc test ($p \leq 0.05$) (Rpackage rstatix version 0.7.2) was used to evaluate the changes in the six traits (SLA, LDMC, THICK_M, CHL_M, Flv_M, and Anth_M) for the three stands (UF, PUF, and NF) during the three sampling periods.

5. Conclusions

Our study emphasizes the importance of leaf traits as indicators of the health status of *Quercus cerris* in both natural and urbanized areas. Additionally, we aimed to identify specific leaf traits suitable for rapid survey assessments to evaluate the stress levels experienced by this species in urban environments. *Quercus cerris* (Turkey oak) is a thermophilic species that is increasingly significant in the context of climate change due to its resilience to climatic extremes and drier conditions in natural habitats across Southern and Central Europe. Our findings highlight the strong adaptability of *Quercus cerris* to diverse climatic and environmental conditions. In urban contexts, we recommend long-term and seasonal monitoring of parameters such as anthocyanin and flavonol content. These traits have demonstrated their utility in detecting specific physiological adaptations to prevailing climatic conditions. They could serve as rapid indicators of *Quercus cerris* responses to climate change, helping to identify potential vulnerabilities and enabling more targeted monitoring strategies. Future work will aim to extend this protocol to other native species characteristic of Mediterranean forest communities that have already investigated by our research group from a phylogenetic point of view [97]. This approach will contribute to the development of practical tools for afforestation and reforestation efforts, particularly in urban and peri-urban settings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants14020285/s1, Table S1: Phytosociological relevés of the sampling stands; Table S2: Climatic data of the three stands in the Campobasso municipality: natural forest (NF), peri-urban forest (PUF), and urban forest (UF). A–M: April–May 2023, J–J: June–July 2023, J–A: July–August 2023; Table S3: EIVE 1.0 indicator values for the niche position of L (light), T (temperature), M (moisture), R (reaction), and N (nitrogen availability) from Dengler et al. [34] for the 70 taxa listed in Table S1.

Author Contributions: Conceptualization, L.Q., P.D.M. and P.F.; Methodology, L.Q., P.D.M. and P.F.; Formal analysis, P.D.M. and L.Q.; Data curation, L.Q.; Writing—original draft preparation, L.Q., P.D.M. and P.F.; Writing—review and editing, L.Q., P.D.M. and P.F.; Supervision, P.F.; Funding acquisition, P.F. All authors have read and agreed to the published version of the manuscript.

Funding: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4—Call for Tender No. 3138 of 16 December 2021, rectified by Decree n. 3175 of 18 December 2021 of the Italian Ministry of University and Research funded by the European Union—NextGenerationEU (Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP: H73C22000300001, project title "National Biodiversity Future Center—NBFC").

Data Availability Statement: The authors will make the raw data supporting this article's conclusions available upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. MedECC. *Climate and Environmental Change in the Mediterranean Basin—Current Situation and Risks for the Future. First Mediterranean Assessment Report*; Zenodo: Geneva, Switzerland, 2020. [CrossRef]
2. Lana-Renault, N.; Morán-Tejeda, E.; Moreno de las Heras, M.; Lorenzo-Lacruz, J.; López-Moreno, N. Chapter 10—Land-Use Change and Impacts. In *Water Resources in the Mediterranean Region*; Zribi, M., Brocca, L., Tramblay, Y., Molle, F., Eds.; Elsevier: Amsterdam, The Netherlands, 2020; pp. 257–296. ISBN 978-0-12-818086-0.
3. Ameztegui, A.; Coll, L.; Cáceres, M.D.; Morán-Ordóñez, A. Disturbance Impacts on Mediterranean Forests across Climate and Management Scenarios. *J. Environ. Manag.* **2024**, *371*, 123193. [CrossRef] [PubMed]
4. Valladares, F.; Benavides, R.; Rabasa, S.G.; Díaz, M.; Pausas, J.G.; Paula, S.; Simonson, W.D. Global Change and Mediterranean Forests: Current Impacts and Potential Responses. In *Forests and Global Change*; Coomes, D.A., Burslem, D.F.R.P., Simonson, W.D., Eds.; Ecological Reviews; Cambridge University Press: Cambridge, UK, 2014; pp. 47–76. ISBN 978-1-107-61480-2.
5. Antrop, M. Landscape Change and the Urbanization Process in Europe. *Landsc. Urban Plan.* **2004**, *67*, 9–26. [CrossRef]
6. Konijnendijk, C.C.; Ricard, R.M.; Kenney, A.; Randrup, T.B. Defining Urban Forestry—A Comparative Perspective of North America and Europe. *Urban For. Urban Green.* **2006**, *4*, 93–103. [CrossRef]
7. de Groot, R.S.; Alkemade, R.; Braat, L.; Hein, L.; Willemen, L. Challenges in Integrating the Concept of Ecosystem Services and Values in Landscape Planning, Management and Decision Making. *Ecol. Complex.* **2010**, *7*, 260–272. [CrossRef]
8. Dobbs, C.; Kendal, D.; Nitschke, C.R. Multiple Ecosystem Services and Disservices of the Urban Forest Establishing Their Connections with Landscape Structure and Sociodemographics. *Ecol. Indic.* **2014**, *43*, 44–55. [CrossRef]
9. Livesley, S.J.; McPherson, E.G.; Calfapietra, C. The Urban Forest and Ecosystem Services: Impacts on Urban Water, Heat, and Pollution Cycles at the Tree, Street, and City Scale. *J. Environ. Qual.* **2016**, *45*, 119–124. [CrossRef]
10. Watts, N.; Adger, W.N.; Ayeb-Karlsson, S.; Bai, Y.; Byass, P.; Campbell-Lendrum, D.; Colbourn, T.; Cox, P.; Davies, M.; Depledge, M.; et al. The Lancet Countdown: Tracking Progress on Health and Climate Change. *Lancet* **2017**, *389*, 1151–1164. [CrossRef] [PubMed]
11. Morabito, M.; Crisci, A.; Gioli, B.; Gualtieri, G.; Toscano, P.; Stefano, V.D.; Orlandini, S.; Gensini, G.F. Urban-Hazard Risk Analysis: Mapping of Heat-Related Risks in the Elderly in Major Italian Cities. *PLoS ONE* **2015**, *10*, e0127277. [CrossRef]
12. Bolund, P.; Hunhammar, S. Ecosystem Services in Urban Areas. *Ecol. Econ.* **1999**, *29*, 293–301. [CrossRef]
13. Savard, J.-P.L.; Clergeau, P.; Mennechez, G. Biodiversity Concepts and Urban Ecosystems. *Landsc. Urban Plan.* **2000**, *48*, 131–142. [CrossRef]

14. Alvey, A.A. Promoting and Preserving Biodiversity in the Urban Forest. *Urban. For. Urban Green.* **2006**, *5*, 195–201. [CrossRef]
15. Bailey, S. Increasing Connectivity in Fragmented Landscapes: An Investigation of Evidence for Biodiversity Gain in Woodlands. *Ecol. Manag.* **2007**, *238*, 7–23. [CrossRef]
16. Gentili, R.; Quaglini, L.A.; Galasso, G.; Montagnani, C.; Caronni, S.; Cardarelli, E.; Citterio, S. Urban Refugia Sheltering Biodiversity across World Cities. *Urban. Ecosyst.* **2024**, *27*, 219–230. [CrossRef]
17. Nesbitt, L.; Hotte, N.; Barron, S.; Cowan, J.; Sheppard, S.R.J. The Social and Economic Value of Cultural Ecosystem Services Provided by Urban Forests in North America: A Review and Suggestions for Future Research. *Urban. For. Urban Green.* **2017**, *25*, 103–111. [CrossRef]
18. Spano, G.; Giannico, V.; Elia, M.; Bosco, A.; Lafortezza, R.; Sanesi, G. Human Health–Environment Interaction Science: An Emerging Research Paradigm. *Sci. Total Environ.* **2020**, *704*, 135358. [CrossRef] [PubMed]
19. Ordóñez Barona, C. Adopting Public Values and Climate Change Adaptation Strategies in Urban Forest Management: A Review and Analysis of the Relevant Literature. *J. Environ. Manag.* **2015**, *164*, 215–221. [CrossRef]
20. Barron, S.; Sheppard, S.R.J.; Condon, P.M. Urban Forest Indicators for Planning and Designing Future Forests. *Forests* **2016**, *7*, 208. [CrossRef]
21. Sukopp, H.; Wurzel, A. Changing Climate and the Effects on Vegetation in Central European Cities. *Arboric. J.* **2000**, *24*, 257–281. [CrossRef]
22. Calfapietra, C.; Peñuelas, J.; Niinemets, Ü. Urban Plant Physiology: Adaptation-Mitigation Strategies under Permanent Stress. *Trends Plant Sci.* **2015**, *20*, 72–75. [CrossRef]
23. Ferrini, F.; Fini, A. Gli Effetti Del Cambiamento Climatico Sugli Alberi in Ambienti Urbani Mediterranei. *Italus Hortus* **2015**, *22*, 59–74.
24. Fantozzi, D.; Montagnoli, A.; Trupiano, D.; Di Martino, P.; Scippa, G.S.; Agosto, G.; Chiatante, D.; Sferra, G. A Systematic Review of Studies on Fine and Coarse Root Traits Measurement: Towards the Enhancement of Urban Forests Monitoring and Management. *Front. Glob. Chang.* **2024**, *7*, 1322087. [CrossRef]
25. Lazzarini, L.; Mahmoud, I.; Pastore, M.C. Urban Planning for Biodiversity. *TeMA J. Land Use Mobil. Environ.* **2024**, *1*, 45–60. [CrossRef]
26. Rivas-Martínez, S.; Sáenz, S.; Penas, A. Worldwide Bioclimatic Classification System. *Glob. Geobot.* **2011**, *1*, 1–634.
27. Lenormand, M.; Papuga, G.; Argagnon, O.; Soubeyrand, M.; Barros, G.D.; Alleaume, S.; Luque, S. Biogeographical Network Analysis of Plant Species Distribution in the Mediterranean Region. *Ecol. Evol.* **2019**, *9*, 237–250. [CrossRef]
28. Blasi, C.; Capotorti, G.; Copiz, R.; Guida, D.; Mollo, B.; Smiraglia, D.; Zavattero, L. *Terrestrial Ecoregions of Italy. Map and Explanatory Notes*; Global Map Srl: Firenze, Italy, 2018.
29. Frigerio, J.; Capotorti, G.; Del Vico, E.; Ouled Larbi, M.; Grassi, F.; Blasi, C.; Labra, M.; Guidi Nissim, W. Tree Tracking: Species Selection and Traceability for Sustainable and Biodiversity-Friendly Urban Reforestation. *Plant Biosyst.* **2023**, *157*, 920–934. [CrossRef]
30. Ellenberg, H. *Zeigerwerte der Gefäßpflanzen Mitteleuropas*; Scripta Geobotanica 9; Erich Goltze KG: Gottingen, Germany, 1974.
31. Ellenberg, H.; Weber, H.E.; Düll, R.; Wirth, V.; Werner, W.; Paulißen, D. *Zeigerwerte von Pflanzen in Mitteleuropa*; Erich Goltze KG: Gottingen, Germany, 1992.
32. Hájek, M.; Dítě, D.; Horsáková, V.; Mikulášková, E.; Peterka, T.; Navrátilová, J.; Jiménez-Alfaro, B.; Hájková, P.; Tichý, L.; Horsák, M. Towards the Pan-European Bioindication System: Assessing and Testing Updated Hydrological Indicator Values for Vascular Plants and Bryophytes in Mires. *Ecol. Indic.* **2020**, *116*, 106527. [CrossRef]
33. Tichý, L.; Axmanová, I.; Dengler, J.; Guarino, R.; Jansen, F.; Midolo, G.; Nobis, M.P.; Van Meerbeek, K.; Aćić, S.; Attorre, F.; et al. Ellenberg-Type Indicator Values for European Vascular Plant Species. *J. Veg. Sci.* **2023**, *34*, e13168. [CrossRef]
34. Dengler, J.; Jansen, F.; Chusova, O.; Hüllbusch, E.; Nobis, M.P.; Meerbeek, K.V.; Axmanová, I.; Bruun, H.H.; Chytrý, M.; Guarino, R.; et al. Ecological Indicator Values for Europe (EIVE) 1.0. *Veg. Classif. Surv.* **2023**, *4*, 7–29. [CrossRef]
35. Kermavnar, J.; Kutnar, L. Three Decades of Understorey Vegetation Change in Quercus-Dominated Forests as a Result of Increasing Canopy Mortality and Global Change Symptoms. *J. Veg. Sci.* **2024**, *35*, e13317. [CrossRef]
36. Santoianni, L.A.; Innangi, M.; Varricchione, M.; Carboni, M.; La Bella, G.; Haider, S.; Stanisci, A. Ecological Features Facilitating Spread of Alien Plants along Mediterranean Mountain Roads. *Biol. Invasions* **2024**, *26*, 3879–3899. [CrossRef]
37. Stefańska-Krzaczek, E.; Krzaczek, R.; Mazurek, N.; Chmura, D. Variability and Determinants of Vascular Plant Species Composition in Patches of Old Managed Oak Forest Stands Dispersed within Scots Pine Monocultures. *Ecosyst* **2024**, *11*, 100235. [CrossRef]
38. Sanczuk, P.; Verheyen, K.; Lenoir, J.; Zellweger, F.; Lembrechts, J.J.; Rodríguez-Sánchez, F.; Baeten, L.; Bernhardt-Römermann, M.; De Pauw, K.; Vangansbeke, P.; et al. Unexpected Westward Range Shifts in European Forest Plants Link to Nitrogen Deposition. *Science* **2024**, *386*, 193–198. [CrossRef] [PubMed]
39. Maynard, D.S.; Bialic-Murphy, L.; Zohner, C.M.; Averill, C.; van den Hoogen, J.; Ma, H.; Mo, L.; Smith, G.R.; Acosta, A.T.R.; Aubin, I.; et al. Global Relationships in Tree Functional Traits. *Nat. Commun.* **2022**, *13*, 3185. [CrossRef] [PubMed]

40. Pérez-Harguindeguy, N.; Díaz, S.; Garnier, E.; Lavorel, S.; Poorter, H.; Jaureguiberry, P.; Bret-Harte, M.S.; Cornwell, W.K.; Craine, J.M.; Gurvich, D.E.; et al. New Handbook for Standardised Measurement of Plant Functional Traits Worldwide. *Aust. J. Bot.* **2013**, *61*, 167–234. [CrossRef]
41. Kraft, N.J.B.; Valencia, R.; Ackerly, D.D. Functional Traits and Niche-Based Tree Community Assembly in an Amazonian Forest. *Science* **2008**, *322*, 580–582. [CrossRef]
42. Li, R.; Zhu, S.; Chen, H.Y.H.; John, R.; Zhou, G.; Zhang, D.; Zhang, Q.; Ye, Q. Are Functional Traits a Good Predictor of Global Change Impacts on Tree Species Abundance Dynamics in a Subtropical Forest? *Ecol. Lett.* **2015**, *18*, 1181–1189. [CrossRef]
43. Valladares, F.; Matesanz, S.; Guilhaumon, F.; Araújo, M.B.; Balaguer, L.; Benito-Garzón, M.; Cornwell, W.; Gianoli, E.; van Kleunen, M.; Naya, D.E.; et al. The Effects of Phenotypic Plasticity and Local Adaptation on Forecasts of Species Range Shifts under Climate Change. *Ecol. Lett.* **2014**, *17*, 1351–1364. [CrossRef]
44. Quaranta, L.; Di Marzio, P.; Di Pietro, R.; Ferretti, F.; Di Salvatore, U.; Fortini, P. Analysis of the Functional Traits of *Quercus cerris* L. Seedlings in the Molise Region (Southern Italy). *Plant Sociol.* **2022**, *59*, 11–24. [CrossRef]
45. Choat, B.; Brodribb, T.J.; Brodersen, C.R.; Duursma, R.A.; López, R.; Medlyn, B.E. Triggers of Tree Mortality under Drought. *Nature* **2018**, *558*, 531–539. [CrossRef]
46. Garnier, E.; Shipley, B.; Roumet, C.; Laurent, G. A Standardized Protocol for the Determination of Specific Leaf Area and Leaf Dry Matter Content. *Funct. Ecol.* **2001**, *15*, 688–695. [CrossRef]
47. Cornelissen, J.H.C.; Cerabolini, B.; Castro-Díez, P.; Villar-Salvador, P.; Montserrat-Martí, G.; Puyravaud, J.P.; Maestro, M.; Werger, M.J.A.; Aerts, R. Functional Traits of Woody Plants: Correspondence of Species Rankings between Field Adults and Laboratory-Grown Seedlings? *J. Veg. Sci.* **2003**, *14*, 311–322. [CrossRef]
48. Castro-Díez, P.; Puyravaud, J.P.; Cornelissen, J.H.C. Leaf Structure and Anatomy as Related to Leaf Mass per Area Variation in Seedlings of a Wide Range of Woody Plant Species and Types. *Oecologia* **2000**, *124*, 476–486. [CrossRef] [PubMed]
49. Díaz, S.; Kattge, J.; Cornelissen, J.H.C.; Wright, I.J.; Lavorel, S.; Dray, S.; Reu, B.; Kleyer, M.; Wirth, C.; Prentice, I.C.; et al. The Global Spectrum of Plant Form and Function: Enhanced Species-Level Trait Dataset. *Sci. Data* **2022**, *9*, 755. [CrossRef]
50. Esperon-Rodriguez, M.; Rymer, P.D.; Power, S.A.; Challis, A.; Marchin, R.M.; Tjoelker, M.G. Functional Adaptations and Trait Plasticity of Urban Trees along a Climatic Gradient. *Urban For. Urban Green.* **2020**, *54*, 126771. [CrossRef]
51. Varricchione, M.; Carranza, M.L.; D'Angeli, C.; de Francesco, M.C.; Innangi, M.; Santoianni, L.A.; Stanisci, A. Exploring the Distribution Pattern of Native and Alien Forests and Their Woody Species Diversity in a Small Mediterranean City. *Plant Biosyst.* **2024**, *158*, 1335–1346. [CrossRef]
52. Kattge, J.; Bönisch, G.; Díaz, S.; Lavorel, S.; Prentice, I.C.; Leadley, P.; Tautenhahn, S.; Werner, G.D.A.; Aakala, T.; Abedi, M.; et al. TRY Plant Trait Database—Enhanced Coverage and Open Access. *Glob. Chang. Biol.* **2020**, *26*, 119–188. [CrossRef] [PubMed]
53. Chalker-Scott, L. Environmental Significance of Anthocyanins in Plant Stress Responses. *Photochem. Photobiol.* **1999**, *70*, 1–9. [CrossRef]
54. Laoué, J.; Fernandez, C.; Ormeño, E. Plant Flavonoids in Mediterranean Species: A Focus on Flavonols as Protective Metabolites under Climate Stress. *Plants* **2022**, *11*, 172. [CrossRef]
55. Daryanavard, H.; Postiglione, A.E.; Mühlemann, J.K.; Muday, G.K. Flavonols Modulate Plant Development, Signaling, and Stress Responses. *Curr. Opin. Plant Biol.* **2023**, *72*, 102350. [CrossRef] [PubMed]
56. Mattila, H.; Valev, D.; Havurinne, V.; Khorobrykh, S.; Virtanen, O.; Antinluoma, M.; Mishra, K.B.; Tyystjärvi, E. Degradation of Chlorophyll and Synthesis of Flavonols during Autumn Senescence—The Story Told by Individual Leaves. *AoB PLANTS* **2018**, *10*, ply028. [CrossRef]
57. Wilson, P.J.; Thompson, K.; Hodgson, J.G. Specific Leaf Area and Leaf Dry Matter Content as Alternative Predictors of Plant Strategies. *New Phytol.* **1999**, *143*, 155–162. [CrossRef]
58. Wright, I.J.; Reich, P.B.; Westoby, M.; Ackerly, D.D.; Baruch, Z.; Bongers, F.; Cavender-Bares, J.; Chapin, T.; Cornelissen, J.H.C.; Diemer, M.; et al. The Worldwide Leaf Economics Spectrum. *Nature* **2004**, *428*, 821–827. [CrossRef]
59. Terzi, M.; Ciaschetti, G.; Fortini, P.; Rosati, L.; Viciani, D.; Di Pietro, R. A revised phytosociological nomenclature for the Italian *Quercus cerris* woods. *Mediterr. Bot.* **2020**, *41*, 101–120.
60. Hammer, O.; Harper, D.; Ryan, P. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontol. Electron.* **2001**, *4*, 4.
61. Vendramini, F.; Díaz, S.; Gurvich, D.E.; Wilson, P.J.; Thompson, K.; Hodgson, J.G. Leaf Traits as Indicators of Resource-Use Strategy in Floras with Succulent Species. *New Phytol* **2002**, *154*, 147–157. [CrossRef]
62. Karavin, N. Effects of Leaf and Plant Age on Specific Leaf Area in Deciduous Tree Species *Quercus cerris* L. var. *cerris. Bangladesh J. Bot.* **2013**, *42*, 301–306. [CrossRef]
63. Dijkstra, P.; Lambers, H. Analysis of Specific Leaf Area and Photosynthesis of Two Inbred Lines of *Plantago major* Differing in Relative Growth Rate. *New Phytol* **1989**, *113*, 283–290. [CrossRef] [PubMed]
64. Garnier, E.; Laurent, G. Leaf Anatomy, Specific Mass and Water Content in Congeneric Annual and Perennial Grass Species. *New Phytol* **1994**, *128*, 725–736. [CrossRef]

65. Ordoñez, J.C.; Van Bodegom, P.M.; Witte, J.-P.M.; Wright, I.J.; Reich, P.B.; Aerts, R. A Global Study of Relationships between Leaf Traits, Climate and Soil Measures of Nutrient Fertility. *Glob. Ecol. Biogeogr.* **2009**, *18*, 137–149. [CrossRef]
66. Karavin, N.; Kilinc, M. Variation in SLA and LMA of Deciduous *Quercus cerris* Var. *cerris and Evergreeen Phillyrea latifolia According to Directional, Seasonal and Climatical Parameters. Ekoloji* **2011**, *20*, 21–29. [CrossRef]
67. Valladares, F.; Martinez-Ferri, E.; Balaguer, L.; Pérez-Corona, M.E.; Manrique, E. Low Leaf-Level Response to Light and Nutrients in Mediterranean Evergreen Oaks: A Conservative Resource-Use Strategy? *New Phytol.* **2000**, *148*, 79–91. [CrossRef] [PubMed]
68. Gratani, L.; Foti, I. Estimating Forest Structure and Shade Tolerance of the Species in a Mixed Deciduous Broad-Leaved Forest in Abruzzo, Italy. *Ann. Bot. Fenn.* **1998**, *35*, 75–83.
69. Reich, P.B.; Walters, M.B.; Ellsworth, D.S. Leaf Age and Season Influence the Relationships between Leaf Nitrogen, Leaf Mass per Area and Photosynthesis in Maple and Oak Trees. *Plant Cell Environ.* **1991**, *14*, 251–259. [CrossRef]
70. Luo, T.; Luo, J.; Pan, Y. Leaf Traits and Associated Ecosystem Characteristics across Subtropical and Timberline Forests in the Gongga Mountains, Eastern Tibetan Plateau. *Oecologia* **2005**, *142*, 261–273. [CrossRef] [PubMed]
71. Milla, R.; Reich, P.B.; Niinemets, Ü.; Castro-Díez, P. Environmental and Developmental Controls on Specific Leaf Area Are Little Modified by Leaf Allometry. *Funct. Ecol.* **2008**, *22*, 565–576. [CrossRef]
72. Wang, J.; Wang, X.; Ji, Y.; Gao, J. Climate Factors Determine the Utilization Strategy of Forest Plant Resources at Large Scales. *Front. Plant Sci.* **2022**, *13*, 990441. [CrossRef] [PubMed]
73. Weisse, M.; Goldman, E.; Carter, S. *Forest Pulse: The Latest on the World's Forests*; World Resources Institute: Washington, DC, USA, 2021; Available online: https://research.wri.org/gfr/forest-pulse (accessed on 30 November 2024).
74. Anderson, C.G.; Bond-Lamberty, B.; Stegen, J.C. Active Layer Depth and Soil Properties Impact Specific Leaf Area Variation and Ecosystem Productivity in a Boreal Forest. *PLoS ONE* **2020**, *15*, e0232506. [CrossRef] [PubMed]
75. Vaieretti, M.V.; Díaz, S.; Vile, D.; Garnier, E. Two Measurement Methods of Leaf Dry Matter Content Produce Similar Results in a Broad Range of Species. *Ann. Bot.* **2007**, *99*, 955–958. [CrossRef]
76. Shomali, A.; Das, S.; Arif, N.; Sarraf, M.; Zahra, N.; Yadav, V.; Aliniaeifard, S.; Chauhan, D.K.; Hasanuzzaman, M. Diverse Physiological Roles of Flavonoids in Plant Environmental Stress Responses and Tolerance. *Plants* **2022**, *11*, 3158. [CrossRef] [PubMed]
77. Camm, E.L.; McCALLUM, J.; Leaf, E.; Koupai-Abyazani, M.R. Cold-Induced Purpling of *Pinus contorta* Seedlings Depends on Previous Daylength Treatment. *Plant Cell Environ.* **1993**, *16*, 761–764. [CrossRef]
78. Murray, J.R.; Smith, A.G.; Hackett, W.P. Differential Dihydroflavonol Reductase Transcription and Anthocyanin Pigmentation in the Juvenile and Mature Phases of Ivy (*Hedera helix* L.). *Planta* **1994**, *194*, 102–109. [CrossRef]
79. Shi, L.; Li, X.; Fu, Y.; Li, C. Environmental Stimuli and Phytohormones in Anthocyanin Biosynthesis: A Comprehensive Review. *Int. J. Mol. Sci.* **2023**, *24*, 16415. [CrossRef]
80. Pei, Z.; Huang, Y.; Ni, J.; Liu, Y.; Yang, Q. For a Colorful Life: Recent Advances in Anthocyanin Biosynthesis during Leaf Senescence. *Biology* **2024**, *13*, 329. [CrossRef] [PubMed]
81. Nagata, N.; Tanaka, R.; Satoh, S.; Tanaka, A. Identification of a Vinyl Reductase Gene for Chlorophyll Synthesis in *Arabidopsis thaliana* and Implications for the Evolution of Prochlorococcus Species. *Plant Cell* **2005**, *17*, 233–240. [CrossRef] [PubMed]
82. Li, Y.; He, N.; Hou, J.; Xu, L.; Liu, C.; Zhang, J.; Wang, Q.; Zhang, X.; Wu, X. Factors Influencing Leaf Chlorophyll Content in Natural Forests at the Biome Scale. *Front. Ecol. Evol.* **2018**, *6*, 64. [CrossRef]
83. Šimková, M.; Vacek, S.; Šimůnek, V.; Vacek, Z.; Cukor, J.; Hájek, V.; Bílek, L.; Prokůpková, A.; Štefančík, I.; Sitková, Z.; et al. Turkey Oak (*Quercus cerris* L.) Resilience to Climate Change: Insights from Coppice Forests in Southern and Central Europe. *Forests* **2023**, *14*, 2403. [CrossRef]
84. Salamanca-Fonseca, M.; Aldana, A.M.; Vargas-Martinez, V.; Acero-Gomez, S.; Fonseca-Tellez, J.; Gutierrez, S.; Hoyos, Y.D.; León, K.M.; Márquez, C.; Molina-R, L.; et al. Effects of Urban, Peri-Urban and Rural Land Covers on Plant Functional Traits around Bogotá, Colombia. *Urban Ecosyst.* **2024**, *27*, 251–260. [CrossRef]
85. Mazrooei, A.; Reitz, M.; Wang, D.; Sankarasubramanian, A. Urbanization Impacts on Evapotranspiration Across Various Spatio-Temporal Scales. *Earths Future* **2021**, *9*, e2021EF002045. [CrossRef]
86. Blasi, C. (Ed.) *La Vegetazione d'Italia. Con Carta Delle Serie di Vegetazione in Scala 1:500,000*; Palombi Editori: Roma, Italy, 2010.
87. Braun-Blanquet, J. *Pflanzensoziologie: Grundzüge der Vegetationskunde*; 3. neubearb. und wesentlich verm. Aufl.; Springer: New York, NY, USA, 1964.
88. Bartolucci, F.; Peruzzi, L.; Galasso, G.; Albano, A.; Alessandrini, A.; Ardenghi, N.M.G.; Astuti, G.; Bacchetta, G.; Ballelli, S.; Banfi, E.; et al. An Updated Checklist of the Vascular Flora Native to Italy. *Plant Biosyst.* **2018**, *152*, 179–303. [CrossRef]
89. Biondi, E.; Blasi, C.; Allegrezza, M.; Anzellotti, I.; Azzella, M.M.; Carli, E.; Casavecchia, S.; Copiz, R.; Del Vico, E.; Facioni, L.; et al. Plant Communities of Italy: The Vegetation Prodrome. *Plant Biosyst.* **2014**, *148*, 728–814. [CrossRef]
90. Canullo, R.; Allegrini, M.C.; Campetella, G. Reference Field Manual for Vegetation Surveys on the CONECOFOR LII Network, Italy (National Programme of Forest Ecosystems Control—UNECE, ICP Forests). *Braun Blanquetia* **2012**, *48*, 5–65.

91. Caprari, C.; Bucci, A.; Ciotola, A.C.; Del Grosso, C.; Dell'Edera, I.; Di Bartolomeo, S.; Di Pilla, D.; Divino, F.; Fortini, P.; Monaco, P.; et al. Microbial Biocontrol Agents and Natural Products Act as Salt Stress Mitigators in *Lactuca sativa* L. *Plants* **2024**, *13*, 2505. [CrossRef]
92. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2012**, *9*, 671–675. [CrossRef] [PubMed]
93. Gottardini, E.; Cristofolini, F.; Cristofori, A.; Pollastrini, M.; Ferretti, M. *Misura Della Fluorescenza Della Clorofilla a, Contenuto Di Clorofilla e Tratti Fogliari: Campionamento, Raccolta e Misurazioni*; Guida per Studi: Campo, CA, USA, 2016. [CrossRef]
94. Abatzoglou, J.T.; Dobrowski, S.Z.; Parks, S.A.; Hegewisch, K.C. TerraClimate, a High-Resolution Global Dataset of Monthly Climate and Climatic Water Balance from 1958–2015. *Sci. Data* **2018**, *5*, 170191. [CrossRef] [PubMed]
95. Kuckartz, U.; Rädiker, S.; Ebert, T.; Schehl, J. *Statistik: Eine verständliche Einführung*; Springer: Berlin/Heidelberg, Germany, 2013; ISBN 978-3-531-19890-3.
96. Chambers, J.M.; Freeny, A.E.; Heiberger, R.M. Analysis of Variance; Designed Experiments. In *Statistical Models in S*; Routledge: Milton Park, UK, 1992; ISBN 978-0-203-73853-5.
97. Di Pietro, R.; Quaranta, L.; Mattioni, C.; Simeone, M.C.; Di Marzio, P.; Proietti, E.; Fortini, P. Chloroplast Haplotype Diversity in the White Oak Populations of the Italian Peninsula, Sicily, and Sardinia. *Forests* **2024**, *15*, 864. [CrossRef]

GENERAL CONCLUSION AND FUTURE PERSPECTIVES

This thesis results has contributed to new phylogenetical and ecological insights into the genus *Quercus*. The plastid DNA of European white oaks have revealed limited genetic differentiation despite their extensive taxonomic diversity. A total of 29 haplotypes were identified, distributed in a structured manner across the Euro-Mediterranean region and the Near East. These findings suggest that climatic and geological events from the Late Tertiary and Quaternary periods influenced the mixing and isolation of haplotypes, providing opportunities for more recent diversification. Southern Europe, in particular, harbours significant yet underexplored genetic variability, with the Italian Peninsula standing out as a crossroads and reservoir of haplotypic diversity. In fact, the research on the chloroplast diversity in white oak populations in Italy, Sicily, and Sardinia confirmed substantial genetic variability, emphasizing the importance of these areas as glacial refugia and centers of diversification. These findings further support the idea that genetic diversity in white oaks is shaped by complex historical and geographical processes. It is my intention to continue this line of research, adding new oak populations collected in countries not yet investigated in eastern Europe to those already studied, in order to gain a more complete insight into the genetic diversity within the genus *Quercus*.

Regarding the ecological study of *Quercus cerris* through the Plant Functional Traits, the research of the Turkey oak seedlings, revealed significant variations in response to different environmental conditions. These variations influence the adaptability and survival of seedlings, underlining the importance of considering functional traits in the conservation and forest management strategies. Furthermore, the analysis of *Quercus cerris* leaf traits in Mediterranean urban context, highlighted that the traits selected can be used as indicators of the urban forest health. Moreover, they provide useful insights for rapidly assessing ecological conditions and planning forest management strategies.

In addition, during my PhD program, I collected the same Functional Traits data along a transect from the Tyrrhenian Sea to the Adriatic Sea with the monitoring of six natural stands distributed between 0 and 1200 m above sea level. The monitored species were: *Abies alba* Mill,, *Acer campestre* L., *Carpinus orientalis* Mill, *Cornus sanuguinea* L., *Crataegus monogyna* Jacq., *Ligustrum vulgare* L:, *Pistacia lentiscus* L., *Quercus cerris* L., *Q. frainetto* Ten, *Q. ilex* L., *Q. petraea* Liebl, *Q. pubescens* Willd, *Ruscus aculeatus* L., *Sorbus torminalis* (L.) Crantz. A dataset with over 300,000 data was obtained. It is my intention to complete and publish this research, surely that the results will be useful in order to obtain other knowledges about species response in different environmental conditions.

Finally, integrating knowledge of the genetic diversity and functional traits offers a more comprehensive understanding of the evolutionary and adaptive processes of these species. This information is crucial for developing sustainable conservation and management strategies for forests, particularly in the context of global environmental changes.

ADDITIONAL ACTIVITIES DURING PhD

During my Doctoral thesis, in addition to research activities related to the phylogenetic and ecological analysis of oaks, I have participated to other botanic activities. In these three years, I had the opportunity to take part into several in-field studies, in particular, I was involved in phytosociological surveys with my research group, investigating the plant diversity of the territories of the "Alto Molise". In this way I have enriched my skills in the identification of plant species of different habitats and learn new knowledge about phytosociological surveys. These activities have also led to several scientific publications as the first floristic reports for the Molise region, of native and alien plant species. In addition, during these three years, I have also taken part in several multidisciplinary works, which have involved the laboratory in which I did my PhD and other research groups of the University of Molise. These works have allowed me to enrich my knowledges and to take part in scientific publications.

plants MDPI

Article

Microbial Biocontrol Agents and Natural Products Act as Salt Stress Mitigators in *Lactuca sativa* L.

Claudio Caprari [1,*], Antonio Bucci [1], Anastasia C. Ciotola [1], Carmine Del Grosso [2,3], Ida Dell'Edera [1], Sabrina Di Bartolomeo [1], Danilo Di Pilla [1], Fabio Divino [1,4], Paola Fortini [1], Pamela Monaco [1], Davide Palmieri [2], Michele Petraroia [1], Luca Quaranta [1], Giuseppe Lima [2] and Giancarlo Ranalli [1]

[1] Department of Biosciences and Territory, University of Molise, C.da Fonte Lappone snc, 86090 Pesche, Italy; antonio.bucci@unimol.it (A.B.); a.ciotola@studenti.unimol.it (A.C.C.); i.delledera@studenti.unimol.it (I.D.); sabrina.dibartolomeo@unimol.it (S.D.B.); d.dipilla1@studenti.unimol.it (D.D.P.); fabio.divino@unimol.it (F.D.); fortini@unimol.it (P.F.); pamela.monaco@unimol.it (P.M.); m.petraroia2@studenti.unimol.it (M.P.); l.quaranta1@studenti.unimol.it (L.Q.); ranalli@unimol.it (G.R.)
[2] Department of Agriculture, Environment and Food Sciences, University of Molise, Via F. De Sanctis snc, 86100 Campobasso, Italy; c.delgrosso2@studenti.unimol.it (C.D.G.); davide.palmieri@unimol.it (D.P.); lima@unimol.it (G.L.)
[3] Institute for Sustainable Plant Protection, National Research Council (CNR), 70126 Bari, Italy
[4] Department of Mathematics and Statistics, University of Jyväskylä, 40014 Jyväskylä, Finland
* Correspondence: claudio.caprari@unimol.it; Tel.: +39-0874404152

Abstract: One of the major problems related to climate change is the increase in land area affected by higher salt concentrations and desertification. Finding economically and environmentally friendly sustainable solutions that effectively mitigate salt stress damage to plants is of great importance. In our work, some natural products and microbial biocontrol agents were evaluated for their long-term effectiveness in reducing salt stress in lettuce (*Lactuca sativa* L. var. *romana*) plants. Fourteen different treatments applied to soil pots, with and without salt stress, were analyzed using biometric (leaf and root length and width), physiological (chlorophyll and proline content), and morphological (microscopic preparations) techniques and NGS to study the microbial communities in the soil of plants subjected to different treatments. Under our long-term experimental conditions (90 days), the results showed that salt stress negatively affected plant growth. The statistical analysis showed a high variability in the responses of the different biostimulant treatments. Notably, the biocontrol agents *Papiliotrema terrestris* (strain PT22AV), *Bacillus amyloliquefaciens* (strain B07), and *Rahnella aquatilis* (strain 36) can act as salt stress mitigators in *L. sativa*. These findings suggest that both microbial biocontrol agents and certain natural products hold promise for reducing the adverse effects of salt stress on plants.

Keywords: abiotic stress; biocontrol; stress mitigation; antagonistic microorganism; plant biostimulation; *Lactuca sativa* var. *romana*

check for updates

Citation: Caprari, C.; Bucci, A.; Ciotola, A.C.; Del Grosso, C.; Dell'Edera, I.; Di Bartolomeo, S.; Di Pilla, D.; Divino, F.; Fortini, P.; Monaco, P.; et al. Microbial Biocontrol Agents and Natural Products Act as Salt Stress Mitigators in *Lactuca sativa* L. *Plants* **2024**, *13*, 2505. https://doi.org/10.3390/plants13172505

Academic Editor: Roberto Barbato

Received: 30 July 2024
Revised: 3 September 2024
Accepted: 4 September 2024
Published: 6 September 2024

1. Introduction

Lettuce (*Lactuca sativa* L. var. *romana*), a member of the Asteraceae family, is one of the most popular vegetables. It is becoming increasingly accepted by consumers because it is healthy and easy to prepare, especially in salads. The most used parts are the leaves, followed by the stems (for juice) and seeds (for seed mixes) [1]. The popularity achieved by lettuce, which is consumed throughout the world, is also because it thrives best at temperatures between 7 and 24 °C. In addition, since lettuce is a long-day plant, varieties that are not affected by the length of the day have been selected to allow rich foliage to grow in all light conditions [1–3]. Lettuce leaves are a healthy source of glycosylated flavonoids, phenolic acids, carotenoids, vitamin B groups, ascorbic acid, tocopherols, sesquiterpene lactones, minerals, and fiber; they are essentially a good source of vitamins and antioxidants. These phytochemicals are very useful and beneficial to the human body. They also have a positive effect on controlling blood cholesterol levels [4–7]. Lettuce plants are moderately sensitive to salinity stress, which negatively affects their productivity [8].

Climate change is becoming a critical factor, since it has a significant impact on all areas of human activity, with negative effects on the environment in many parts of the world, on the life and health of people, and on various sectors of the economy [9]. Agriculture is particularly vulnerable to the impact of climate change, as it is one of the most weather-dependent sectors of the economy. The negative effects of climate change, coupled with the rapid melting of glaciers and the prolonged periods of drought, have led to a significant decline in agricultural development worldwide, especially in semi-arid regions [10,11].

Among plant abiotic stresses, salinity is particularly harmful to lettuce plants, since these plants are moderately sensitive to salinity stress; high salinity negatively affects lettuce productivity. Salinity causes significant damage to biodiversity, ecosystems, human health, and natural resources, posing a serious threat to modern agriculture [12,13]. Salinity stress affects plants in several ways: (1) it decreases the water potential at the rhizosphere level, resulting in a plant water deficit; (2) it induces phytotoxicity due to the accumulation of ions such as Na^+ and Cl^-, usually leading to the abundant production of reactive oxygen species (ROS) with the impairment of physiological processes, especially photosynthesis and protein synthesis [14,15]; and (3) it decreases plant nutrient uptake and transport [16]. However, the primary factor contributing to salt accumulation in agricultural soils is the high concentration of salts in irrigation water [17]. Finally, increased soil salinity can also result from plant transpiration or evaporation from the soil surface, leading to higher water evapotranspiration and further soil salinization [9]. To make effective decisions for sustainable agricultural management, it is essential to consider these climate changes and assess the impact of air temperature on soil salinity [18].

Over the last 5 years (2020–2024), a constantly increasing number of papers on biostimulants and plant growth have been published (from 193 in 2020 to 277 in 2024, according to Scopus 6/2024), which confirms the interest in this matter. Plant growth-promoting microorganisms (PGPMs) are a potential source of both alternative agrochemicals (fertilizers and pesticides) and environmental stress mitigators [19–21]. The same authors adopted lettuce as a plant model to describe the biocontrol activity of *Azotobacter chroococcum* against fungal infection. They hypothesized a double-mode action for this organism as both an antagonist agent and a biostimulant [19]. Moreover, an interesting research field is the biological control of plant growth, including the suppression of pathogens by beneficial microorganisms that have been isolated and adopted in several studies, both in vitro and in vivo. In recent years (2020–2024), a constant number of papers that have used “biological control” and “plant growth” as keywords have been published (1591 in 2020; 1719 in 2021; 1706 in 2022; 1730 in 2023; and 900 in 2024, according to Scopus 6/2024) [22].

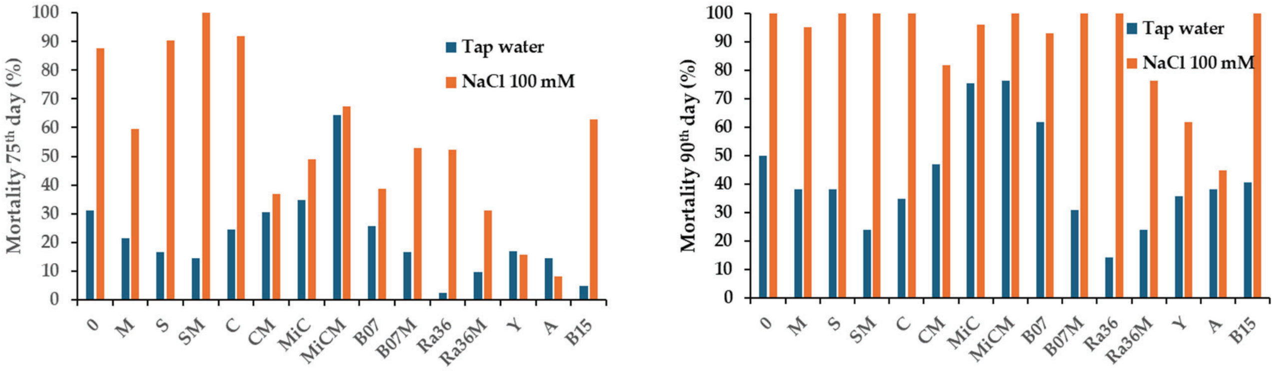
Microbial biocontrol agents (BCAs) represent a promising alternative to chemical control agents, with several BCA formulations already available for practical use [23,24]. Recently, newly characterized lipopeptides from *Bacillus amyloliquefaciens* exhibited strong inhibitory activity against *Fusarium oxysporum*, indicating a potential role in biocontrol activity [25]. In a recent study, the biocontrol agent *Papiliotrema terrestris*, strain PT22AV, was developed as the active component of a granular formulation already tested as a biological fungicide in several trials [26]. Moreover, the endophytic rhizobacterium *Rahnella aquatilis*, strain 36, controlled the root-infecting fungal pathogen *F. oxysporum* [27].

This work represents a cohort (also called “follow-up”) study based on 14 different treatments carried out over a long period of time (up to 90 days) on *L. sativa* var. *romana* plants with and without NaCl salinity stress. Specifically, the overall objectives of our work were to compare several natural products and to verify whether three previously characterized microbial biocontrol agents (*P. terrestris*, strain PT22AV; *B. amyloliquefaciens*, strain B07; and *R. aquatilis*, strain 36) could act as salt stress-mitigating agents on *L. sativa*. This study involved the following steps: (i) verifying the effects of different microbial biocontrol agents, natural products, and biostimulant applications on lettuce growth and mortality; (ii) determining the attenuation by these applications of induced salt stress by measuring physiological performances; and (iii) analyzing the microbial community in the lettuce's peaty substrate, both treated and untreated with salt.

2. Results

2.1. Mortality Analysis

Plant mortality was evaluated in terms of cumulative incidence, i.e., the number of plants that died within a given time divided by the total number of plants at the beginning of the study, expressed as a percentage. Specifically, the 75th and 90th days were considered significant time points. Figure 1 summarizes the mortality data of the plants on the 75th and 90th days subjected to different treatments with protein hydrolysates and cultures of microorganisms (bacteria and yeasts). The 75th day was considered because it was the time of 100% mortality for the first plot of plants (SM treatment), whereas the 90th day was the last monitoring time of the experiment.



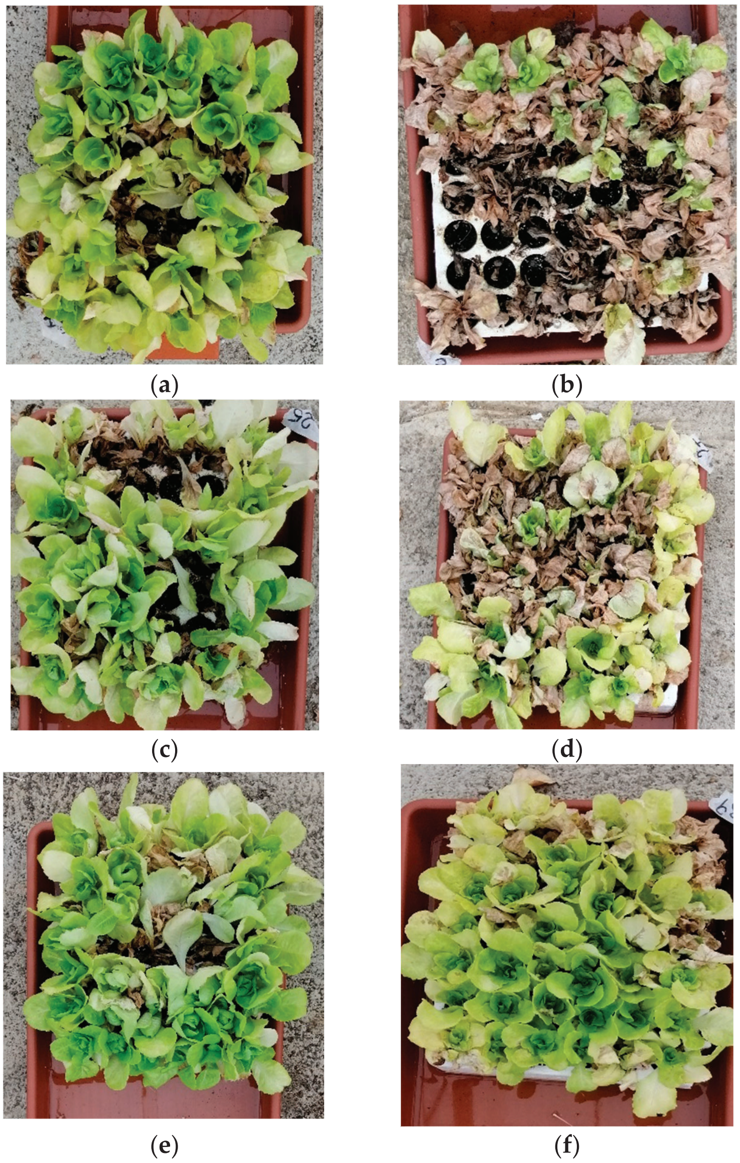
(**a**) (**b**)

Figure 1. Mortality cumulative incidence of lettuce plants on (**a**) 75th and (**b**) 90th days after exposure to saline stress (controls vs. treatments). Legend: 0 = control without treatments; M = molasses; S: soil; SM = S + M; C = compost; CM = C + M; MiC = micro compost mix; MiCM = MiC + M; B07 = *B. amyloliquefaciens* strain B07; B07M = B07 + M; Ra36 = *R. aquatilis* strain 36; Ra36M = Ra36 + M; Y = *P. terrestris* strain PT22AV; A = Activeg; B15 = Bioalga 15.

As an example, Figure 2c–f show the results of the treatments of *L. sativa* with the microbial strain B07 and *P. terrestris* PT22AV. These two treatments showed a marked mitigating effect against salt stress on day 75. The survival was still high compared to the control plants (Figure 2a,b).

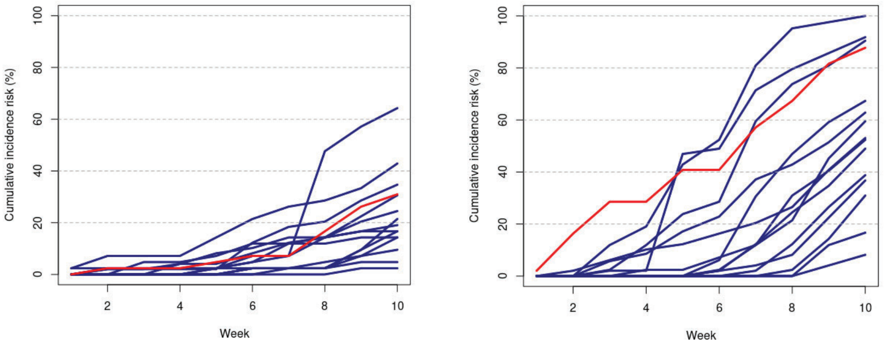
In Figure 3, the blue lines represent the cumulative incidence risk (CIR, %) trajectories for each treatment, which were reported across a time span of ten weeks under the experimental conditions with only tap water (panel a) and with the addition of NaCl (panel b). In both panels of Figure 3, the CIRs calculated with respect to the respective control treatment (denoted by j = 0) are reported in the color red. Note that the higher the cumulative incidence risk of mortality, the lower the level of protection of the respective treatment.

Then, we considered the Z test to control for the potential differences between the CIRs of each treatment and the respective control CIRs across the ten weeks. In other words, with respect to the plots in Figure 3, we controlled how significantly higher or lower the levels of the blue lines were each week compared to the levels of the respective red line.



(**a**) (**b**) (**c**) (**d**) (**e**) (**f**)

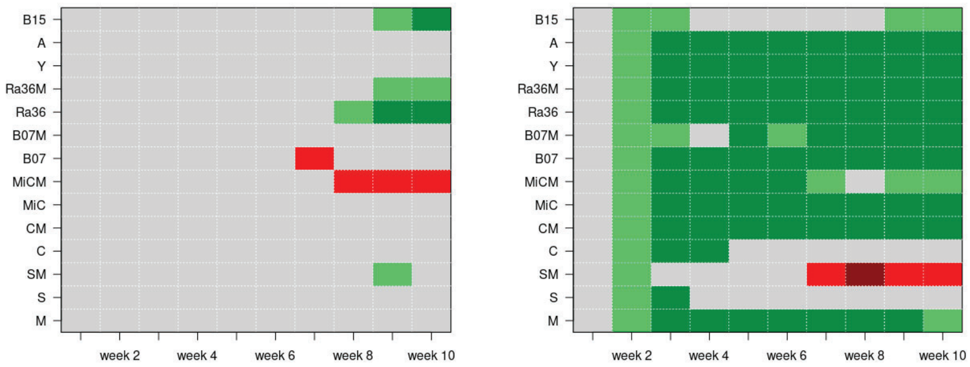
Figure 2. Mortality of lettuce plants on 75th day: (**a**) tap water as control, (**b**) 100 mM NaCl solution as control, (**c**) B07 treatment with tap water, (**d**) B07 treatment with 100 mM NaCl, (**e**) *P. terrestris* strain PT22AV treatment with tap water, and (**f**) *P. terrestris* strain PT22AV treatment with 100 mM NaCl.



(**a**) (**b**)

Figure 3. In blue, the CIR trajectories for each treatment are shown, while in red, the CIR trajectories of the respective control treatment are shown, across the ten weeks, under the experimental conditions with only tap water (**a**) and with the addition of NaCl (**b**).

In Figure 4, panel (a) shows the data for only tap water and panel (b) shows the data for the addition of NaCl. The levels of significance of the Z tests are reported as colored images for each treatment and each week: the pixels are in dark green and dark red when the CIR_{jt} was, respectively, lower or higher than the CIR_{0t} with a significance of 0.001; the pixels are in light green and light red when the CIR_{jt} was, respectively, lower or higher than the CIR_{0t} with a significance of 0.01; and finally, the pixels are in gray when there was no significant difference between the CIR_{jt} and CIR_{0t}.



(**a**) (**b**)

Figure 4. The levels of significance of the Z tests, across the ten weeks, under the experimental conditions with only tap water (**a**) and with the addition of NaCl (**b**): the pixels in dark green and dark red show the CIRs, respectively, lower or higher than the control CIRs (significance 0.001); the pixels in light green and light red show the CIRs, respectively, lower or higher than the control CIRs (significance 0.01); and the pixels in gray show no significant difference between the treatment and control CIRs. Legend: M = molasses; S: soil; SM = S + M; C = compost; CM = C + M; MiC = micro compost mix; MiCM = MiC + M; B07 = *B. amyloliquefaciens* strain B07; B07M = B07 + M; Ra36 = *R. aquatilis* strain 36; Ra36M = Ra36 + M; Y = *P. terrestris* strain PT22AV; A = Activeg; B15 = Bioalga 15.

Further, to better assess the effectiveness of each treatment, the respective CIRs (in percentages) of the treatments and controls are reported in Table 1 for the 10th week under the experimental conditions with only tap water and with the addition of NaCl.

Table 1. Respective CIRs (in percentage) of treatments and controls.

Test	0	M	S	SM	C	CM	MiC	MiCM	B07	B07M	Ra36	Ra36M	Y	A	B15
H_2O	31.0	21.4	16.7	14.3	24.5	30.6	34.7	64.3	42.9	16.7	2.4	9.5	19.0	14.3	4.8
NaCl 100 mM	87.8	59.5	90.5	100.0	91.8	36.7	49.0	67.3	38.8	53.1	52.4	31.0	16.7	8.2	62.9

Legend: 0 = control without treatments; M = molasses; S: soil; SM = S + M; C = compost; CM = C + M; MiC = micro compost mix; MiCM = MiC + M; B07 = *B. amyloliquefaciens* strain B07; B07M = B07 + M; Ra36 = *R. aquatilis* strain 36; Ra36M = Ra36 + M; Y = *P. terrestris* strain PT22AV; A = Activeg; B15 = Bioalga 15.

From this Table 1, it can be seen that only a few treatments were effective at mitigating NaCl-induced stress. Among the best were the two treatments with the biostimulant Activeg (CIR = 8.2%) and the treatment with the biocontrol agent Y (*P. terrestris*). The latter had a CIR of 16.7%. The other treatments, such as B07 (*B. amyloliquefaciens*) and RA36M (*R. aquatilis* strain 36 + M), were less effective. Molasses (M) added alone to the compost treatments induced a CIR reduction.

The experimental design (Section 4.1) included fourteen different treatments. The treatments were applied to both the control and salt-stressed lettuce plants. We focused on the three biocontrol agents and Activeg for the subsequent in vivo analysis because not all treatments proved effective. We also reported on the molasses treatment because it was an additional part of the biocontrol agent treatments.

2.2. Biometric Measures

The biometric data (see Section 4.3.2) of the whole lettuce plants were obtained in a non-destructive analysis by measuring the leaf size, height, and width on the 60th day, as shown in Table 2.

Table 2. The biometric data of the whole lettuce plants through the measurement of the leaf size at 60 days. Plants were exposed to NaCl 100 mM, to tap water (control), and to several mitigation treatments. The values are means ± SD (n = 13). Significance was evaluated by Student's *t*-test. Different letters in the same line indicate statistical differences at $p < 0.05$.

Codes	Biometric Data of Lettuce			
	Height (cm), Mean (±SD)		**Width (cm), Mean (±SD)**	
	Tap Water	**NaCl 100 mM**	**Tap Water**	**NaCl 100 mM**
0	8.0 (1.1) a	6.7 (0.9) b	3.1 (0.8) a	2.4 (0.4) b
M	8.8 (1.2) a	7.5 (1.0) b	3.6 (1.0) a	3.2 (0.9) a
B07	7.0 (0.8) a	7.1 (0.6) a	2.9 (0.4) a	3.0 (0.4) a
B07M	10.7 (1.4) a	6.9 (0.8) b	3.7 (1.0) a	3.8 (1.1) a
Ra36	12.7 (1.9) a	8.0 (1.1) b	4.3 (1.2) a	3.8 (1.0) a
Ra36M	12.1 (1.7) a	9.0 (1.3) b	3.9 (1.0) a	3.2 (0.7) b
Y	10.0 (1.1) a	11.2 (1.7) b	3.7 (0.6) a	3.7 (0.8) a
A	9.3 (1.2) a	11.7 (1.8) b	3.1 (0.5) a	4.1 (0.4) b

Legend: 0 = control without treatments; M = molasses; B07 = *B. amyloliquefaciens* strain B07; B07M = B07 + M; Ra36 = *R. aquatilis* strain 36; Ra36M = Ra36 + M; Y = *P. terrestris* strain PT22AV; A = Activeg.

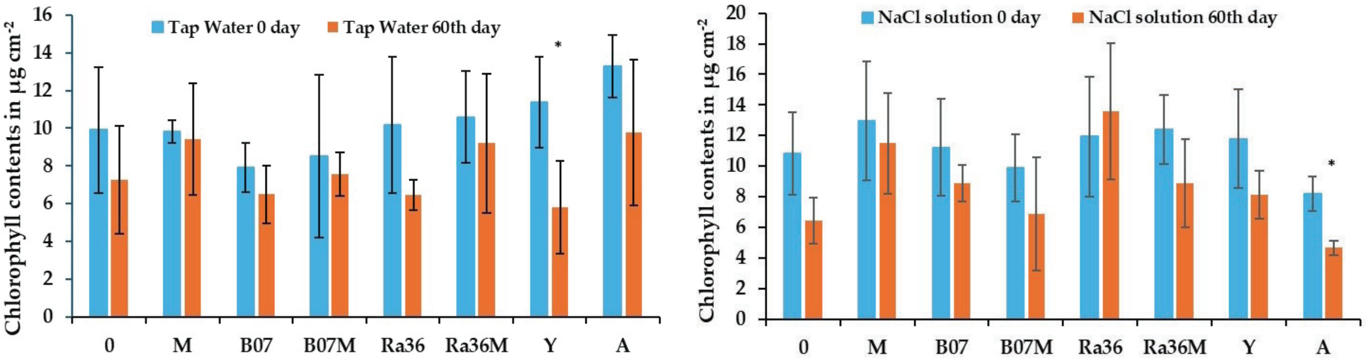
Student's *t*-test showed significant effects of the salinity level and biostimulant applications, but not for all of the growth parameters evaluated. The B07M, Ra36, and Ra36M treatments showed a significant reduction in the height of the lettuce leaves, while only the Activeg and Y treatments induced a significant increase in the height when comparing the control treatment with the salt treatment. Non-significant variations were recorded in most of the tests regarding the width of the lettuce leaves. The Ra36M and Activeg treatments were the exceptions.

2.3. Chlorophyll Content Analysis

The relative chlorophyll content in the leaves of the water-only controls and of the NaCl-treated plants on the 60th day of exposure is shown in Figure 5a,b. The chlorophyll content decreased with time under both the stress and non-stress conditions. Decreases in the chlorophyll content of up to several percentage points occurred under both conditions, but only the *P. terrestris* treatment (Figure 5a) was found to be statistically significant in the water-only samples. For the salt-stressed samples, only the treatment with Activeg was statistically significant (Figure 5b). The Ra36 sample showed an increase in the chlorophyll content in the salt-stressed samples, although the difference was not significant, contrary to the general trend. Finally, regarding the decrease in the chlorophyll content caused by the action of salt stress, the best performance was observed for the treatment with Ra36. The sample treated with *P. terrestris* showed a good effect in buffering the decrease in the chlorophyll content.

2.4. Proline Assay

A proline assay was performed on lettuce leaves only at the end of the long-term study (on day 90), according to the initial experimental design, which included non-destructive parameters and analyses. Under our conditions, in many of the lettuce leaves subjected to the treatments, the proline assays showed values close to or below the limit of the sensitivity of the calibration curve (<0.17 μg/mL). However, the proline assay on the lettuce leaves associated with the Ra36M, Y, and A biotreatments showed slightly higher values ranging from 0.90 to 4.5 μg/mL, but no statistical differences were recorded. These results suggest that to obtain useful information on the true effects of salinity stress and the real efficacy of the compared biostimulants, performing a proline assay in lettuce leaves after the late stage of plant growth (90 days) is not suitable.



(**a**) (**b**)

Figure 5. Chlorophyll contents in μg cm^{-2}, mean ± SD (n = 3), of treated plants and their response to salt stress and control treatments. Tap water (**a**) and 100 mM NaCl (**b**), at two times (0 and 60th day). Significant difference of 0 day-treated plants with respect to 60th day-treated plants, based on Student's *t*-test (* $p \leq 0.05$). Legend: 0 = control without treatments; M = molasses; B07 = *B. amyloliquefaciens* strain B07; B07M = B07 + M; Ra36 = *R. aquatilis* strain 36; Ra36M = Ra36 + M; Y = *P. terrestris* strain PT22AV; A = Activeg.

2.5. Confocal Microscope Observations

The autofluorescence of lettuce leaf samples was also observed using a confocal microscope to evaluate the modulation of the tissue structure in response to the different conditions. As shown in Figure 6b, the leaves of NaCl-treated plants presented an abnormal stomatal structure and a different fluorescence pattern compared to the control plants on the 75th day (Figure 6a), reflecting the response of leaves to the strong salt stress. However, these effects appeared to be strongly mitigated when the plants were grown in the presence of the microbial agents B07 (Figure 6d) and *P. terrestris* (Figure 6f) compared to their tap water controls (Figures 6c and 6e, respectively).

2.6. Root Analysis

Root observations on the 75th and 90th days are reported in Figures 7 and S1.

The roots of the lettuce seedlings grown with H_2O alone (control) compared to all of the treatments showed the following (Figures 7a and S1a): (a) In 12 cases, a root length between 1.1 and 2.0 cm was observed and (b) only the B07 treatment revealed a root length between 2.1 and 3.0 cm. The color of the root system was generally cream except for the B07-treated roots, which appeared white (Figures 7a and S1c). Furthermore, root branching in 10 treatments showed values between 0.6 and 1.0 cm. The remaining three treatments (M, B07M, and Ra36M) showed higher branching (1.1–2.0 cm). These values indicate a healthy root system. On the other hand, the lettuce roots treated with 0.1 M NaCl showed high variability in their length between treatments. A value of less than 0.5 cm was observed for the treatment with NaCl alone (Figures 7a and S1b); a value between 0.6 and 1.0 cm was observed for the treatment with M. Values between 1.1 and 2.0 cm were recorded for the B07 and B07M treatments (Figures 7a and S1d). Finally, the highest root length values were observed for the Ra36, Ra36M, Y, and A treatments. Similarly, higher values of root branching (2.1–3.0 cm) appeared in the Ra36, Ra36M, and Y treatments, where the roots were light or dark brown in color, unbranched, and attached to the bottom of the polystyrene tray, indicating a suboptimal state of the root system, as shown in Figure 7a,b. The results in Figure 7b show further deterioration in the parameters considered (length, branching, and color) for the lettuce roots on day 90 of the saline treatments, compared with the data from day 75.

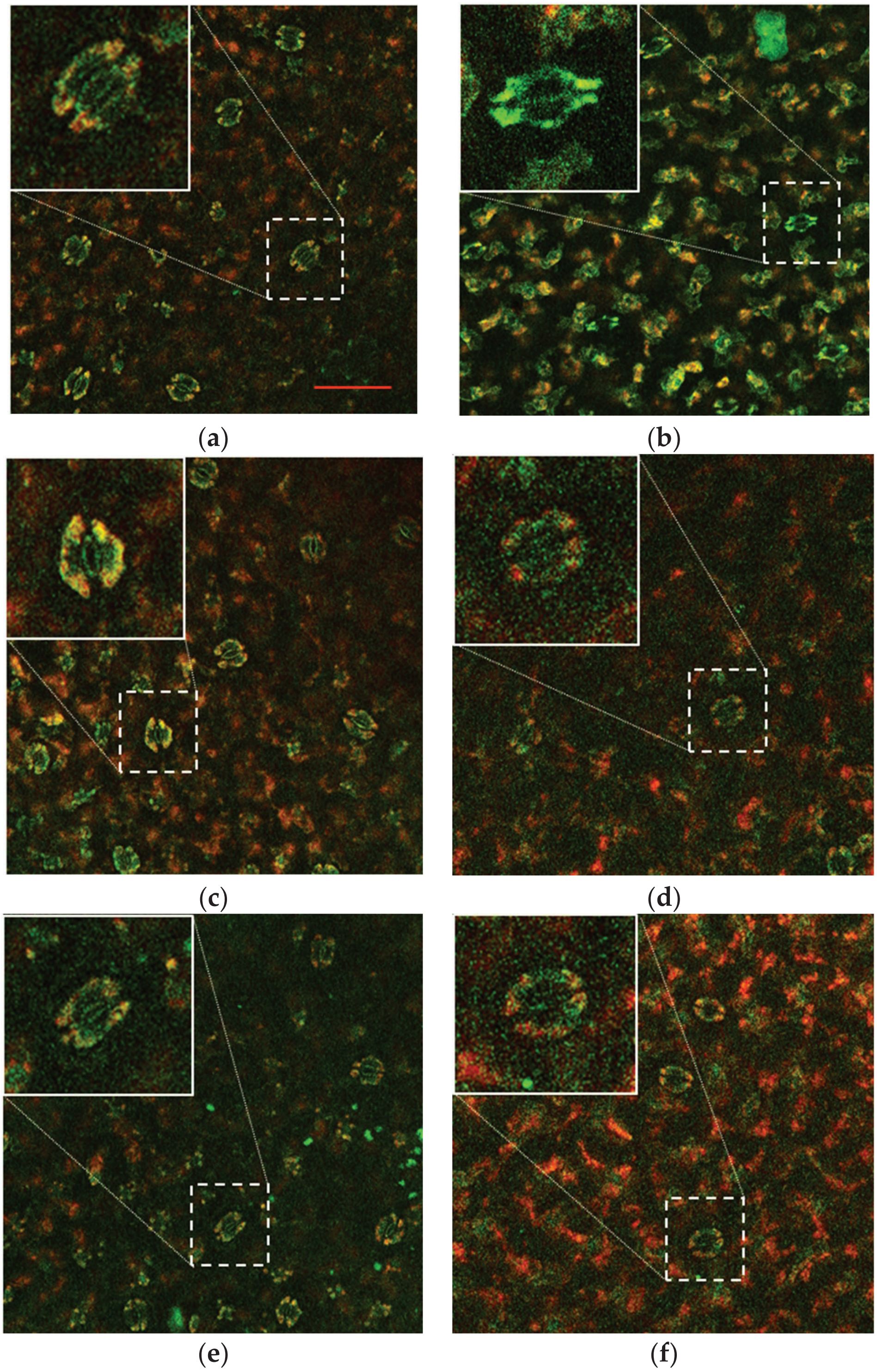
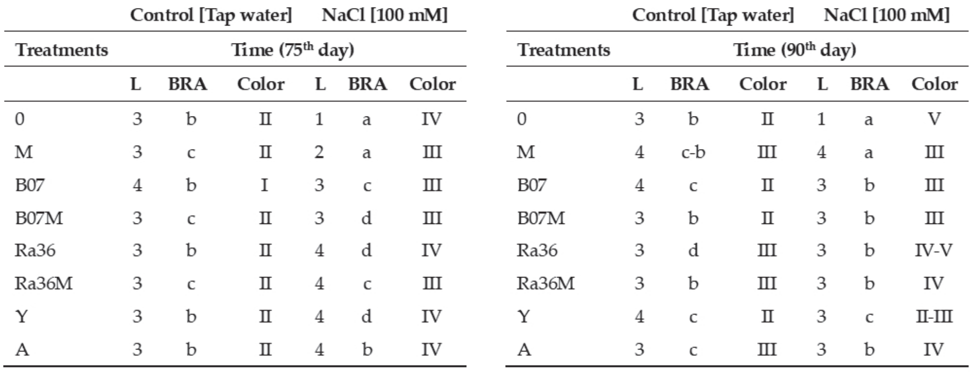


Figure 6. Confocal microscope observations of lettuce leaves: (**a**) tap water control, (**b**) 100 mM NaCl solution control, (**c**) B07 treatment with tap water, (**d**) B07 treatment with 100 mM NaCl, (**e**) *P. terrestris* strain PT22AV treatment with tap water, and (**f**) *P. terrestris* strain PT22AV treatment with 100 mM NaCl. Red and green fluorescence signals were captured after 400–600 nm laser excitation of plants pigments. Representative images of at least 10 fields are shown. Scale bar: 100 µm. Inset shows 3× magnification of stomata.



	Control [Tap water]			NaCl [100 mM]		
Treatments	Time (75th day)					
	L	BRA	Color	L	BRA	Color
0	3	b	II	1	a	IV
M	3	c	II	2	a	III
B07	4	b	I	3	c	III
B07M	3	c	II	3	d	III
Ra36	3	b	II	4	d	IV
Ra36M	3	c	II	4	c	III
Y	3	b	II	4	d	IV
A	3	b	II	4	b	IV

(**a**)

	Control [Tap water]			NaCl [100 mM]		
Treatments	Time (90th day)					
	L	BRA	Color	L	BRA	Color
0	3	b	II	1	a	V
M	4	c-b	III	4	a	III
B07	4	c	II	3	b	III
B07M	3	b	II	3	b	III
Ra36	3	d	III	3	b	IV-V
Ra36M	3	b	III	3	b	IV
Y	4	c	II	3	c	II-III
A	3	c	III	3	b	IV

(**b**)

Figure 7. Length, branching, and root color of lettuce plants in response to salt stress (tap water and 100 mM NaCl) on 75th day (**a**) and on 90th day (**b**). L = length (1 < 0.5 cm; 2 = 0.6–1.0 cm; 3 = 1.1–2.0 cm; 4 = 2.1–3.0 cm; 5 > 3.1 cm). BRA = branching (a < 0.5 cm; b = 0.6–1.0 cm; c = 1.1–2.0 cm; d = 2.1–3.0 cm; e > 3.1 cm). Color (I, white; II, creme; III, light brown; IV, dark brown; V, black). Treatments: 0 = control without treatments; M = molasses; B07 = *B. amyloliquefaciens* strain B07; B07M = B07 + M; Ra36 = *R. aquatilis* strain 36; Ra36M = Ra36 + M; Y = *P. terrestris* strain PT22AV; A = Activeg.

2.7. Viable Microbial Count

The viable microbial count results for the pot soil are reported in Table 3.

Table 3. The microbial counts (as log CFU g^{-1} ± SD) analyzed in the treated pot soil exposed to both tap water and salt solution (NaCl 100 mM) on the 90th day. The results are expressed as the mean of three replicates separately analyzed ± SD. The exact value of (*p*) was evaluated by Student's *t*-test.

Treatments	Microbial Groups	Tap Water Log CFU g^{-1} (±SD)	NaCl 100 mM Log CFU g^{-1} (±SD)	*p*
0	TVBC	5.9 (0.35)	5.0 (0.30)	0.03309
	F and Y	3.4 (0.21)	3.6 (0.26)	0.26481
	SFB	5.2 (0.25)	4.3 (0.30)	0.01960
M	TVBC	5.0 (0.40)	6.4 (0.25)	0.05988
	F and Y	4.7 (0.28)	5.4 (0.40)	0.05635
	SFB	3.6 (0.35)	3.9 (0.36)	0.29379
B07	TVBC	6.7 (0.38)	6.0 (0.46)	0.50818
	F and Y	4.5 (0.43)	4.7 (0.64)	0.60456
	SFB	6.2 (0.47)	5.3 (0.38)	0.07152
B07M	TVBC	6.5 (0.32)	5.6 (0.65)	0.11194
	F and Y	4.4 (0.25)	4.1 (0.24)	0.27961
	SFB	6.0 (0.66)	5.0 (0.40)	0.09997
Ra36	TVBC	7.0 (0.65)	6.7 (0.55)	0.63783
	F and Y	5.5 (0.20)	5.7 (0.30)	0.29601
	SFB	5.2 (0.35)	6.0 (0.40)	0.50334
Ra36M	TVBC	7.2 (0.55)	6.8 (0.40)	0.42308
	F and Y	5.4 (0.22)	5.8 (0.35)	0.13245
	SFB	4.8 (0.21)	6.2 (0.20)	0.00097
Y	TVBC	6.7 (0.65)	6.7 (0.22)	0.90566
	F and Y	6.0 (0.49)	6.9 (0.26)	0.03573
	SFB	6.6 (0.95)	4.5 (0.30)	0.02349
A	TVBC	5.1 (0.35)	6.8 (0.40)	0.00467
	F and Y	4.7 (0.28)	5.4 (0.40)	0.05635
	SFB	3.6 (0.35)	4.9 (0.36)	0.09610

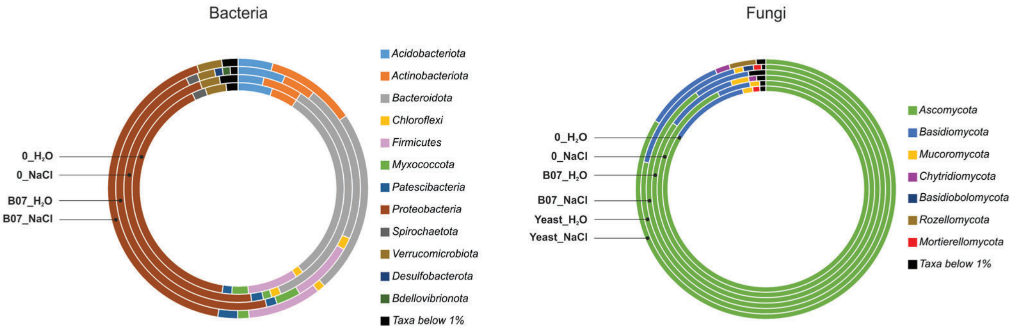
Legend: 0 = control without treatments; M = molasses; B07 = *B. amyloliquefaciens* strain B07; B07M = B07 + M; Ra36 = *R. aquatilis* strain 36; Ra36M = Ra36+ M; Y = *P. terrestris* strain PT22AV; A = Activeg. TVBC = Total Viable Aerobic Bacteria; F and Y = fungi and yeasts; SFB = spore-forming bacteria.

Considering all ofmthe treatments, a significant difference was shown only for the 0 (control) and A (Activeg) treatments for the total viable bacterial count (Table 3). The control showed a lower number of microbes in the NaCl-treated sample. In the Activeg treatment, we observed a higher value of the TVBC with a low amount of the treatment. Only the 0, Ra36M, and Y (*P. terrestris*) treatments showed significant differences in the number of spore-forming bacteria. The control and Y treatments followed the same trend, with a reduction in the microbial count in the stressed samples; otherwise, an increased value was found for the stressed Ra36M treatment, and high values were obtained for both tap water and the saline solution. Finally, when *P. terrestris* was added to the NaCl treatment, the result was a significant increase in the number of yeast and fungal cells, and the final microbe number was close to 10^7 CFU g^{-1} fw^{-1}.

2.8. Microbial Community Analysis

Soil bacterial communities of lettuce seedlings subjected to salt stress (samples: 0_NaCl_2 and 0_NaCl_3) and of stressed plants treated with the B07 strain (samples: B07_NaCl_1, B07_NaCl_2, and B07_NaCl_3) were examined using an NGS (next-generation sequencing) analysis of 16S rRNA gene amplicons (V3-V4 regions). The corresponding control samples (0_H_2O_1, 0_H_2O_3 and B07_H_2O_2, B07_H_2O_3, respectively) were also analyzed. The total number of final reads for each of these samples is reported in Table S2.

To examine the taxonomic composition of the lettuce soil bacterial communities according to the treatment type, the relative abundances of the different taxa were averaged for each experimental group. Overall, twelve bacterial phyla showed relative abundance values higher than 1%. With percentages between 40.45 and 46.81% and between 21.14 and 34.61%, respectively, *Proteobacteria* and *Bacteroidota* were among the most represented phyla, followed by *Firmicutes*, *Acidobacteriota*, and *Actinobacteriota* (Figure 8a).



(**a**) (**b**)

Figure 8. Bacterial (**a**) and fungal (**b**) community composition in controls and treatments at phylum level.

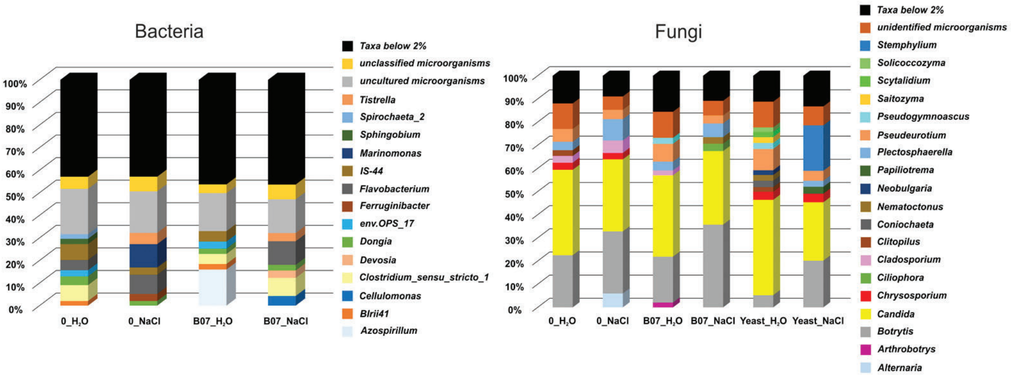
An important reduction in the relative abundance of *Bacteroidota* was observed in the soil microbial communities of lettuce seedlings treated with the B07 strain (B07_H_2O and B07_NaCl groups) compared to the 0_H_2O- and 0_NaCl-associated bacterial communities. Indeed, the relative abundance of this phylum in the 0_H_2O and 0_NaCl samples was 30.26% and 34.61%, respectively, whereas in the samples B07_H_2O and B07_NaCl, it decreased to 21.14% and 22.91%, respectively.

With regards to *Firmicutes* and *Actinobacteriota*, interesting differences were observed based on the experimental groups. The *Firmicutes* percentage was significantly lower in the soil samples of salt-stressed plants (0_NaCl group = 0.62%) compared to the other sample groups (0_H_2O = 7.68%; B07_H_2O = 8.26%; B07_NaCl = 9.14%).

The *Actinobacteriota* phylum—which showed a relative abundance between 4.00 and 5.59% in the 0_H_2O, 0_NaCl, and B07_H_2O samples—reached 11.23% in the soil of stressed *L. sativa* L. seedlings treated with the B07 strain (B07_NaCl).

Acidobacteriota exhibited higher values of relative abundance in the samples not subjected to salt stress, with a difference (Δ) of 1.61% between the 0_H_2O and 0_NaCl groups and 1.96% between the $B07\_H_2O$ and B07_NaCl groups.

An analysis of the microbial community composition at the genus taxonomic level revealed that uncultured and unclassified bacteria ranked first in all of the examined groups of lettuce soil (Figure 9a). More specifically, the average relative abundance of uncultured genera ranged between 14.94 and 20.10%, with a progressive reduction moving from samples 0_H_2O (20.10%) to 0_NaCl (18.41%), $B07\_H_2O$ (16.85%), and B07_NaCl (14.94%). The bacteria not classified at the genus level varied between 3.88% ($B07\_H_2O$) and 6.55% (B07_NaCl).



(**a**) (**b**)

Figure 9. Bacterial (**a**) and fungal (**b**) community composition in controls and treatments at genus level.

Flavobacterium, *Dongia*, *Devosia*, and *Bauldia* were among the most represented genera in all of the analyzed groups. The relative abundance of *Flavobacterium* in the salt-stressed samples was higher than in the non-stressed ones, reaching percentages of 8.55% and 10.47% in the NaCl- and B07_NaCl-associated microbial communities, compared to 4.60% for 0_H_2O and 1.39% for $B07\_H_2O$. For both *Devosia* and *Bauldia*, greater relative abundance values were observed in the B07_NaCl group (3.27% and 1.99%, respectively). The 0_H_2O control group showed the highest percentage of bacteria belonging to the *Dongia* (3.91%) and IS-44 (7.01%) genera, as well as a relatively high abundance of *Clostridium* species (≈7.01%). Regarding the latter genus, it is interesting to note that although it was very poorly represented in the 0_NaCl samples (average relative abundance = 0.02%), it reached a percentage of ≈7.98% in the soil bacterial communities of salt-stressed seedlings treated with the B07 strain.

On the contrary, the *Marinomonas* genus, which was absent in the 0_H_2O, $B07\_H_2O$, and B07_NaCl samples, was among the most represented genera in the 0_NaCl group. Similarly, *Azospirillum* (absent or with a relative abundance of <0.4% in the other groups) constituted about 16% of the bacterial communities of the lettuce soil samples treated with the B07 strain (Figure 9a).

In addition to the bacterial communities, the fungal communities of 18 pot soil samples collected from *L. sativa* L. seedlings subjected to the treatments summarized in Table S1 were analyzed using ITS2 region sequencing. The total number of final reads ranged between 38,252 (sample $B07\_H_2O\_2$) and 74,014 (sample Yeast_NaCl_2; Table S3).

For both bacteria and fungi, the average relative abundance values of the different taxa were calculated for each experimental group. The NGS analysis revealed the presence of a limited number of phyla with relative abundance percentages above 1% (see Figure 8b).

Ascomycota was the dominant phylum in all of the examined samples, with average abundance values between 78.51% (yeast sample group) and 92.67% (NaCl sample group). Overall, fungi belonging to the *Ascomycota* phylum were more abundant in the soil mycobiota of salt-stressed plants (the 0_NaCl, B07_NaCl, and Yeast_NaCl samples) compared to the corresponding controls (0_H_2O, $B07\_H_2O$, and $Yeast\_H_2O$, respectively).

A different trend was observed for fungi belonging to the *Basidiomycota* phylum, which showed lower relative abundance values in the soil samples of seedlings subjected to salt stress. However, *Basidiomycota* ranked second out of all of the investigated fungal communities, with percentages ranging between 4.95% (0_NaCl) and 17.35% (Yeast_H_2O).

Candida, *Botrytis*, *Pseudeurotium*, *Plectosphaerella*, *Chrysosporium*, *Cladosporium*, and *Scytalidium* were among the fungal genera with a relative abundance greater than 1% in all of the examined groups (see Figure 9b). *Candida* and *Botrytis* represented an important fraction of the lettuce soil fungal communities. Indeed, fungi belonging to the *Candida* genus contributed 25.21–41.18% to the mycobiota composition, with a reduction in their relative abundance values in the salt-stressed samples (considerable in the case of yeast-treated seedlings). The genus *Botrytis* varied between 5.26 and 35.73%, showing higher percentages in the soil samples of lettuce treated with NaCl. As can be seen in Figure 9b, a part of the mycobiota (5.75–11.28%) was represented by fungi unidentified at the genus level.

Compared to the other samples, *Pseudeurotium* and *Plectosphaerella* were more copious in the Yeast_H_2O and 0_NaCl sample groups, respectively, with relative abundance values of 9.20% and 9.23%.

Interestingly, the *Stemphylium* genus (which was overall poorly represented) reached a percentage of 19.63% in the Yeast_NaCl group. Similarly, *Alternaria*, which constituted 5.99% of the 0_NaCl sample's mycobiota, showed an average relative abundance of <1% in the other soil samples. The rarefaction analysis, a measure used to estimate the alpha diversity in samples and gauge whether or not the sequencing efforts captured the microbial diversity, showed a higher fungal biodiversity in the soils not subjected to salt stress (average Shannon index values for the groups 0_H_20, B07_H_20, and Yeast_H_20 ranging from 4.76 to 5.28) compared to those treated with NaCl at a concentration of 100 mM (average Shannon index values for the groups 0_NaCl, B07_NaCl, and Yeast_NaCl ranging from 3.94 to 4.45).

The principal coordinate analysis (PCoA) results based on the Jaccard metric highlighted the variation among the experimental groups, although intra-group variability/dispersion was also observed, as shown in Figure 10.

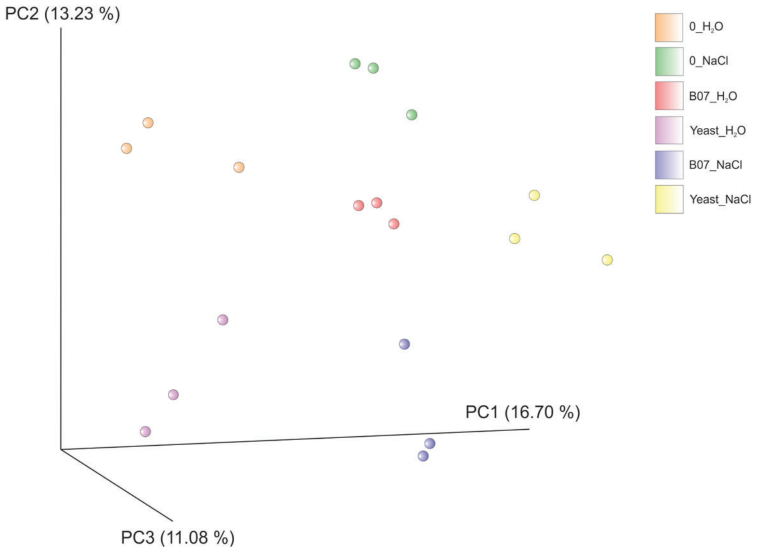


Figure 10. Principal coordinate analysis (PCoA) of fungal communities. The plot was generated using the Jaccard metric.

2.9. Environmental Impact, CO_2-Equivalent Emissions, and Mitigation Actions

According to previous works, here, we report an evaluation of the environmental impact related to this study, the CO_2-equivalent emissions, and the mitigation actions by new plantations [28,29].

The evaluation of the environmental impact of this manuscript, in terms of the social cost and CO_2-equivalent emissions, resulted in EUR 12.0 and about 300 kg CO_2 equivalent, respectively. This influenced us to provide a new plantation of *Carpinus* plants for mitigation actions. *C. betulus* and *C. orientalis* were used, due to them both being autochthonous species with an environmental adaptation ability. A QR code tag linked to this manuscript was added to each tree planted in Green Park—Campus Unimol (41°60′76″ N, 14°26′50″ E).

3. Discussion

The defined experimental design was conservative. It did not include destructive investigations such as the determination of plant biomass or the extraction of chlorophyll using chemical methods. Therefore, our experimental design favored the use of other biometric–vegetative indicators, such as (i) leaf width and length; (ii) root system length, branching, and coloration; (iii) the cumulative incidence risk (CIR), which was obtained by measuring the weekly mortality cumulative incidence of the lettuce seedlings; and (iv) the chlorophyll content, which was determined using the AtLeaf CHL PLUS tool (FT Green LLC, Wilmington, DE, USA). Confocal microscopy was also performed on a representative aliquot of lettuce leaves from each Styrofoam cassette. Following the experiments, the microbial communities of the pot soil used as a growing medium for the lettuce seedlings were analyzed. The analyses performed on epigeal (leaf) and hypogean (root and soil) fractions are discussed below.

3.1. Effects of Microbial Biocontrol Agents and Biostimulants on Reducing Mortality and Salinity Stress in Lettuce Leaves

The use of the cumulative incidence risk (CIR) showed a clear and positive performance for the biostimulant Activeg (Figures 3 and 4). In addition, the CIR indicated that the biocontrol agents, sometimes enriched with molasses, played an additional potential role in mitigating salt stress, but at a lower intensity compared to the other biostimulants tested in this work [30,31]. Furthermore, recent studies have proposed using legume protein hydrolysates not only as biostimulants, but also as potential functional foods with minimal environmental impact [32].

Among the biometric indicators adopted, a positive performance in terms of the development of the lettuce plants was recorded on day 60 after the addition of the biostimulant Activeg (A) (see Table 2). In fact, under salt stress, the *Fabaceae* extract solution provided statistically significant increases in leaf height (increases of more than 20%), in comparison to both the control (H_2O only) and the other treatments. In contrast, significant reductions in lettuce leaf height were observed with the use of other biostimulants (M, S, MS, and C) and biocontrol agents (B07, Ra36, Ra36M, and Y) [30,33–36].

Under the experimental conditions we used, the results showed a significant decrease in the chlorophyll content only in the samples subjected to salt stress, such as the salt control and the treatment with Activeg. The data showed a decrease in the chlorophyll content ranging from 40% to 43% (Figure 5). The results obtained here agree with the data already reported by other authors [37–39]. The lettuce seedlings treated with *P. terrestris* showed a decrease in their chlorophyll content of 49 percent in the control sample with the source water, while it was only 31 percent in the sample treated with the saline solution. These results agree with those of Yasar et al. [40], who stated that growth retardation, a typical symptom of salinity damage to plants, is due to inhibited cell elongation. In addition, a comparison of salt-sensitive and salt-resistant pumpkin genotypes [41] hypothesized the role of chlorophyllase activity and oxidative membrane damage induced by salt stress.

The Ra36 treatment resulted in a non-significant increase in the chlorophyll content compared to the H_2O control. These data are consistent with the increase in leaf biometric measurements. The four treatments with molasses as the biostimulant (M, CM, SM, and MiCM) also showed no significant change. The data on the chlorophyll content in the molasses (M) treatments did not result in reduced plant mortality on the 75th day compared to the treatments with biocontrol agents such as Ra36, B07, and Y. The exception was the treatment with Activeg, which has already been reported in the literature [42] and which resulted in the best survival of the lettuce plants on both the 75th and 90th days of the saline treatment (Figures 1 and 2). Furthermore, the biostimulant Activeg led to a reduction in transplant stress in the field [43–45]. Therefore, the biometric data on leaf height reported in Table 2 confirm the positive effect of the mitigating and biostimulating treatments [43,46]. Under our experimental conditions, the total chlorophyll content did not appear to be a useful marker for assessing the long-term response of salt-stressed lettuce plants. Most of the changes in the concentration did not appear to be significantly correlated with the CIR.

Salt stress is one of the most damaging environmental stresses; it affects the water balance through stomatal conductance [47]. In glycophytes, salinity stress reduces stomatal conductance, leading to a decrease in the photosynthetic and transpiration rates [48]. In halophytes, this reduction is less [49], resulting in their resistance to stress. In our experiments, there seemed to be a clear difference between the tap water control and the 100 mM NaCl treatment (see Figure 6a,b). Figure 6b shows stomatal closure and reduced red fluorescence. This may have been due to the reduced chlorophyll content. In contrast, the samples treated with the microbial biocontrol agents *B. amyloliquefaciens* (B07) (Figure 6c,d) and *P. terrestris* (Y) (Figure 6e,f) showed a similar chlorophyll content under the two experimental conditions with and without salt stress. The stomatal guard cells were visibly turgid, indicating the opening of the stomata [49], as shown in Figure 6d,f. These results indicate that *B. amyloliquefaciens* and *P. terrestris* had a positive effect on the lettuce plants under the salt stress condition.

3.2. Effects of Microbial Biocontrol Agents and Biostimulants on Lettuce Roots as Salt Stress Mitigators

The biometric measurements of lettuce root length, branching, and color showed the best values with Activeg (A) as the salt stress mitigant (see Figures 7a and S1). The results obtained showed that in the control (0, tap water only) and in the B07, B07M, and Ra36M treatments, higher values were recorded for lettuce root length and branching. Salt stress mitigation, albeit at different intensities, was observed in response to the Ra36, Y, and A treatments. The lack of a salt stress-mitigating effect was recorded by analyzing both branching (lateral roots) and root surface coloration in the M, S, SM, C, CM, MiC, and MiCM treatments. Only the treatments with microbial biocontrol agents showed root length, branching, and coloration parameters that were in line with those of the tap water control on the 90th day (Figure 7b).

Our results agree with the literature on salt-stressed plant root morphology [50–53]. Under our experimental conditions, the microbial biocontrol agents B07, Y, and Ra36 (the latter alone or with molasses added) showed a good ability to mitigate the effects of salt stress (NaCl 100 mM) on root system morphology on both the 75th and 90th days of lettuce seedling growth.

3.3. Effects of Microbial Biocontrol Agents as Salt Stress Mitigators on Lettuce Soil Microbial Communities

To show whether NaCl stress could alter the microbial biodiversity communities of the lettuce soil pots, we performed an NGS analysis. The NGS results showed that *Proteobacteria* and *Bacteroidota* were among the most representative phyla in the samples under analysis. These were followed by *Firmicutes*, *Acidobacteriota*, and *Actinobacteriota* in lower proportions (Figure 8a). The bacterial phyla mentioned above are widely distributed in different ecosystems. For example, some authors have indicated that *Proteobacteria*, *Bacteroidota*, *Firmicutes*, and *Actinobacteriota* are the dominant phyla in the oral cavity of *Japalura sensu lato* [54]. Other authors have pointed to the sequential involvement of *Bacteroidota* and *Firmicutes* in the degradation of cellulose from rice straw [55]. Furthermore, some researchers have found that in the rumen of sheep treated by gavage with a mixed bacterial suspension of acid-producing strains isolated from cattle rumen [56], the abundance of *Bacteroidota*, *Actinobacteriota*, *Acidobacteriota*, and *Proteobacteria* significantly increased, whereas bacteria of the phylum *Firmicutes* were much less represented. In the examined pot soils, the relative abundance of *Firmicutes* and *Acidobacteriota* decreased with the salt treatment (group 0_NaCl). In the soils that received the strain B07 and NaCl, an increase in *Firmicutes* and a less drastic reduction in *Acidobacteriota* were observed. In general, 54% of *Firmicutes*, 39% of *Proteobacteria*, and 7% of *Actinobacteria* belong to the salt-tolerant PGPBs evaluated in 40 articles [57]. The most dominant genera of halotolerant PGPBs are *Bacillus* and *Pseudomonas*. PGPBs can provide cross-protection against various stresses and increase plant growth through various direct and indirect mechanisms, including by altering the root morphology; obtaining nutrients; synthesizing exopolysaccharides, phytohormones, volatile compounds, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase; altering ion homeostasis; inducing the aggregation of antioxidants and compatible solutes; inducing systemic tolerance; and modulating stress-sensitive genes [57]. Our experiments are consistent with those of authors who included *B. amyloliquefaciens* among the PGPRs [58]. Our experiments showed that the B07 treatment resulted in a reduction in the *Bacteroidota* abundance, whereas the B07 treatment with 100 mM NaCl increased *Actinobacteriota*.

The genus *Flavobacterium* represents a significant fraction of the root- and leaf-associated microbiome in a wide range of plant species [59], and from our experimental data, it appears that *Flavobacterium*, *Dongia*, *Devosia*, and *Bauldia* were among the most represented genera in all of the groups analyzed. In our experiments, the most abundant taxa in the B07 salt treatment samples were the genera *Flavobacterium*, *Devosia*, and *Bauldia*, which are Gram-negative soil bacteria and useful microorganisms in bioremediation [60,61]. For example, treatment with *Flavobacterium crocinum* HYN0056T under drought and salt stress conditions resulted in the enhanced up-regulation of several drought- and salt-inducible genes in *Arabidopsis* [62], and treatment with *Flavobacterium* sp. strain GJW24 improved the resistance of *Arabidopsis* and *Brassica* plants to drought and drought-related salt stress [63]. In general, microorganisms of the genus *Flavobacterium* are also producers of several molecules that can improve plant health [64]. Regarding the genus *Devosia*, there are studies in the literature on the beneficial effect of the flavonoids and microbe-based material (CFS: cell-free supernatant) containing active compounds secreted by *Devosia* sp. SL43, improving the growth of soybean plants under salt stress [65,66]. *Clostridium* reached an average relative abundance of ≈7.98% in soil treated with B07 and salt. For *Clostridium*, Peng et al. [67] observed the stress-induced enrichment of mRNAs encoding osmoprotectants, such as ABC transporters for choline, betaine, glycine betaine, proline betaine, carnitine, and betaine biosynthesis.

The fungal community analysis focused on the two taxonomic levels of the phylum and the genus. *Ascomycota* were the most abundant in the salt-treated samples. In contrast, *Basidiomycota* were relatively abundant in the tap water control samples (Figure 8b). The most abundant genera were *Candida*, *Botrytis*, *Pseudeurotium*, *Plectosphaerella*, *Chrysosporium*, *Cladosporium*, and *Scytalidium* (Figure 9b). The highest concentration of *Candida* was found in the tap water control samples. However, this yeast was still present in the salt-treated samples. This is not surprising, as other authors have studied *Candida* species that only tolerate salt stress [68,69]. In our experiments, the biocontrol agent *P. terrestris* further restricted *Candida*, although it still represented the most abundant genus in the Yeast_NaCl group. In addition to *Candida*, *Botrytis*, *Stemphylium*, *Pseudeurotium*, and *Chrysosporium* were the most abundant genera in the samples treated with *P. terrestris* and salt. Some of the species belonging to the genus *Stemphylium* include microorganisms of a marine origin, which have adapted to grow in salt-rich environments. Other species (e.g., *Stemphylium lycopersici*) have a positive interaction with *Zea mays* roots that allows for an improved yield under saline stress conditions [70]. In both cases, this could explain the surprisingly high relative abundance of the genus under salt stress with the *P. terrestris* treatment as the result of a useful synergic interaction [71]. *Pseuderotium* sp. has also been found on tomato roots as an endophytic and beneficial fungus [72].

The microbiota expression of the total viable microbial community (bacteria, yeasts, fungi, and spore-forming bacteria) is shown in Table 3. The total microbial counts reached higher values in the enrichment treatments (bioaugmentation) with the microbial strains that were selected as biocontrol agents. Therefore, the effects of adding pure yeast cultures induced positive interactions among the microorganisms in the pot soil. The treatment with *P. terrestris* showed an ability to adapt to the biotic and abiotic conditions of the soil over time. This was confirmed by the counts shown in Table 3.

4. Materials and Methods

4.1. Experimental Design

The trials were carried out using the roots, peaty substrate, and leaves of *L. sativa* L. var *romana* (Figure 11) following the experimental design shown in Figure S2. Lettuce is a species of dicotyledonous angiosperm plant belonging to the family Asteraceae. Its main morphological appearance shows generally thin taproots; basal or root leaves (with the latter forming a basal rosette); and cauline leaves, arranged alternately in the following pattern: the basal ones are short petiolate, and the others are sessile.

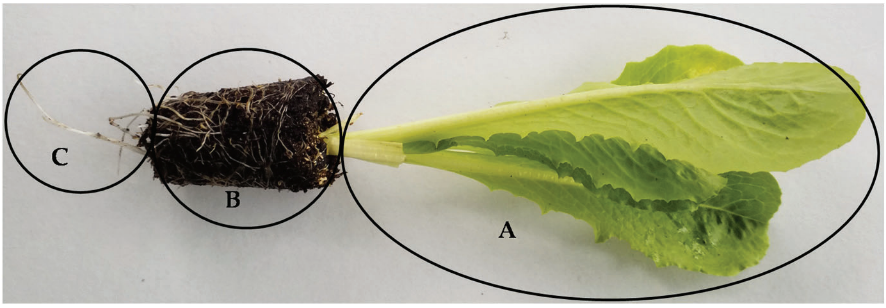


Figure 11. The three different parts of the lettuce plants under investigation: (**A**) leaves, (**B**) peaty substrate, and (**C**) roots.

The tests were set up according to the following two-factor scheme: (i) the first one was associated with the presence/absence of NaCl, denoted as 0 (tap water, no NaCl) and 1 (NaCl at 100 mM); (ii) the second factor was the type of treatment, and included 14 treatments in addition to the control (tap water), as described in Table 4.

Table 4. List of treatments adopted for experimental trials with lettuce plants.

Codes	Treatments	Details
0	Control	No treatments; only tap water or 0.1M salt
M	Molasses	Sugar industry by-product (*Beta vulgaris*)
S	Soil mix	Soil sample extract
SM	Soil mix + M	Soil sample extract + M
C	Compost mix	Commercial compost extract
CM	Compost mix + M	Commercial compost extract + M
MiC	Micro compost mix	Bacterial mixture from compost (C)
MiCM	Micro compost mix + M	Bacterial mixture from compost (C) + M
B07	B07 bacterial strain	Pure culture Dpt. AAA, Unimol, Italy, [73] *B. amyloliquefaciens*
B07M	B07 bacterial strain +M	Pure culture + M
Ra36	Ra36 bacterial strain	Pure culture Dpt. AAA, Unimol, Italy, [27] *R. aquatilis* strain 36
Ra36M	Ra36 bact. strain + M	Pure culture + M
Y	Yeast strain	Pure culture Dpt. AAA, Unimol, Italy, [26] *P. terrestris* PT22AV.
A	Activeg®	Commercial biostimulant through enzymatic hydrolysis from *Fabaceae*
B15	Bioalga 15®	Commercial biostimulant through extraction from *Ascophillum nodosus*

The design included about 1400 young lettuce plants in Styrofoam cassettes containing 90 lettuce seedlings. The seedlings were initially grown for 4 weeks post-germination on brown peat substrate enriched with 0.5% of a slow-release fertilizer (NPK: 12-12-12) and furnished by Consorzio Agrario di Isernia, Isernia, Italy. The main physicochemical characteristics of the new soil included a pH of 7.40, an EC of 2.10 mS, available nitrogen in the amount of 18.25 mg kg^{-1}, available phosphorus in the amount of 9.20 mg kg^{-1}, and available potassium in the amount of 11.50 mg kg^{-1}. Each of the thirty treatment blocks was settled with around 45 plants. The experiment was carried out for 4 months in a greenhouse with dimensions of about 30 × 8 × 4 m (long, wide, high), with 240 m^2 of useful surface; the roof was glass and the lateral walls were transparent methacrylate panels, and there was no heating/cooling control system inside (Figure 12). The ambient conditions during the experimental period were 16–23 °C, with a relative humidity between 70 and 90%; no nutrients were added directly to the substrate. Portable data loggers (RHiLog Escort Data Loggin System Ltd., Auckland, New Zealand) constantly monitored these parameters at the on-site greenhouse at the Dept. Bioscience and Territory, University of Molise, Italy.

Figure 12. A view of the experimental trial using lettuce growth plots under greenhouse conditions.

The NaCl solution, at a concentration of 100 mM [corresponding to 5.0 Ohm and 1.80 mS m^{-1} of electrical resistivity (ER) and conductivity (EC)], was prepared in a laboratory and obtained by adding 5.80 g L^{-1} (1.30 dS m^{-1}) of NaCl to tap water (12.0 Ohm). The defined salt solution can be regarded as stress on the growth of the selected lettuce. The saline solution and biostimulants were administered at regular 7-day intervals, providing a volume of 3.0 L/Styrofoam cassette. The positions of the treatment blocks of the lettuce plants were randomized within the greenhouse, considering their exposure to sunlight, as shown in Figure 12. At the beginning of the experiment and in accordance with the experimental design, 3 L of water was added to each saucer to promote its initial acclimatization in the greenhouse. In this trial, the seedlings were grown under the salt stress produced by 100 mM NaCl. The treatments included microbial suspensions, commercial biostimulants, molasses, compost, and soil extracts that were added at intervals of time to mitigate the effects of saline stress.

4.2. Preparation of Biostimulant Solutions and Biocontrol Products

4.2.1. Commercial Biostimulant Solutions

The stock solution of Activeg® (Hydro Fert srl, Bari, Italy) was made with 5.0 g L^{-1} of Activeg in tap water. This biostimulant was obtained through enzymatic hydrolysis from the biomass of *Fabaceae* and was made from high-quality, low-racemization protein hydrolysates containing triacontanol and L-free amino acids, which are active substances ready for use by crops.

The stock solution of Bioalga 15® (Hydro Fert srl, Bari, Italy) was made with 10.0 g L^{-1} of Bioalga 15 in tap water. Bioalga 15 is a growth promoter made up of 15% seaweeds (*Aschophyllum nodosum*), and it also contains natural PGRs (plant growth regulators), enzymes, proteins, vitamins, amino acids, and polysaccharides.

4.2.2. Molasses, Compost, Micro Compost, and Soil Extract Solution

A beet molasses (M) solution, produced by Zuccherificio del Molise, Portocannone, Italy, was prepared as a dilution of 50.0 g L^{-1} in tap water just before use. Its chemical composition was 78.3% dry matter, 13.5% crude protein, and 48% sucrose.

A compost solution was prepared as follows: bio-composted green residues (5.0 kg) (Bio Compost, Compo Group, Munster, Germany) were added to a PVC tank (total volume: 20.0 L) containing 10.0 L of tap water, and the solution was frequently mixed with a rod mixer until 20 min before its use. Then, the supernatant of the compost solution (300 mL) was diluted in 3.0 L of tap water. The diluted solution was used for the treatments.

A micro compost mix (MiC) was created using an enrichment technique, as follows: A total of 100 mL of the compost solution previously created was preliminary subjected to 80 °C for 10 min to induce sporulation within the primitive microbial community. Then, a viable aerobic bacterial count was determined using a 10^{-1} to 10^{-8} serial dilution on plate count agar (PCA, BD Difco, Milan, Italy) that was incubated at 37 °C for 48–72 h. Randomly, five bacterial colonies were selected from the growth on the higher-dilution plates (10^{-7}), transferred to fresh PCA media, and stored at 4 °C. When required, the bacterial strains were mixed and inoculated into a flask containing 10 L of sterilized plate count broth and incubated under aerobic conditions at 37 °C overnight. The bacterial growth on the medium was centrifuged at 7000 rpm for 10 min, and the viable bacterial cells were recovered and resuspended in 10 L of tap water before use.

A micro compost mix + M (MiCM) was created by adding a beet molasses solution, as previously reported.

A soil solution was prepared. An agricultural soil sample (Villa Vanda farm, Rosciano, Italy) was collected at a depth of 2–20 cm and sifted with a sterilized sieve (diameter of 2 mm) to remove gravel and plant residues; it was then stored at 4 °C. The soil characteristics were a pH of 7.30, an organic carbon content of 18.40 g kg^{-1}, and a Kjeldahl nitrogen content of 0.90 g kg^{-1}. The soil (5.0 kg) was added to a PVC tank (total volume of 20.0 L) containing 10.0 L of tap water and frequently mixed with a rod mixer until 20 min before use. Then, the soil supernatant (300 mL) mixed with 3.0 L of tap water was used for the treatments.

4.2.3. Microbial Biocontrol Product Preparation

Biocontrol product solutions based on the B07 and RA36 bacterial strains and the PT22AV yeast strain used in the experiments were prepared according to the references reported in Table 4.

4.3. Monitoring Parameters

4.3.1. Mortality Analysis

To evaluate the effectiveness of each treatment in terms of mortality protection, we considered the cumulative incidence risks (CIRs) across the first ten weeks. The cumulative incidence risk at a certain time point is an epidemiological measure, defined as the ratio between the number of deaths within that time point, or the cumulative incidence cases, and the total number of individuals at risk since the beginning of the study, or the population at risk [74]. In epidemiology, the cumulative incidence risk estimates the probability of death within a certain time point.

Concerning our study, for each treatment j (j = 0, 1, …, 14) and for each week t (t = 1, …, 10), we used Y_{jt} to denote the number of plants that died within week t under treatment j and N_j to denote the total number of plants under treatment j at the beginning of the experiment. Then, each cumulative incidence risk (CIR) was given by the ratio of the proportion of plants that died within week t under treatment j.

$$\mathrm{CIR}_{jt} = \frac{\mathrm{Y}_{jt}}{\mathrm{N}_j}$$

As a second step, we controlled how significantly different the CIRs of each treatment j were from the CIRs of the control treatment across the ten weeks; that is, for each week t, we proposed the null hypothesis H_0: $CIR_{jt} = CIR_{0t}$. The statistical test considered was the classical normal Z test, which is generally used to control the difference between two proportions [75]. We considered two levels of significance, 0.01 and 0.001, to accurately differentiate the effectiveness of each treatment.

4.3.2. Biometric Measures

The lettuce plants were tested in non-destructive analyses at intervals of time; in parallel, leaves and then fresh leaf tissues were immediately stored at −80 or −20 °C until use for biochemical analyses. Biometric evaluations, such as the evaluation of a leaf's growth, were carried out on 13 plants per plot, following an X-shaped design; the biometric measures included the length and width of all of the leaves on each plant, with the mean and standard deviation. This technique guaranteed the continuous growth of the lettuce plants and the respect of our initial experimental design. Therefore, destructive analyses on plants, such as a dry biomass determination or proline assays, were not carried out.

4.3.3. Chlorophyll Content Measurement

The relative chlorophyll content was obtained using the AtLeaf CHL PLUS tool (FT Green LLC, Wilmington, DE, USA), a non-destructive indirect method based on the spectral absorption of the leaves [76,77]. The measurements were taken along a diagonal for three points of each seedling block in the middle of the right side of the leaves (Figure 13a,b). The measurements were repeated twice at different times: on the 60th day after the experiment started and from the fourth week after the lettuce plant's germination (0).

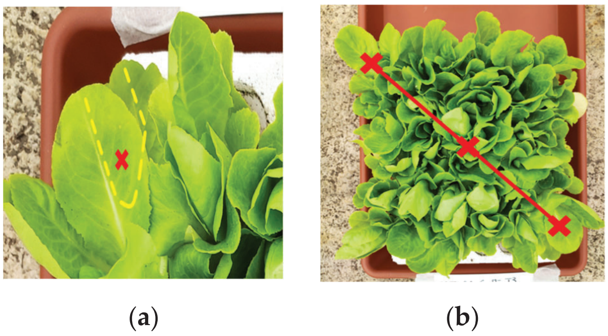

(**a**) (**b**)

Figure 13. Example of chlorophyll measurement. (**a**) Particular of the leaf portion measured; (**b**) diagonal of the three measures indicated with red crosses.

4.3.4. Free Proline Assay

A free proline assay on lettuce leaves was performed using the colorimetric method according to Bates et al. [78,79]. Briefly, a proline standard (60 mg, Sigma-Aldrich, St. Louis, MI, USA), glacial acetic acid, toluene as the extracting solution, and ninhydrin were used. A spectrophotometer was used at 520 nm (UV-1600PC model, VWR®, Milan, Italy), with a standard curve ranging from 0.17 to 35 µg/mL.

4.3.5. Morphological and Visual Evaluation of Leaves and Roots

Leaf samples from the lettuce seedlings were collected and fixed on a slide. The autofluorescent images were acquired with a confocal microscope (TCS SP8; Leica, Wetzar, Germany) equipped with 20, 40, and 63× magnification objectives and Leica LAS X software 3.5.5 (Leica Microsystems, Buccinasco, Italy). Visible light (400–600 nm) was used for pigment excitation. A magnification of 20× was employed to visualize the leaf stomata.

The roots were observed at different times; a visual assessment was made by lifting the tray of polystyrene containing the seedlings (see Figure S1). The roots were photographed at each observation time.

Length (1 < 0.5 cm; 2 = 0.6–1.0 cm; 3 = 1.1–2.0 cm; 4 = 2.1–3.0 cm; 5 > 3.1 cm). BRA = branching (a < 0.5 cm; b = 0.6–1.0 cm; c = 1.1–2.0 cm; d = 2.1–3.0 cm; e > 3.1 cm). Color (I, white; II, cream; III, light brown; IV, dark brown; V, black).

Laboratory and portable models for optical microscopy (OM)—Nikon Eclipse E600 model (Nikon Instruments Europe B.V. Amsterdam, The Netherlands)—and stereo microscopy (SM)—Zeiss AxioScope (Carl Zeiss Spa, Milan, Italy)—connected to high-resolution digital cameras were utilized. Samples of leaves and roots obtained at pre-set intervals from the growth trials were examined.

4.3.6. Viable Microbial Count

For the determination of the total viable bacterial count (TVBC), plate count agar (PCA, BD Difco) was used at 37.0 °C for 48–72 h. The spore-forming bacteria (SFB) sample dilutions were preliminary tested at 80 °C for 10 min; they were then inoculated onto Petri dishes with the addition of PCA and incubated at 37 °C for 48–72 h. For the fungal and yeast counts, peptone dextrose agar (PDA, BD Difco) and yeast extract agar (YEA, Biolife Italiana, Milan, Italy) were used at 28 °C for 48 h [80].

4.3.7. Extraction of Genomic DNA from Soil Samples

To assess the bacterial diversity and taxonomic composition through next-generation sequencing (NGS), DNA was extracted at the end of the experimentation from soil samples subjected to the different treatments by using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) and following the manufacturer's instructions. For the analysis of fungal communities, DNA was extracted by using the DNeasy 96 PowerSoil Pro QIAcube HT Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

4.3.8. 16S rRNA Gene and ITS2 Region Amplicon Library Preparation, Sequencing, and Bioinformatics Analysis

Next-generation sequencing was performed at BMR Genomics srl, Padova, Italy, according to [29]. The sequences generated in the present study were deposited in the NCBI Sequence Read Archive under BioProject PRJNA1140535.

For the fungal communities, the biodiversity within a given sample (alpha diversity) was calculated with the Shannon index and analyzed through the Kruskal–Wallis test. The similarity between samples (beta diversity) was calculated using the Jaccard metric.

A PCoA representation of the beta diversity was performed using QIIME2.

Overall, 18 pot soil samples associated with the lettuce seedlings subjected to different treatments (three replicates for each experimental group) were considered for the study of the micro- and mycobiota (Table S1).

4.4. Environmental Impact

To contribute to the reduction in the environmental impacts originating from these experiments, a preliminary assessment of the inputs (energy consumption, reagents, and materials at the lab level) and outputs was initially converted into CO_2 equivalents (CO_2 eq.), and then into partial mitigation action through the allocation of the planting of additional plants, according to our previous work [28].

4.5. Statistical Analysis

The statistical analysis of the data was conducted in three distinct phases (see Figure S2). The first phase concerned the definition of the experimental design for the collection of data. In this sense, all of the sampling blocks were defined in relation to the treatments (biostimulants or biocontrol agents) and experimental conditions (salt levels), while trying to maintain balance with respect to the number of plants included in each block. All of the results presented in this study are expressed as means ± SD (standard deviation), and an unpaired Student's *t*-test was used to compare the experimental groups. The statistical analysis was carried out using GraphPad, accessed at https://www.graphpad.com/quickcalcs/ttest2/ (accessed on 23 August 2024) and https://www.socscistatistics.com/tests/studentttest/default2.aspx (accessed on 18 July 2024).

The second phase concerning data analysis was conducted using the free-license computing environment R [81]. The analysis mainly concerned the exploratory aspects with reference to the mortality curve and colorimetric indicators.

The third phase, as shown in Figure S2, represents the final stage, and includes the Environmental Impact, Results, and Discussion Sections.

5. Conclusions

The objective of our experiment was to evaluate the capability of selected natural products and microbial biocontrol agents to provide long-term mitigation of salt stress (NaCl 100 mM) in lettuce (*L. sativa* L. var *romana*). This experiment was conducted using Styrofoam cassettes, with each containing 45 lettuce plants. Fourteen different treatments, with and without a salt addition, were analyzed over the long term (90 days) using non-destructive techniques. The results were subjected to a statistical analysis.

The results showed that the biocontrol agents (*P. terrestris*, strain PT22AV; *B. amyloliquefaciens*, strain B07; and *R. aquatilis*, strain 36) and natural compounds, such as some of the biostimulants and molasses, can reduce/mitigate salt stress in *L. sativa* L var. *romana*.

A multifunctional intervention would be able to ensure appropriate responses of the selected biocontrol agents to the following: (i) transplanting stress in the field (efficacy as a biostimulating agent); (ii) environmental stresses, such as climate change, increased salinity, or reduced water availability for crops (efficacy as a bio-attenuating agent); and (iii) antagonistic activity against phytopathogens of horticultural crops (efficacy as a bio-suppressing agent of plant diseases).

Collectively, our data show some aspects of novelty and biological/application interest. In fact, a single biotreatment based on appropriately selected, tested, and effective microorganisms or natural compounds will be used and adopted to ensure growth and plant protection. This will, therefore, be an effective, economically sustainable, and environmentally friendly solution.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants13172505/s1; Table S1: Codes assigned to soil samples collected from *L. sativa* L. seedlings for microbial community analyses; Table S2: Number of final reads obtained from NGS analysis for studying taxonomic composition of lettuce soil bacterial communities; Table S3: The number of final reads obtained from the NGS analysis for the study of the taxonomic composition of lettuce soil fungal communities. Figure S1: Example of roots observations of lettuce plants in response to salt stress; Figure S2: Schematic diagram of experimental design.

Author Contributions: Conceptualization, C.C., G.L., F.D. and G.R.; methodology, F.D., C.C., P.F., S.D.B. and G.R.; software, A.C.C., I.D. and F.D.; validation, A.C.C., I.D. and F.D.; formal analysis, F.D., P.M. and A.B.; investigation, A.C.C., C.D.G., I.D., D.D.P., D.P., M.P. and L.Q.; resources, C.C. and G.R.; data curation, C.C.; writing—original draft preparation, C.C. and G.R.; writing—review and editing, C.C., G.R., A.B., P.M., P.F., S.D.B., D.P., C.D.G. and G.L.; supervision, G.R.; funding acquisition, G.R. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by (i) IRIS-DIBT2023, grant number PROGET_20242025_IRIS-DIBT_2023—A digital forest—new Green Park—as compensation and traceability for the environmental impact of annual scientific research; (ii) DEMETRA, grant number PROJET 67374_MiPAAF_IT_2018—The design and validation of multifunctional and diversified production systems based on the integration between plant and animal production in the marginal areas of central-southern Italy.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Acknowledgments: We sincerely thank Immacolata Doganieri and Sandra Ciccone for their support with the administration of the funding and procurement management.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Rusu, T.; Moraru, P.I.; Mintas, O.S. Influence of environmental and nutritional factors on the development of lettuce (*Lactuca sativa* L.) microgreens grown in a hydroponic system: A review. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2021**, *49*, 12427. [CrossRef]
2. Jenni, S. Rib Discoloration: A Physiological Disorder Induced by Heat Stress in Crisphead Lettuce. *HortScience* **2005**, *40*, 2031–2035. [CrossRef]
3. Wei, S.; Yang, X.; Huo, G.; Ge, G.; Liu, H.; Luo, L.; Hu, J.; Huang, D.; Long, P. Distinct Metabolome Changes during Seed Germination of Lettuce (*Lactuca sativa* L.) in Response to Thermal Stress as Revealed by Untargeted Metabolomics Analysis. *Int. J. Mol. Sci.* **2020**, *21*, 1481. [CrossRef]
4. Shin, Y.K.; Bhandari, S.R.; Jo, J.S.; Song, J.W.; Cho, M.C.; Yang, E.Y.; Lee, J.G. Response to Salt Stress in Lettuce: Changes in Chlorophyll Fluorescence Parameters, Phytochemical Contents, and Antioxidant Activities. *Agronomy* **2020**, *10*, 1627. [CrossRef]
5. Abdelkader, M.; Voronina, L.; Shelepova, O.; Puchkov, M.; Loktionova, E.; Zhanbyrshina, N.; Yelnazarkyzy, R.; Tleppayeva, A.; Ksenofontov, A. Monitoring Role of Exogenous Amino Acids on the Proteinogenic and Ionic Responses of Lettuce Plants under Salinity Stress Conditions. *Horticulturae* **2023**, *9*, 626. [CrossRef]
6. Baslam, M.; Pascual, I.; Sánchez-Díaz, M.; Erro, J.; García-Mina, J.M.; Goicoechea, N. Improvement of Nutritional Quality of Greenhouse-Grown Lettuce by Arbuscular Mycorrhizal Fungi Is Conditioned by the Source of Phosphorus Nutrition. *J. Agric. Food Chem.* **2011**, *59*, 11129–11140. [CrossRef] [PubMed]
7. Shi, M.; Gu, J.; Wu, H.; Rauf, A.; Bin Emran, T.; Khan, Z.; Mitra, S.; Aljohani, A.S.M.; Alhumaydhi, F.A.; Al-Awthan, Y.S.; et al. Phytochemicals, Nutrition, Metabolism, Bioavailability, and Health Benefits in Lettuce—A Comprehensive Review. *Antioxidants* **2022**, *11*, 1158. [CrossRef]
8. Ünlükara, A.; Cemek, B.; Karaman, S.; Erşahin, S. Response of lettuce (*Lactuca sativa* var. *crispa*) to salinity of irrigation water. *N. Z. J. Crop Hortic. Sci.* **2008**, *36*, 265–273. [CrossRef]
9. Corwin, D.L. Climate change impacts on soil salinity in agricultural areas. *Eur. J. Soil Sci.* **2021**, *72*, 842–862. [CrossRef]
10. Sun, X.; Wang, K.; Kang, S.; Guo, J.; Zhang, G.; Huang, J.; Cong, Z.; Sun, S.; Zhang, Q. The role of melting alpine glaciers in mercury export and transport: An intensive sampling campaign in the Qugaqie Basin, inland Tibetan Plateau. *Environ. Pollut.* **2017**, *220*, 936–945. [CrossRef]
11. Leng, P.; Zhang, Q.; Li, F.; Kulmatov, R.; Wang, G.; Qiao, Y.; Wang, J.; Peng, Y.; Tian, C.; Zhu, N.; et al. Agricultural impacts drive longitudinal variations of riverine water quality of the Aral Sea basin (Amu Darya and Syr Darya Rivers), Central Asia. *Environ. Pollut.* **2021**, *284*, 117405. [CrossRef] [PubMed]
12. Youssef, M.H.M.; Raafat, A.; El-Yazied, A.A.; Selim, S.; Azab, E.; Khojah, E.; El Nahhas, N.; Ibrahim, M.F.M. Exogenous Application of Alpha-Lipoic Acid Mitigates Salt-Induced Oxidative Damage in Sorghum Plants through Regulation Growth, Leaf Pigments, Ionic Homeostasis, Antioxidant Enzymes, and Expression of Salt Stress Responsive Genes. *Plants* **2021**, *10*, 2519. [CrossRef]
13. Payen, S.; Basset-Mens, C.; Núñez, M.; Follain, S.; Grünberger, O.; Marlet, S.; Perret, S.; Roux, P. Salinisation impacts in life cycle assessment: A review of challenges and options towards their consistent integration. *Int. J. Life Cycle Assess.* **2016**, *21*, 577–594. [CrossRef]
14. Hossain, M.S.; Dietz, K.-J. Tuning of Redox Regulatory Mechanisms, Reactive Oxygen Species and Redox Homeostasis under Salinity Stress. *Front. Plant Sci.* **2016**, *7*, 548. [CrossRef] [PubMed]
15. Freitas, D.; Campos, D.; Gomes, J.; Pinto, F.; Macedo, J.A.; Matos, R.; Mereiter, S.; Pinto, M.T.; Polónia, A.; Gartner, F.; et al. O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype. *eBioMedicine* **2019**, *40*, 349–362. [CrossRef]
16. Alsamadany, H.; Mansour, H.; Elkelish, A.; Ibrahim, M.F.M. Folic Acid Confers Tolerance against Salt Stress-Induced Oxidative Damages in Snap Beans through Regulation Growth, Metabolites, Antioxidant Machinery and Gene Expression. *Plants* **2022**, *11*, 1459. [CrossRef] [PubMed]
17. Ahmad, R.; Anjum, M.A. Physiological and molecular basis of salinity tolerance in fruit crops. In *Fruit Crops*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 445–464.
18. Khamidov, M.; Ishchanov, J.; Hamidov, A.; Donmez, C.; Djumaboev, K. Assessment of Soil Salinity Changes under the Climate Change in the Khorezm Region, Uzbekistan. *Int. J. Environ. Res. Public Health* **2022**, *19*, 8794. [CrossRef]
19. Triantafyllou, A.; Kamou, N.; Papadopoulou, A.; Leontidou, K.; Mellidou, I.; Karamanoli, K. Evaluation of the biocontrol potential of PGPB strains isolated from drought-tolerant tomatoes against fungal pathogens. *J. Plant Pathol.* **2023**, *105*, 1013–1029. [CrossRef]
20. Gebashe, F.; Gupta, S.; Van Staden, J. Disease management using biostimulants. In *Biostimulants for Crops from Seed Germination to Plant Development*; Elsevier: Amsterdam, The Netherlands, 2021; pp. 411–425.
21. Borhannuddin Bhuyan, M.H.M.; Mohsin, S.M.; Mahmud, J.A.; Hasanuzzaman, M. Use of Biostimulants for Improving Abiotic Stress Tolerance in Brassicaceae Plants. In *The Plant Family Brassicaceae*; Springer: Singapore, 2020; pp. 497–531.
22. Witkowicz, R.; Skrzypek, E.; Gleń-Karolczyk, K.; Krupa, M.; Biel, W.; Chłopicka, J.; Galanty, A. Effects of application of plant growth promoters, biological control agents and microbial soil additives on photosynthetic efficiency, canopy vegetation indices and yield of common buckwheat (*Fagopyrum esculentum* Moench). *Biol. Agric. Hortic.* **2021**, *37*, 234–251. [CrossRef]
23. Qiao, R.; Xu, M.; Jiang, J.; Song, Z.; Wang, M.; Yang, L.; Guo, H.; Mao, Z. Plant growth promotion and biocontrol properties of a synthetic community in the control of apple disease. *BMC Plant Biol.* **2024**, *24*, 546. [CrossRef]

24. Palmieri, D.; Ianiri, G.; Del Grosso, C.; Barone, G.; De Curtis, F.; Castoria, R.; Lima, G. Advances and Perspectives in the Use of Biocontrol Agents against Fungal Plant Diseases. *Horticulturae* **2022**, *8*, 577. [CrossRef]
25. Romano, A.; Vitullo, D.; Di Pietro, A.; Lima, G.; Lanzotti, V. Antifungal Lipopeptides from *Bacillus amyloliquefaciens* Strain BO7. *J. Nat. Prod.* **2011**, *74*, 145–151. [CrossRef] [PubMed]
26. Castoria, R.; Miccoli, C.; Barone, G.; Palmieri, D.; De Curtis, F.; Lima, G.; Heitman, J.; Ianiri, G. Molecular Tools for the Yeast *Papiliotrema terrestris* LS28 and Identification of Yap1 as a Transcription Factor Involved in Biocontrol Activity. *Appl. Environ. Microbiol.* **2021**, *87*, e02910-20. [CrossRef]
27. Palmieri, D.; Vitale, S.; Lima, G.; Di Pietro, A.; Turrà, D. A bacterial endophyte exploits chemotropism of a fungal pathogen for plant colonization. *Nat. Commun.* **2020**, *11*, 5264. [CrossRef]
28. Palmieri, M.; Lasserre, B.; Marino, D.; Quaranta, L.; Raffi, M.; Ranalli, G. The Environmental Footprint of Scientific Research: Proposals and Actions to Increase Sustainability and Traceability. *Sustainability* **2023**, *15*, 5616. [CrossRef]
29. Caprari, C.; Bucci, A.; Divino, F.; Giovacchini, S.; Mirone, E.; Monaco, P.; Perrella, G.; Quaranta, L.; Scalabrino, S.; Ranalli, G. Collection methods of wild barn owl pellets at low environmental contamination and proposals of microbiological and ecological investigations. *Ann. Microbiol.* **2024**, *74*, 14. [CrossRef]
30. El-Tokhy, F.; Tantawy, A.; El-Shinawy, M.; Abou-Hadid, A. Effect of Sugar Beet Molasses and Fe-Edhha on Tomato Plants Grown under Saline Water Irrigation Condition. *Arab Univ. J. Agric. Sci.* **2019**, *26*, 2297–2310. [CrossRef]
31. Tallarita, A.V.; Vecchietti, L.; Golubkina, N.A.; Sekara, A.; Cozzolino, E.; Mirabella, M.; Cuciniello, A.; Maiello, R.; Cenvinzo, V.; Lombardi, P.; et al. Effects of Plant Biostimulation Time Span and Soil Electrical Conductivity on Greenhouse Tomato 'Miniplum' Yield and Quality in Diverse Crop Seasons. *Plants* **2023**, *12*, 1423. [CrossRef]
32. Azman, A.T.; Mohd Isa, N.S.; Mohd Zin, Z.; Abdullah, M.A.A.; Aidat, O.; Zainol, M.K. Protein Hydrolysate from Underutilized Legumes: Unleashing the Potential for Future Functional Foods. *Prev. Nutr. Food Sci.* **2023**, *28*, 209–223. [CrossRef]
33. Wise, K.; Williams, L.B.; Selby-Pham, S.; Wright, P.F.A.; Simovich, T.; Gill, H.; Gupta, A.; Puri, M.; Selby-Pham, J. Supplementation of fertiliser with the biostimulant molasses enhances hemp (*Cannabis sativa*) seed functional food antioxidant capacity by induction of stress responses. *Sci. Hortic.* **2024**, *334*, 113299. [CrossRef]
34. Zuzunaga-Rosas, J.; Calone, R.; Mircea, D.M.; Shakya, R.; Ibáñez-Asensio, S.; Boscaiu, M.; Fita, A.; Moreno-Ramón, H.; Vicente, O. Mitigation of salt stress in lettuce by a biostimulant that protects the root absorption zone and improves biochemical responses. *Front. Plant Sci.* **2024**, *15*, 1341714. [CrossRef]
35. Rouphael, Y.; Colla, G. Synergistic Biostimulatory Action: Designing the Next Generation of Plant Biostimulants for Sustainable Agriculture. *Front. Plant Sci.* **2018**, *9*, 426696. [CrossRef] [PubMed]
36. Sabatino, L.; Consentino, B.B.; Rouphael, Y.; De Pasquale, C.; Iapichino, G.; D'Anna, F.; La Bella, S. Protein Hydrolysates and Mo-Biofortification Interactively Modulate Plant Performance and Quality of 'Canasta' Lettuce Grown in a Protected Environment. *Agronomy* **2021**, *11*, 1023. [CrossRef]
37. Chandramohanan, K.T. A Study on the Effect of Salinity Stress on the Chlorophyll Content of Certain Rice Cultivars of Kerala State of India. *Agric. For. Fish.* **2014**, *3*, 67. [CrossRef]
38. Taïbi, K.; Taïbi, F.; Ait Abderrahim, L.; Ennajah, A.; Belkhodja, M.; Mulet, J.M. Effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidant defence systems in *Phaseolus vulgaris* L. *S. Afr. J. Bot.* **2016**, *105*, 306–312. [CrossRef]
39. Bacha, H.; Tekaya, M.; Drine, S.; Guasmi, F.; Touil, L.; Enneb, H.; Triki, T.; Cheour, F.; Ferchichi, A. Impact of salt stress on morpho-physiological and biochemical parameters of *Solanum lycopersicum* cv. *Microtom* leaves. *S. Afr. J. Bot.* **2017**, *108*, 364–369. [CrossRef]
40. Yasar, F.; Ellialtioglu, S.; Yildiz, K. Effect of salt stress on antioxidant defense systems, lipid peroxidation, and chlorophyll content in green bean. *Russ. J. Plant Physiol.* **2008**, *55*, 782–786. [CrossRef]
41. Sevengor, S.; Yasar, F.; Kusvuran, S.; Ellialtioglu, S. The effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidative enzymes of pumpkin seedling. *Afr. J. Agric. Res.* **2011**, *6*, 4920–4924. [CrossRef]
42. Cristofano, F.; El-Nakhel, C.; Rouphael, Y. Biostimulant Substances for Sustainable Agriculture: Origin, Operating Mechanisms and Effects on Cucurbits, Leafy Greens, and Nightshade Vegetables Species. *Biomolecules* **2021**, *11*, 1103. [CrossRef]
43. Dong, C.; Wang, G.; Du, M.; Niu, C.; Zhang, P.; Zhang, X.; Ma, D.; Ma, F.; Bao, Z. Biostimulants promote plant vigor of tomato and strawberry after transplanting. *Sci. Hortic.* **2020**, *267*, 109355. [CrossRef]
44. Krinis, D.I.; Kasampalis, D.S.; Siomos, A.S. Biostimulants as a Means to Alleviate the Transplanting Shock in Lettuce. *Horticulturae* **2023**, *9*, 968. [CrossRef]
45. Loconsole, D.; Cristiano, G.; De Lucia, B. Biostimulant Application, under Reduced Nutrient Supply, Enhances Quality and Sustainability of Ornamental Containerized Transplants. *Agronomy* **2023**, *13*, 765. [CrossRef]
46. Ma, Y. Abiotic Stress Responses and Microbe-Mediated Mitigation in Plants. *Agronomy* **2023**, *13*, 1844. [CrossRef]
47. Mickelbart, M.V.; Hasegawa, P.M.; Bailey-Serres, J. Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nat. Rev. Genet.* **2015**, *16*, 237–251. [CrossRef]
48. Chaves, M.M.; Costa, J.M.; Zarrouk, O.; Pinheiro, C.; Lopes, C.M.; Pereira, J.S. Controlling stomatal aperture in semi-arid regions—The dilemma of saving water or being cool? *Plant Sci.* **2016**, *251*, 54–64. [CrossRef]
49. Hedrich, R.; Shabala, S. Stomata in a saline world. *Curr. Opin. Plant Biol.* **2018**, *46*, 87–95. [CrossRef]
50. Nadeeka, P.; Seran, T. The effects of goat manure and sugarcane molasses on the growth and yield of beetroot (*Beta vulgaris* L.). *J. Agric. Sci. Belgrade* **2020**, *65*, 321–335. [CrossRef]

51. Zhang, X.; He, P.; Guo, R.; Huang, K.; Huang, X. Effects of salt stress on root morphology, carbon and nitrogen metabolism, and yield of Tartary buckwheat. *Sci. Rep.* **2023**, *13*, 12483. [CrossRef] [PubMed]
52. Acosta-Motos, J.; Ortuño, M.; Bernal-Vicente, A.; Diaz-Vivancos, P.; Sanchez-Blanco, M.; Hernandez, J. Plant Responses to Salt Stress: Adaptive Mechanisms. *Agronomy* **2017**, *7*, 18. [CrossRef]
53. Zhang, Y.; Zhang, Y.; Zhang, R.; Song, Y.; Li, G.; Song, Y.; Ma, G.; Guo, H. Adaptability of root morphology and growth of two forage grass species in response to salt stress. *Front. Environ. Sci.* **2024**, *12*, 1406778. [CrossRef]
54. Tian, Z.; Pu, H.; Cai, D.; Luo, G.; Zhao, L.; Li, K.; Zou, J.; Zhao, X.; Yu, M.; Wu, Y.; et al. Characterization of the bacterial microbiota in different gut and oral compartments of splendid japalure (*Japalura sensu lato*). *BMC Vet. Res.* **2022**, *18*, 205. [CrossRef] [PubMed]
55. Huang, J.; Gao, K.; Yang, L.; Lu, Y. Successional action of *Bacteroidota* and *Firmicutes* in decomposing straw polymers in a paddy soil. *Environ. Microbiome* **2023**, *18*, 76. [CrossRef] [PubMed]
56. Yu, J.; Li, C.; Li, X.; Liu, K.; Liu, Z.; Ni, W.; Zhou, P.; Wang, L.; Hu, S. Isolation and functional analysis of acid-producing bacteria from bovine rumen. *PeerJ* **2023**, *11*, e16294. [CrossRef] [PubMed]
57. Zamanzadeh-Nasrabadi, S.M.; Mohammadiapanah, F.; Hosseini-Mazinani, M.; Sarikhan, S. Salinity stress endurance of the plants with the aid of bacterial genes. *Front. Genet.* **2023**, *14*, 1049608. [CrossRef]
58. Kazerooni, E.A.; Maharachchikumbura, S.S.N.; Adhikari, A.; Al-Sadi, A.M.; Kang, S.-M.; Kim, L.-R.; Lee, I.-J. Rhizospheric *Bacillus amyloliquefaciens* Protects *Capsicum annuum* cv. Geumsugangsan from Multiple Abiotic Stresses via Multifarious Plant Growth-Promoting Attributes. *Front. Plant Sci.* **2021**, *12*, 669693. [CrossRef]
59. Kolton, M.; Erlacher, A.; Berg, G.; Cytryn, E. The *Flavobacterium* Genus in the Plant Holobiont: Ecological, Physiological, and Applicative Insights. In *Microbial Models: From Environmental to Industrial Sustainability*; Springer: Singapore, 2016; pp. 189–207.
60. Staley, J.T.; Vasilyeva, L.; Yee, B. Bauldia. In *Bergey's Manual of Systematics of Archaea and Bacteria*; Wiley: Hoboken, NJ, USA, 2019; pp. 1–5.
61. Talwar, C.; Nagar, S.; Kumar, R.; Scaria, J.; Lal, R.; Negi, R.K. Defining the Environmental Adaptations of Genus *Devosia*: Insights into its Expansive Short Peptide Transport System and Positively Selected Genes. *Sci. Rep.* **2020**, *10*, 1151. [CrossRef]
62. Kim, J.; Woo, O.-G.; Bae, Y.; Keum, H.L.; Chung, S.; Sul, W.J.; Lee, J.-H. Enhanced Drought and Salt Stress Tolerance in Arabidopsis by *Flavobacterium crocinum* HYN0056T. *J. Plant Biol.* **2020**, *63*, 63–71. [CrossRef]
63. Kim, H.; Woo, O.-G.; Bin Kim, J.; Yoon, S.-Y.; Kim, J.-S.; Sul, W.J.; Hwang, J.-Y.; Lee, J.-H. *Flavobacterium* sp. strain GJW24 ameliorates drought resistance in Arabidopsis and Brassica. *Front. Plant Sci.* **2023**, *14*, 1257137. [CrossRef]
64. Seo, H.; Kim, J.H.; Lee, S.-M.; Lee, S.-W. The Plant-Associated Flavobacterium: A Hidden Helper for Improving Plant Health. *Plant Pathol. J.* **2024**, *40*, 251–260. [CrossRef]
65. Shah, A.; Subramanian, S.; Smith, D.L. Flavonoids and *Devosia* sp SL43 cell-free supernatant increase early plant growth under salt stress and optimal growth conditions. *Front. Plant Sci.* **2022**, *13*, 1030985. [CrossRef]
66. Monjezi, N.; Yaghoubian, I.; Smith, D.L. Cell-free supernatant of *Devosia* sp. (strain SL43) mitigates the adverse effects of salt stress on soybean (*Glycine max* L.) seed vigor index. *Front. Plant Sci.* **2023**, *14*, 1071346. [CrossRef] [PubMed]
67. Peng, J.; Wegner, C.-E.; Liesack, W. Short-Term Exposure of Paddy Soil Microbial Communities to Salt Stress Triggers Different Transcriptional Responses of Key Taxonomic Groups. *Front. Microbiol.* **2017**, *8*, 400. [CrossRef] [PubMed]
68. Jacobsen, M.D.; Beynon, R.J.; Gethings, L.A.; Claydon, A.J.; Langridge, J.I.; Vissers, J.P.C.; Brown, A.J.P.; Hammond, D.E. Specificity of the osmotic stress response in *Candida albicans* highlighted by quantitative proteomics. *Sci. Rep.* **2018**, *8*, 14492. [CrossRef] [PubMed]
69. García, M.J.; Ríos, G.; Ali, R.; Bellés, J.M.; Serrano, R. Comparative physiology of salt tolerance in *Candida tropicalis* and *Saccharomyces cerevisiae*. *Microbiology* **1997**, *143*, 1125–1131. [CrossRef]
70. Ali, R.; Gul, H.; Rauf, M.; Arif, M.; Hamayun, M.; Husna; Khilji, S.A.; Ud-Din, A.; Sajid, Z.A.; Lee, I.-J. Growth-Promoting Endophytic Fungus (*Stemphylium lycopersici)* Ameliorates Salt Stress Tolerance in Maize by Balancing Ionic and Metabolic Status. *Front. Plant Sci.* **2022**, *13*, 890565. [CrossRef]
71. Ben Ali, W.; Navarro, D.; Kumar, A.; Drula, E.; Turbé-Doan, A.; Correia, L.O.; Baumberger, S.; Bertrand, E.; Faulds, C.B.; Henrissat, B.; et al. Characterization of the CAZy Repertoire from the Marine-Derived Fungus *Stemphylium lucomagnoense* in Relation to Saline Conditions. *Mar. Drugs* **2020**, *18*, 461. [CrossRef]
72. Manzotti, A.; Bergna, A.; Burow, M.; Jørgensen, H.J.L.; Cernava, T.; Berg, G.; Collinge, D.B.; Jensen, B. Insights into the community structure and lifestyle of the fungal root endophytes of tomato by combining amplicon sequencing and isolation approaches with phytohormone profiling. *FEMS Microbiol. Ecol.* **2020**, *96*, fiaa052. [CrossRef]
73. Vitullo, D.; Di Pietro, A.; Romano, A.; Lanzotti, V.; Lima, G. Role of new bacterial surfactins in the antifungal interaction between *Bacillus amyloliquefaciens* and *Fusarium oxysporum*. *Plant Pathol.* **2012**, *61*, 689–699. [CrossRef]
74. Woodward, M. *Epidemiology*, 3rd ed.; Chapman and Hall: London, UK; CRC: New York, NY, USA, 2013; ISBN 9780429196263.
75. Triola, M.; Triola, M.; Roy, J. *Biostatistics for the Biological and Health Sciences*, 2nd ed.; Pearson: London, UK, 2017; ISBN 9780134039015.
76. Zhu, J.; Tremblay, N.; Liang, Y. Comparing SPAD and atLEAF values for chlorophyll assessment in crop species. *Can. J. Soil Sci.* **2012**, *92*, 645–648. [CrossRef]
77. Novichonok, E.V.; Novichonok, A.O.; Kurbatova, J.A.; Markovskaya, E.F. Use of the atLEAF+ chlorophyll meter for a nondestructive estimate of chlorophyll content. *Photosynthetica* **2016**, *54*, 130–137. [CrossRef]

78. Bates, L.S.; Waldren, R.P.; Teare, I.D. Rapid determination of free proline for water-stress studies. *Plant Soil* **1973**, *39*, 205–207. [CrossRef]
79. Kalhor, M.S.; Aliniaeifard, S.; Seif, M.; Asayesh, E.J.; Bernard, F.; Hassani, B.; Li, T. Title: Enhanced salt tolerance and photosynthetic performance: Implication of ɤ-amino butyric acid application in salt-exposed lettuce (*Lactuca sativa* L.) plants. *Plant Physiol. Biochem.* **2018**, *130*, 157–172. [CrossRef] [PubMed]
80. Aquilano, C.; Baccari, L.; Caprari, C.; Divino, F.; Fantasma, F.; Saviano, G.; Ranalli, G. Effects of EOs vs. Antibiotics on *E. coli* Strains Isolated from Drinking Waters of Grazing Animals in the Upper Molise Region, Italy. *Molecules* **2022**, *27*, 8177. [CrossRef] [PubMed]
81. Iacus, S.M.; Masarotto, G. *Laboratorio di Statistica con R*; Workbooks; McGraw-Hill: New York, NY, USA, 2003; ISBN 8838660840.

Caprari *et al. Annals of Microbiology* (2024) 74:14
https://doi.org/10.1186/s13213-024-01759-6

Annals of Microbiology

ORIGINAL ARTICLE **Open Access**

Collection methods of wild barn owl pellets at low environmental contamination and proposals of microbiological and ecological investigations

Claudio Caprari[1], Antonio Bucci[1], Fabio Divino[1], Simone Giovacchini[1], Enrico Mirone[1], Pamela Monaco[1], Giada Perrella[1], Luca Quaranta[1], Simone Scalabrino[1] and Giancarlo Ranalli[1*]

Abstract

Background The barn owl *Tyto alba* is a medium-sized nocturnal raptor, predator of small mammals, birds, reptiles, and insects, distributed all over the world. Environmentally uncontaminated wild barn owl pellets are hard and rare to find and provide a natural matrix for biological investigations.

Results Different solutions for collecting wild barn owl pellets at low environmental contamination are proposed. These solutions are based on a daily sanitized surface for sample retrieval, followed by rapid analysis. As an example of the possible investigations that can be carried out on these matrices, with the aim to highlight the relevance of the obtained results also from a biotechnological perspective. Two rare pellet samples from wild barn owl *T. alba*, appropriately collected in a rural area of central Italy, were analyzed using culture-dependent, molecular (Next-Generation Sequencing), and enzyme profile analysis techniques. The osteological observations of the bone remains provided useful information for identifying the prey, mainly rodents (*Apodemus* sp.). Under our experimental conditions, the results revealed both a wide heterogeneity between the pellet microbiota and a great percentage of uncultured bacteria not classified at the species level. Furthermore, microbial cultures of *Malbranchea albolutea*, *Debaryomyces hansenii*, and *Lactobacillus* sp. were isolated and studied.

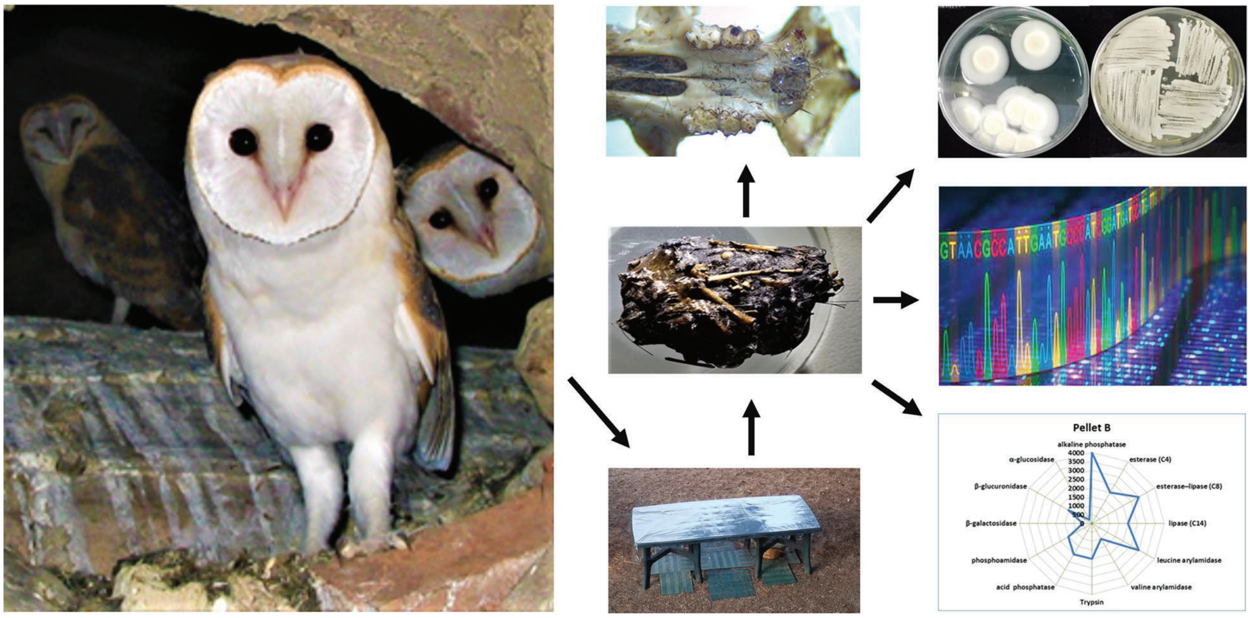
Finally, we quantified the environmental impact of our experimental work in terms of CO_2 equivalent release. To compensate for the release of 300 kg/CO_2 eq., three *Taxus. baccata* L. were planted.

Conclusions This work provides a starting point for developing an effective strategy to study and characterize wild barn owl pellets at low environmental contamination. It presents a simple and easy technique/protocol for collecting the pellets. The microbiota heterogeneity found in the two analyzed samples suggests that barn owl pellets can represent a potential natural reservoir for the discovery of new microorganisms to be used in various biotechnological applications. This could open the way for further large-scale studies on a greater number of samples and populations.

---

*Correspondence:
Giancarlo Ranalli
ranalli@unimol.it
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

The barn owl (*Tyto alba*) is a medium-sized nocturnal raptor representing the most widespread owl species on Earth (Kross et al. 2016). It is distributed in Europe, Africa, Southern Asia, Oceania, North and South America, both on mainland and on islands (BirdLife International 2019; Viganò et al. 2020). This raptor plays a very important role in the ecological balance of a territory. Beyond its ecological importance as a top predator, *T. alba* may be considered a biological control agent of rodent pests in agriculture (Marti et al. 1979; Wood and Fee 2003; Whelan et al. 2008; Meyrom et al. 2009; Kross et al. 2016, 2018).

Barn owls readily occupy human-made structures, persist in agricultural settings better than many bird species and, with enough nests, several breeding pairs may be present in one km^2 (Taylor 2003; Kasprzykowski and Goławski 2006; Meyrom et al. 2009; Roulin 2020). These factors, together with their ability to remove large quantities of rodents and the abundance of rodent pests within agroecosystems, make them an attractive option for farmers to control pests (Durant et al. 2004; Marti 2010; Kross et al. 2016; Johnson et al. 2018; St. George and Johnson 2021).

Raptors have a varied diet, some rely on insects, others on small birds, many on small vertebrates (Teta et al. 2012; Roulin and Dubey 2012; Roulin and Christe 2013). In the raptor stomach, the small mammal is subjected to the action of digestive juices that destroy the tissue leaving the skin and skeleton intact. These residues, under the action of a rotational movement, aggregate together until they gradually form an ovoid mass, which the bird of prey will regurgitate after a few hours. This small elongated greyish ball, often pointed at its extremities, is called "pellet" or "rejection ball". The formation of pellets avoids a long and difficult digestion of the unassimilated elements by the raptor (mammal hair and bones, insect chitin, insect larvae hair, etc.). Raptors usually reject one pellet per day, rarely two or three, which may contain the remains of several small mammals. The pellets accumulate at the foot of nocturnal raptor nests, in church steeples, in isolated attics, in hollow trees, on cliffs and in cave entrances (Chaline et al. 1974). Since the analysis of pellet content is relatively easy and cheap, barn owl diet has been thoroughly investigated, especially in Europe and America (Jaksic et al. 1982; Bellocq 2000; Love et al. 2000; Cagnacci et al. 2012; Milana et al. 2016, 2018; Romano et al. 2020). Barn owl diet can offer a source of information on the state of conservation of the environment surrounding their settlement (Chaline et al. 1974). However, despite the several studies carried out on barn owl pellets, almost no one has focused on the analysis of pellet associated microbial communities. The study of microbial community dynamics is important for many aspects, including ecological and scientific discovery, biotechnological development, sustainable agriculture, environmental protection, and human health (Bucci et al. 2017).

It is well known that nature offers great potential for the study of both microbial biodiversity and functional ecology. The study of animals living in captive conditions are quick, convenient, easy, and more easily attributable to a specific individual (e.g. age, sex, etc.). Indeed, the collection of residues and wastes (feces, urine, litter, etc.) for example from cages and aviaries is a usual practice. However, the condition of captive life (e.g., feeding regimen, instinctive behavior, etc.) induces profound alterations in animals compared to the wildlife (Rawat and Agarwal 2015; Feckler et al. 2023).

Obtaining "genuine" samples of residues and wastes from animals in the wild free from environmental contamination is very difficult and complex. In fact, wild animals release their residues randomly and very unpredictably. Therefore, the collection of samples from free-living animals in natural matrices (e.g., soil, plant surfaces, water, etc.) is tedious, time-consuming, and often fails to yield useful results. The environment rapidly induces contamination of free-living animal residues altering the genuineness of the samples (Holt and Miller 2010). In view of this, how to collect wad samples at low environmental contamination? What kind of useful information could be derived from samples obtained from wild raptors? (Hegedus et al. 2023).

To address these questions we propose: (I) a feasible solution for recovering residual pellet samples from wild nocturnal raptors; (II) microbiological investigations to characterize *T. alba* wad microbiota and to unravel the potential presence of novel and uncharacterized strains of biotechnological interest; III) a method to acquire information on the barn owl diet through wad osteological observations; IV) finally, an evaluation of the environmental impact of our experiments, in terms of CO_2 equivalent release, and how to compensate it.

Materials and methods

Experimental design

The experimental design adopted in this research (Fig. S1) has been developed with the aim to provide an effective strategy to collect and study from a microbiological and ecological perspective wild barn owl pellets, at low environmental contamination.

Sampling site

The sampling site of this study is located at a rural area (42° 20′ 59.23″ N 14° 01′ 55.38″ E) of Rosciano municipality, Abruzzo region, (Central Italy). The area is at 20 km from Adriatic Sea, where the territory is mainly hilly (150–300 m altitude) at a transitional zone from Mediterranean to temperate bioclimate (Blasi et al. 2014). The area is characterized by i) a mosaic of croplands, mainly herbaceous (cereals) and horticultural crops used in succession as sheep pasture; ii) uncultured areas with Mediterranean and sub-Mediterranean arboreal and shrubby vegetation, hedges and coppices composed of mixed *Quercus pubescens* Willd. and riparian vegetation of the valley floor; iii) large area with destination both to traditional and specialized orchards (olive cultures) and vineyards (Montepulciano d'Abruzzo cultivar). The landscape composition was confirmed using Google Earth (®) (last accessed July 2023) and landscape elements were analyzed within a 1 km radius around the nest site of barn owls (Fig. S2a and b).

Pellet collecting systems

Based on previous long-term observations of the area where the active wild barn owls' nest was located (less than 20 m), we noticed that adults released their pellets by night. It was noticed that remains of pellets were sometimes found in a narrow area, under the tree canopy, in the garden. Therefore, four different sample collecting systems were proposed, tested, and compared (Fig. 1a and b).

System 1. A hard, flat surface of about 2.5 m^2, like a Table (2.40 m × 1.0 m; height from the ground, 90 cm) in PVC, green coloured, was adopted. A daily checked table surface covered with a transparent plastic sheet was cleaned before nightfall by a hygienic water solution of alkyldimethylbenzylammonium 10% w/v (Neo-Desogen®, Teleflex Medical S.r.l., Varedo, Italy) and rinsed with sterile water. The two main reasons for the operating conditions described above are: (a) to avoid contamination by soil microorganisms and, (b) to define the date of recovery of samples.

System 2. A perforated (1.0 × 1.0 cm), rigid PVC net suspended two meters above the ground; tension is provided by tie rods on 4 posts driven into the ground, placed at the corners of the rectangular-shaped net (2.0 m × 5.0 m). Every day, the surface of the net is inspected and cleaned of plant residues that may have settled inside, fallen by gravity or atmospheric events (rain, wind, etc.). Before sunset, the upper surface of the net is washed and disinfected from above with a spray bar containing a 2% benzalkonium chloride solution in water, followed by a final rinse.

System 3. A perforated (1.0 × 1.0 cm), roll-up net in nylon, suspended two meters above the ground; tension is provided by tie rods on 4 posts driven into the ground, placed at the corners of the rectangular-shaped net, followed by sanitisation phase, as above (system 2);

(**a**) (**b**)

(**c**) (**d**)

Fig. 1 Images of wild barn owls *T. alba* living under a roof of Villa Vanda house, at Rosciano, Italy: **a** System 1; **b** System 2, at low environmental contamination. **c** One individual, in the position of alarm and/or fear; **d** Three individuals living in the same nest, in rest position

System 4. A vegetable roll-up fabric net (natural hemp, jute, cotton, etc.), adopted like the above system 2 and 3.

Samples and sampling

Two pellet samples (henceforth reported as A and B) were collected in Rosciano municipality. After sampling, A and B pellets were quickly subjected to the following laboratory activities: partial sterile dissection for optical observation and morphological analyses; inoculation in different media, for microbial growth, isolation, and characterization of isolates; DNA extraction for molecular investigations of the microbial communities. The pellets A and B from wild barn owls (*T. alba*) were freshly collected in the same site on 15th and 30th December 2022, respectively. Both pellets were collected under a 40-year-old tree (*Pinus nigra* J.F. Arnold). Then, the pellets were handled with latex gloves, immediately placed in sterile conical centrifuge tubes (volume 50 mL) (Corning Pharmaceutical Glass, SPA, Pisa, Italy), and transferred to the laboratory under refrigerated conditions for subsequent analyses, as quickly as possible, and no later than 48 h.

The collection surface is located less than 20 m from an active nest located under the roof of a historic building dating from the late 18th century (Villa Vanda, Rosciano, Italy) (Fig. 1a and b). The nest location allowed us to know the beginning and end of nocturnal predation activity. Therefore, images were acquired manually with a digital camera (Nikon, Coolpix L19 model) at first light when the raptors returned to the nest (Fig. 1c and d).

Pellet analyses and morphological observations

At laboratory scale, sterile conditions were adopted to avoid contaminations during the main phases of pellet analyses. Preliminary, pellets were weighted by a lab balance (Extend mod., Sartorius, Goettingen, Germany), under a bunsen flame. Then, the pellets were processed and disassembled, under sterile cabinet, to obtain sub-samples (in small pieces) both for microbial cultivation and molecular investigations of bacterial and fungal communities. Measures of pH and conductivity were performed with HI 763100 and HI11310 probes, respectively (Edge mod., Hanna Instruments, Woonsocket, RI, USA). The pH values were determined in ratio 1:10 w/v a physiological solution (0.9 M NaCl).

The remaining, almost complete pellets were submitted to optical observation by stereomicroscope (Axioplan mod., Zeiss, Germany) at 10x, 20x and 35x magnifications to identify barn owl prey. Records of images and morphological data (shape and dimension) with bar scale were acquired (Fig. 2a and b). The osteological parts were observed, and the identification was performed at the lowest possible taxonomic level (species level) comparing the undigested bone rests with the reference literature (Chaline et al. 1974; Di Palma and Massa 1981; Erome 1982; Brom 1986; Nappi 2001; Paolucci and Bon 2022). According to McDowell and Medlin 2009, the minimum number of individuals obtained by counting the most common diagnostic osteological remnants (element) of each species of small mammal in each pellet determined the prey number (McDowell and Medlin 2009).

Media and cultural techniques

The microbial growth tests were performed under sterile conditions. From both A and B pellets, several sub-samples were taken separately, cutting each pellet with a thin metal blade, previously sterilized under the flam.

Each pellet sample was cut into small pieces, randomly collected, and weighed into a sterile test tube. Then, 1.0 g of pellet was added to 9.0 mL of sterile saline (0.9% NaCl w/v). Samples ranged from 10 to diluted to 1.0×10^{-8} were inoculated into agar culture media: TSA (Tryptic Soya Agar -Biolife Italiana, Milano, Italy) for bacteria determination and PDA (Potato Dextrose Agar -BD Difco™, Milano, Italy) added with 100 µg/mL ampicillin (Fisher BioReagents) for fungi. The plates were incubated at 37 °C for 24–72 h to allow the growth of bacteria, and at 28 °C for 4–5 days for fungi.

C-EC agar (Biolife Italiana, Milano, Italy) was used for discriminating between total and fecal coliforms and *Escherichia coli* (Aquilano et al. 2022).

Bacillus spp. determination was performed by a total spore count. The saline sample mixture was heat shocked at 80 °C for ten minutes on a water bath. Then, 0.1 mL of the sample mixture was plated onto TSA and incubated overnight at 37 °C (Murray and American Society for Microbiology 1995). *Lactobacillus* spp. determination was carried out on MRS medium, incubation at 30 °C for 72 h (de Man et al. 1960; DSMZ 2023). All microbial counts were expressed as Colony Forming Unit (CFU) per 1.0 g of fresh weight (f.w.). The statistical data were expressed in terms of mean ± Standard Error (SE).

Microbial isolation

Morphology of individual colonies supported us in the strain isolation. Colonies with different color, size, border shape, and texture were chosen and inoculated in TSA and PDA. Then, the purity of each isolate was confirmed, and the isolated strains were observed under optical microscope.

In addition, preliminary identification tests were performed, including morphological properties under microscopic observations and physiological and biochemical tests for bacteria by a Gram reaction and API-Systems (Biomerieux Italia, Firenze), according to Bergey's Manual (Bergey et al. 2000).

To confirm the identity of microbial isolates, the yeast and fungal strains were sequenced for the ITS rDNA (ITS1, 5.8S, and ITS2 28S rRNA gene) (Raja et al. 2017) whereas bacterial strains were subjected to the partial sequencing (500 bp) of the 16S rRNA gene (Drancourt et al. 2000). Sequencing was performed by BMR Genomics (Padua, Italy). The obtained sequences were submitted to GenBank in the National Center for Biotechnology Information (Zhang et al. 2000; Morgulis et al. 2008). Bacteria isolated from barn owl pellets were stored at −80 °C in cryovials (Microbank), whereas fungi and yeasts were preserved at 4 °C on PDA slants (Jurado et al. 2021).

(a) (b)

Fig. 2 Imagine of wild barn owl pellet: **a** shape and dimension of an analyzed pellet with bar scale; **b** View entire pellet under stereomicroscope

Enzymatic activities

Multiple enzymatic activities of pellet as a whole and of isolated microorganisms were determined by the API-ZYM system (bio-Merieux Italia, Rome, Italy). A semi-quantitative evaluation of the activities of 19 hydrolytic enzymes [alkaline phosphatase, esterase (C4), esterase–lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cistine arylamidase, trypsin, α-chymotripsin, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetil-b-glucosamidase, α-mannosidase, and α-fucosidase] was performed (Ranalli et al. 2001; Viti et al. 2006). All determinations were made on three replicates. The reproducibility was > 95%. Results are reported in figures by MS Excel, radar function.

16S rRNA gene and ITS2 region amplicon library preparation and sequencing

Next Generation Sequencing analysis was performed at BMR Genomics company (Padova, Italy), following the protocol previously used by Monaco and colleagues (Monaco et al. 2020, 2021) for bacterial community investigation. The V3–V4 regions of the 16S rRNA gene were amplified using the primer pair 16S_univFor and 16S_univRev, modified with universal tails (Nadkarni et al. 2002).

The ITS2 regions of fungi were amplified using the modified primers ITS3_KYO2 (Toju et al. 2012) and ITS4r (White et al. 1990). PCR products were purified with Thermolabile Exonuclease I (NEB) and amplified with Nextera XT Index (Illumina) on a second PCR step. Amplicons were then normalized with SequalPrep (Thermo Fisher) and multiplexed. The pool was purified with Agencourt XP 1X magnetic beads. The library was run on the Illumina MiSeq and sequenced with V3 chemistry – 300PE strategy. Bioinformatics analysis was performed using QIIME2 tools version 2021.4 (Caporaso et al. 2010; Bokulich et al. 2018).

The obtained reads were cleaned of primers by using the Cutadapt software (v. 2021.4) and then processed with the denoised paired plugin of the DADA2 software (Callahan et al. 2016). Briefly, sequences have been trimmed at the 3′ end and filtered by quality and length. Then, they were de-replicated and merged to obtain unique sequences, and chimeras were eliminated. The Amplicon Sequence Variants (ASVs) with a frequency < 0.01% were not considered. All reads from bacterial communities were classified to the lowest possible taxonomic rank using a reference dataset from the SILVA database (version 138). On the other hand, all reads obtained from fungal communities were classified to the lowest possible taxonomic rank using a reference dataset from the UNITE database (version 8.3). Sequences generated in the present study were deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA957615.

Alpha-diversity was calculated by using the Shannon index whereas similarity between the investigated bacterial and fungal communities (beta-diversity) was calculated by using Bray–Curtis dissimilarity index (Ärje et al. 2016; Caprari et al. 2023).

Predictive functional analysis on sequencing data

The predictive functional profiling/analysis of microbial communities (taxa bacteria) responds to the question: who does what! For the functional ecological rules of microbial communities 16S rRNA marker gene sequences were analyzed (Langille et al. 2013; Xia et al. 2019) by the python script "collapse_table.py", Python 3 environment by FAPROTAX database (version 1.2.6, Available online:https://pages.uoregon.edu/slouca/LoucaLab/archive/FAPROTAX/lib/php/index.php, accessed on 13 December 2023) (Zhang et al. 2023; Liu et al. 2023). Such a script works in two steps. Firstly, it maps the list of prokaryotic clades detected in the previously mentioned NGS analysis to the respective metabolic or ecologically relevant functions. To do this, it relies on the FAPROTAX database, which consolidates information from the literature by explicitly providing such mappings. Second, it counts the occurrences of the functions and reports. As a result, we obtained a list of functions with the respective number of occurrences. We executed the script for our two samples (A and B). We plot the obtained results using a heatmap in which the rows represent the detected functions, the columns represent the samples (A and B), and each cell contains a circle sized proportionally to the number of prokaryotic clades detected in the sample (column) for the function (row). We only report functions for which we observed at least one occurrence.

Environmental impact, CO_2 equivalent emission and mitigation actions

In order to assess, reduce and mitigate the greenhouse gas generated by the laboratory activities originating from these researches, we adopted the protocol and indicators (Table 1) of a previous work (Palmieri et al. 2023).

Results

Comparison methods for collecting wild raptor pellets, at low environmental contamination.

The results among the four tested systems to collect wild raptors pellets, comparing the advantages and limits, are described in Table 2.

Because of the detailed notes above reported, method 1 was evaluated positively and adopted. Therefore, the

Table 1 Indicators selected to calculate the environmental footprint originated by a research activity, impacts and green mitigation acts. Amount x K conversion = CO_2 eq. Data by Palmieri et al. 2023 modified (Palmieri et al. 2023)

Type Consumptions	Evaluation of CO_2 eq. emission				
	Parameter	**Unit**	**Amount**	**K conversion to CO_2**	**CO_2 yield (kg)**
Energy (electric power by fossil)	Equipments (instruments, steril hoods, fans, cooling, sterilization, others).	kWh		0.224	
	Tap water	m^3		0.32	
Chemical products	Reagents (dried cultural media; acid and basic solutions, others).	kg, liter		1.47	
	Toxic, hazard (solvents).	kg		0.62	
Wastes	Plastic	kg		1.74	
	Glass	kg		0.85	
	Paper	kg		2.42	
	Effluents	m^3		0.29	
Transports personnel and samples/materials		km		0.121	
CO_2 eq. total emissions (kg)					
Social Costs (€)					
Mitigation Action	N° of plants to be planted K conversion kg CO_2 eq. to number of plant, 100:1	Which type; when, where; how; who provide; control and guarantee in the time.			

Table 2 Advantages and limits of methods for collecting and sanitizing wild raptor pellets, at low environmental contamination. - not easy, not fast; +/- easy, not fast; + easy and fast; + + very easy and fast; + + + very easy and very fast

N°	Methods	Set-up	Recovery	Sanitisation
1	Hard, flat surface (like table)	Easy to assemble and transport; cumbersome. Only for flat land	+ + +	+ + +
2	Perforated roll-up net in PVC + 4 poles for land support	Easy to fix; less complex to move, cumbersome. Even for non-flat land	+ +	+ +
3	Perforated roll-up net in nylon + 4 poles for land support	Easy to fix; less complex to move, not cumbersome. Even for non-flat land	+ +	+
4	Vegetable fabric net roll-up + 4 poles for land support (like hammock)	Easy to fix and to move, not cumbersome. Even for non-flat land	+/-	-

collection of wild barn owl pellets was carried out using a table surface (about 2.5 m^2), daily sanitized. It can be considered useful and adequate for sampling recovery on the site selected.

Table 3 Main characteristics of the analyzed A and B pellets

Main Characteristics	A	B
Weight (g)	2.16	2.14
Dimension (length, mm)	33	37
Color	Brown with bone remains	
Appearance	Translucent on surface	
pH	7.3	7.5
Conductivity (µS)	16.5	12.0

Pellet characteristics

Results of preliminary analyses on *T. alba* pellets are shown in Table 3.

Both samples appeared fresh, intact, in good condition, of brown color and shiny appearance with some bony elements on the surface alongside, others deepening. The two pellet samples were characterized by small size (< 40 mm) and weight less than 3.0 g each.

Morphological observations

Results of the prey identification at the lowest possible taxonomic level (Fig. 3a and b) were: *Apodemus sylvaticus* and *Apodemus* spp. for pellet A, and *Apodemus flavicollis* and *Rattus rattus* for pellet B.

Microbial count and isolated microorganisms

Regarding bacterial viable counts (Table 4), high values were obtained for both pellet samples, with average

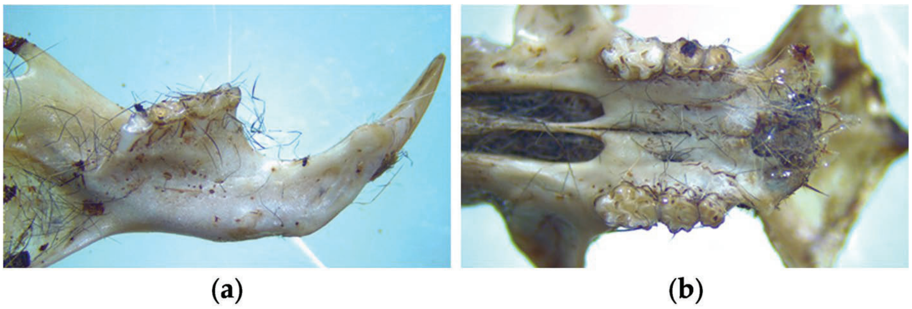


Fig. 3 Example of undigested bones used as diagnostic elements to identify *T. alba* preys: lower jaw (**a**) and skull (**b**) of a mouse belonging to *Apodemus* spp

Table 4 Microbial counts on analyzed pellet samples (data expressed as log CFU/g ± SE)

Microbial group	Pellet A	Pellet B
Total viable aerobic heterotrophic bacteria	6.5 ± 0.5	6.4 ± 0.4
Fungi & Yeasts	5.1 ± 0.3 0.2 ± 0.05	4.6 ± 0.2 nd[a]
Enterobacteriaceae	2.0 ± 0.2	2.9 ± 0.4
E. coli	1.5 ± 0.2	1.8 ± 0.3
Spore-forming bacteria	3.5 ± 0.1	3.0 ± 0.2
Lactobacillus spp.	2.2 ± 0.3	3.1 ± 0.5

[a] *nd* not detected

values of 10^6—10^7 CFU/g f.w. At the same time, spore-forming bacteria showed a colony amount ranging from 10^3 to 10^4 CFU/g f.w. Results of fungal counts varied from 10^4 to 10^5 CFU/g f.w. The presence of yeasts was observed only in pellet A. In both pellets, similar amounts of *Enterobacteriaceae* colonies, including, *E. coli* were detected.

In Fig. 4a and b, one yeast colony and one fungal colony were reported, respectively. Table 5 shows the identification of microorganisms isolated from the analyzed pellets based on the comparison with DNA sequences available in reference databases.

Information databases using the internal transcribed spacer region from the fungi type and reference material showed that isolate strain 3B_1 had 100% identity with *Malbranchea albolutea* and the isolate strain AS4 had 100% identity with *Penicillium* sp. However, the ITS marker alone for identification does not discriminate at the species level in certain fungal genera such as *Penicillium* sp., and it may be necessary to sequence one or more protein-coding genes to obtain a more precise identification at the species level (Raja et al. 2017).

The isolate A5 showed an identity of 100% with both *Debaryomyces* sp. isolate FBFY22 and *Debaryomyces hansenii* clone Turkey strain M 6.3.

Analogs analyses with bacterial strains revealed that the strain N2 showed 100% of identity with *E. coli* strain LWY24; NB1 strain showed 100% of identity with *E. coli*

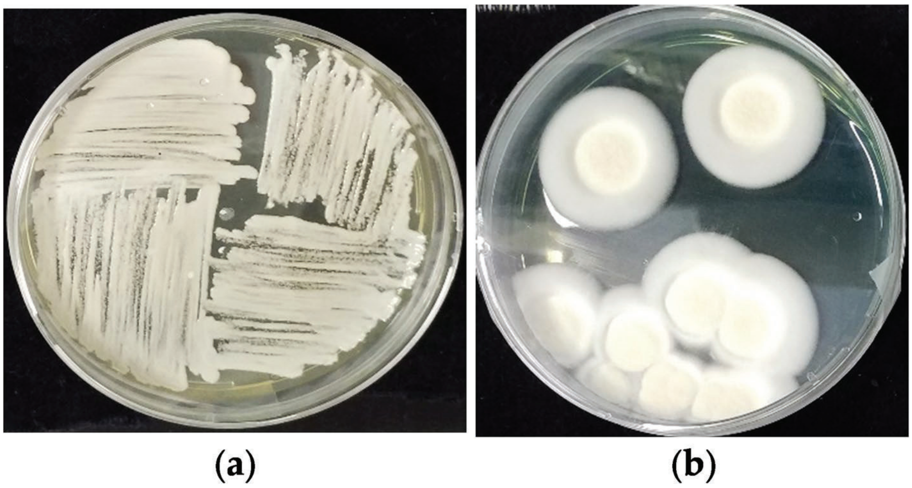


Fig. 4 Microbial growth of *D. hansenii* (**a**) and *M. albolutea* (**b**) on PDA medium, respectively

Table 5 Molecular identification of some of the strains isolated from the investigated wild barn owl pellets

Strains	Accession number	Closest affiliations	Identification and Accession Number (% Identity)
Bacteria			
N2	OR044424	*E. coli* strain LWY24	CP054556.1 (100)
NB1	OR044425	*E. coli* strain AVS0973	CP124471.1 (100)
NB2	OR044426	*E. ludwigii* strain RHB47	CP099325.1 (100)
NB3	OR044427	*P. fragi* strain NMC25	CP021132.1 (100)
Fungi			
3B_1	OR044530	*M. albolutea*	OW988116.1 (100)
AS4	OR044532	*Penicillium* sp.	MW662654.1 (100)
Yeast			
A5	OR044531	*D. hansenii* clone Turkey strain M6.3	MT004747.1 (100)

strain AVS0973; NB2 strain showed 100% identity with *Enterobacter ludwigii* strain RHB47; NB3 strain showed 100% of identity with *Pseudomonas fragi* strain NMC25.

Enzymatic profiles

Figure 5a and b show two enzymatic profiles obtained by the API ZYM assay on both pellet A and B. As expected, pellet A showed many enzyme activities (15 out of 19), while a lower number we recorded in pellet B (12 out 19) reflecting differences in microbial communities.

Figure 5c and d show the enzymatic profiles obtained by isolated yeast *D. hansenii* and bacterial strain *P. fragi*, respectively. The yeast *D. hansenii* shows 7 out of 19 profiles, with the peculiar presence of esterase-lipase (C8) and α-galactosidase enzymes, although with a low activity.

In contrast, the enzyme profile of the isolated bacterial strain of *P. fragi* reported here shows a greater number of enzymes (13 out of 19), with the peculiar presence of trypsin and β-glucuronidase enzymes.

Microbial community composition

NGS analysis provided interesting information about the composition of microbial communities associated with barn owl pellets. With regards to *Bacteria*, sequencing of the V3–V4 regions of the 16S rDNA resulted in 106 ASVs (Amplicon Sequence Variants) for sample A and in 104 ASVs for sample B, with a total number of 33,211 and 27,396 final reads, respectively. For fungal communities, the ITS2 region sequencing allowed 39,942 final reads and 33 ASVs for pellet A, and 37,963 final reads and 51 ASVs in the case of pellet B.

Overall, the microbial communities of the two wild barn owl pellets consisted of a few dominant phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, *Bacteroidota*, and *Desulfobacterota* for Bacteria and *Ascomycota*, *Mucoromycota*, *Basidiomycota*, and *Mortierellomycota* for Fungi (Fig. 6). In more detail, the analysis of the bacterial community structure revealed that *Firmicutes* were the main phylum in both wild pellets, with a relative abundance of 61.3% in sample A and 90.8% in sample B.

Proteobacteria showed a percentage of 30.4% in pellet A (with *Gammaproteobacteria* class particularly abundant), but they were poorly represented in pellet B (0.2%). The relative abundance of *Actinobacteriota* varied from 5.5 to 8.1%, whereas *Bacteroidota* ranged between 0.2 and 2.4%. *Desulfobacterota*, not found in pellet A, was among the top phyla of pellet B (0.9%).

Regarding mycobiota composition, *Ascomycota* phylum was the most represented in both investigated wads (relative abundance 78.4% in sample A and 83.1% in sample B), followed by *Mucoromycota*, which showed percentages between 14.7 (sample B) and 21.4% (sample A), and *Basidiomycota* (0.1% sample A; 2.1% sample B).

Fungi belonging to *Mortierellomycota* phylum were not retrieved in wad A, whereas they were found in pellet B even if in a very low percentage (0.1%).

At genus taxonomic level (Fig. 7), the similarity based on Bray–Curtis index between the investigated bacterial communities was 43.5%. *Lactobacillus* was the most abundant genus in both samples but in the wad B it was predominant accounting nearly 90% of the total reads, with *L. oris* (26.5%) and *L. gasseri* (15.3%) among the most represented species. *Enterorhabdus* (3.3%), *Muribaculaceae* members (2.3%), *Desulfovibrio* (0.9%), *Corynebacterium* (0.9%), and few other bacterial genera with relative abundance values ≤ 0.5% completed the composition of the microbiota B.

In pellet A, unclassified species of *Lactobacillus* genus accounted for 38.8%, followed by species belonging to the genera *Yersinia* (21.0%), mainly *Y. enterocolitica* (20.1%), *Streptococcus* (15.8%), *Escherichia-Shigella*

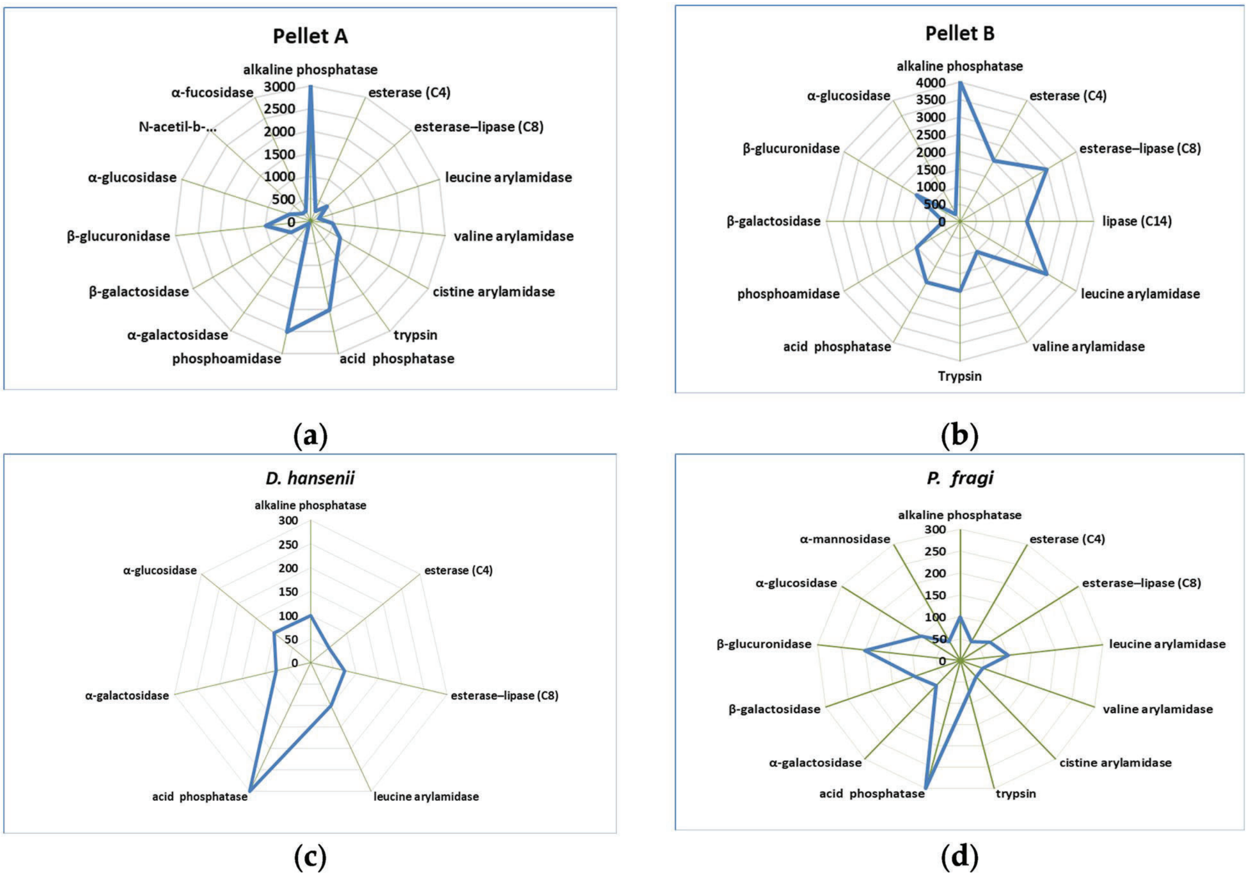


Fig. 5 Enzymatic profiles (in nanomoles) on pellets A (**a**) and B (**b**); *D. hansenii* isolate (**c**) and *P. fragi* isolate (**d**)

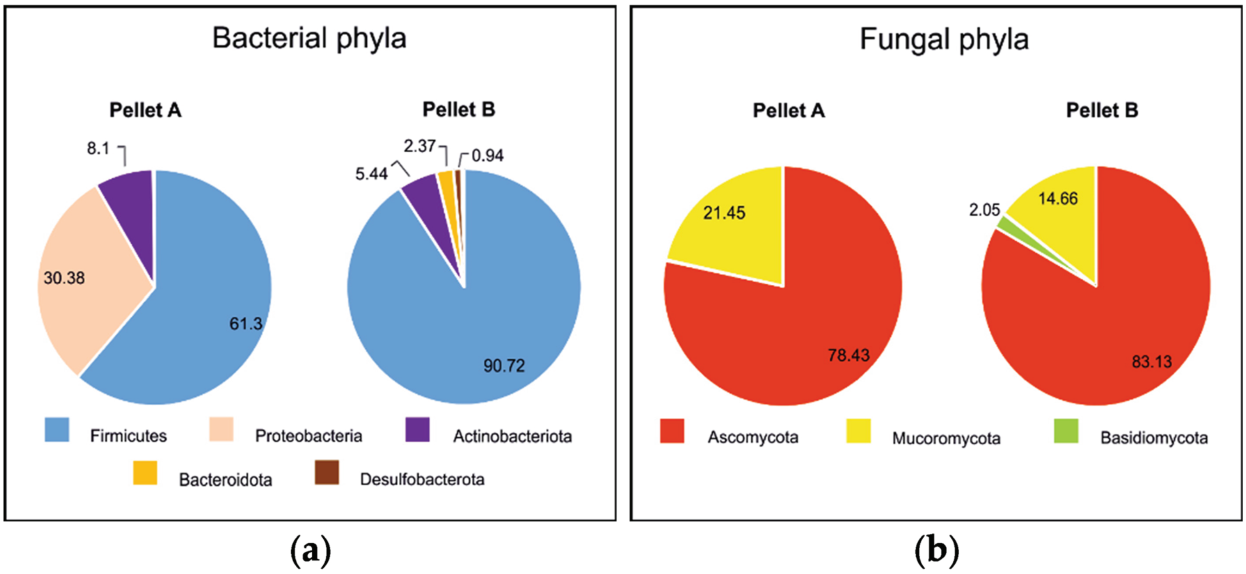
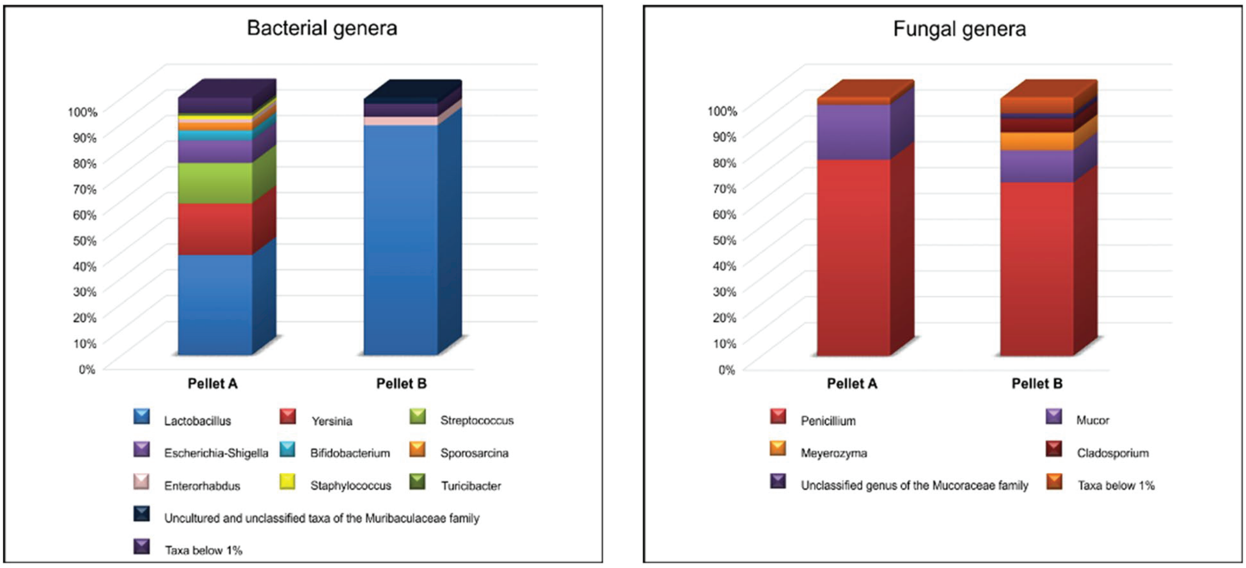


Fig. 6 Microbial communities of barn owl pellets at Phylum level; bacterial phyla (**a**) and fungal phyla (**b**)



(a) **(b)**

Fig. 7 Microbial communities of barn owl pellets at Genus level; bacterial genera (**a**) and fungal genera (**b**)

(8.8%), *Bifidobacterium* (3.9%), *Sporosarcina* (3.0%), *Enterorhabdus* (1.9%), *Staphylococcus* (1.2%), *Turicibacter* (1.1%), and *Corynebacterium* (0.8%).

Overall, a high percentage of uncultured and unclassified bacteria at species level was detected both in sample A (77.1%) and in sample B (56.0%).

As regards the mycobiota, the similarity percentage at genus level was 79.7%. In sample A, *Penicillium* and *Mucor* dominated fungal communities, with percentages of 75.9 and 21.4%, respectively. In pellet B, in addition to these two genera that represented 79.6% of the mycobiota, other fungal genera such as the yeast *Meyerozyma* (6.8%) and the mold *Cladosporium* (5.4%) showed high relative abundance values (Fig. 7).

The alpha diversity was estimated through the Shannon relative index (h). The obtained results for bacterial communities were 0.59 (pellet A) and 0.47 (pellet B) whereas for fungal communities the obtained values were 0.49 (pellet A) and 0.44 (pellet B).

Predictive functional analysis

The predictive ecological and functional data of microbial communities and microorganisms (bacteria) found on two pellets are reported in Fig. 8. The identified bacterial functional groups were 17, both for pellet A and pellet B. In the two samples, great amounts of bacterial sequences were assigned to fermentation and chemoheterotrophy (anaerobic predominance). Furthermore, in pellet A, invertebrate parasites and animal parasites or symbionts were detected. A low number of bacterial sequences were assigned to aerobic chemoheterotrophy, human pathogens, nitrate reduction and ureolysis.

In pellet B, different predictive ecological-functional characteristics were noticed with abundance assigned to human gut, human associated, mammal gut and animal parasites or symbionts. The function of sulfate respiration and respiration of sulfur compounds were detected only in pellet B. The functions of human pathogens pneumonia, plant pathogen, and aromatic compounds degradation were rare in both pellets.

Environmental impact, CO_2 eq. emission and mitigation actions

Here we report the evaluation of environmental impact related to this study, the CO_2 equivalent emission, and mitigation actions by new plantations, according to the Mat e Meth section. The evaluation of the environmental impact of this manuscript, in terms of social cost and CO_2 equivalent emission, resulted in 12.0 euro and about 300 kg CO_2 eq., respectively. This bring us to provide a new plantation by tree *Taxus baccata* L. plants for mitigation actions and a QRcode tag linked to this manuscript was added to each tree planted in the Green Campus – Unimol (41° 60′ 76″ N 14° 26′ 50″ E).

Discussion

Concerning the daily observations and collection of fresh pellets when present, our experimental protocol guarantees the rapid analyses in the lab. The sanitized table represents a valid and simple solution to study wild barn

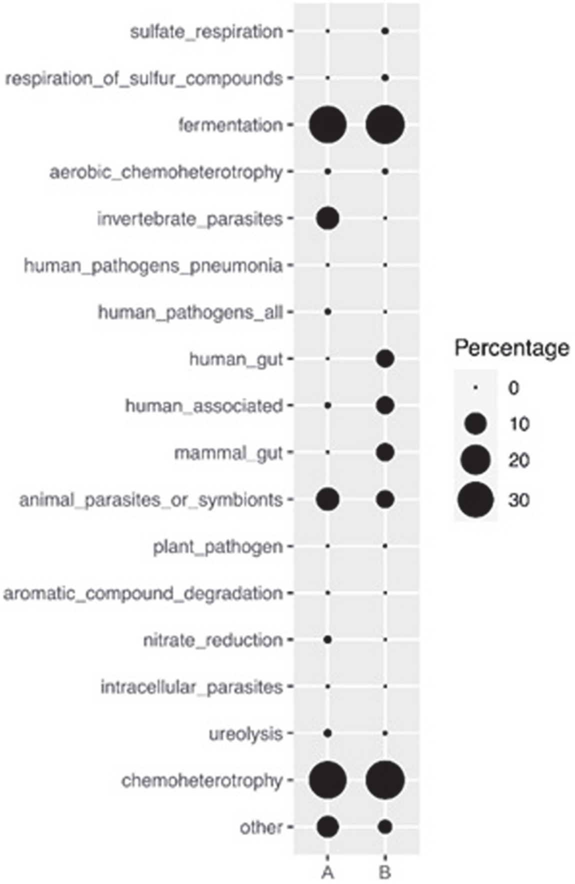


Fig. 8 Predictive functional profiling of bacteria (bacterial communities) present in pellets **A** and **B**

owl pellets, and minimizes potential environmental contamination. Since the rarity of the phenomenon, in four months we could observe only two samples, therefore our protocol should be considered as a valid model to collect and analyze wild barn owl pellets.

The determined pH values of these pellets were not acidic as expected but slightly above neutral. The data appeared to be markedly different from values reported in previous research, where pellets were characterized by acidic pH values. A large amount of reflux of alkaline pancreatic or intestinal fluid into the stomach could explain our observations (Leprince et al. 1979).

Ecology of barn owl prey

Based on the remains found in the analyzed pellets, barn owl prey can be fitted in two main groups of species. The first comprised *A. sylvaticus* and *R. rattus* that are adapted to almost all habitat types in agroecosystems due to their generalist behavior and their characteristics that allow them to exploit the spatial niches provided by ecotone-rich landscapes (Tattersall et al. 2001; Amori et al. 2008; Loy et al. 2017). The second group included *A. flavicollis* which is mainly related to highly heterogeneous landscapes in which the presence of natural patches of shrub or forest habitats is remarkable (Paniccia et al. 2022). This ecological information on the prey species is consistent with the environmental features of the territory in which pellets were collected. A mosaic of wide agricultural areas surrounded by native oak edges can host a diverse micromammal community. The different pellet composition we observed is related to different hunting events conducted by barn owls in the habitats of the study area. Indeed, in agro-forestal environments barn owls have home ranges not very vast (average 6.6 km^2) and tend to prey in habitats located not so far from their nest (Séchaud et al. 2021).We believe our pellet are composed by preys hunted on agricultural patches and on woody edges, where different mice species live.

Wild barn owl pellet microbial communities and predictive functional analysis

To date, little is known about microbiological aspects of wild barn owl pellets (Orosz-Coghlan et al. 2022). Consequently, we have examined the microbiota of the two wild *T. alba* pellets collected on a sanitized surface. Information on the structure and composition of the bacterial and fungal community is just one example of information that can be obtained from samples at low environmental contamination. To achieve this goal, both traditional cultivation techniques (culture-dependent methods) and molecular investigations (Next-Generation Sequencing) have been employed.

NGS analyses of the two wild barn owl samples revealed differences in the structure and composition of microbial communities and the predominance of a limited number of bacterial and fungal taxa. Regarding the bacterial communities, most of the sequences were assigned to enteric bacteria, first *Lactobacillus* species (*Firmicutes* phylum), which are considered the most important probiotic bacteria of the gut microbiota (Afrin et al. 2021; Rastogi and Singh 2022). Moreover, pellet A contained a relatively high percentage of bacteria belonging to the genus *Bifidobacterium*, gut colonization obligate anaerobes widely used as probiotics (Sharma et al. 2021). However, in addition to lactobacilli and bifidobacteria, pathogenic bacteria such as *Yersinia enterocolitica* have also been found in the analyzed pellets. This species, which in pellet A reached a relative abundance of 21.0%, is widely distributed in nature and it can be isolated from terrestrial and aquatic habitats, as well as from the gut of numerous mammals, avian species, and cold-blooded animals. The presence of fecal indicator bacteria and enteric organisms implicated in human infections within barn owl pellets was also reported by Orosz-Coghlan and colleagues, who therefore recommended handling with caution pellets used for educational purposes (Orosz-Coghlan et al. 2022). It is likely that the enteric bacteria found in the examined pellets may derive from *T. alba* prey (mice and rats), rather than from the barn owl itself. This is further supported by the presence of bacteria typically associated with mouse gut, including species belonging to *Enterorhabdus* genus and *Muribaculaceae* family (Clavel et al. 2009, 2010; Lagkouvardos et al. 2019). Moreover, it seems that the abundance of some taxa, such as *Turicibacter*, one of the main bacterial genera of mammalian gut microbiota, that in sample A showed a percentage of 1.1%, may vary in relation to host sex and age (Zhou et al. 2019; Wu et al. 2022). Consequently, the investigation of pellet microbial communities, especially if extended to a larger number of samples, could indirectly provide information about raptor diet and prey, useful to integrate morphological analysis data.

With regards to mycobiota analysis, *Penicillium* and *Mucor* were the main genera shared between the two *T. alba* pellets. *Penicillium* fungi are ubiquitous ascomycetes commonly found in soil, air, as well as in extreme environments, in certain food products, or associated with plants.

It is noteworthy that a high percentage of unclassified and/or uncultured microorganisms (including *Bifidobacterium*, *Enterorhabdus*, *Lactobacillus*, and *Muribaculaceae* members for bacteria and *Penicillium* species for fungi) was detected in both *T. alba* pellets. Consequently, barn owl pellets could also be an important source of new, yet un-described microorganisms with further interesting potential applications (probiotic strains, biocontrol agents, producers of biologically active compounds, food-associated molds, etc.).

Microbial ecological functions refer to the natural processes carried out by microorganisms in the environment (Zhao et al. 2023). The preliminary results of our predictive functional analysis suggested an involvement of the pellet microbiota both on *T. alba* diet and its digestive processes.

Potentiality of isolated strains

The aim of isolating microorganisms potentially useful in the field of biotechnology was largely achieved. Among the isolated strains, there was *D. hansenii*, a halotolerant yeast, which showed esterase-lipase (C8) and α-galactosidase activities (Nguyen et al. 2009). Due to their enzymatic activities, *D. hansenii* strains could be not only of considerable biotechnological and commercial importance (in food, dairy, detergent, and pharmaceutical industries), but they could also be used in biorestoration field to remove unwanted residues like fats and lipids on altered artworks (Banik 2003; Papagora et al. 2013; Ranalli and Zanardini 2021).

Based on the enzyme profile in Fig. 5d and the metabolic versatility of the genus *Pseudomonas*, *P. fragi* could be adopted in various biotechnological applications like bioremediation and degradation of various xenobiotic compounds in soil and polluted wastewater (Viti et al. 2006; Bosch-Roig et al. 2016). The peculiar presence of trypsin enzyme could be associated with a potential use in new strategies to pest control (Pilon et al. 2017). Furthermore, β-glucuronidase activity can lead to a reduction in glucuronide conjugation, which causes xenobiotic-induced toxicity in humans (Dashnyam et al. 2018).

Among fungi, *M. albolutea* was also isolated. As reported by (Díaz-Rojas et al. 2021) *M. albolutea* Sigler & Carmich [*Onygenaceae*; syn. *Auxarthron alboluteum* Sigler & Hambleton] is a keratinophilic fungus isolated from soil worldwide (Sigler and Carmichael 1976). Recently, the same authors affirmed that no chemical and pharmacological investigations on *M. albolutea* were carried out to biosynthesize different classes of bioactive compounds. For these purposes, a first report of a great interest were received on *M. albolutea* for the ability to produce alboluteins A-C (1–3), ardeemins and sartoryglabrins analogs; these compounds are involved in the PTP1B inhibitions, a negative modulator of insulin and leptin signaling, highly validated pharmacological target against insulin resistance and obesity (Kerru et al. 2018). For these reasons, the importance to future deep study and comparison among several *M. albolutea* strains, on the possible effects that alboluteins A-C have antitumoral potential since PTP1B is like a positive regulator of tumor progression (Díaz-Rojas et al. 2021).

Conclusions

T. alba pellets represent a natural matrix for analyzing wild barn owl diet and associated microbial communities. For this reason, we developed a strategy to properly collect and analyze these rare and precious matrices. The main conclusions of this paper can be summarized as follows:

1) Under our experimental conditions, the collection system 1 proved to be a viable and simple solution for both recovering wild barn owl pellets and minimizing potential environmental contamination.
2) Microbiological analyses can unravel the biodiversity existing in these poorly investigated matrices and lead to the discovery of microorganisms that could play a key role in biotechnological processes. Moreover, the results obtained can be considered as a starting point to develop more effective cultivation- based techniques for the isolation, the growth, and the characterization of a larger number of microorganisms still unidentified in barn owl pellets of wildlife specimens.

3) Osteological analyses can be considered a useful tool to identify the barn owl preys, and when coupled with microbiological investigations, may provide more detailed information on their diet.
4) The environmental impact in terms of CO_2 eq. was evaluated and mitigation actions were performed by new tree plantation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-024-01759-6.

Additional file 1: Fig. S1. Schematic protocol adopted in this work.

Additional file 2: Fig. S2. a View of the area around the nest; b QR code for panoramic view by drone

Acknowledgements
The authors would like to acknowledge Paola Fortini supporting the selection of trees for mitigation action (Garden of the Apennine Flora of Capracotta—Museo Giardino della Flora Appenninica, Capracotta, Italy). Furthermore, Chiara Paniccia for wild barn owl pellet examination consultancy and to the project OpenMICE. The authors would like to acknowledge the Vanda Mili family (Rosciano, Italy) owners of the house where part of the research activities took place. We sincerely thank Immacolata Doganieri and Sandra Ciccone for their support with the administration of the funding and procurement management.

Data availability statement

Sequences deposited in the NCBI GenBank database on Sequence Read Archive (SRA), accession number PRJNA957615.

Authors' contributions

Investigation: C.C. and G.R.; data curation: A.B., C.C., F.D., S.G., E.M., P.M., G.P., L.Q., S.S. and G.R.; writing—original draft preparation: A.B., C.C., M.P., and G.R.; writing—review and editing: A.B., C.C., M.P., and G.R.; funding acquisition: C.C. and G.R. All authors have read and agreed to the published version of the manuscript.

Funding
This research received internal funding from the University of Molise, titled *Nature, environment, and forest: from theory to practice, years* 2022–2023. DiBT 27/22.

Availability of data and materials

The numerical data used to support the findings of this study are included in the article. The datasets generated and analyzed during the current study are available in the NCBI repository, the link used for data upload: https://www.ncbi.nlm.nih.gov/, and accession numbers are: OR044424; OR044425; OR044426; OR044427; OR044530; OR044531; OR044532.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

Author details

[1]Department of Biosciences and Territory, University of Molise, C. da Fonte Lappone Snc, Pesche, IS 86090, Italy.

Received: 21 December 2023 Accepted: 28 March 2024

References

Afrin S, Akter S, Begum S, Hossain MN (2021) The prospects of *Lactobacillus oris* as a potential probiotic with cholesterol-reducing property from mother's milk. Front Nutr 8:619506. https://doi.org/10.3389/fnut.2021.619506

Amori G, Contoli L, Nappi A (2008) Mammalia II. Erinaceomorpha, Soricomorpha, Lagomorpha, Rodentia. Il Sole 24 ORE Business Media Srl (ed) Collana "Fauna d'Italia." Edizioni Calderini, Milano, p 752

Aquilano C, Baccari L, Caprari C et al (2022) Effects of EOs vs. antibiotics on *E. coli* strains isolated from drinking waters of grazing animals in the upper Molise region, Italy. Molecules 27:8177. https://doi.org/10.3390/molecules27238177

Ärje J, Choi KP, Divino F et al (2016) Understanding the statistical properties of the percent model affinity index can improve biomonitoring related decision making. Stoch Environ Res Risk Assess 30:1981–2008. https://doi.org/10.1007/S00477-015-1202-6

Banik G (2003) Removal of starch paste adhesives and relinings from paper-based objects by means of enzyme poultices. In: Cremonesi P (ed) Materiali tradizionali ed innovativi nella pulitura dei dipinti e delle opere policrome mobili. Il Prato, Piazzola sul Brenta (Padova), Italy, p 33–38

Bellocq MI (2000) A review of the trophic ecology of the Barn Owl in Argentina. J Raptor Res 34:108–119

Bergey DH, David H, Holt JG (2000) Bergey's manual of determinative bacteriology. Lippinccott Williams & Wilkins, Philadelphia

BirdLife International (2019) *Tyto alba* (amended version of 2016 assessment). IUCN Red List Threat Species 2019. ISSN 2307-8235 (online) IUCN. 2019: T22688504A155542941

Blasi C, Capotorti G, Copiz R et al (2014) Classification and mapping of the ecoregions of Italy. Plant Biosyst - an Int J Deal with All Asp Plant Biol 148:1255–1345. https://doi.org/10.1080/11263504.2014.985756

Bokulich NA, Kaehler BD, Rideout JR et al (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:90. https://doi.org/10.1186/s40168-018-0470-z

Bosch-Roig P, Decorosi F, Giovannetti L et al (2016) Connecting phenome to genome in *Pseudomonas stutzeri* 5190: an artwork biocleaning bacterium. Res Microbiol 167:757–765. https://doi.org/10.1016/j.resmic.2016.09.003

Brom TG (1986) Microscopic identification of feathers and feather fragments of palearctic birds. Bijdr Tot Dierkd 56:181–204. https://doi.org/10.1163/26660644-05602001

Bucci A, Petrella E, Celico F, Naclerio G (2017) Use of molecular approaches in hydrogeological studies: the case of carbonate aquifers in southern Italy. Hydrogeol J 25:1017–1031. https://doi.org/10.1007/s10040-017-1562-8

Cagnacci F, Bolzoni L, Rosà R et al (2012) Effects of deer density on tick infestation of rodents and the hazard of tick-borne encephalitis. I: empirical assessment. Int J Parasitol 42:365–372. https://doi.org/10.1016/j.ijpara.2012.02.012

Callahan BJ, McMurdie PJ, Rosen MJ et al (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583. https://doi.org/10.1038/nmeth.3869

Caporaso JG, Kuczynski J, Stombaugh J et al (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303

Caprari C, Fantasma F, Monaco P et al (2023) Chemical profiles, in vitro antioxidant and antifungal activity of four different *Lavandula angustifolia* L. Eos Molecules 28:392. https://doi.org/10.3390/molecules28010392

Chaline J, Baudvin H, Jammot D, Saint Girons MC (1974) Les Proies Des Rapaces. Petit Memmiferes et Leur Environnement, Paris

Clavel T, Charrier C, Braune A et al (2009) Isolation of bacteria from the ileal mucosa of TNFdeltaARE mice and description of *Enterorhabdus mucosicola* gen. nov., sp. nov. Int J Syst Evol Microbiol 59:1805–1812. https://doi.org/10.1099/ijs.0.003087-0

Clavel T, Duck W, Charrier C et al (2010) *Enterorhabdus caecimuris* sp. nov., a member of the family *Coriobacteriaceae* isolated from a mouse model of spontaneous colitis, and emended description of the genus *Enterorhabdus* Clavel et al. 2009. Int J Syst Evol Microbiol 60:1527–1531. https://doi.org/10.1099/ijs.0.015016-0

Dashnyam P, Mudududdla R, Hsieh T-J et al (2018) β-Glucuronidases of opportunistic bacteria are the major contributors to xenobiotic-induced toxicity in the gut. Sci Rep 8:16372. https://doi.org/10.1038/s41598-018-34678-z

de Man JC, Rogosa M, Sharpe ME (1960) Medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130–135

Di Palma MG, Massa B (1981) Contributo Metodologico per Lo Studio Dell'alimentazione Dei Rapaci. Atti I Convegno Italiano di Ornitologia, Aulla, pp 69–76

Díaz-Rojas M, Raja H, González-Andrade M et al (2021) Protein tyrosine phosphatase 1B inhibitors from the fungus *Malbranchea albolutea*. Phytochemistry 184:112664. https://doi.org/10.1016/j.phytochem.2021.112664

Drancourt M, Bollet C, Carlioz A et al (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol 38:3623–3630. https://doi.org/10.1128/JCM.38.10.3623-3630.2000

DSMZ (2023) List of media for microorganisms. https://www.dsmz.de/collection/catalogue/microorganisms/culture-technology/list-of-media-for-microorganisms. Accessed 22 Apr 2023

Durant JM, Gendner J-P, Handrich Y (2004) Should I brood or should I hunt: a female barn owl's dilemma. Can J Zool 82:1011–1016. https://doi.org/10.1139/z04-078

Erome GAS (1982) Contribution à l'identification des proies des rapaces. Le Bièvre 4:129–135

Feckler A, Wolfram J, Schulz R, Bundschuh M (2023) Reducing pollution to levels not harming biodiversity and ecosystem functions: a perspective on the post-2020 Global Biodiversity Framework. Curr Opin Environ Sci Heal 35:100495. https://doi.org/10.1016/j.coesh.2023.100495

Hegedus C, Andronie L, Uiuiu P et al (2023) Pets, genuine tools of environmental pollutant detection. Animals 13:2923. https://doi.org/10.3390/ani13182923

Holt EA, Miller SW (2010) Bioindicators: using organisms to measure environmental impacts. Nat Educ Knowl 3:8

Jaksic FM, Seib RL, Herrera CM (1982) Predation by the Barn Owl (*Tyto alba*) in Mediterranean habitats of Chile, Spain and California: a comparative approach. Am Midl Nat 107:151. https://doi.org/10.2307/2425196

Johnson MD, Wendt C, St. George D et al (2018) Can barn owls help control rodents in winegrape vineyard landscapes? A review of key questions and suggested next steps. Proc Vertebr Pest Conf 28:360. https://doi.org/10.5070/V42811037

Jurado V, Gonzalez-Pimentel JL, Hermosin B, Saiz-Jimenez C (2021) Biodeterioration of Salón de Reinos, Museo Nacional del Prado, Madrid. Spain Appl Sci 11:8858. https://doi.org/10.3390/app11198858

Kasprzykowski Z, Goławski A (2006) Habitat use of the Barn Owl *Tyto alba* and the Little Owl Athene noctua in central-eastern Poland. Biol Lett 43:33–39

Kerru N, Singh-Pillay A, Awolade P, Singh P (2018) Current anti-diabetic agents and their molecular targets: a review. Eur J Med Chem 152:436–488. https://doi.org/10.1016/j.ejmech.2018.04.061

Kross SM, Bourbour RP, Martinico BL (2016) Agricultural land use, barn owl diet, and vertebrate pest control implications. Agric Ecosyst Environ 223:167–174. https://doi.org/10.1016/j.agee.2016.03.002

Kross SM, Ingram KP, Long RF, Niles MT (2018) Farmer perceptions and behaviors related to wildlife and on-farm conservation actions. Conserv Lett 11:e12364. https://doi.org/10.1111/conl.12364

Lagkouvardos I, Lesker TR, Hitch TCA et al (2019) Sequence and cultivation study of *Muribaculaceae* reveals novel species, host preference, and functional potential of this yet undescribed family. Microbiome 7:28. https://doi.org/10.1186/s40168-019-0637-2

Langille MGI, Zaneveld J, Caporaso JG et al (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31:814–821. https://doi.org/10.1038/nbt.2676

Leprince P, Dandrifosse G, Schoffeniels E (1979) The digestive enzymes and acidity of the pellets regurgitated by raptors. Biochem Syst Ecol 7:223–227. https://doi.org/10.1016/0305-1978(79)90053-X

Liu J, Wu F, Xiang T et al (2023) Differences of airborne and mural microorganisms in a 1,500-year-old Xu Xianxiu's Tomb, Taiyuan, China. Front Microbiol 14:1253461. https://doi.org/10.3389/fmicb.2023.1253461

Love RA, Webon C, Glue DE et al (2000) Changes in the food of British Barn Owls (*Tyto alba*) between 1974 and 1997. Mamm Rev 30:107–129. https://doi.org/10.1046/j.1365-2907.2000.00060.x

Loy A, Cassini MH, Colangelo P, Di Febbraro M (2017) Distribution, spatial interaction and niche analysis in three species of European moles (genus *Talpa, Soricomorpha*: *Mammalia*) in Italy. Biol J Linn Soc 122:872–882. https://doi.org/10.1093/biolinnean/blx085

Marti CD (2010) Dietary trends of Barn Owls in an agricultural ecosystem in Northern Utah. Wilson J Ornithol 122:60–67. https://doi.org/10.1676/09-025.1

Marti CD, Wagner PW, Denne KW (1979) Nest Boxes for the Management of Barn Owls. Wildl Soc Bull 7:145–148

McDowell MC, Medlin GC (2009) Using the diet of the barn owl (*Tyto alba*) as an indicator of small vertebrate abundance in the Channel Country, south-western Queensland. Aust Mammal 31:75. https://doi.org/10.1071/AM08116

Meyrom K, Motro Y, Leshem Y et al (2009) Nest-Box use by the Barn Owl *Tyto alba* in a biological pest control program in the Beit She'an Valley, Israel. Ardea 97:463–467. https://doi.org/10.5253/078.097.0410

Milana G, Lai M, Maiorano L et al (2016) Geographic patterns of predator niche breadth and prey species richness. Ecol Res 31:111–115. https://doi.org/10.1007/s11284-015-1319-6

Milana G, Luiselli L, Amori G (2018) Forty years of dietary studies on barn owl (*Tyto alba*) reveal long term trends in diversity metrics of small mammal prey. Anim Biol 68:129–146. https://doi.org/10.1163/15707563-17000095

Monaco P, Divino F, Naclerio G, Bucci A (2020) Microbial community analysis with a specific statistical approach after a record breaking snowfall in Southern Italy. Ann Microbiol 70:63. https://doi.org/10.1186/s13213-020-01604-6

Monaco P, Bucci A, Naclerio G, Mello A (2021) Heterogeneity of the white truffle *Tuber magnatum* in a limited geographic area of Central-Southern Italy. Environ Microbiol Rep 13:591–599. https://doi.org/10.1111/1758-2229.12956

Morgulis A, Coulouris G, Raytselis Y et al (2008) Database indexing for production MegaBLAST searches. Bioinformatics 24:1757–1764. https://doi.org/10.1093/bioinformatics/btn322

Murray P, American Society for Microbiology (1995) Manual of Clinical Microbiology. ASM Press, Ann Arbor

Nadkarni MA, Martin FE, Jacques NA, Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257–266. https://doi.org/10.1099/00221287-148-1-257

Nappi A (2001) I micromammiferi d'Italia. Esselibri-Simone, Napoli

Nguyen H-V, Gaillardin C, NeuvÃ©glise C (2009) Differentiation of *Debaryomyces hansenii* and *Candida famata* by rRNA gene intergenic spacer fingerprinting and reassessment of phylogenetic relationships among *D. hansenii, C. famata, D. fabryi, C. flareri* (=*D. subglobosus*) and *D. prosopidis*: descriptio. FEMS Yeast Res 9:641–662. https://doi.org/10.1111/j.1567-1364.2009.00510.x

Orosz-Coghlan P, Sullivan L, Gerba C, Schmidt R (2022) The microbiology of barn owl pellets, with special reference to pellets used in educational settings. https://doi.org/10.13140/RG.2.2.15224.11523

Palmieri M, Lasserre B, Marino D et al (2023) The environmental footprint of scientific research: proposals and actions to increase sustainability and traceability. Sustainability 15:5616. https://doi.org/10.3390/su15075616

Paniccia C, Laura Carranza M, Frate L et al (2022) Distribution and functional traits of small mammals across the Mediterranean area: landscape composition and structure definitively matter. Ecol Indic 135:108550. https://doi.org/10.1016/j.ecolind.2022.108550

Paolucci P, Bon M (2022) Mammiferi terrestri d'Italia. Riconoscimento, ecologia e tricologia, WBA HANDBO. WBA-HANDBOOKS, Verona

Papagora C, Roukas T, Kotzekidou P (2013) Optimization of extracellular lipase production by *Debaryomyces hansenii* isolates from dry-salted olives using response surface methodology. Food Bioprod Process 91:413–420. https://doi.org/10.1016/j.fbp.2013.02.008

Pilon FM, da Silva C, R, Visôtto LE, et al (2017) Purification and characterization of trypsin produced by gut bacteria from *Anticarsia gemmatalis*. Arch Insect Biochem Physiol 96:e21407. https://doi.org/10.1002/arch.21407

Raja HA, Miller AN, Pearce CJ, Oberlies NH (2017) Fungal identification using molecular tools: a primer for the natural products research community. J Nat Prod 80:756–770. https://doi.org/10.1021/acs.jnatprod.6b01085

Ranalli G, Zanardini E (2021) Advanced biocleaning system for historical wall paintings. Microorganisms in the deterioration and preservation of cultural heritage. Springer International Publishing, Cham, pp 217–233

Ranalli G, Bottura G, Taddei P et al (2001) Composting of solid and sludge residues from agricultural and food industries. Bioindicators of monitoring and compost maturity. J Environ Sci Heal Part A 36:415–436. https://doi.org/10.1081/ESE-100103473

Rastogi S, Singh A (2022) Gut microbiome and human health: Exploring how the probiotic genus *Lactobacillus* modulate immune responses. Front Pharmacol 13:1042189. https://doi.org/10.3389/fphar.2022.1042189

Rawat US, Agarwal NK (2015) Biodiversity: concept, threats and conservation. Environ Conserv J 16:19–28

Romano A, Séchaud R, Roulin A (2020) Global biogeographical patterns in the diet of a cosmopolitan avian predator. J Biogeogr 47:1467–1481. https://doi.org/10.1111/jbi.13829

Roulin A (2020) Barn Owls: evolution and ecology. Cambridge University Press, Cambrige

Roulin A, Christe P (2013) Geographic and temporal variation in the consumption of bats by European Barn Owls. Bird Study 60:561–569. https://doi.org/10.1080/00063657.2013.847051

Roulin A, Dubey S (2012) The occurrence of reptiles in Barn Owl diet in Europe. Bird Study 59:504–508. https://doi.org/10.1080/00063657.2012.731035

Séchaud R, Schalcher K, Machado AP et al (2021) Behaviour-specific habitat selection patterns of breeding barn owls. Mov Ecol 9:18. https://doi.org/10.1186/s40462-021-00258-6

Sharma M, Wasan A, Sharma RK (2021) Recent developments in probiotics: an emphasis on *Bifidobacterium*. Food Biosci 41:100993. https://doi.org/10.1016/j.fbio.2021.100993

Sigler L, Carmichael JW (1976) Taxonomy of *Malbranchea* and some other *Hyphomycetes* with arthroconidia. Mycotaxon 4:349–488

St. George DA, Johnson MD (2021) Effects of habitat on prey delivery rate and prey species composition of breeding barn owls in winegrape vineyards. Agric Ecosyst Environ 312:107322. https://doi.org/10.1016/j.agee.2021.107322

Tattersall FH, Macdonald DW, Hart BJ et al (2001) Habitat use by wood mice (*Apodemus sylvaticus*) in a changeable arable landscape. J Zool 255:487–494. https://doi.org/10.1017/S095283690100156X

Taylor I (2003) Barn Owls: Predator-Prey Relationships and Conservation. Cambridge University Press, Cambridge (UK)

Teta P, Hercolini C, Cueto G (2012) Variation in the diet of Western Barn Owls (*Tyto alba*) along an Urban-Rural Gradient. Wilson J Ornithol 124:589–596. https://doi.org/10.1676/11-173.1

Toju H, Tanabe AS, Yamamoto S, Sato H (2012) High-coverage ITS primers for the DNA-based identification of *Ascomycetes* and *Basidiomycetes* in environmental samples. PLoS One 7:e40863. https://doi.org/10.1371/journal.pone.0040863

Viganò M, Ancillotto L, Agnelli P et al (2020) Frequency of occurrence and ingested biomass of different prey of the Barn Owl *Tyto alba* in an island ecosystem. Birds 1:5–12. https://doi.org/10.3390/birds1010002

Viti C, Mini A, Ranalli G et al (2006) Response of microbial communities to different doses of chromate in soil microcosms. Appl Soil Ecol 34:125–139. https://doi.org/10.1016/j.apsoil.2006.03.003

Whelan CJ, Wenny DG, Marquis RJ (2008) Ecosystem services provided by birds. Ann N Y Acad Sci 1134:25–60. https://doi.org/10.1196/annals.1439.003

White T, Bruns T, Lee S et al (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. PCR protocols: a guide to methods and applications. Academic Press, Inc, Cambridge, pp 315–322

Wood BJ, Fee CG (2003) A critical review of the development of rat control in Malaysian agriculture since the 1960s. Crop Prot 22:445–461. https://doi.org/10.1016/S0261-2194(02)00207-7

Wu Y, Peng X, Li X et al (2022) Sex hormones influence the intestinal microbiota composition in mice. Front Microbiol 13:964847. https://doi.org/10.3389/fmicb.2022.964847

Xia F, Zhou X, Liu Y et al (2019) Composition and predictive functional analysis of bacterial communities inhabiting Chinese Cordyceps insight into conserved core microbiome. BMC Microbiol 19:105. https://doi.org/10.1186/s12866-019-1472-0

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7:203–214. https://doi.org/10.1089/10665270050081478

Zhang Y, Su M, Wu F et al (2023) Diversity and composition of culturable microorganisms and their biodeterioration potentials in the sandstone of Beishiku Temple, China. Microorganisms 11:429. https://doi.org/10.3390/microorganisms11020429

Zhao D, Hou H, Liu H et al (2023) Microbial community structure and predictive functional analysis in reclaimed soil with different vegetation types: the example of the Xiaoyi Mine Waste Dump in Shanxi. Land 12:456. https://doi.org/10.3390/land12020456

Zhou W, Xu H, Zhan L et al (2019) Dynamic development of fecal microbiome during the progression of diabetes mellitus in Zucker diabetic fatty rats. Front Microbiol 10:232. https://doi.org/10.3389/fmicb.2019.00232

Publisher's Note

Italian Botanist 16: 89–103 (2023)
doi: 10.3897/italianbotanist.16.114657
https://italianbotanist.pensoft.net

RESEARCH ARTICLE

Italian Botanist
Published by Società Botanica Italiana

Notulae to the Italian native vascular flora: 16

Fabrizio Bartolucci[1], Gianniantonio Domina[2], Michele Adorni[3], Silvia Assini[4], Ilaria Brugellis[4], Giovanni Buccomino[5], Francesca Carruggio[6], Fabio Conti[1], Emanuele Costanzo[7], Emanuele Del Guacchio[8], Francesco Falcinelli[9], Francesco Festi[10], Luca Fontanabona[11], Luigi Forte[6], Paola Fortini[12], Gabriele Galasso[13], Emanuele Genduso[6], Luigi Ghillani[14], Günter Gottschlich[15], Duilio Iamonico[16], Valentina Lucia Astrid Laface[17], Michele Lonati[18], Francesco Mascia[19], Giacomo Mei[20], Ginevra Nota[18], Paola Palazzolo[21], Gaetano Pazienza[6], Giuseppe Pellegrino[6], Giovanna Potenza[22], Luca Quaranta[12], Emanuele Repetto[23], Leonardo Rosati[22], Francesco Santi[11], Grazia Secci[24], Giuseppe Nicola Silletti[25], Adriano Stinca[26], Gianmarco Tavilla[27], Valeria Tomaselli[6], Giancarlo Tondi[28], Lorenzo Lastrucci[29]

1 *Centro Ricerche Floristiche dell'Appennino (Università di Camerino - Parco Nazionale del Gran Sasso e Monti della Laga), Via Prov.le km 4,2 - San Colombo, 67021 Barisciano (L'Aquila), Italy* **2** *Dipartimento di Scienze Agrarie, Alimentari e Forestali (SAAF), Università di Palermo, Viale delle Scienze, edificio 4, 90128 Palermo, Italy* **3** *Sistema Museale di Ateneo, Orto Botanico di Parma, Strada Luigi Carlo Farini 90, 43121 Parma, Italy* **4** *Dipartimento di Scienze della Terra e dell'Ambiente, Università di Pavia, via S. Epifanio 14, 27100 Pavia, Italy* **5** *Via Sagunto 20, 00174 Roma, Italy* **6** *Dipartimento di Bioscienze, Biotecnologie e Ambiente (DBBA), Campus Universitario "Ernesto Quagliariello", Università degli Studi di Bari "Aldo Moro", Via Orabona 4, 70125 Bari, Italy* **7** *LifeWatch ERIC Service Centre con sede in Lecce, Via Per Monteroni, C/O LAB BIOforIU, Lecce, Italy* **8** *Dipartimento di Biologia, Università di Napoli Federico II, c/o Orto Botanico, Via Foria 223, 80139 Napoli, Italy* **9** *Reparto Carabinieri Biodiversità di Assisi, viale Umberto I 5, 06081 Assisi (Perugia), Italy* **10** *Fondazione Museo Civico di Rovereto / Società Museo Civico di Rovereto, Largo S. Caterina 41, 38068, Rovereto, Trento, Italy* **11** *BIOME Lab, Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Alma Mater Studiorum, Università di Bologna, Via Irnerio 42, 40126 Bologna, Italy* **12** *Dipartimento di Bioscienze e Territorio, Università degli Studi del Molise, Via Hertz s.n.c., 86090 Pesche (Isernia), Italy* **13** *Sezione di Botanica, Museo di Storia Naturale di Milano, Corso Venezia 55, 20121 Milano, Italy* **14** *Via Carlo Casalegno 6, 43123 Parma, Italy* **15** *Hermann-Kurz-Straße 35, 72074 Tübingen, Germany* **16** *Sapienza Università di Roma, Dipartimento di Biologia Ambientale, Piazzale Aldo Moro 5, I-00185 Roma, Italy* **17** *Dipartimento di Agraria, Università "Mediterranea" di Reggio Calabria, Feo di Vito snc, 89122 Reggio Calabria, Italy* **18** *Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco (Torino), Italy* **19** *Dipartimento di Scienze della Vita, Università di Siena, Via Aldo Moro 2, 53100 Siena, Italy* **20** *Facoltà di Scienze Agrarie, Ambientali e Alimentari, Libera Università di Bolzano, Piazza Università 5, 39100 Bolzano, Italy* **21** *Aree protette del Po piemontese, Grangia di Pobietto, 15025 Morano sul Po (Alessandria), Italy* **22** *Scuola di Scienze Agrarie, Forestali, Alimentari e Ambientali, Università della Basilicata, Via dell'Ateneo Lucano 10, 85100 Potenza, Italy* **23** *EURAC Research, Istituto per l'ambiente alpino, Viale Druso 1, 39100 Bolzano, Italy* **24** *Via G. Matteotti 10, Siliqua (Sud Sardegna), Italy*

---

25 *Via Gioia 80, 70029 Santeramo in Colle (Bari), Italy* **26** *Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Università della Campania Luigi Vanvitelli, Via Antonio Vivaldi 43, I-81100 Caserta, Italy* **27** *Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Catania, 95131 Catania, Italy* **28** *Via F. D'Ovidio 89, 00137 Roma, Italy* **29** *Sistema Museale di Ateneo, Università di Firenze, Via G. La Pira 4, 50121 Firenze, Italy*

Corresponding author: Fabrizio Bartolucci (fabrizio.bartolucci@gmail.com)

Academic editor: Lorenzo Peruzzi | Received 26 October 2023 | Accepted 14 November 2023 | Published 23 November 2023

Citation: Bartolucci F, Domina G, Adorni M, Assini S, Brugellis I, Buccomino G, Carruggio F, Conti F, Costanzo E, Del Guacchio E, Falcinelli F, Festi F, Fontanabona L, Forte L, Fortini P, Galasso G, Genduso E, Ghillani L, Gottschlich G, Iamonico D, Laface VLA, Lonati M, Mascia F, Mei G, Nota G, Palazzolo P, Pazienza G, Pellegrino G, Potenza G, Quaranta L, Repetto E, Rosati L, Santi F, Secci G, Silletti GN, Stinca A, Tavilla G, Tomaselli V, Tondi G, Lastrucci L (2023) Notulae to the Italian native vascular flora: 16. Italian Botanist 16: 89–103. https://doi.org/10.3897/italianbotanist.16.114657

Abstract

In this contribution, new data concerning the distribution of native vascular flora in Italy are presented. It includes new records, confirmations, and exclusions to the Italian administrative regions. Nomenclatural and distribution updates, published elsewhere, and corrigenda are provided as supplementary material.

Keywords
Endemic taxa, Floristic data, Italy

How to contribute

The text for the new records, exclusions, and confirmations should be submitted electronically to Lorenzo Lastrucci (lorenzo.lastrucci@unifi.it). The corresponding specimen along with its scan or photograph have to be sent to FI Herbarium: Sezione di Botanica "Filippo Parlatore" del Museo di Storia Naturale, Via G. La Pira 4, 50121 Firenze (Italy). Those texts concerning nomenclatural novelties and typifications (only for accepted names) should be submitted electronically to: Fabrizio Bartolucci (fabrizio.bartolucci@gmail.com). Each text should be within 1,000 characters (spaces included).

Floristic records of native taxa

Alchemilla effusa Buser (Rosaceae)

\+ **UMB**: Castelluccio di Norcia (Norcia, Perugia) (WGS84: 42.781561°N, 13.195514°E), Piano Grande, prato pingue presso i Mergani; suolo calcareo, 1272 m, 7 July 2021, leg. *F. Falcinelli*, det. *F. Festi* (FI, Herb. Tondi). – Species new for the flora of Umbria.

F. Falcinelli, F. Festi, G. Tondi

Alchemilla marsica Buser (Rosaceae)

+ **MOL**: Capracotta (Isernia) (WGS84: 41.830136°N, 14.293988°E), Habitat 6210*, prateria semi-mesofila secondaria polispecifica perenne, copertura 100%, a dominanza di *Bromopsis erecta* (Huds.) Fourr., *Carex flacca* Schreb. subsp. *erythrostachys* (Hoppe) Holub, *Brachypodium rupestre* (Host) Roem. & Schult., 1332 m s.l.m. esposizione sud sud-est, 6 June 2021, leg. *P. Fortini*, det. *G. Tondi* (FI, IS). – Species new for the flora of Molise.

P. Fortini, G. Tondi

Alchemilla reniformis Buser (Rosaceae)

+ **UMB**: Monte Coscerno (S. Anatolia di Narco, Perugia) (WGS84: 42.718762°N, 12.885913°E), pascolo, versante N; suolo calcareo, 1635 m, 15 July 2021, leg. *F. Falcinelli*, det. *G. Tondi*, rev. *F. Festi* (FI, Herb. Tondi). – Species new for the flora of Umbria.

F. Falcinelli, F. Festi, G. Tondi

Anemone hortensis L. subsp. *hortensis* (Ranunculaceae)

+ **PIE**: Gavi (Alessandria), calanchi dello Scrivia (sponda sinistra) (WGS84: 40.090440°N, 53.303100°E), 483 m, 3 March 2022, *E. Repetto* (FI). – Species new for the flora of Piemonte.

We observed this species in a dry grassland of a badland on the left bank of the Scrivia River. The species grows together with *Brachypodium rupestre* (Host) Roem. & Schult., *Carex caryophyllea* Latourr., *Globularia bisnagarica* L., and *Lotus dorycnium* L.

E. Repetto, S. Assini

Cornus sanguinea L. subsp. *hungarica* (Kárpáti) Soó (Cornaceae)

+ **MOL**: Pesche (Isernia), (WGS84: 41.602233°N, 14.271280°E), cespuglieto. 695 m s.l.m. esposizione sud sud-est, 17 May 2023, *P. Fortini*, *L. Quaranta* (IS). – Subspecies new for the flora of Molise.

P. Fortini, L. Quaranta

Crassula tillaea Lest.-Garl. (Crassulaceae)

+ **PIE**: Trecate (Novara), (WGS84: 45.4372860°N, 8.8065930°E), pratello arido acidofilo del Ticino (sponda destra), 135 m, 26 April 2023, leg. *S. Assini*, det. *P. Palazzolo* (FI). – Species new for the flora of Piemonte.

This species was reported for Piemonte as "recorded by mistake" (Bartolucci et al. 2018), but we observed large populations occurring in pioneer siliceous dry grasslands, located between the Polo Industriale San Martino and the right bank of the Ticino River. This species grows together with *Aira caryophyllea* L., *Tuberaria guttata* (L.) Fourr., *Spergularia rubra* (L.) J.Presl & C.Presl, *Carex caryophyllea* Latourr., and *Aphanes arvensis* L.

S. Assini, P. Palazzolo

Desmazeria sicula **(Jacq.) Dumort. (Poaceae)**

+ **PUG**: Lesina (Foggia), l'Isola, tra Cauto e Santa Maria, (WGS84: 41.906388°N, 15.497954°E), pratello terofitico su suolo sabbioso, 4 m s.l.m., 30 May 2023, leg. *V. Tomaselli*, *F. Carruggio*, det. *V. Tomaselli*, *F. Carruggio*, *E. Genduso*, (FI, BI Nos 57968, 57969). − Species new for the flora of Puglia.

E. Genduso, F. Carruggio

Euphorbia palustris **L. (Euphorbiaceae)**

+ **PUG**: Chieuti (Foggia), Marina di Fantina, (WGS84: 41.916602°N, 15.215413°E), fragmiteto in pineta costiera (rimboschimento a *Pinus halepensis* Mill.), 3 m s.l.m., 22 April 2023, leg. *E. Costanzo*, *E. Genduso*, det. *V. Tomaselli*, *F. Carruggio*, *E. Genduso* (FI, BI Nos 58007). − Species confirmed for the flora of Puglia.

Formerly reported by Fiori (1926) in the area of "Golfo di Taranto lungo il fiume Lato nelle Menasciole", but its presence was never confirmed.

E. Genduso, V. Tomaselli

Festuca sicula **C.Presl (Poaceae)**

+ **MOL**: Carovilli (Isernia) (WGS84: 41.665589°N, 14.274823°E), prato da sfalcio mesofilo, 986 m s.l.m., 20 June 2022, *P. Fortini*, *L. Quaranta* (IS). – Species new for the flora of Molise.

P. Fortini, L. Quaranta

Festuca stricta **Host subsp.** ***trachyphylla*** **(Hack.) Patzke ex Pils (Poaceae)**

+ **MOL**: Pescopennataro (Isernia), (WGS84: 41.5157755°N, 14.193816°E), prateria mesofila continua su suolo profondo, 955 m s.l.m., 15 June 2022, *P. Fortini*, *L. Quaranta* (FI, IS). – Subspecies new for the flora of Molise.

P. Fortini, L. Quaranta

Hieracium diaphanoides **Lindeb. subsp.** ***pseudumbrosum*** **Zahn (Asteraceae)**

+ **EMR**: Tizzano Val Parma (Parma), pendici di Monte Fuso sopra Rusino (WGS84: 44.50554°N, 10.26450°E), bosco misto mesofilo, 1080 m, 26 June 2013, leg. *M. Adorni*, *L. Ghillani*, det. *G. Gottschlich* (FI). – Subspecies new for the flora of Emilia-Romagna.

M. Adorni, L. Ghillani, G. Gottschlich

Hieracium tenuiflorum **Arv.-Touv. subsp.** ***tenuiflorum*** **(Asteraceae)**

+ **MAR**: Rotella (Ascoli Piceno), Monte Ascensione (WGS84: 42.926439°N, 13.553891°E), margini di bosco, 700−1100 m, 7 May 2008, leg. *C. D'Angeli*, *F. Conti*, det. *G. Gottschlich* (APP No. 39706). − Subspecies new for the flora of Marche.

Hieracium tenuiflorum was recently recorded for Marche without indication of the subspecies (Canzoneri et al. 2022).

G. Gottschlich, F. Conti

Lysimachia loeflingii F.J.Jiménez-López & M.Talavera (Primulaceae)

+ **PUG**: Bari, Campus Università degli studi di Bari Aldo Moro (WGS84: 41.109738°N, 16.882152°E) vegetazione sinantropica mesofila degli incolti e delle aree prative e ruderali in prossimità delle aree di cantiere 12 m, 3 April 2023, leg. *G. Mei*, det. *G. Mei*, *A. Stinca* (FI, *Herb. G. Mei*); Bari, Istituto di Selvicoltura ed ecologia Forestale (WGS84: 41.111358°N, 16.882177°E) vegetazione degli incolti nelle aree più fresche e in prossimità dei passaggi pedonali 15 m, 3 April 2023, leg. *G. Mei*, det. *G. Mei*, *A. Stinca* (*Herb. G. Mei*). – Species confirmed for the flora of Puglia.

This finding confirms the only historical report for the Italian peninsula; in fact the presence of *L. loeflingii* in Italy is known for Sardegna, and Sicilia (Barone et al. 2023; Gianguzzi et al. 2023), while it was reported as doubtfully occurring in Puglia (Bartolucci et al. 2018, 2022).

G. Mei, A. Stinca

Melomphis arabica (L.) Raf. (Asparagaceae)

+ **CAL**: San Ferdinando (Reggio Calabria), Località Torre, (WGS84: 38.495195°N, 15.920087°E), a bordo strada e nei terreni incolti circostanti, 5 m, 6 May 2023, *V.L.A. Laface*, *G. Tavilla* (REGGIO, CAT). – Species confirmed for the flora of Calabria.

During fieldwork around the Metropolitan City of Reggio Calabria, we discovered a well-established population of the species near the village of San Ferdinando on uncultivated land.

V.L.A. Laface, G. Tavilla

Montia arvensis Wallr. (Montiaceae)

+ **PUG**: Gravina in Puglia (Bari), "Bosco Difesa Grande", Lago Matera, (WGS84: 40.753235°N, 16.382895°E), prati umidi terofitici con *Ranunculus sardous* e *Lotus parviflorus*, 438 m s.l.m., 17 March 2023, leg. *V. Tomaselli*, det. *V. Tomaselli*, *G. Pazienza*, (BI No. 57778). – Species confirmed for the flora of Puglia.

V. Tomaselli, G. Pazienza

Najas major All. (Hydrocharitaceae)

+ **BAS**: Pignola (Potenza), loc. Lago Pantano (WGS84:, 40.587943°N, 15.751694°E), in the lake, 760 m a.s.m., 12 August 2022, *L. Rosati*, *G. Potenza* (FI). – Species new for the flora of Basilicata.

During the monitoring of macrophyte vegetation at Lake Pantano, we detected several small patches of a *Najas major*-dominated community, at a depth of 0,6–1,3 m, close to the shore.

G. Potenza, L. Rosati

Narcissus tazetta L. subsp. *italicus* (Ker Gawl.) Baker (Amaryllidaceae)

+ **SAR**: Curcuris (Oristano), loc. Riu canali, prati umidi ai margini del Corso d'acqua (WGS84: 39.756013°N, 8.824960°E), 141 m s.l.m., 16 January 2023, *F. Mascia* (CAG); Siliqua (Sud Sardegna), loc. Su tidili, Margini e capezzagne di coltivi, prati umidi (WGS84: 39.284911°N, 8.795614°E), 66 m s.l.m., 13 February 2023, *G. Secci & Mascia* (FI, CAG); loc. Tzinnigas, incolti umidi (WGS84: 39.251558°N, 8.767128°E), 144 m s.l.m., 13 February 2023 *G. Secci*; Villanovaforru (Sud Sardegna), loc. Funtana Iannus, radure umide ai margini della lecceta, margini di sentieri (WGS84: 39.636856°N, 8.859135°E), 318 m s.l.m.,15 January 2023, *F. Mascia* (CAG). – Subspecies confirmed for the flora of Sardegna.

The first records for Sardegna refer to material collected in Jerzu by Bornemann (Barbey 1884) and by Bonomi (*sine loco*, 1895, FI). These records were never confirmed thereafter.

F. Mascia, G. Secci

Sagina alexandrae Iamonico (Caryophyllaceae)

+ **PIE**: Trecate (Novara), prati aridi del Ticino (sponda destra) (WGS84: 45.440868°N, 8.805930°E), 112 m, 26 April 2023, leg. *I. Brugellis*, det. *S. Assini* (FI). – Species confirmed for the flora of Piemonte.

We observed the species occurring in a siliceous dry grassland, which can be classified as Habitat 6210*, located between the Polo Industriale San Martino and the right bank of the Ticino River.

S. Assini, I. Brugellis

Salsola tragus L. (Amaranthaceae)

+ **LAZ**: Tarquinia (Viterbo), Riserva Naturale Statale Saline di Tarquinia (WGS84: 42.210163°N, 11.708960°E), dune, 2 m, 6 November 2022, *G. Buccomino*, *D. Iamonico* (FI); Roma, Tenuta Presidenziale di Castel Porziano, litorale interno, November 1987, *B. Anzalone*, rev. *D. Iamonico* 1 June 2023 (RO-HA); Latina, Isola di Ponza, 26 June 1966, *B. Anzalone*, rev. *D. Iamonico* 1 June 2023 (RO-HA); Latina, Isola di Ponza, pressi di Ponza, 16–19 October 1966, *B. Anzalone*, rev. *D. Iamonico* 1 June 2023 (RO-HA). – Species new for the flora of Lazio.

In addition to our findings, we found three further specimens collected by B. Anzalone in the provinces of Rome and Latina and previously identified as *Salsola kali* L. (see also Anzalone et al. 2010: 265). Further specimens annotated as *S. kali* are preserved at RO, but they cannot be surely identified as they are lacking mature fruits.

D. Iamonico, G. Buccomino

Sisymbrium austriacum **Jacq. subsp.** ***austriacum*** **(Brassicaceae)**

– **CAM**. Species to be excluded from the flora of Campania.
– **MOL**. Species to be excluded from the flora of Molise.

The indications of *Sisymbrium austriacum* for Campania and Molise, i.e., Montevergine (province of Avellino) and Matese massif (Campania and Molise) (e.g., Fiori 1924; Pignatti 1982; Lucchese 1995) apparently rely only on Tenore (1831) and were never confirmed (cf. also Santangelo et al. 2008). However, due to the incomplete material, Tenore himself (1835–1836, under the name *S. pannonicum* Jacq. var. "*Jacobaeefolium*") questioned whether it could instead represent a variety of *S. altissimum* L. or even a new species. The only pertinent material in Tenore's herbarium (NAP, barcode NAP0001829!) consists of a few fruiting scapes apparently collected in Abruzzo by Cecchetti (see Tenore 1831). The label, handwritten by Tenore, reports his aforementioned doubts. In any case, this material was already identified by L. Grande as *S. orientale* L., which makes the reports for Campania and Molise unreliable.

E. Del Guacchio

Tragopogon eriospermus **Ten. (Asteraceae)**

+ **PUG**: Laterza (Taranto), Fontana Imperatore, (WGS84: 40.669799°N, 16.755913°E), prateria a *Lolium arundinaceum* (Schreb.) Darbysh. subsp. *arundinaceum*, *Phalaris paradoxa* L. e *Poa palustris* L, 350 m s.l.m., 4 June 2023, leg. *G. Silletti*, *E. Costanzo*, det. *G. Silletti*, *V. Tomaselli* (FI, BI Nos 58198-58206). – Species confirmed for the flora of Puglia.

This species had been historically reported by Tenore (1831) for the "Tavoliere".

G.N. Silletti, E. Costanzo, V. Tomaselli

Trigonella infesta **(Guss.) Coulot & Rabaute (Fabaceae)**

+ **PUG**: Ginosa (Taranto), Gravina di Ginosa (WGS84: 40.582500°N, 16.760556°E), incolto arido pietroso, 200 m, 9 June 2023, leg. *G. Pellegrino*, det. *G. Pellegrino*, *L. Forte* (BI No. 58194). – Species new for the flora of Puglia.

This is a western Mediterranean species (Coulot and Rabaute 2013), which reaches the Ionian Islands in Greece to the east (Dimopoulos et al. 2013). For southern peninsular Italy, the species was reported with certainty only for Basilicata (Portal to the Flora of Italy 2023).

G. Pellegrino, L. Forte

Vicia melanops Sm. (Fabaceae)

+ **EMR**: San Lazzaro di Savena (Bologna), Parco dei Gessi Bolognesi (WGS84: 44.444876°N, 11.375468°E), prato su affioramenti gessosi, 205 m, 24 April 2022, leg. *L. Fontanabona*, det. *L. Fontanabona*, *F. Santi.*, *A. Zarantonello* (FI). − Species confirmed for the flora of Emilia-Romagna.

This species was already reported in Emilia-Romagna by Mattei (1886), who cites a record by Odoardo Beccari dating back to 1864 for the hills surrounding Bologna. The same record is also reported in Betti (1909).

L. Fontanabona, F. Santi

Vicia pannonica Crantz subsp. *striata* (M.Bieb.) Nyman (Fabaceae)

+ **BAS**: Pignola (Potenza), Pantano (WGS84: 40.588492°N, 15.741264°E), margine seminativo di grano, 785 m, 27 May 2023, leg. *G. Potenza*, *L. Rosati*; det. *G. Potenza*, *L. Rosati* (FI). – Subspecies new for the flora of Basilicata.

In the southern Italian peninsula, this taxon has been previously reported only for Puglia (Bartolucci et al. 2018). It is a Mediterranean element considered as a segetal species (Fanfarillo et al. 2020) but also observed in shrublands and forest edges Pignatti (1982). In the NE Italian administrative regions it is considered as an alien taxon.

G. Potenza, L. Rosati

Floristic records of regional alien taxa

Viburnum tinus L. subsp. *tinus* (Viburnaceae)

+ (NAT) **PIE**: Comune di Torino (TO), collina di Torino presso il Colle della Maddalena (WGS84: 45.030988°N, 7.720587°E), versante termofilo a *Quercus pubescens* e *Q. cerris*, 704 m s.l.m., 14 April 2023, *M. Lonati*, *G. Nota* (FI). – Naturalized regional alien taxon new for the flora of Piemonte.

In the area where *V. tinus* was found, this species forms a small population with specimens in the reproductive stage and many young seedlings, probably originating from the older plants.

G. Nota, M. Lonati

Nomenclatural and distribution updates from other literature sources

Nomenclatural and distribution updates, and corrigenda to Bartolucci et al. (2018) and subsequent updates summarised in the Portal to the Flora of Italy (2023) according to Bacchetta et al. (2007), Bracchi and Romani (2010), Fici (2014), Gristina et al. (2014), Hernández-Ledesma et al. (2015), Banasiak et al. (2016), Kadereit et al. (2016), Bartolucci et al. (2020, 2023a, 2023b), Kreutz et al. (2020), Maglio (2021), Conti and Bartolucci (2022), Gestri et al. (2022, 2023), Joffard et al. (2022), Prosser et al. (2022), Selvaggi et al. (2022, 2023), Stace (2022), Bovio (2022), Brusa (2022), Aedo (2023), Alessandrini et al. (2023), Applequist (2023), Böhnert and Del Guacchio (2023), Brullo et al. (2023), Buldrini et al. (2023), Conti et al. (2023a, 2023b, 2023c, 2023d), De Santis (2023), Fraser-Jenkins et al. (2023), Gargano et al. (2023), Gianguzzi et al. (2023), Gregor et al. (2023), Iamonico et al. (2023a, 2023b), Laghi and Pica (2023), Lazzeri et al. (2023), Longo (2023); Longo and Ottonello (2023), Longo et al. (2023a, 2023b), Maglio and Roccia (2023), Manni et al. (2023), Nicolella et al. (2023a, 2023b), Pica and Laghi (2023), Pica et al. (2023), Romero Zarco (2023), Sciuto et al. (2023), Skubic et al. (2023), Tiburtini et al. (2023), Zepigi et al. (2023) are provided in Suppl. material 1.

F. Bartolucci, G. Galasso

Acknowledgements

We gratefully acknowledge the following colleagues who provided distribution, nomenclatural, and taxonomic advice: Acta Plantarum staff, Alessandro Alessandrini, Lorenzo Peruzzi, Antonio Pica, Lorenzo Pinzani.

References

Aedo C (2023) A Monograph of the genus *Geranium* L. (Geraniaceae). Consejo Superior de Investigaciones Científicas, Madrid, 898 pp.

Alessandrini A, Montanari S, Ardenghi NMG, Banfi E, Bruschi T, Ceroni P, Coli M, De Vivo R, Faggi G, Federici A, Fenaroli F, Fornasari L, Frascari M, Gobbato M, Gottschlich G, Grillini P, Lazzeri V, Longo D, Lorenzini G, Morelli V, Morolli S, Ottonello M, Polverelli L, Romani E, Rossetti G, Sirotti M, Uhlich H, Zambrini A (2023) Terza rassegna di novità per la flora dell'Emilia-Romagna segnalate nel forum Acta Plantarum, con una entità da eliminare. ActaPlantarum Notes 9: 66–79.

Anzalone B, Iberite M, Lattanzi E (2010) La Flora vascolare del Lazio. Informatore Botanico Italiano 42(1): 187–317.

Applequist WL (2023) Report of the Nomenclature Committee for Vascular Plants: 74. Taxon 72(4): 908–922. https://doi.org/10.1002/tax.12993

Bacchetta G, Pontecorvo C, Vacca R (2007) The flora of Monte Arcuentu (SW Sardinia). Webbia 62(2): 175–204. https://doi.org/10.1080/00837792.2007.10670822

Banasiak Ł, Wojewódzka A, Baczyński J, Reduron J, Piwczyński M, Kurzyna-Młynik R, Gutaker R, Czarnocka-Cieciura A, Kosmala-Grzechnik S, Spalik K (2016) Phylogeny of Apiaceae subtribe Daucinae and the taxonomic delineation of its genera. Taxon 65(3): 563–585. https://doi.org/10.12705/653.8

Barbey W (1884) Florae Sardoae Compendium. Georges Bridel Editeur, Lousanne.

Barone G, Bajona E, Bartolucci F, Cancellieri L, Caruso G, Conti F, Domina G, Fascetti S, Franzoni, Laface VLA, Pinzani L, Rosati L, Scoppola A, Stinca A, Tilia A, Crisafulli A (2023) Contribution to the floristic knowledge of Lipari and Panarea Islands (Sicilia, Italy). Italian Botanist 16: 59–71. https://doi.org/10.3897/italianbotanist.16.113415

Bartolucci F, Domina G, Bagella S, Barberis G, Briozzo I, Calbi M, Caria MC, Cavallaro V, Chianese G, Cibei C, Conti F, Dagnino D, Esposito A, Galasso G, Giacanelli V, Forte L, Gottschlich G, Lattanzi E, Longo D, Mei G, Merli M, Orsenigo S, Pau GB, Pazienza G, Peccenini S, Pisanu S, Rivieccio G, Roma-Marzio F, Scafidi F, Selvi F, Stinca A, Turcato C, Nepi C (2020) Notulae to the Italian native vascular flora: 10. Italian Botanist 10: 47–55. https://doi.org/10.3897/italianbotanist.10.60743

Bartolucci F, Peruzzi L, Galasso G, Albano A, Alessandrini A, Ardenghi NMG, Astuti G, Bacchetta G, Ballelli S, Banfi E, Barberis G, Bernardo L, Bouvet D, Bovio M, Cecchi L, Di Pietro R, Domina G, Fascetti S, Fenu G, Festi F, Foggi B, Gallo L, Gottschlich G, Gubellini L, Iamonico D, Iberite M, Jiménez-Mejías P, Lattanzi E, Marchetti D, Martinetto E, Masin RR, Medagli P, Passalacqua NG, Peccenini S, Pennesi R, Pierini B, Poldini L, Prosser F, Raimondo FM, Roma-Marzio F, Rosati L, Santangelo A, Scoppola A, Scortegagna S, Selvaggi A, Selvi F, Soldano A, Stinca A, Wagensommer RP, Wilhalm T, Conti F (2018) An updated checklist of the vascular flora native to Italy. Plant Biosystems 152(2): 179–303. https://doi.org/10.1080/11263504.2017.1419996

Bartolucci F, Domina G, Andreatta S, Argenti C, Astuti G, Ballelli S, Ballestrin S, Banfi E, Barberis D, Bernardo L, Bertolli A, Bonali F, Bonini F, Bruschi T, Buccomino G, Caldarella O, Cancellieri L, Caputo P, Conti F, Crisanti A, Del Guacchio E, Falcinelli F, Festi F, Ferri V, Filibeck G, Galasso G, Gestri G, Gigante D, Gubellini L, Gottschlich G, Guarino R, Hofmann N, Király G, Laghi P, Lazzeri V, Lonati M, Luchino F, Lupoletti J, Mei G, Merli M, Pagitz K, Paura B, Pennesi R, Perrino EV, Pica A, Pierini B, Pinzani L, Pittarello M, Praleskouskaya S, Prosser F, Roma-Marzio F, Santi F, Saiani D, Sebellin A, Soldano A, Spilli T, Stinca A, Terzi M, Tiburtini M, Tomasi G, Venanzoni R, Lastrucci L (2022) Notulae to the Italian native vascular flora: 13. Italian Botanist 13: 67–84. https://doi.org/10.3897/italianbotanist.13.86403

Bartolucci F, Domina G, Adorni M, Bacchetta G, Bajona E, Banfi E, Barbadoro F, Biscotti N, Bonsanto D, Conti F, Da Pozzo M, Dagnino D, De Fine G, Del Guacchio E, Forte L, Di Gristina E, Galasso G, Ghillani L, Gottschlich G, Gubellini L, Laface VLA, Lasen C, Lonati M, Mainetti A, Mascia F, Mazzacuva G, Musarella CM, Orsenigo S, Passalacqua NG, Pazienza G, Pinzani L, Rodi ES, Santi F, Sáez L, Selvaggi A, Tomaselli V, Torino L, Zidorn C, Lastrucci L (2023a) Notulae to the Italian native vascular flora: 15. Italian Botanist 15: 91–109. https://doi.org/10.3897/italianbotanist.15.105796

Bartolucci F, Domina G, Conti F (2023b) (2979) Proposal to conserve the name *Ranunculus isthmicus* against *R. schowii* (Ranunculaceae). Taxon 72(4): 941–942. https://doi.org/10.1002/tax.13012

Betti S (1909) Supplemento alla Flora Bolognese. Rivista Italiana di Scienze Naturali (Siena), 20: 15–19, 30–32, 56–58, 109–117.

Böhnert T, Del Guacchio E (2023) The type and correct authorship of the name *Muscari neglectum* (Asparagaceae). Taxon. https://doi.org/10.1002/tax.13025

Bovio M (Ed.) (2022) Note di aggiornamento al volume Flora vascolare della Valle d'Aosta – 8. Revue Valdotaine d'Histoire Naturelle 76: 89–98.

Bracchi G, Romani E (2010) Checklist aggiornata e commentata della flora della Provincia di Piacenza. Museo Civico di Storia Naturale di Piacenza, Piacenza.

Brullo S, Brullo C, Cambria S, Tomaselli V, Crisafulli A, Siracusa G, Minissale P, Giusso del Galdo G (2023) Taxonomic and ecological remarks on *Solenopsis bivonae* species complex (Campanulaceae). PhytoKeys 229: 77–111. https://doi.org/10.3897/phytokeys.229.104324

Brusa G (2022) Contributo all'incremento delle conoscenze floristiche in Valle d'Aosta. Revue Valdôtaine d'Histoire Naturelle 76: 55–68.

Buldrini F, Gianaroli I, Bosi G, Alessandrini A, Santini C (2023) Spontaneous vascular flora of the historical monumental cemetery of Modena (N-Italy). Italian Botanist 15: 111–136. https://doi.org/10.3897/italianbotanist.15.102589

Canzoneri A, Carbini D, Domina G, Faggi G, Giovannini G, Gottschlich G, Lazzeri V, Ottonello M, Paganelli G, Peccenini S, Sassi D (2022) Rassegna di segnalazioni notevoli riguardanti le Marche comparse nel forum Acta Plantarum. Acta Plantarum Notes 8: 166–169.

Conti F, Bartolucci F (2022) La Flora vascolare del Parco Nazionale d'Abruzzo, Lazio e Molise. Edizioni del Parco, FastEdit (Acquaviva Picena, Ascoli Piceno).

Conti F, Oberprieler C, Dorfner M, Schabel E, Bartolucci F (2023a) *Pedicularis rostratospicata* subsp. *marsica* (P. Sect. Rostratae, Orobanchaceae), a new subspecies from the Central Apennines (Italy). Plants 12: 2614. https://doi.org/10.3390/plants12142614

Conti F, Falcinelli F, Giacanelli V, Santucci B, Miglio M, Manzi A, Bartolucci F (2023b) New floristic data of vascular plants from central Italy. Natural History Sciences 10(1): 51–56. https://doi.org/10.4081/nhs.2023.636

Conti F, Palermo D, Santucci B, Miglio M, Paolucci M, De Santis E, Giacanelli V, Bartolucci F (2023c) Additions to the vascular flora of the central and southern Italy. Flora Mediterranea 33: 83–89. https://doi.org/10.7320/FlMedit33.083

Conti F, Cangelmi G, Da Valle J, De Santis E, Giacanelli V, Gubellini L, Hofmann N, Masin R, Miglio M, Palermo D, Santucci B, Bartolucci F (2023d) Additions to the vascular flora of Italy. Flora Mediterranea 33: 177–191. https://doi.org/10.7320/FlMedit33.177

Coulot P, Rabaute P (2013) Monographie des Leguminosae de France. Tome 3. Tribù des Trifolieae Société Botanique du Centre-Ouest, Jarnac, 760 pp.

De Santis E (2023) La Flora del Monte Campitelli (Anagni, FR). Contributo alla conoscenza della Flora vascolare dei Monti Ernici. ActaPlantarum Notes 9: 80–88.

Dimopoulos P, Raus Th, Bergmeier E, Constantinidis Th, Iatrou G, Kokkini S, Strid A, Tzanoudakis D (2013) Vascular plants of Greece: an annotated checklist. Berlin; Athens: Botanic Garden and Botanical Museum Berlin-Dahlem; Hellenic Botanical Society.

Fanfarillo E, Latini M, Iberite M, Bonari G, Nicolella G, Rosati L, Salerno G, Abbate A (2020) The segetal flora of winter cereals and allied crops in Italy: species inventory with chorological, structural and ecological features. Plant Biosystems 154(6): 935–946. https://doi.org/10.1080/11263504.2020.1739164

Fici S (2014) A taxonomic revision of the *Capparis spinosa* group (Capparaceae) from the Mediterranean to Central Asia. Phytotaxa 174(1): 1–24. http://dx.doi.org/10.11646/phytotaxa.174.1.1

Fiori A (1924) Nuova flora analitica d'Italia, vol. 1(4). Tipografia di M. Ricci, Firenze.

Fiori A (1926) Nuova flora analitica d'Italia, vol. 2(2). Tipografia di M. Ricci, Firenze.

Fraser-Jenkins CR, Gibby M, Rumsey FJ, Ekrt L, Evans AJ, Boudrie M, Pigott AC, Golding R (2023) (2957) Proposal to conserve the name *Dryopteris affinis* (Lowe) Fraser-Jenk. against *D. affinis* Kinahan (Pteridophyta: Dryopteridaceae). Taxon 72(3): 665–666. https://doi.org/10.1002/tax.12966

Gargano D, Franzoni J, Luqman H, Fior S, Rovito S, Peruzzi L (2023) Phenotypic correlates of genetic divergence suggest at least three species in the complex of *Dianthus virgineus* (Caryophyllaceae). Taxon. https://doi.org/10.1002/tax.13073

Gestri G, Pierini B, Peruzzi L, Festi F (2022) Aggiornamento sulla distribuzione del genere *Alchemilla* L. (Rosaceae) in Toscana (Italia centrale). Atti della Società Toscana di Scienze Naturali di Pisa, Memorie Serie B 129: 47–51.

Gestri G, Pierini B, D'Antraccoli M, Bernardini A, Peruzzi L (2023) An updated inventory of the vascular flora of the Cerbaie hills (Tuscany, Italy). Italian Botanist 15: 165–175. https://doi.org/10.3897/italianbotanist.15.105302

Gianguzzi L, Guarino R, Bazan G, Di Pietro R, Acosta ATR, Bajona E, Bolliger P, Bonomi C, Camuffo A, Console C, Fascetti S, Fortini P, Frattaroli A, Mei G, Mondello F, Olivari S, Rizzieri M, Rosati L, Sarmati S, Scuderi L, Simonazzi M, Spampinato G, Viegi L, Stinca A (2023) Itineraries of the Working Group for Vegetation Science of the Italian Botanical Society – 1 (2022): Excursion to the Egadi Islands, Mount San Giuliano and Mount Cofano (Trapani, western Sicily, Italy). Italian Botanist 16: 1–57. https://doi.org/10.3897/italianbotanist.16.103989

Gregor T, Kasperek G, Raabe U (2023) *Festuca guestfalica* ein westfälischer Lokalendemit oder eine in Mittel- und Westeuropa weit verbreitete Sippe? Kochia 16: 109–119. https://doi.org/10.21248/kochia.v16.168

Gristina AS, Fici S, Siragusa M, Fontana I, Garfì G, Carimi F (2014) Hybridization in *Capparis spinosa* L.: Molecular and morphological evidence from a Mediterranean island complex. Flora 209(12): 733–741. https://doi.org/10.1016/j.flora.2014.09.002

Hernández-Ledesma P, Berendsohn WG, Borsch T, von Mering S, Akhani H, Arias S, Castañeda-Noa I, Eggli U, Eriksson R, Flores-Olvera H, Fuentes-Bazán S, Kadereit G, Klak C, Korotkova N, Nyffeler R, Ocampo G, Ochoterena H, Oxelman B, Rabeler RK, Sanchez A, Schlumpberger BO, Uotila P (2015) A taxonomic backbone for the global synthesis of FlorItaly 69 species diversity in the angiosperm order Caryophyllales. Willdenowia 45(3): 281–383. https://doi.org/10.3372/wi.45.45301

Iamonico D, Domina G, Conti F (2023a) On the identity of *Rumex uliginosus* and the occurrence of *R. dentatus* in Italy (Polygonaceae). Phytotaxa 619(1): 105–110. https://doi.org/10.11646/phytotaxa.619.1.6

Iamonico D, Guglielmone L, Del Guacchio E (2023b) The genus *Sagina* (Caryophyllaceae) in Italy: nomenclatural remarks. Plants 12: 3169. https://doi.org/10.3390/plants12173169

Joffard N, Buatois B, Arnal V, Véla E, Montgelard C, Schatz B (2022) Delimiting species in the taxonomically challenging orchid section *Pseudophrys*: Bayesian analyses of genetic and phenotypic data. Frontiers in Ecology and Evolution 10: 1058550. https://doi.org/10.3389/fevo.2022.1058550

Kadereit JW, Albach DC, Ehrendorfer F, Galbany-Casals M, Garcia-Jacas N, Gehrke B, Kadereit G, Kilian N, Klein JT, Koch MA, Kropf M, Oberprieler C, Pirie MD, Ritz CM, Röser M, Spalik K, Susanna A, Weigend M, Welk E, Wesche K, Zhang L-B, Dillenberger MS (2016) Which changes are needed to render all genera of the German flora monophyletic? Willdenowia 46(1): 39–91. https://doi.org/10.3372/wi.46.46105

Kreutz K, Klaver JMI, Bioagioli M (2020) *Ophrys metaurensis*, a new species from the Marche region (Central Italy). GIROS Orchidee Spontanee d'Europa 63(1): 137–151.

Laghi P, Pica A (2023) Segnalazioni floristiche (n. 165–167): 167. *Epipactis purpurata* Sm. Quaderno di Studi e Notizie di Storia Naturale della Romagna 57: 255.

Lazzeri V, Campus G, Cardini A, Gestri G, Nicolella G, Tognazzi F (2023) Note floristiche tosco-sarde V: novità regionali per le regioni Toscana e Sardegna. ActaPlantarum Notes 9: 94–103.

Longo D (2023) Alpi Liguri 2022 - Il XIII° Raduno Annuale di Acta Plantarum. ActaPlantarum Notes 9: 44–65.

Longo D, Ottonello M (2023) *Narcissus pseudonarcissus* L. subsp. *provincialis* (Pugsley) J.-M. Tison: conferma della presenza in Italia e in Liguria. ActaPlantarum Notes 9: 89–93.

Longo D, Airale A, Bellone G, Faggi G, Gottschlich G, Lazzeri V, Ottonello M, Prosser F, Tison J-M (2023a) Rassegna di segnalazioni notevoli per il Piemonte e Valle d'Aosta comparse nel forum Acta Plantarum III. ActaPlantarum Notes 9: 18–30.

Longo D, Ardenghi NMG, Banfi E, Blanca M, Ferrando U, Gottschlich G, Ottonello M, Pavone S (2023b) Rassegna di segnalazioni notevoli per la Liguria comparse nel forum Acta Plantarum III. ActaPlantarum Notes 9: 9–17.

Lucchese F (1995) Elenco preliminare della flora spontanea del Molise. Annali di Botanica (Rome) 53, Suppl. 12: 1–386.

Mattei GE (1886) Aggiunte alla flora bolognese. Tip. Azzoguidi, Bologna.

Maglio M (2021) Contribution à la connaissance de *Pinguicula arvetii* Genty (Lentibulariaceae) dans les Alpes sud-occidentales italiennes. Bulletin Mensuel de la Société Linnéenne de Lyon 90 (3–4) : 79–93.

Maglio M, Roccia A (2023) Typification des noms *Pinguicula variegata* Arv.-Touv. et *Pinguicula arvetii* Genty (Lentibulariaceae) et observations sur les récoltes de Casimir Arvet-Touvet. Bulletin Mensuel de la Société Linnéenne de Lyon 92 (5–6) : 148–164.

Manni QG, Gennaio R, Pellegrino G (2023) An overview on the presence of *Iris unguicularis* (Iridaceae) in Salento (Apulia, southern Italy) and a new record of *Iris unguicularis* subsp. *carica* for the Italian vascular flora. Plant Biosystems 157(4): 890–898. https://doi.org/10.1080/11263504.2023.2229849

Nicolella G, Alessandrini A, Buono V, Canzoneri A, Longo D, Zepigi M [Eds] (2023a) Noterelle 0394–0425. Novità per la Flora Italiana e segnalazioni floristiche regionali. Acta Plantarum Notes 9: 222–139.

Nicolella N, Ardenghi NMG, Antonini M, Bamonte R, De Santis E, Iamonico D, Ottonello M, Palermo D, Palmieri M, Rossi F (2023b) Rassegna di segnalazioni notevoli riguardanti alcune regioni dell'Italia centro-meridionale comparse nel forum Acta Plantarum II. ActaPlantarum Notes 9: 31–43.

Pica A, Laghi P (2023) Atlante delle Orchidee del Parco Nazionale delle Foreste Casentinesi, Monte Falterona e Campigna. Guida alle specie e chiavi di riconoscimento. P. N. delle

Foreste Casentinesi, Monte Falterona e Campigna. Premiato Stabilimento Tipografico dei Comuni, Santa Sofia (FC), 356 pp.

Pica A, Berardi D, Ciaschetti G (2023) Prime segnalazioni di *Anacamptis berica* in Abruzzo e in Molise. GIROS Orchidee Spontanee d'Europa 66(1): 78–85.

Pignatti S (1982) Flora d'Italia, vol. 1. Edagricole, Bologna.

Portal to the Flora of Italy (2023) Portale della Flora d'Italia/Portal to the Flora of Italy. 2023.1. https://dryades.units.it/floritaly/ [accessed 03.08.2023 and 09.08.2023]

POWO (2023) Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. http://www.plantsoftheworldonline.org/ [Accessed 13.10.2023]

Prosser F, Bertolli A, Festi F, Tomasi G, Andreatta S, Costantini L, Dal Corso G, Di Carlo F, Menini F, Ovatoli M, Pighi E, Scortegagna S, Trenchi M (2022) Segnalazioni Floristiche veronesi III. Bollettino del Museo Civico di Storia Naturale di Verona. Botanica Zoologia 46: 5–28.

Romero Zarco C (2023) *Bromus cincinnatus* (Poaceae): perennial oat-grass or annual brome-grass? Mediterranean Botany 44: e84748. https://doi.org/10.5209/mbot.84748

Santangelo A, Bronzo E, Croce A, Salvati C, Strumia S (2008) Base di dati per le ricerche floristiche: un esempio per il Matese campano. Informatore Botanico Italiano 40(1): 59–71.

Selvaggi A, Soldano A, Pascale M, Dellavedova R [Eds] (2022) Note floristiche piemontesi n. 1082–1180. Rivista Piemontese di Storia Naturale 43: 175–209.

Selvaggi A, Soldano A, Pascale M, Dellavedova R [Eds] (2023) Note floristiche piemontesi n. 1181–1242. Rivista Piemontese di Storia Naturale 44: 227–252.

Sciuto K, Wolf MA, Sfriso A, Brancaleoni L, Iberite M, Iamonico D (2023) Molecular and morphometric update on italian *Salicornia* (Chenopodiaceae), with a focus on the species *S. procumbens* s.l. Plants 12: 375. https://doi.org/10.3390/plants12020375

Skubic M, Záveská E, Frajman B (2023) Meeting in Liguria: Hybridisation between Apennine endemic *Euphorbia barrelieri* and western Mediterranean *E. nicaeensis* led to the allopolyploid origin of *E. ligustica*. Molecular Phylogenetics and Evolution 185: 107805. https://doi.org/10.1016/j.ympev.2023.107805.

Stace CA (2022) Conspectus of and key to the world's species of *Vulpia* C.C. Gmel. (Poaceae: Loliinae) and seven related genera. British & Irish Botany 4(2): 74–94. https://doi.org/10.33928/bib.2022.04.074

Tenore M (1831) Sylloge plantarum vascularium florae neapolitanae. Ex Typographia Fibreni, Neapoli.

Tenore M (1835–1836) Flora napolitana, vol. 5. Dalla Stamperia e Cartiera del Fibreno, Napoli.

Tiburtini M, Bacchetta G, Sarigu M, Cambria S, Caputo P, De Luca D, Domina G, Turini A, Peruzzi L (2023) Integrative taxonomy of *Armeria* taxa (Plumbaginaceae) endemic to Sardinia and Corsica. Plants 12: 2229. https://doi.org/10.3390/plants12112229

Zepigi M, Ardenghi NMG, Bellio R, Colombo S, Federici A, Ferrando U, Galasso G, Giordana F, Lazzeri V, Leoni G, Maggioni L, Mazzi G, Mazzoli A, Menini F, Merli M, Parrino N, Perino P, Siega Vignut P, Truzzi A (2023) Rassegna di segnalazioni notevoli per alcune regioni del nord Italia comparse nel forum Acta Plantarum. ActaPlantarum Notes 9: 113–121.

Supplementary material 1

Categories concerning the occurrence status of taxa follow Bartolucci et al. (2018)

Authors: Fabrizio Bartolucci, Gabriele Galasso

Data type: pdf

Explanation note: 1. Nomenclatural updates; 2. Note updates; 3. Distribution updates; 4. Synonyms, misapplied or included names.

Link: https://doi.org/10.3897/italianbotanist.16.114657.suppl1

Italian Botanist 17: 13–21 (2024)
doi: 10.3897/italianbotanist.17.126693
https://italianbotanist.pensoft.net

RESEARCH ARTICLE

Italian Botanist
Published by Società Botanica Italiana

Notulae to the Italian native vascular flora: 17

Fabrizio Bartolucci[1], Gianniantonio Domina[2], Sandro Ballelli[3], Fabio Conti[1], Paola Fortini[4], Emanuele Del Guacchio[5], Emanuela Di Iorio[5], Gabriele Galasso[6], Leonardo Gubellini[7], Nicole Hofmann[8], Valentina Lucia Astrid Laface[9], Michele Lonati[10], Giuliana Mazzacuva[9], Ginevra Nota[10], Simone Pesaresi[8], Lorenzo Pinzani[11], Filippo Prosser[12], Luca Quaranta[4], Federico Selvi[13], Manuel Tiburtini[14], Robertino Tiburtini[15], Thomas Wilhalm[16], Lorenzo Lastrucci[17]

1 *Centro Ricerche Floristiche dell'Appennino (Università di Camerino - Parco Nazionale del Gran Sasso e Monti della Laga), Via Prov.le km 4,2 - San Colombo, 67021 Barisciano (L'Aquila), Camerino, Italy* **2** *Dipartimento di Scienze Agrarie, Alimentari e Forestali (SAAF), Università di Palermo, Viale delle Scienze, edificio 4, 90128 Palermo, Italy* **3** *Herbarium Universitatis Camerinensis, Scuola di Bioscienze e Medicina Veterinaria - Unità di Diversità vegetale e Gestione degli ecosistemi, Università di Camerino, Via Pontoni 5, 62032 Camerino (Macerata), Italy* **4** *Laboratorio di Floristica, Dipartimento di Bioscienze e Territorio, Università degli Studi del Molise, 86090 Pesche (Isernia), Italy* **5** *Dipartimento di Biologia, Università di Napoli Federico II, c/o Orto Botanico, Via Foria 223, 80139 Napoli, Italy* **6** *Sezione di Botanica, Museo di Storia Naturale di Milano, Corso Venezia 55, 20121 Milano, Italy* **7** *Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini", Provincia di Pesaro e Urbino, Via Barsanti 18, 61122 Pesaro, Italy* **8** *Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy* **9** *Dipartimento di Agraria, Università "Mediterranea" di Reggio Calabria, Feo di Vito snc, 89122 Reggio Calabria, Italy* **10** *Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco (Torino), Italy* **11** *Dipartimento di Scienze, Università di Roma Tre, Viale G. Marconi 446, 00146 Roma, Italy* **12** *Fondazione Museo Civico di Rovereto, Largo S. Caterina 41, 38068 Rovereto, Italy* **13** *Dipartimento di Scienze e Tecnologie Agrarie, Alimentari, Ambientali e Forestali, Università di Firenze, Piazzale delle Cascine 28, I-50144 Firenze, Italy* **14** *PLANTSEED Lab, Dipartimento di Biologia, Università di Pisa, Via Derna 1, 56126, Pisa, Italy* **15** *Via Cunicchio 9, 63811 Sant'Elpidio a Mare (Fermo), Italy* **16** *Museo di Scienze Naturali dell'Alto Adige, Via Bottai 1, 39100 Bolzano, Italy* **17** *Sistema Museale di Ateneo, Università di Firenze, Via G. La Pira 4, 50121 Firenze, Italy*

Corresponding author: Fabrizio Bartolucci (fabrizio.bartolucci@unicam.it)

Academic editor: Lorenzo Peruzzi | Received 3 May 2024 | Accepted 4 May 2024 | Published 22 May 2024

Citation: Bartolucci F, Domina G, Ballelli S, Conti F, Fortini P, Del Guacchio E, Di Iorio E, Galasso G, Gubellini L, Hofmann N, Laface VLA, Lonati M, Mazzacuva G, Nota G, Pesaresi S, Pinzani L, Prosser F, Quaranta L, Selvi F, Tiburtini M, Tiburtini R, Wilhalm T, Lastrucci L (2024) Notulae to the Italian native vascular flora: 17. Italian Botanist 17: 13–21. https://doi.org/10.3897/italianbotanist.17.126693

Abstract

In this contribution, new data concerning the distribution of native vascular flora in Italy are presented. It includes new records, confirmations, and exclusions to the Italian administrative regions for taxa in the genera *Anacyclus*, *Anthyllis*, *Bolboschoenus*, *Catapodium*, *Festuca*, *Hordeum*, *Lavandula*, *Ophrys*, *Pseudopodospermum*, *Rosa*, *Sorbus* and *Spergula*. Nomenclatural and distribution updates, published elsewhere, and corrigenda are provided as Suppl. material 1.

Keywords

Endemic taxa, Floristic data, Italy

How to contribute

The text for the new records, exclusions, and confirmations should be submitted electronically to Lorenzo Lastrucci (lorenzo.lastrucci@unifi.it). The corresponding specimen along with its scan or photograph have to be sent to FI Herbarium: Sezione di Botanica "Filippo Parlatore" del Museo di Storia Naturale, Via G. La Pira 4, 50121 Firenze (Italy). Those texts concerning nomenclatural novelties and typifications (only for accepted names) should be submitted electronically to: Fabrizio Bartolucci (fabrizio.bartolucci@gmail.com). Each text should be within 1,000 characters (spaces included).

Floristic records of native taxa

Anacyclus clavatus (Desf.) Pers. (Asteraceae)

+ **LIG**: Arma di Taggia (Imperia), parcheggio Piazzale Sant'Erasmo, lungo la ciclabile Imperia-San Remo (WGS84: 43.832754°N, 7.856074°E), aiuole incolte sul margine del parcheggio, 7 m, 1 June 2023, *M. Lonati, G. Nota* (FI). – Species new for the flora of Liguria.

This species forms a well-established population on an uncultivated area of over one ha, with several hundreds of individuals in the flowering stage.

M. Lonati, G. Nota

Anthyllis apennina F.Conti & Bartolucci (Fabaceae)

+ **MAR**: Conca S. Lorenzo, costone rupestre, 1360 m, 14 July 1960, *V. Marchesoni* (FI, CAME); Pendici meridionali orientali del M. Cetrognola, pascoli xerici, substrato calcareo, 1300–1400 m, 30 June 1986, *S. Ballelli* (CAME); M. Gioco del Pallone, vers. E-NE, pascoli pingui e seslerieti, 1100–1200 m, 13 July 1995, *S. Ballelli* (CAME); *ibidem*, pascoli xerici di vetta, 1180 m, 13 July 1995, *S. Ballelli* (CAME); *ibidem*, rupi e pascoli a *Sesleria apennina*, 1110 m, 13 July 1995, *S. Ballelli* (CAME); M. Gioco del Pallone, vers. W, pascoli rocciosi e bosco sottostante, 1100–1200 m, 13 July 2001, *S. Ballelli* (CAME); Pendici SE del M. Vettoretto, Pié Vettore, cespuglieti a *Juniperus* e *Arctostaphylos*, substr. calcareo, 1400–1450 m, 29 July 1992, *S. Ballelli* (CAME);

ibidem, seslerieti e arctostafileti, substr. calcareo, 1500–1800 m, 29 July 1992, *S. Ballelli* (CAME); M. Costa, pendici nord (a nord di Monte Spina di Gualdo), esp. NE, bosco di faggio e pascoli sovrastanti, substr. calcareo, 9 June 2001, *S. Ballelli* (CAME); Castelsantangelo sul Nera (Macerata), Spina di Gualdo, presso la Forca, pascoli, substr. calcareo, 1320 m ca., 19 June 2003, *S. Ballelli* (CAME); Valle dell'Infernaccio lungo il sentiero nella valle, prati e pascoli aridi, substr. calcareo, 4 June 1995, *S. Ballelli* (CAME). − Species new for the flora of Marche.
+ **UMB**: M. Castellaccio, Pian Grande, xerogramineti a *Stipa*, 1350–1450 m, 20 July 1962, *V. Marchesoni* (FI, CAME); M. Castello (sopra inghiottitoio di Pian Grande), esp. E, boschi e pascoli, substr. calcareo, 13 June 1997, *S. Ballelli* (CAME); *ibidem*, pascoli a *Sesleria*, 1 July 2003, *S. Ballelli* (CAME); Colle delle Cupaie (M. Ventosola), esp. SE, pascoli rupestri, substr. calcareo, 1250–1350 m, 4 June 2002, *S. Ballelli* (CAME); sotto Costa Precino (M. Ventosola), esp. E, pascoli sassoso-rupestri, substr. calcareo, 19 June 2020, *S. Ballelli* (CAME). − Species new for the flora of Umbria.

Anthyllis apennina was so far recorded for Abruzzo and Lazio (Conti and Bartolucci 2022).

F. Conti, S. Ballelli

Bolboschoenus planiculmis (F.Schmidt) T.V.Egorova (Cyperaceae)

+ **TOS**: Pelago (Firenze), Il Fossato (WGS84: 43.781428°N, 11.447483°E), sponda fluviale, 90 m, 4 September 2023, leg. *L. Pinzani*, det. *L. Lastrucci*, *L. Pinzani* (FI, *Herb. Pinzani*). – Species confirmed for the flora of Toscana.

According to Di Natale et al. (2020), this species is documented in Toscana only by a single ancient specimen (Padule di Massaciuccoli, Feb 1864, *O. Beccari*, FI058805 sub *Scirpus maritimus*; rev. L. Pignotti 09/1998, sub *Scirpus maritimus*). At the site of discovery, this species forms a small population in a temporarily wet depression along the Sieve River.

L. Pinzani, L. Lastrucci

Catapodium pauciflorum (Merino) Brullo, Giusso, Miniss. & Spamp. (Poaceae)

+ **MAR**: Marina Palmense (Fermo), Habitat: Prateria subsalsa. Area di Volo di Marina Palmense, nei pressi del Camping "Spinnaker" (WGS84: 43.1557288°N, 13.81091827°E), 8 May 2023, *M. Tiburtini, R. Tiburtini* (FI). – Species new for the flora of Marche.

Along the Adriatic coast, this species shows a patchy distribution (Bartolucci et al. 2024), and this may due to identification problems with similar species (Brullo et al. 2003).

M. Tiburtini, R. Tiburtini

Festuca circummediterranea Patzke (Poaceae)

+ **TAA**: Brentonico (Trento), M. Baldo, ca. 100 m a N di Malga Postemon (WGS84: 45.773300°N, 10.9199990°E), pascolo su calcare, 1390 m, 12 June 2007, *F. Prosser,*

A. Bertolli, det. *F. Prosser*, rev. *T. Wilhalm*, conf. *B. Foggi* (FI). – Species new for the flora of Trentino-Alto Adige.
+ **VEN**: Malcesine (Verona), M. Baldo, versante W del M. Altissimo al Dosso dei Roveri (WGS84: 45.814932°N, 10.868885°E), zona erbosa su calcare, 1060 m, 3 August 2005, *F. Prosser*, rev. *T. Wilhalm*, conf. *B. Foggi* (FI, ROV 48119). – Species confirmed for the flora of Veneto.

The specimens from Monte Baldo were noticed because they resembled an (octoploid) *F. laevigata* Gaudin (Arndt 2005), but leaf section and sheath corresponded to that of (diploid) *F. circummediterranea* (Ardenghi et al. 2016). The stomata length also clearly indicated a diploid plant (approx. 25 µm, Fischer et al. in prep.). The specimen from Trentino-Alto Adige comes from an isolated tussock, that from the Veronese part from a limited individual-rich population in a barely impacted area. The present records extend the range of this Mediterranean species to southern Alps.

F. Prosser, T. Wilhalm

Hordeum geniculatum All. (Poaceae)

+ **MAR**: Staffolo (Ancona), loc. Coste di Staffolo (WGS84: 33T 43.4529256°N, 13.2201362°E), stradello argilloso sopra al calanco, suolo argilloso-limoso, c. 250 m, 17 May 2002, leg. et det. *S. Pesaresi* (sub *Hordeum hystrix* Roth, PESA); Pesaro (Pesaro e Urbino), tra Candelara e Ginestreto, margini di campo lungo la strada della Blilla (WGS84: 43.852420°N, 12.850836°E), suolo argilloso, humus nullo, c. 155 m, 29 May 2012, leg. et det. *L. Gubellini* (PESA, FI). – Species new for the flora of Marche.

L. Gubellini, S. Pesaresi

Lavandula latifolia Medik. (Lamiaceae)

– **CAM.** Species to be excluded from the flora of Campania.

The occurrence of this species in Campania is exclusively based on an indication for Alburni massif (Salerno) by Caputo et al. (1977): “Rupi presso «Il Figliolo» (m 1300)”. According to our field surveys and pertinent specimens [*Herb. Del Guacchio* (NAP)], this indication is to be attributed to the recently described *L. austroapennina* N.G.Passal., Tundis & Upson. Accordingly, *L. latifolia* should be excluded from the regional flora (see also Grande 1924).

E. Del Guacchio, E. Di Iorio

Ophrys fusca Link subsp. *forestieri* (Rchb.f.) Kreutz (Orchidaceae)

+ **CAL**: Motta San Giovanni (Reggio Calabria), località Pitea (WGS84: 38.008475°N, 15.734279°E), in prossimità di un impianto di *Pinus* sp. con presenza di formazioni rocciose, 826 m, 11 February 2024, leg. *V.L.A. Laface, G. Mazzacuva*, det. *V.L.A. Laface, G. Mazzacuva, L. Torino* (REGGIO, FI). – Subspecies new for the flora of Calabria.

Many individuals have been observed growing in the pine forest and near the rock formations. This subspecies is easily distinguished by its early flowering, beginning to bloom in January with full flowering in February/March.

V.L.A. Laface, G. Mazzacuva

***Pseudopodospermum hispanicum* (L.) Zaika, Sukhor. & N.Kilian subsp. *neapolitanum* (Grande) Bartolucci, Galasso & F.Conti (Asteraceae)**

+ **TOS**: Roccalbegna (Grosseto), margini di boscaglia lungo la strada fra Usi e Rocconi nella valle dell'Albegna (WGS84: 42.740556°N, 11.472238°E), 530 m ca., suolo argilloso, raro, 12 June 1999, leg. *F. Selvi* 0341, vid. *F. Bartolucci* (FI); Castell'Azzara (Grosseto), scendendo alla Sforzesca, erbosi su calanco argilloso (WGS84: 42.774027°N, 11.703910°E), 600 m ca., raro, 18 May 2007, *F. Selvi 2843* (FI); Usi (Grosseto) radure e margini boschivi lungo la strada verso Murci (WGS84: 42.740556°N, 11.472238°E), suolo argilloso, 500 m ca., 9 May 2009, leg. *F. Selvi 3132*, vid. *F. Bartolucci* (FI). – Subspecies new for the flora of Toscana.

The specimens of *Scorzonera hispanica* L. mentioned in Selvi (2010) belong to this subspecies, endemic to central and southern Italy (Bartolucci et al. 2020). The Tuscan populations mark the northern distribution limit of this taxon.

F. Selvi

***Rosa nitidula* Besser (Rosaceae)**

+ **UMB**: Gubbio (Perugia), Monte Petria (WGS84: 43.428747°N, 12.661019°E), margini di bosco e arbusteti, suolo calcareo, 875 m, 1 June 2022, leg. *L. Gubellini et N. Hofmann*, det. *L. Gubellini* (PESA, FI). – Species new for the flora of Umbria.

L. Gubellini, N. Hofmann

+ **MOL**: Fragnete (Isernia), Colle Santa Maria (WGS84: 41.98472°N, 14.31583°E, cespuglieto, ca. 460 m, 16 May 2005, leg. *V. Viscosi*, det. *E Lattanzi* (IS); Fornelli (Isernia) (WGS84: 41.61410°N, 14.13828°E), siepe su marna argillosa, ca. 500 m, 25 May 2005, leg. *S. Pietrunti*, det. *S. Pietrunti, E. Lattanzi, A. Tilia* (IS); Colli al Volturno (Isernia), strada provinciale da Colli al Volturno a Fornelli (WGS84: 41.59928°N, 14.10741°E), cespuglieto, ca. 450 m, 18 July 2006, leg. *S. Pietrunti*, det. *S. Pietrunti, E Lattanzi* (FI, IS). – Species new for the flora of Molise.

P. Fortini, L. Quaranta

***Sorbus chamaemespilus* (L.) Crantz (Rosaceae)**

– **CAM**. Species to be excluded from the flora of Campania.

– **PUG**. Species to be excluded from the flora of Puglia.

Tenore (1831–1833: 70, under the name *Pyrus chamaemespylus*, see also Tenore 1831) indicated this species for Montevergine (Avellino, Campania) and Gargano

(Foggia, Apulia), where it has been no longer found. By the expression "Tenore, 1827 (Monte di Mezzo, in subalpinis)", Fenaroli (1970) merely quoted that indication. Bartolucci et al. (2024) does not indicate this species for Campania and report it as doubtful for Puglia (see also Pignatti 1982, Conti and Bartolucci 2015). As Tenore (1831–1833: 278) himself deleted all the localities of southern Italy (not represented at NAP), the reports for Campania and Puglia are considered erroneous.

E. Del Guacchio

Spergula arvensis **L. (Caryophyllaceae)**

+ **MAR**: Castelsantangelo sul Nera (Macerata), Monti Sibillini, al Piano perduto (WGS84: 42.839953°N, 13.196694°E), luoghi erbosi incolti, suolo calcareo, humus nullo o subnullo, c. 1310 m, 8 October 2023, leg. et det. *L. Gubellini* (PESA, FI) – Species confirmed for the flora of Marche.

This species, recorded at the end of the 19th century for the Pesaro area by Paolucci (1890), was recently found in the southern sector of Marche region at Pian Perduto (Mts. Sibillini), where it forms a population of about a hundred individuals in resting fields.

L. Gubellini, N. Hofmann

Nomenclatural and distribution updates from other literature sources

Nomenclatural and distribution updates, and corrigenda to Bartolucci et al. (2024) according to Klaver and Rossi (2011), Pignatti et al. (2017), Hertel and Presser (2021), Tison et al. (2021), Iamonico et al. (2022), Adamo et al. (2023), Martini et al. (2023), Parolo and Della Ferrera (2023), Uhlemann (2023), Gianguzzi et al. (2024), Gottschlich (2024), Iamonico and Del Guacchio (2024), Kozlowski et al. (2024); Sennikov (2024), Sennikov and Tikhomirov (2024), Španiel et al. (2024), Valle et al. (2024) are provided in Suppl. material 1.

F. Bartolucci, G. Galasso

Acknowledgements

We gratefully acknowledge the following colleagues who provided distribution, nomenclatural, and taxonomic advice: Acta Plantarum ODV, Bruno Foggi, Duilio Iamonico, Giacomo Mei, Lorenzo Peruzzi.

References

Adamo M, Skokanová K, Bobo-Pinilla J, Giaccone E, Peñas de Giles J, Mucciarelli M (2023) Molecular evidence and environmental niche evolution at the origin of the disjunct distribution in three mountain endemic *Tephroseris* (Asteraceae) of the Mediterranean basin. Alpine Botany 133: 117–133. https://doi.org/10.1007/s00035-023-00300-w

Ardenghi NMG, Fiorini G, Rossi G, Foggi B (2016) Chromosome numbers and karyomorphology of *Festuca circummediterranea* Patzke group (Poaceae, Loliinae). Phytotaxa 263(3): 255–269. https://doi.org/10.11646/phytotaxa.263.3.6

Arndt S (2005) Systematik der *Festuca valesiaca* und *Festuca laevigata*-Gruppe in den Westalpen. PhD Thesis, Friedrich-Schiller-Universität Jena, Jena.

Bartolucci F, Galasso G, Conti F (2020) Nomenclatural novelties and typification of names in *Scorzonera sensu lato* (Asteraceae, Cichorieae) for the Italian vascular flora. Phytotaxa 437(5): 279–290. https://doi.org/10.11646/phytotaxa.437.5.2

Bartolucci F, Peruzzi L, Galasso G, Alessandrini A, Ardenghi NMG, Bacchetta G, Banfi E, Barberis G, Bernardo L, Bouvet D, Bovio M, Calvia G, Castello M, Cecchi L, Del Guacchio E, Domina G, Fascetti S, Gallo L, Gottschlich G, Guarino R, Gubellini L, Hofmann N, Iberite M, Jiménez-Mejías P, Longo D, Marchetti D, Martini F, Masina RR, Medagli P, Peccenini S, Prosser F, Roma-Marzio F, Rosati L, Santangelo A, Scoppola A, Selvaggi A, Selvi F, Soldano A, Stinca A, Wagensommer RP, Wilhalm T, Conti F (2024) A second update to the checklist of the vascular flora native to Italy. Plant Biosystems 158(2): 219–296. https://doi.org/10.1080/11263504.2024.2320126

Brullo S, Galdo G, Minissale P, Spampinato G (2003). Considerazioni tassonomiche sui generi *Catapodium* Link, *Desmazeria* Dumort. e *Castellia* Tineo (Poaceae) in Italia. Informatore Botanico Italiano 35: 158–170.

Caputo G, Ricciardi M, Moggi G (1977) Nuovi reperti floristici per il Monte Alburno (Appennino Campano-Lucano). Webbia 31(2): 295–311.

Conti F, Bartolucci F (2015) The vascular flora of the National Park of Abruzzo, Lazio and Molise (Central Italy). An annotated checklist. Geobotany Studies. Cham, Springer. https://doi.org/10.1007/978-3-319-09701-5

Conti F, Bartolucci F (2021) *Anthyllis apennina* (Fabaceae), a new species from central Apennine (Italy). PhytoKeys 176: 111–129. https://doi.org/10.3897/phytokeys.176.62774

Di Natale S, Lastrucci L, Hroudová Z, Viciani D (2022) A review of *Bolboschoenus* species (Cyperaceae) in Italy based on herbarium data. Plant Biosystems 156(1): 261–270. https://doi.org/10.1080/11263504.2020.1852330

Fenaroli L (1970) Florae garganicae prodromus – pars altera. Webbia 24(2): 435–578. https://doi.org/10.1080/00837792.1970.10669916

Fischer MA, Adler W, Oswald K (in prep.) Exkursionsflora für Österreich und die gesamten Ostalpen. Biologiezentrum der Oberösterreichischen Landesmuseen.

Gianguzzi L, Di Gristina E, Barone G, Domina G (2024) A new species of the *Centaurea busambarensis* complex (Asteraceae) from th Nebrodi Mountains in Sicily (Central Mediterranean region). Phytotaxa 646(2) : 169–180. https://doi.org/10.11646/phytotaxa.646.2.6

Grande L (1924) Note di floristica. Nuovo Giornale Botanico Italiano n. s. 31: 105–160.

Hertel S, Presser H (2021) Weitere Erkenntnisse zur Vielfalt der Epipactis-Arten im südlichen Italien. Berichte aus den Arbeitskreisen Heimische Orchideen 38(1): 122–162.

Iamonico D, Del Guacchio E (2024) Loeflingia hispanica L. In: Raab-Straube E von, Raus T (Eds) Euro+Med-Checklist Notulae, 17. Willdenowia 54(1): 13. https://doi.org/10.3372/wi.54.54101

Iamonico D, Sciuto K, Sfrisio A, Argenti C, Wolf MA (2022) *Blitum venetum* (Chenopodiaceae), a new species from the north-eastern Dolomites (Italian Eastern Alps). Acta Botanica Croatica 81(2): 121–128. https://doi.org/10.37427/botcro-2022-009

Klaver JMI, Rossi D (2011) Contributo alla conoscenza delle Orchidee della Provincia di Pesaro e Urbino. GIROS Notizie 46: 30–36.

Kozlowski G, Fragnière Y, Clément B, Gilg O, Sittler B, Lang J, Eidesen PB, Lang SI, Wasowicz P, Meade C (2024) Genome size in the *Arenaria ciliata* species complex (Caryophyllaceae), with special focus on northern Europe and the Arctic. Plants 13: 635. https://doi.org/10.3390/plants13050635

Martini F, Bertani G, Boscutti F, Bruna A, Danelutto A, Pavan R, Peruzovich C (2023) Flora del Friuli Venezia Giulia. Repertorio critico diacronico e atlante corologico. Udine: Forum. Italian.

Paolucci L (1890) Flora Marchigiana. Tip. Federici, Pesaro.

Parolo G, Della Ferrera E (2023) *Rumex nivalis* Hegetschw. (Polygonaceae), una nuova specie per la flora d'Italia, in prossimità del confine svizzero. Bollettino della Società ticinese di scienze naturali 111: 93–100.

Pignatti S (1982) Flora d'Italia, Vols. 1–3. Edagricole, Bologna.

Pignatti S, Guarino R, La Rosa M (2017) Flora d'Italia 2, Ed. 2. Edagricole, Bologna.

Selvi F (2010) A critical checklist of the vascular flora of Tuscan Maremma (Grosseto province, Italy). Flora Mediterranea 20: 47–139.

Sennikov AN, Tikhomirov VN (2024) Atlas Florae Europaeae notes, 35. Further critical notes on *Cytisus* sect. *Tubocytisus* (Fabaceae) in Europe. PhytoKeys 238: 199–230. https://doi.org/10.3897/phytokeys.238.118032

Sennikov AN, Tikhomirov VN (2024) Atlas Florae Europaeae notes, 33. Taxonomic synopsis of East European species of the *Cytisus ratisbonensis* group (Fabaceae). PhytoKeys 238: 157–197. https://doi.org/10.3897/phytokeys.238.118031

Španiel S, Mártonfiová L, Zozomová-Lihová J (2024) An unexpected occurrence of *Alyssum rossetii* (Brassicaceae) in the Pyrenees, a new species for the Spanish flora. Willdenowia 53: 297–307. https://doi.org/10.3372/wi.53.53307

Tenore M (1831) Sylloge plantarum vascularium florae neapolitanae hucusque detectarum. Tipografia del Fibreno, Neapoli [Naples].

Tenore M (1830 [1830–1833]) Flora napolitana [Vol. 2(2)]. Dalla Stamperia Francese, Napoli.

Tison JM, Abdulhak S, Bock B, Boudrie M, Fridlender A, Roccia A, Van Es J, Véla E (2021) Combinaisons nouvelles requises dans la seconde édition de Flora Gallica. Evaxiana 8: 220–225.

Uhlemann I (2023) *Taraxacum* section *Crocea* (= *Taraxacum* section *Fontana*, Asteraceae, Crepidinae) in the European Alps. Neilreichia 13–14(2022–2023): 195–226. https://doi.org/10.5281/zenodo.10120059

Valle B, Eustacchio E, Gallo GR, Beretta M, Bonelli M, Zanzottera A, Gianfranceschi L, Federici G, Mangili F, Mangili L, Perico G, Traini M, Caccianiga M (2024) *Campanula bergomensis* (Campanulaceae), a new species from Bergamo Prealps (Northern Italy). Phytotaxa 637(2): 120–132. https://doi.org/10.11646/phytotaxa.637.2.1

Supplementary material 1

Supplementary data

Authors: Fabrizio Bartolucci, Gabriele Galasso

Data type: species data (PDF file)

Explanation note: 1. Nomenclatural updates; 2. Distribution updates; 3. Synonyms, misapplied or included names.

Link: https://doi.org/10.3897/italianbotanist.17.126693.suppl1

Italian Botanist 18: 155–166 (2024)
doi: 10.3897/italianbotanist.18.141896
https://italianbotanist.pensoft.net

RESEARCH ARTICLE

Italian Botanist
Published by Società Botanica Italiana

Notulae to the Italian alien vascular flora: 18

Gabriele Galasso[1], Gianniantonio Domina[2,3], Michele Adorni[4],
Alessandro Alessandrini[5], Nicola M. G. Ardenghi[6], Gianluigi Bacchetta[7],
Giulio Barone[2,3], Fabrizio Bartolucci[8], Dario Boker[9], Giacomo Calvia[10],
Alba Cuena-Lombraña[7], Adriana De Luca[11], Emanuele Del Guacchio[12],
Mauro Fois[7], Paola Fortini[13], Andrea Lallai[7], Michele Lonati[14], Vanessa Lozano[15],
Giacomo Marengo[3,14], Francesco Mascia[16], Giacomo Mei[10], Enzo Meneguzzo[17],
Ginevra Nota[14], Ines Orrù[18], Lina Podda[7], Luca Quaranta[13], Riccardo Rocca[2],
Marco Sarigu[7], Adriano Stinca[19], Francesca Valentini[20], Lorenzo Lastrucci[21]

1 *Sezione di Botanica, Museo di Storia Naturale di Milano, Corso Venezia 55, 20121 Milano, Italy* **2** *Dipartimento di Scienze Agrarie, Alimentari e Forestali (SAAF), Università di Palermo, Viale delle Scienze, edificio 4, 90128 Palermo, Italy* **3** *National Biodiversity Future Center (NBFC), Piazza Marina 61, 90133 Palermo, Italy* **4** *Orto Botanico, Sistema Museale di Ateneo, Università di Parma, Strada L.C. Farini 90, 43121 Parma, Italy* **5** *Via G. Pilati 19, 40018 San Pietro in Casale (Bologna), Italy* **6** *Orto Botanico, Sistema Museale di Ateneo, Università di Pavia, Via Sant'Epifanio 14, 27100 Pavia, Italy* **7** *Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente (DISVA), Università di Cagliari, Viale Sant'Ignazio da Laconi 13, 09123 Cagliari, Italy* **8** *Centro Ricerche Floristiche dell'Appennino (Università di Camerino - Parco Nazionale del Gran Sasso e Monti della Laga), San Colombo, 67021 Barisciano (L'Aquila), Italy* **9** *Vicolo Crocefisso 44, 38056 Levico Terme (Trento), Italy* **10** *Facoltà di Scienze Agrarie, Ambientali e Alimentari, Libera Università di Bolzano, Piazza Università 5, 39100 Bolzano, Italy* **11** *Orto Botanico, Università di Napoli Federico II, Via Foria 223, 80139 Napoli, Italy* **12** *Dipartimento di Biologia, Università di Napoli Federico II, c/o Orto Botanico, Via Foria 223, 80139 Napoli, Italy* **13** *Dipartimento di Bioscienze e Territorio, Università del Molise, Via H. Hertz s.n.c., 86090 Pesche (Isernia), Italy* **14** *Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), Università di Torino, Largo P. Braccini 2, 10095 Grugliasco (Torino), Italy* **15** *Dipartimento di Agraria, Università di Sassari, Viale Italia 39a, 07100 Sassari, Italy* **16** *Dipartimento di Scienze della Vita, Università di Siena, Via P.A. Mattioli 4, 53100 Siena, Italy* **17** *Via San Francesco 29, 21018 Sesto Calende (Varese), Italy* **18** *Corso di Laurea in Conservazione e Gestione della Natura e dell'Ambiente, Università di Cagliari, Cittadella Universitaria Monserrato, S.P. Monserrato-Sestu km 0,700, 09042 Monserrato (Cagliari), Italy* **19** *Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Università della Campania Luigi Vanvitelli, Via A. Vivaldi 43, 81100 Caserta, Italy* **20** *Museo delle Scienze di Trento (MUSE), Corso del Lavoro e della Scienza 3, 38122 Trento, Italy* **21** *Sistema Museale di Ateneo, Università di Firenze, Via G. La Pira 4, 50121 Firenze, Italy*

Corresponding author: Gabriele Galasso (gabriele.galasso@comune.milano.it)

Academic editor: Lorenzo Peruzzi | Received 15 November 2024 | Accepted 25 November 2024 | Published 29 November 2024

Citation: Galasso G, Domina G, Adorni M, Alessandrini A, Ardenghi NMG, Bacchetta G, Barone G, Bartolucci F, Boker D, Calvia G, Cuena-Lombraña A, De Luca A, Del Guacchio E, Fois M, Fortini P, Lallai A, Lonati M, Lozano V, Marengo G, Mascia F, Mei G, Meneguzzo E, Nota G, Orrù I, Podda L, Quaranta L, Rocca R, Sarigu M, Stinca A, Valentini F, Lastrucci L (2024) Notulae to the Italian alien vascular flora: 18. Italian Botanist 18: 155–166. https://doi.org/10.3897/italianbotanist.18.141896

Abstract

In this contribution, new data concerning the distribution of vascular flora alien to Italy are presented. It includes new records, exclusions, and status changes from casual to naturalized or invasive for Italy or for Italian administrative regions for taxa in the genera *Acacia*, *Akebia*, *Allocasuarina*, *Aloë*, *Brachychiton*, *Convolvulus*, *Freesia*, *Gleditsia*, *Hesperocyparis*, *Kalanchoë*, *Liriope*, *Mazus*, *Melia*, *Phyla*, *Platycladus*, *Prunus*, and *Retama*. Nomenclatural and distribution updates, published elsewhere, and corrections are provided as Suppl. material 1.

Keywords

Alien species, floristic data, Italy, nomenclature

How to contribute

The text for the new records, status changes from casual to naturalized or invasive, exclusions, and confirmations should be submitted electronically to Lorenzo Lastrucci (lorenzo.lastrucci@unifi.it). The corresponding specimen along with its scan or photograph has to be sent to FI Herbarium: Museo di Storia Naturale (Botanica), Sistema Museale di Ateneo, Via G. La Pira 4, 50121 Firenze (Italy). Those texts concerning nomenclatural novelties and typifications (only for accepted names) should be submitted electronically to Gabriele Galasso (gabriele.galasso@comune.milano.it). Each text should be within 1,000 characters (spaces included).

Floristic records

Acacia melanoxylon R.Br. (Fabaceae)

+ (INV) **ITALIA** (**SAR**): San Teodoro (Gallura Nord-Est Sardegna), Monte Nieddu (WGS84: 40.750272°N, 9.589148°E), invasiva in ambiente ripariale, 370 m, 15 July 2024, *M. Fois, A. Cuena-Lombraña* (FI, CAG). – Status change from naturalized to invasive alien for the flora of Italy; status change from casual to invasive alien for the flora of Sardegna.

Despite being recently reported as a casual alien (Galasso et al. 2024), this species has to be considered invasive in Sardegna. Several individuals, in all life stages, have been observed spreading in some natural environments, especially under humid and shady conditions, in Rio Leni (Villacidro, Sud Sardegna province), Monte Arci (Pau, Oristano province), Monte Nieddu (San Teodoro, Gallura Nord-Est Sardegna province), and Berchida (Siniscola, Nuoro province).

M. Fois, A. Cuena-Lombraña

Akebia quinata (Thunm. ex Houtt.) Decne. (Lardizabalaceae)

+ (NAT) **ITALIA** (**TAA**): Castelnuovo (Trento), scarpata incolta al margine settentrionale della strada che collega la loc. Spagolle (Castelnuovo) con Via G. Gozzer (Borgo Valsugana), a E del ponte che attraversa il Torrente Moggio (WGS84: 46.05056°N, 11.47202°E), scarpata incolta, 380 m, SSE, 14 April 2024, leg. *F. Valentini, D. Boker, L.M. Boker*, det. *F. Valentini* (FI, TR). – Status change from casual to naturalized alien for the flora of Italy (Trentino-Alto Adige).

Akebia quinata was previously reported as a casual alien in Trentino-Alto Adige by Wilhalm (2011). This new population was first seen in 2023. It consists of a large population, with individuals growing from the soil to the upper part of the trees. In 2024 numerous flowering individuals were detected.

F. Valentini, D. Boker

Allocasuarina verticillata (Lam.) L.A.S.Johnson (Casuarinaceae)

+ (NAT) **ITALIA** (**SAR**): Domus de Maria (Sud Sardegna), antica strada romana (WGS84: 38.909013°N, 8.899185°E), naturalizzata nella macchia, 81 m, 5 May 2024, *A. Lallai, M. Sarigu, L. Podda* (FI, CAG). – Status change from casual to naturalized alien for the flora of Italy (Sardegna).

This species was introduced in Sardegna in the second half of the 19th century (Gennari 1874) and has been used as an ornamental tree in urban greenery and for reforestation in degraded contexts. The area where the species was found has been subject to reforestation by the Regional Forestry Agency since the end of the 1950s to the 1970s with several non-native species, such as *Acacia saligna* (Labill.) H.L.Wendl., *A. pycnantha* Benth., and *Brachychiton populneus* (Schott & Endl.) R.Br. The population recorded here covers a surface of about 100 m^2, each with at least fifty individuals, including adults, juveniles, and seedlings, arranged in two groups of individuals. Accordingly, *Allocasuarina verticillata* is to be considered naturalized.

A. Lallai, M. Sarigu

Aloë microstigma Salm-Dyck subsp. *microstigma* (Asphodelaceae)

+ (NAT) **ITALIA** (**SIC**): Lampedusa e Linosa (Agrigento), Isola di Lampedusa, Cala Francese (WGS84: 35.496217°N, 12.626316°E), rocky uncultivated land, 10 m, 20 March 2024, *G. Domina* (FI, SAF). – Naturalized alien species new for the flora of Italy (Sicilia).

This species is native to Cape Province (South Africa), and is used in horticulture as low maintenance garden plant. The new population probably comes from maintenance pruning remains from nearby houses. An additional population of a few individuals has been observed in Favignana (Trapani province) at Cala del Passo, 30 m a.s.l. on a cliff overhanging the sea. Plant samples were identified according to Klopper et al. (2023).

G. Barone, G. Domina

Brachychiton populneus **(Schott & Endl.) R.Br. (Malvaceae)**

+ (NAT) **ITALIA** (**SAR**): Domus de Maria (Sud Sardegna), antica strada romana (WGS84: 38.913738°N, 8.907798°E), naturalizzata nella macchia, 30 m, 5 May 2024, *L. Podda, G. Bacchetta* (FI, CAG). – Status change from casual to naturalized alien for the flora of Italy (Sardegna).

Since the first report in 2018 (Ruggero et al. 2024), several individuals of various ages have been observed naturalized within the coastal scrub vegetation together with other non-native species (*Allocasuarina verticillata* [Lam.] L.A.S.Johnson, *Acacia saligna* [Labill.] H.L.Wendl., and *A. pycnantha* Benth.) in an area subjected to reforestation by the Regional Forestry Agency until the 1970s.

L. Podda, G. Bacchetta

Convolvulus tricolor **L. subsp.** ***tricolor*** **(Convolvulaceae)**

– **CAM**. – Alien species to be excluded from the flora of Campania.

The individuals reported as *Convolvulus tricolor* s.l. from the Phlegrean Fields (Napoli province) (Terracciano 1917) are to be referred to *C. tricolor* subsp. *cupanianus* (Tod.) Cavara & Grande (Bartolucci et al. 2024). The plants from the island of Capri (Cerio 1939) were described as intermediate between the same native subspecies and the autonym, non-native, subspecies. As Cerio (1939) was not able to assess if the plant was escaped or indigenous, and no specimen collected by him is available (*Herbarium I. Cerio*, pers. obs.), *C. tricolor* subsp. *tricolor* is to be excluded from the flora of Campania.

E. Del Guacchio, A. De Luca

Freesia leichtlinii **Klatt subsp.** ***alba*** **(G.L.Mey.) J.C.Manning & Goldblatt (Iridaceae)**

+ (NAT) **SIC**: Castellammare del Golfo (Trapani), Monte Inici, Contrada Belvedere (WGS84: 38.030211°N, 12.873714°E), side road, 190 m, 16 March 2024, *R. Rocca* (FI). – Status change from casual to naturalized alien for the flora of Sicilia.

In Sicilia, this species was previously reported as a casual alien. The report of *Freesia refracta* (Jacq.) Eckl. ex Klatt on the Island of Pantelleria (Domina and Mazzola 2008) refers to this taxon. In Castellammare del Golfo we observed an abundant population, comprising numerous individuals of various ages, many seeding, extending along the road, spanning approximately 50 m^2. Not far from the main population two smaller nuclei were observed, less than 1 km away. Similarly, another population consisting of several dozens of individuals on a road embankment on the road SS514, in southeastern Sicilia (Chiaramonte Gulfi, Ragusa province, WGS84: 37.057117°N, 14.649669°E) was observed.

R. Rocca, G. Barone

Gleditsia triacanthos L. (Fabaceae)

+ (NAT) **MOL**: Campobasso (Campobasso), poco a S di Via Campania (WGS84: 41.557252°N, 14.677393°E), lungo un sentiero, 657 m, 8 May 2024, *P. Fortini, L. Quaranta* (FI, IS). – Naturalized alien species new for the flora of Molise.

Around 10 individuals approximately 10–12 m tall were found along the path in a peripheral area of Campobasso. At least five individuals are mature and abundantly producing fruit and a notable renewal of young plants has been observed all around.

P. Fortini, L. Quaranta

Hesperocyparis arizonica (Greene) Bartel (Cupressaceae)

+ (NAT) **UMB**: Orvieto (Terni), nei pressi del Pozzo di San Patrizio (WGS84: 42.722664°N, 12.120481°E), pareti rocciose verticali che dalla città digradano verso il basso, 260 m, 11 February 2024, *G. Marengo, M. Lonati* (FI). – Status change from casual to naturalized alien for the flora of Umbria.

This species grows in vertical cliffs near the urban area of Orvieto, and probably has originated from individuals cultivated in the nearby urban parks. The population is stable, consisting of several shrubby and fruit-bearing individuals.

G. Marengo, M. Lonati

Kalanchoë ×houghtonii D.B.Ward (Crassulaceae)

+ (NAT) **SIC**: Palermo (Palermo), Monte Pellegrino, W slope (WGS84: 38.158301°N, 13.345287°E), pine grove, 70 m, 14 April 2024, *R. Rocca* (FI). – Status change from casual to naturalized alien for the flora of Sicilia.

This nothotaxon was first reported as a casual alien in Sicilia in 2017 (Di Gregorio et al. 2017). We observed an expanding population in Monte Pellegrino, reproducing both sexually and through leaf propagules. Two additional populations were recorded: one on a cliff in the harbour of Terrasini (Palermo province, WGS84: 38.15599°N, 13.08188°E), the other in Palermo along the motorway spur route connecting to Tommaso Natale/Sferracavallo (WGS84: 38.191133°N, 13.283020°E). In 2017, numerous individuals of *Kalanchoë ×houghtonii* were observed on the roofs of the historic center of Palermo (G. Galasso pers. comm).

R. Rocca, G. Barone

Liriope spicata (Thunb.) Lour. (Asparagaceae)

+ (NAT) **SAR**: Senorbì (Sud Sardegna), loc. Funtana ‘e Canna (WGS84: 39.541880°N, 9.134429°E), area ruderale, 202 m, July 2024, *I. Orrù, F. Mascia* (FI). – Naturalized alien species new for the flora of Sardegna.

Individuals of the species have been found in ruderal areas, along the edges of the railroad, and originated from dispersed propagules of plants that were cultivated over 10 years ago in the garden of a cantonment house and no longer present today.

I. Orrù, F. Mascia

***Mazus pumilus* (Burm.f.) Steenis (Mazaceae)**

+ (NAT) **EMR**: Parma (Parma), Basilica di Santa Maria della Steccata (WGS84: 44.802658°N, 10.327379°E), tra le fessure del selciato, 53 m, 1 July 2024, *M. Adorni, A. Alessandrini* (FI). – Naturalized alien species new for the flora of Emilia-Romagna.

M. Adorni, A. Alessandrini

***Melia azedarach* L. (Meliaceae)**

+ (NAT) **SAR**: Oristano (Oristano), strada SP56 (WGS84: 39.92388°N, 8.58032°E), incolto, substrato sabbioso, presente lungo i bordi stradali, 9 m, 29 July 2024, leg. *V. Lozano, G. Brundu*, det. *G. Brundu* (FI, *Herb. Uniss Agraria*). – Status change from casual to naturalized alien for the flora of Sardegna.

The species was observed also in: Selargius (Cagliari) (WGS84: 39.253887°N, 9.173048°E), uncultivated land, 10 m, 9 June 2024, *L. Podda*; San Vito (Sud Sardegna), road SS125 'Orientale Sarda' (WGS84: 39.520588°N, 9.595178°E), roadside, 8 m, 15 June 2024, *L. Podda*; Cagliari (Cagliari), Viale G. Marconi (WGS84: 39.243090°N, 9.152730°E), roadside, 6 m, 5 July 2024, *L. Podda*; Oristano (Oristano), road SP81, Viale Repubblica 23 (WGS84: 39.90775°N, 8.56968°E), roadside, 9 m, 29 July 2024, *V. Lozano, G. Brundu*. The observed individuals probably originated from plants cultivated as ornamentals in adjacent gardens. In recent years, we have observed several naturalized adult individuals and seedlings in ecosystems subject to anthropogenic disturbance including the southern (particularly Campidano), central eastern (Sarrabus and Ogliastra) and central western parts (Oristano) of Sardegna.

V. Lozano, L. Podda

***Phyla canescens* (Kunth) Greene (Verbenaceae)**

+ (NAT) **LOM**: Gerenzano (Varese), Via R. Lepetit 34 (WGS84: 45.632838°N, 8.966914°E), verde curato all'interno dell'Insubrias BioPark, 227 m, 28 September 2016, *E. Meneguzzo* (FI, *Herb. E. Meneguzzo*). – Status change from casual to naturalized alien for the flora of Lombardia.

In recent years this population of *Phyla canescens* has expanded to occupy an area of approximately 200 m^2.

Enzo Meneguzzo, Nicola M.G. Ardenghi

***Platycladus orientalis* (L.) Franco (Cupressaceae)**

+ (NAT) **TAA**: Merano (Bolzano), loc. Quarazze (WGS84: 46.684801°N, 11.149760°E), vegetazione sinantropica di recupero delle scarpate e delle aree più disturbate della vegetazione forestale, 365 m, 8 March 2024, leg. *G. Mei, E. Grande*, det. *G. Mei, A. Stinca* (FI, *Herb. G. Mei*). – Status change from casual to naturalized alien for the flora of Trentino-Alto Adige.

The discovery of a population characterized by individuals of different ages, renewal and seedlings in the municipality of Merano, highlights how the species has now become naturalized in Trentino-Alto Adige.

G. Mei, A. Stinca

***Prunus amygdalus* Batsch (Rosaceae)**

+ (NAT) **SAR**: Quartu Sant'Elena (Cagliari), loc. Simbirizzi, incolti del lago (WGS84: 39.264670°N, 9.197378°E), naturalizzata negli incolti, 47 m, 25 July 2024, *G. Calvia, F. Mascia, G. Bacchetta* (FI, CAG). – Status change from casual to naturalized alien for the flora of Sardegna.

This species has been cultivated throughout the island since a long time, exhibiting little to no tendency to become wild. At most, it would often spread around the planted trees through vegetative propagation. However, in recent years we have observed adult and subadult specimens in areas far from old plantations, with regeneration occurring through seed propagation as well. This naturalization has been noted in several areas of the island, including both the southern (particularly Campidano and Marmilla) and northern (Logudoro and Mejlogu) parts.

G. Calvia, F. Mascia

***Retama monosperma* (L.) Boiss. subsp. *monosperma* (Fabaceae)**

+ (NAT) **ITALIA** (**LIG**): Ventimiglia (Imperia), fraz. Latte, lungo la strada SS1 Aurelia (WGS84: 43.795199°N, 7.571592°E), margine della strada, negli interstizi del muro di contenimento, 37 m, 20 February 2024, *M. Lonati, G. Nota* (FI). – Status change from casual to naturalized alien for the flora of Italy (Liguria).

The species colonizes uncultivated fields and abandoned terraces in the area between Latte and Bordighera (Imperia province), also settling in cracks of walls and sidewalks of urban infrastructures.

M. Lonati, G. Nota

Nomenclatural and distribution updates from other literature sources

Nomenclatural, status, and distribution updates according to Negri (1943), Schroeder (1972), Roma-Marzio et al. (2016), Paton et al. (2019), Moroni and O'Leary (2020), Knapp and Naczi (2021), Alterio et al. (2023), Jesus et al. (2023), Andreone and Guazzone (2024), Antonietti and Dellavedova (2024a, 2024b), Antonietti et al. (2024a, 2024b, 2024c), Bajona et al. (2024), Bonivento et al. (2024), Borghesan et al. (2024), Brusa (2024), Chepinoga et al. (2024), Đurović et al. (2024), Elvebakk and Bjerke

(2024), Fiaschi et al. (2024), Freire et al. (2024), Missouri Botanical Garden (2024 [onwards]), Pascale (2024), Pasta and Badalamenti (2024), Picco and Deandrea (2024), POWO (2024a [onwards], 2024b [onwards], 2024c [onwards], 2024d [onwards], 2024e [onwards], 2024f [onwards], 2024g [onwards], 2024h [onwards]), 2024i [onwards]), Roma-Marzio et al. (2024), Röser and Tkach (2024), Rota and Olivero (2024), Selvaggi and Dellavedova (2024), Sukhorukov et al. (2024), Verloove et al. (2024), Pasta et al. (2025), and corrections to Galasso et al. (2024), available at the Portal to the Flora of Italy (2024), are provided in Suppl. material 1.

G. Galasso, F. Bartolucci

Acknowledgements

We gratefully acknowledge the Acta Plantarum staff, Enrico Banfi, Lorenzo Peruzzi, Francesco Roma-Marzio, Rizzieri R. Masin, and Alberto Selvaggi who provided useful information.

References

Alterio E, Argenti C, Baro E, Bordin G, Campagnaro T, Camuffo A, Elerdini M, Favaro G, Filesi L, Iacopino S, Lattanzi E, Masin R, Perfetti M, Scariot A, Scortegagna S, Sitzia T, Trentanovi G, Villani M (2023) Segnalazioni floristiche venete: Tracheofite 595–631, Briofite 32–43. Natura Vicentina 23[2021–2022]: 71–95.

Andreone F, Guazzone M (2024) Nota n. 1304. *Hyacinthoides* ×*massartiana* Geerinck (Asparagaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 217.

Antonietti A, Dellavedova R (2024a) Nota n. 1286. *Nassella tenuissima* (Trin.) Barkworth (Poaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 213.

Antonietti A, Dellavedova R (2024b) Nota n. 1290. *Hosta ventricosa* Stearn (Asparagaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 214–215.

Antonietti A, Ardenghi NMG, Dellavedova R (2024a) Nota n. 1308. *Vitis* ×*novae-angliae* Fernald (Vitaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 218.

Antonietti A, Clemente F, Dellavedova R (2024b) Nota n. 1284. *Thladiantha dubia* Bunge (Cucurbitaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 212.

Antonietti A, Dellavedova R, Selvaggi A (2024c) Nota n. 1283. *Parthenocissus inserta* (A.Kern.) Fritsch (Vitaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 211–212.

Bajona E, Spadaro V, Raimondo FM (2024) From urban greenery and security hedging uses to naturalization: current and new status of some woody alien species in Sicily. In: 119°

Congresso della Società Botanica Italiana. X International Plant Science Conference (IPSC). Teramo, 11–13 September 2024, 45.

Bartolucci F, Domina G, Buccomino G, Ciaschetti G, Conti F, Costanza N, De Luca A, Del Guacchio E, Falcinelli F, Forte L, Galasso G, Ganz C, Iamonico D, Lonati M, Marengo G, Mei G, Nota G, Orsenigo S, Pazienza G, Pellegrino G, Pinzani L, Stinca A, Tavilla G, Tilia A, Tomaselli V, Tondi G, Venanzoni R, Lastrucci L (2024) Notulae to the Italian native vascular flora: 18. Italian Botanist 18: 97–108. https://doi.org/10.3897/italianbotanist.18.140958

Bonivento E, Selvaggi A, Cravello C, Stinca A, Soldano A (2024) Nota n. 1285. *Oxalis articulata* Savigny (Oxalidaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 212.

Borghesan S, Fasano F, Crippa A, Quaglini LA, Citterio S, Banfi E, Galasso G, Gentili R (2024) First record of *Klasea quinquefolia* (M.Bieb. ex Willd.) Greuter & Wagenitz (Asteraceae) in Italy. BioInvasions Records 13(3): 577–588. https://doi.org/10.3391/bir.2024.13.3.02

Brusa G (2024) I bambù negli habitat naturali e seminaturali: specie, distribuzione ed ecologia. Natura Bresciana 46[2023]: 5–46.

Cerio E (1939) Note sulla flora caprese. Archivio Botanico e Biogeografico Italiano 15(2): 134–147.

Chepinoga VV, Barkalov VY, Ebel AL, Knyazev MS, Baikov KS, Bobrov AA, Chkalov AV, Doronkin VM, Efimov PG, Friesen NV, German DA, Gontcharov AA, Grabovskaya-Borodina AE, Gureyeva II, Ivanenko YA, Kechaykin AA, Korobkov AA, Korolyuk EA, Kosachev PA, Kupriyanov AN, Luferov AN, Melnikov DG, Mikhailova MA, Nikiforova OD, Orlova LV, Ovchinnikova SV, Pinzhenina EA, Poliakova TA, Shekhovstsova IN, Shipunov AB, Shmakov AI, Smirnov SV, Tkach N, Troshkina VI, Tupitsyna NN, Vasjukov VM, Vlasova NV, Verkhozina AV, Anenkhonov OA, Efremov AN, Glazunov VA, Khoreva MG, Kiseleva TI, Krestov PV, Kryukova MV, Kuzmin IV, Lashchinskiy NN, Pospelov IN, Pospelova EB, Zolotareva NV, Sennikov AN (2024) Checklist of vascular plants of Asian Russia. Botanica Pacifica 13(sp.iss.): 3–310. https://doi.org/10.17581/bp.2024.13S01

Di Gregorio G, Bellone G, Cassanego E, Longo D, Montalto S, Montoleone E, Tomasello G (2017) Rassegna di segnalazioni notevoli riguardanti la Sicilia. Acta Plantarum Notes 5: 70–74.

Domina G, Mazzola P (2008) Flora ornamentale delle isole circumsiciliane. Quaderni di Botanica Ambientale e Applicata 19: 107–119.

Đurović SZ, Temunović M, Schönswetter P, Frajman B (2024) Polyploidisation and niche differentiation drive the diversification of the *Euphorbia epithymoides* group (Euphorbiaceae) in southeastern Europe. Perspectives in Plant Ecology, Evolution and Systematics 65: 125825. https://doi.org/10.1016/j.ppees.2024.125825

Elvebakk A, Bjerke JW (2024) *Papaver* recircumscribed: a review of neighbouring Papaveraceae genera, including *Afropapaver* nom. et stat. nov. and *Oreomecon*, a large, Arctic-Alpine genus. PhytoKeys 248: 105–188. https://doi.org/10.3897/phytokeys.248.121011

Fiaschi T, Bonari G, Frignani F, Gizzi G, Landi M, Magrini S, Quilghini G, Pafumi E, Scoppola A, Angiolini C (2024) Vascular flora of the isthmus of Feniglia (southern Tuscany, Italy). Italian Botanist 17: 77–101. https://doi.org/10.3897/italianbotanist.17.122982

Freire SE, Grossi MA, Bayón ND, Monti C, Iharlegui L, Migoya MA (2024) Taxonomic revision and morphometric analysis of *Gamochaeta* (Asteraceae, Gnaphalieae). Annals of the Missouri Botanical Garden 109: 244–320. https://doi.org/10.3417/2024836

Galasso G, Conti F, Peruzzi L, Alessandrini A, Ardenghi NMG, Bacchetta G, Banfi E, Barberis G, Bernardo L, Bouvet D, Bovio M, Castello M, Cecchi L, Del Guacchio E, Domina G, Fascetti S, Gallo L, Guarino R, Gubellini L, Guiggi A, Hofmann N, Iberite M, Jiménez-Mejías P, Longo D, Marchetti D, Martini F, Masin RR, Medagli P, Musarella CM, Peccenini S, Podda L, Prosser F, Roma-Marzio F, Rosati L, Santangelo A, Scoppola A, Selvaggi A, Selvi F, Soldano A, Stinca A, Wagensommer RP, Wilhalm T, Bartolucci F (2024) A second update to the checklist of the vascular flora alien to Italy. Plant Biosystems 158(2): 297–340. https://doi.org/10.1080/11263504.2024.2320129

Gennari P (1874) La guida dell'Orto Botanico della R. Università di Cagliari. Tip. Edit. dell'Avvenire di Sardegna, Cagliari.

Jesus A, Bonhomme V, Evin A, Soteras R, Jacomet S, Bouby L, Antolin F (2023) Morphometrics of waterlogged archaeological seeds give new insights into the domestication and spread of *Papaver somniferum* L. in western Europe. PLOS One 18(5): e0286190. https://doi.org/10.1371/journal.pone.0286190

Klopper RR, Grace OM, Klopper AW, Smith GF, Wyk AEV (2023) A taxonomic revision of *Aloe* sect. *Purpurascentes* (Asphodelaceae subfam. Alooideae). Phytotaxa 628(1): 1–64. https://doi.org/10.11646/phytotaxa.628.1.1

Knapp WM, Naczi RFC (2021) Vascular plants of Maryland, USA. A comprehensive account of the state's botanical diversity. Smithsonian Contributions to Botany 113: 1–151. https://doi.org/10.5479/si.14605674

Missouri Botanical Garden (2024) [onwards] *Amelanchier lamarckii*. In: Missouri Botanical Garden. Plant Finder. Missouri Botanical Garden, St. Louis. https://www.missouribotanicalgarden.org/PlantFinder/PlantFinderDetails.aspx?kempercode=a403 [Accessed 02.10.2024]

Moroni P, O'Leary N (2020) Insights into the systematics of tribe Duranteae (Verbenaceae) II: a taxonomic revision of the New World genus *Duranta*. Annals of the Missouri Botanical Garden 105(4): 502–577. https://doi.org/10.3417/2020581

Negri G (1943) A proposito di alcune nuove località di *Loranthus europaeus* L. in Italia. Nuovo Giornale Botanico Italiano, n.s. 50(1–2): 149–152. https://doi.org/10.1080/11263504309439862

Pascale M (2024) Nota n. 1282. *Iris pallida* Lam. (Iridaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 211.

Pasta S, Badalamenti E (2024) Found but lost? The short-lived success of *Crataegus coccinea* L. (Rosales: Rosaceae) in north-eastern Sicily (Italy). Natural History Sciences 11(2): 65–69. https://doi.org/10.4081/nhs.2024.758

Pasta S, Lo Cascio P, Badalamenti E (2025) Additional data on the ongoing naturalization of the non-native woody plant *Duranta erecta* (Verbenaceae) in Sicily, Italy. Acta Botanica Croatica 84(1). [e-first] https://doi.org/10.37427/botcro-2025-009

Paton AJ, Mwanyambo M, Govaerts RHA, Smitha K, Suddee S, Phillipson PB, Wilson TC, Forster PI, Culham A (2019) Nomenclatural changes in *Coleus* and *Plectranthus* (Lamiaceae): a tale of more than two genera. PhytoKeys 129: 1–158. https://doi.org/10.3897/phytokeys.129.34988

Picco F, Deandrea G (2024) Nota n. 1309. *Acalypha australis* L. (Euphorbiaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 218–219.

Portal to the Flora of Italy (2024) Portale della Flora d'Italia/Portal to the Flora of Italy. 2024.2. https://dryades.units.it/floritaly/ [Accessed 06.10.2024]

POWO (2024a) [onwards] *Anisodontea* × *hypomadara* (Sprague) D.M.Bates. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:558945-1 [Accessed 27.06.2024]

POWO (2024b) [onwards] *Bambusa vulgaris* Schrad. ex J.C.Wendl. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:392574-1 [Accessed 17.06.2024]

POWO (2024c) [onwards] *Brachychiton populneus* (Schott & Endl.) R.Br. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:822268-1 [Accessed 27.06.2024]

POWO (2024d) [onwards] *Hibiscus ×rosa-sinensis* L. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:560756-1 [Accessed 27.06.2024]

POWO (2024e) [onwards] *Paeonia ×suffruticosa* Andrews. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:711877-1 [Accessed 24.06.2024]

POWO (2024f) [onwards] *Panicum gattingeri* Nash. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. [Accessed 01.10.2024]

POWO (2024g) [onwards] *Papaver somniferum* subsp. *somniferum*. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:111359-3 [Accessed 05.11.2024]

POWO (2024h) [onwards] *Phyllostachys edulis* (Carrière) J.Houz. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:415998-1 [Accessed 17.06.2024]

POWO (2024i) [onwards] *Prunus amygdalus* Batsch. In: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:60439867-2 [Accessed 15.10.2024]

Roma-Marzio F, Ardenghi NMG, Argenti E, Banfi E, Campagnolo G, Ceschin S, D'Ascanio M, Di Lernia D, Falcidia G, Fanfarillo E, Fiaschi T, Galasso G, Giardini M, Gurau M, Kleih M, Iamonico D, Olivieri N, Meneguzzo E, Pelella E, Perolini D, Pinzani L, Lastrucci L (2024) Nuove segnalazioni floristiche italiane 17. Flora vascolare (189–215). Notiziario della Società Botanica Italiana 8: 1–7. [e-first]

Roma-Marzio F, Bedini G, Müller JV, Peruzzi L (2016) A critical checklist of the woody flora of Tuscany (Italy). Phytotaxa 287(1): 1–135. https://doi.org/10.11646/phytotaxa.287.1.1

Röser M, Tkach N (2024) Delimitation and nomenclature of *Agrostis*, *Polypogon* and related grasses (Poaceae subfamily Pooideae). Schlechtendalia 41: 63–67. https://doi.org/10.25673/116223

Rota F, Olivero G (2024) Nota n. 1280. *Leonurus sibiricus* L. (Lamiaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 210.

Ruggero A, Trainito E, Bacchetta G, Podda L, Lallai A, Mascia F, Manca M, Bertotto G, Calvia G (2024) Contribution to a new vascular flora of Sardinia (Italy): I (1–30). Flora Mediterranea 34: 13–46. https://doi.org/10.7320/FlMedit34.013

Schroeder F-G (1972) *Amelanchier*-Arten als Neophyten in Europa. Abhandlungen des Naturwissenschaftlichen Vereins zu Bremen 37(3): 287–419.

Selvaggi A, Dellavedova R (2024) Nota n. 1281. *Liriodendron tulipifera* L. (Magnoliaceae). In: Selvaggi A, Soldano A, Pascale M, Dellavedova R (Eds) Note floristiche piemontesi n. 1243–1314. Rivista Piemontese di Storia Naturale 45: 210–211.

Sukhorukov AP, Kushunina MA, Stepanova NY, Kalmykova OG, Golovanov YM, Sennikov AN (2024) Taxonomic inventory and distributions of Chenopodiaceae (Amaranthaceae s.l.) in Orenburg Region, Russia. Biodiversity Data Journal 12: e121541. https://doi.org/10.3897/BDJ.12.e121541

Terracciano N (1917) Aggiunta alla "Flora dei Campi Flegrei". Atti del Real Istituto di Incoraggiamento alle Scienze Naturali di Napoli, s. 7 68[1916]: 269–450.

Verloove F, Prosser F, Busnardo G, Leliaert F (2024) Morphological and molecular data confirm the first European record of *Sagina maxima* (Caryophyllaceae) in Bassano del Grappa (Veneto region, northeastern Italy). BioInvasions Records 13(2): 305–317. https://doi.org/10.3391/bir.2024.13.2.02

Wilhalm T (2011) Ergänzungen und Korrekturen zum Katalog der Gefäßpflanzen (4). Gredleriana 11: 71–82.

Supplementary material 1

Supplementary data

Authors: Gabriele Galasso, Fabrizio Bartolucci

Data type: pdf

Explanation note: 1. Nomenclatural updates; 2. Note updates; 3. Distribution updates; 4. Synonyms, misapplied or included names.

Link: https://doi.org/10.3897/italianbotanist.18.141896.suppl1

sustainability

Article

The Environmental Footprint of Scientific Research: Proposals and Actions to Increase Sustainability and Traceability

Margherita Palmieri [1], Bruno Lasserre [1], Davide Marino [1], Luca Quaranta [1], Maxence Raffi [2] and Giancarlo Ranalli [1,*]

[1] Department of Bioscience and Territory, University of Molise, C.da Fonte Lappone snc, 86090 Pesche, Italy
[2] Sustainability Strategy Senior Consultant, 14 Rue du Grenier, Saint Lazare, 75003 Paris, France
* Correspondence: ranalli@unimol.it; Tel.: +39-0874404155

Abstract: In their work, scientists are responsible for stating a purpose, defining experimental conditions, producing data, describing and analyzing these data by statistical means, arguing by comparison with the literature, and drawing conclusions. When a manuscript is submitted for publication, no assessment of the direct and indirect environmental impacts of producing the work is reported. In this context, the aim of our study is to suggest schematic methods to assess, reduce, and mitigate the impact of greenhouse gas (GHG) emissions generated by three different papers (two scientific articles and a conference proceeding) previously published by the same authors. The results reported here show proposals and actions to contribute to the reduction of environmental impacts: a preliminary assessment of the inputs and outputs was initially converted into CO_2 equivalents (CO_2 eq.) and, subsequently, into partial mitigation action through the allocation of the planting of additional plants. Furthermore, real traceability and an opportunity to verify the close connection between initial works (papers and conference book) causing the environmental impact and new green life are suggested. Finally, we propose a new label (*S-Paper to T-Plant*) for eco-friendly guidelines.

Keywords: environmental footprint; scientific research; CO_2 equivalent; mitigate impact; social costs of carbon; traceability

Citation: Palmieri, M.; Lasserre, B.; Marino, D.; Quaranta, L.; Raffi, M.; Ranalli, G. The Environmental Footprint of Scientific Research: Proposals and Actions to Increase Sustainability and Traceability. *Sustainability* **2023**, *15*, 5616. https://doi.org/10.3390/su15075616

Academic Editors: Kyle Maurice Woosnam and Barbara Sacchi

Received: 17 January 2023
Revised: 2 March 2023
Accepted: 21 March 2023
Published: 23 March 2023

1. Introduction

1.1. Foreword

As a scientific researcher that works daily … "I live, I consume, I am, I am a worker; if I am a worker involved in research on climate change, I consume myself." Therefore, I ask myself the following: "Is everything allowed to me if I work in research on climate change issues as well?" Climate change is a global phenomenon; working as a scientist in a laboratory also contributes to (negative) impacts on a global level through the use of natural resources such as water, energy, and materials, and the production of entropy heat, waste, CO_2, etc. As a scientific researcher working in this field, is everything allowed? Further, are all activities allowable without any prior reflection or analysis of the possible environmental impacts of my actions? For example, is any evaluation between different laboratory analytical methods required? Is all of this markedly imbued with ambiguity and hypocrisy disguised as true and scientific aims? Just because I am a researcher, is everything permissible?

Limitations and restrictions are present today in the ethical field on the use of animal and human organisms. The environmental impacts and downstream implications of scientific research work are not addressed, even when the results are made public (publications, conferences, patents, etc.). Any analysis upstream, during, or downstream of the related environmental impact is omitted. Even more limited are any calculations or quantifications of potential, desirable, necessary, and due environmental relief as forms of mitigation [1].

Although scientists recognize the importance of being more efficient in the use of resources and avoiding the negative impact of the overall research effort [2,3], their work is concerned with outlining the scope, defining experimental conditions, producing data, describing and analyzing these data by statistical means, arguing against the literature, and drawing conclusions. At the time of publication, ethical requirements must be stated and met. Nevertheless, when a manuscript is submitted for publishing, no assessment of the direct and indirect environmental impacts of carrying out the work is reported.

As a rule, in the Section 2, we describe in detail the reagents required, the supporting equipment, and the references. However, at first, there is no critical analysis of the choice between different methods from the point of view of environmental sustainability, consideration of the upstream effects of reagents (production, toxicity, etc.), or consideration of downstream effects in the disposal phase after use. In addition, attention is not given to the consumption of exhaustible environmental resources, such as energy and water, litter (from paper, glass, plastic, etc.), toxic waste, and wastewater. In a globalized world, every human action contributes to the impact on the environment in terms of energy consumption, CO_2 emissions, the use of natural resources, potentially dangerous substances, and waste, so even research works are not excluded, and their effects on sustainable development and climate change should be highly regarded.

Here, we express our position on this matter, and we report proposals that include evaluations of the environmental fingerprint following scientific work (ready to publish) and conference papers. These are not considered as a way to "make peace with it, make penance," but instead are a way to "pay" with activities that are designed to mitigate the negative environmental impact of our previous work damage.

An environmental footprint can be a scientific academic term. Evidence of the impact that a business's activities have on the environment include its resource environment and pollution emissions. Examples include (i) the depletion of natural resources; (ii) noise and aesthetic impacts; (iii) residual air and water emissions; (iv) long-term waste disposal; (v) uncompensated health effects; and (vi) changes in the local quality of life. The environmental footprint cited here by the authors of this paper, although starting from a complex scientific concept, is treated in the existing research as a common environmental footprint and includes the water footprint, carbon footprint, etc.

1.2. Background: The Environmental Impact of Scientific Research (the Context)

The environmental and social implications of climate change depend not only on the Earth's systemic responses but also on impacts generated by human activities [4]. The impacts of such activities influence the planetary boundaries that define the safe operating space for humanity with respect to Earth's system. In particular, the concentration of carbon dioxide is negatively affecting climate change; it increased from a pre-industrial value of 280 ppm to 398.5 ppm in 2015, with a proposed threshold limit of 350 ppm [5].

In fact, the IPCC (*Intergovernmental Panel on Climate Change*) emphasizes the aim of decarbonization of economic processes, and more recently (in 2019), the European Commission adopted the Green Deal with the ambitious goal of achieving climate neutrality by 2050 [6].

While scientific research, which encompasses numerous disciplines, has the task of advancing society, it also causes high environmental costs due to laboratory activities. Indeed, research and the activities of researchers also contribute to greenhouse gas (GHG) emissions and resource consumption [7]. A laboratory, for example, requires a large amount of energy as its consumption per square meter is 5 to 10 times that of office buildings [8]. Some of the most common laboratory equipment in use in scientific research (e.g., fume hoods, ultra-low-temperature freezers, and autoclaves) are, in fact, among the largest consumers of energy [9].

Biological, medical, and agricultural research activities also have an impact on the environment: in 2014, 20,500 institutions engaged in this field produced around 5.5 million tons of plastic waste annually [10]. Regarding research conducted in a university context, Achten et al. in 2013 estimated the carbon footprint of a PhD project to be 21.5 tons of CO_2 equivalents (tCO_2 eq.), of which emissions caused by transport accounted for 75%, conference attendance for 20%, and finally, infrastructure for 5% [3].

The pharmaceutical industry globally generates 55% more carbon than the automotive industry [11]. Healthcare sector research (which includes both hospitals and laboratories) generated a climate footprint of 2 giga tCO_2 emissions in 2019, accounting for 4.4% of emissions globally [12]. A literature study not only highlighted the importance of estimating the C eq. footprint generated by clinical laboratory activities, but it also suggested methods to improve environmental performance [13].

Globally, there are several voluntary (nonprofit) initiatives that encourage the adoption of low-environmental-impact measures in laboratories, such as the International Institute for Sustainable Laboratories (I2SL), S-Labs, and My Green Lab (the latter pathway was undertaken in 2020 by the pharmaceutical company Astrazeneca) [2]. The transition to sustainability by laboratories should include a more responsible approach to science by adopting measures that limit negative externalities on the environment in terms of consumption and pollution. Sharing space and equipment, for example, can save energy (fewer building utilities, less ventilation equipment, etc.), as well as the production, disposal, and transportation of multiple types of equipment and waste at the end of their lives [13].

Research conducted in laboratories that have embarked on low-environmental-impact paths may, therefore, also be more efficient at optimizing the financial resources on which research is based. Optimizing energy and equipment costs means not only having less impact on the environment but also investing in human resources (scientists and researchers) and increasing the quality of scientific production. Funding lines could be directed toward those laboratories that implement ecological standards and, thus, optimize the use of different resources (environmental, human, and financial).

In the context of applied research in the field of biorestoration, sustainable restoration and conservation practices are becoming increasingly popular, not only to contain the effect of climate change on the environment but also to ensure greater operator safety with fewer hazardous products and greater respect for works of art [14,15]. In this sense, the use of more sustainable strategies in the field of restoration stems from the Cultural Heritage reflections inspired by the Venice Charter in 1964. The Strategic Innovation and Research Agenda (SIRA) of the Bio-Based Industry Consortium (2017) also emphasizes the transition toward products and practices that consider environmental, social, and economic aspects [16].

The use of more sustainable experimental approaches is now widely shared by restorers around the world. In a survey conducted in the United States, it was found that more than 60% of restorers believed their working practices were potentially harmful to the environment, with the greatest concern being the use and disposal of solvents, chemicals, and hazardous materials [17].

Today, there are a few studies that use different methodologies for assessing the carbon footprint of research activities. For example, a team of researchers in France developed a tool (open source) to allow laboratories to determine their carbon footprint [18]. A frequently used method is LCA (life cycle assessment) for assessing the life cycle of processes [19]. The scale of analysis also differs. For example, some research has assessed the impacts of departments [20,21], with others assessing those related to conferences [22–24] or individual research projects [3]. In addition, there are a few studies proposing instruments to mitigate the impact of research activities and the technology sector in general [25,26].

Based on this context, we propose an experimental and innovative model to quantify CO_2 emissions and compensate for impacts through the planting of trees with a traceability system (QR code).

1.3. Objective of this Paper

The objective of this paper is to propose a methodological path to carry out a critical analysis of the environmental impacts resulting from both laboratory activities (the results of which are the subject of a scientific publication) and conference proceedings.

This analysis accounts for impacts expressed in CO_2 eq. emissions resulting from the comparison of different laboratory analytical methodologies, instrumentation, the use (or non-use) of chemicals (e.g., reagents), resources (e.g., water), waste production, and energy consumption.

At the conclusion of the assessment, the analysis involves a method for compensating for environmental damage by calculating a conversion calculation for the number of trees to be planted to sequester the quantities of CO_2 eq. emitted into the atmosphere. Knowing the amount of CO_2 eq. emitted is also useful for estimating both the damage and related social costs caused by pollutant emissions and the compensation in terms of carbon credits.

We develop and apply this to real cases with two methodological frameworks for assessing CO_2 eq. emissions in two contexts: laboratory activities (Section 2.1) and a conference proceeding (Section 2.2). Moreover, we propose how to assess, reduce, and mitigate the impacts of the works. We indicate actions to increase the full sustainability of the works carried out using examples of real mitigation tracking and the creation of QR codes linked to planting interventions. Finally, we propose a new label to illustrate the actions described above.

2. Materials and Methods

2.1. Methodological Framework for Assessing Impacts: Scientific Research

We proposed a methodological framework for assessing the impacts of experimental laboratory activities that could be replicated and adapted to different research fields. A step-by-step scheme applicable to laboratory research is shown in Figure 1.

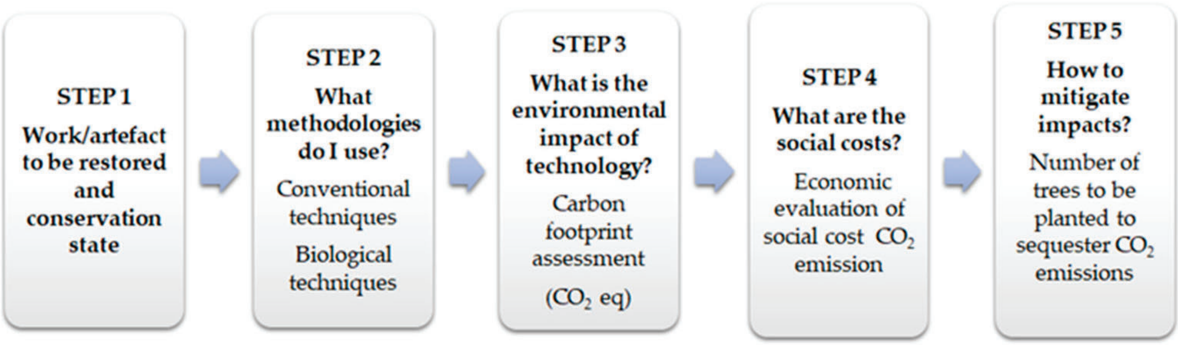


Figure 1. Methodological framework for the evaluation of CO_2 emissions applied to scientific research.

Step 1 was to clarify the objective of the investigation and, in this specific case, the type of work or artifact to be restored and its state of conservation (step 1).

This made it possible to identify the most appropriate restoration and conservation interventions based on the characteristics and problems related to the artifact (step 2).

Step 3 involved assessing the environmental impact of each type of intervention in terms of CO_2 emissions from the consumption of goods and resources and the production of waste (step 3). We considered waste from scientific laboratories (chemical, biological, etc.), which is generally classified as hazardous waste (according to safety standards) and is not suitable for recycling.

For the economic evaluation of CO_2 emissions (step 4), there were two approaches: one was based on the social cost of carbon (SCC), and the other on the market value of emission permits. Specifically, SCC is defined as the marginal cost caused by the emission of one ton of greenhouse gas (carbon dioxide) in one year. This cost also represents the value of damages avoided by reducing or compensating for emissions, i.e., the benefit of reducing atmospheric CO_2 emissions [27]. Ricke et al. estimated that the SCC globally was between United States Dollar (USD) 177 and 805 per ton of CO_2 (tCO_2) (average value of USD 417) [28]. The market value is the price established by the market for emission permits, and, according to the European Climate Exchange, the price ranged from USD 153 per tCO_2 in 2008 to USD 84.01 per tCO_2 in 2022 (January 2022). In this paper, we estimated the social cost of carbon considering EPA estimates (2020) [27] as equal to USD 42 (EUR 40.05 discounted to 2022). This value was multiplied by the CO_2 eq. tons emitted from laboratory activities and from conferences.

Finally, an estimate was made of the number of trees to be planted in compensation for the CO_2 emissions produced by the experimental laboratory activities (step 5).

To make this framework applicable, we proposed a survey sheet (Table 1) useful for the inventory of each identified methodology and the different parameters of laboratory activities, which could be traced to energy consumption, chemicals, equipment, and waste. For each parameter, the unit of measurement and the value taken should be indicated, as this was information that could be translated into CO_2 eq. emissions and was useful for estimating the social costs and the mitigation of impacts in terms of tree planting.

Table 1. Survey sheet for data collection.

Type Consumptions	Evaluation of CO_2 eq. Emission				
	Parameter	Unit	Amount	K Conversion to CO_2	CO_2 Yield (kg)

2.2. Methodological Framework for Assessing Impacts: A Conference

To assess CO_2 eq. conference emissions, a GHG protocol was used in the bibliography [29,30], which differentiated three scopes: GHG emissions from sources controlled by the facility or organization (scope 1); indirect emissions related to energy that was not produced within the structure (scope 2); and finally, other indirect emissions, including travel, catering, and waste generation (scope 3).

From this context, to evaluate the emissions of a conference, we developed a methodological framework divided into four steps (Figure 2).

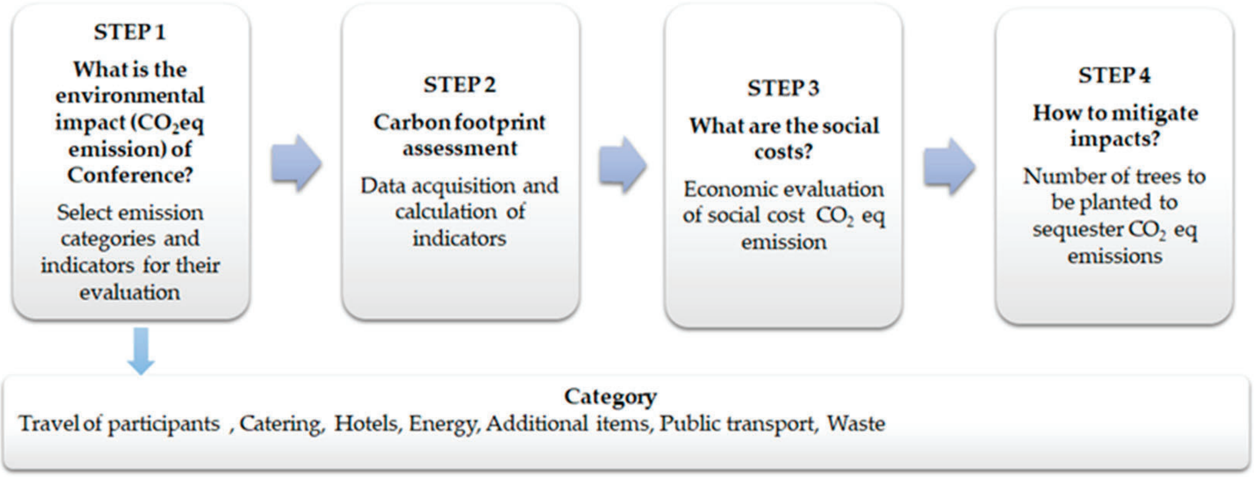


Figure 2. Methodological framework suggested for the evaluation of CO_2 emissions applied to a conference.

Step 1 provided for the selection of impact categories identified by the GHG protocol and related indicators. The selection of these categories depended on the availability of the data underlying the calculation of the indicators.

Step 2 involved quantifying the carbon footprint based on available data. Specifically, according to Table 1, emissions were calculated by multiplying activity data (e.g., passenger travel kilometers, electricity consumption, fuel consumption, etc.) by the appropriate emission factors. The last two steps followed the same methodological procedure described in Figure 1 (Section 2.1). In particular, after estimating the SCC by multiplying CO_2 emissions by the economic coefficient [27] (step 3), we quantified the number of trees to be planted to sequester the CO_2 emitted by the conference (step 4).

3. Results

3.1. Case Studies: Two Scientific Manuscripts Recently Published by the Authors

The results of the application of the above approaches on how to assess, reduce, and mitigate the environmental impacts for two case studies are reported in Tables 2 and 3.

Table 2. Main indicators suggested for calculating the environmental footprint originating from a research activity, impacts, and green mitigation acts. Data applied to a paper by Ranalli et al., 2021 [1], Department of Biosciences and Territory (DiBT), University of Molise, Pesche, Italy.

Type Consumptions	**Evaluation of CO_2 eq. Emission**				
	Parameter	**Unit**	**Amount**	**K Conversion to CO_2**	**CO_2 Yield (kg)**
Energy (electric power by fossil)	Equipment (instruments, sterile hoods, fans, cooling, sterilization, others).	kWh	600	0.224 [31]	134.40
	Tap water	m^3	12.5	0.32 [32]	4.00
Chemical products	Reagents (dried cultural media; antibiotics, acid and basic solutions, others).	kg, liter	35	1.47 [32]	51.40
	Toxic, hazard (solvents).	kg	2	0.62 [32]	1.24
Wastes	Plastic	kg	20	1.74 [32]	34.80
	Glass	kg	4	0.85 [32]	3.40
	Paper	kg	2	2.42 [32]	4.84
	Effluents	m^3	11.0	0.29 [33]	3.20
Transports personnel and samples/materials [34] (Bus and Car, diesel)		km	850	0.121 [35]	102.85
CO_2 eq. total emissions (kg)		340.13 (0.34 t)			
Social Costs (€)		13.62			
Mitigation Action [36]	N° of plants to be planted: 4 41°60′74.57″ N 14°26′46.43″ E	Which type (*Ilex aquifolium* L.); when (autumn); where (DiBT, Unimol, Pesche, IT); how (manually); who provides (expert gardeners); control and guarantee of the time (Green Service, DiBT).			

The methodology suggested in Table 2 and Figure 3 is applied to scientific research carried out on both laboratory and onsite scales, published by Ranalli et al., 2021 [1].

(**a**) *Ilex aquifolium* tree (**b**) QR code by Ranalli et al., 2021.

Figure 3. (**a**) Example of an *I. aquifolium* (var. *argentea marginata*) planted; (**b**) QR code traceability between environmental impact of manuscript [1] and mitigation action by *I. aquifolium* plantation.

3.1.1. Plant Mitigation Actions 1

Plants offer great biodiversity. Supported by botanic experts, we selected the following in case 1:

- *Ilex aquifolium* L. (var. *argentea marginata*) species (called Agrifoglio), belonging to the Aquifoliaceae Family. This is an evergreen tree or shrub that grows up to 10 m tall with shiny, dark green, decorative, variegated foliage that does not renew simultaneously. The reddish-colored fruits provide a decorative contrast to the color of the leathery, spiny-margined leaves on the lower branches of young plants. It contains saponins, the xanthine theobromine, and a yellow pigment, ilexanthine [37]. Nowadays, it is rarely used in herbal medicine due to its toxicity; however, it has diuretic, febrifuge, and laxative properties [38].
- K conversion kg CO_2 eq. to the number of plants, 100:1 [36].

Table 3 and Figure 4 reports data from the same methodology when applied to the scientific laboratory research of Aquilano et al., 2022 [39].

(**a**) *Taxus baccata* tree (**b**) QR code by Aquilano et al., 2022.

Figure 4. (**a**) Example of a *T. baccata* planted; (**b**) QR code for traceability between environmental impact of manuscript [21] and mitigation action by *T. baccata* plantation.

Table 3. Main indicators suggested for calculating the environmental footprint originating from a research activity, impacts, and green mitigation acts. Data applied to paper by Aquilano et al., 2022 [39], DiBT, University of Molise, Pesche, Italy.

Type Consumptions	**Evaluation of CO_2 eq. Emission**				
	Parameter	**Unit**	**Amount**	**K Conversion to CO_2**	**CO_2 Yield (kg)**
Energy (electric power by fossil)	Equipment (instruments, sterile hoods, fans, cooling, sterilization, others)	kWh	800	0.224 [31]	179.20
	Tap water	m^3	22.0	0.32 [32]	7.04
Chemical products	Reagents (dried cultural media; antibiotics, acid and basic solutions, others)	kg, liter	50	1.47 [32]	73.00
	Toxic, hazard (solvents)	kg	1	0.62 [32]	0.62
Wastes	Plastic (Petri dishes)	kg	30	1.74 [32]	52.20
	Glass	kg	2	0.85 [32]	1.70
	Paper	kg	2	2.42 [32]	4.84
	Effluents	m^3	20.0	0.29 [33]	5.80
Transports personnel and samples/materials (Car, gasoline) [34]		km	800	0.12 [35]	96.80
CO_2 eq. total emissions (kg)		421.20 (0.421 t)			
Social Costs (€)		17.00			
Mitigation Action [36]	N° of plants to be planted: 4 41°60′75.90″ N 14°26′49.46″ E	Which type (*Taxus baccata* L.); when (autumn); where (DiBT, Unimol, IT); how (manually); who provides (expert gardeners); control and guarantee of the time (Green Service, DiBT).			

3.1.2. Plant Mitigation Actions 2

Plants offer great biodiversity. Supported by botanic experts, we selected the following in case 2:

- *Taxus baccata* L. is a tree of the conifer order, widely used as an ornamental hedge or isolated plant. It is also known as the "tree of death." The active ingredient responsible for the toxicity of branches, leaves, and seeds, where it is present in percentages varying between 0.5 and 2%, is an alkaloid taxin. It has a narcotic and paralyzing effect on humans and many domestic animals. The organs that contain the most of it are the old leaves.
- K conversion kg CO_2 eq. to the number of plants, 100:1 [36].

3.2. Case Study: A Conference

The selected case is a conference titled "Lo Stato dell'Arte 20" (The State of Art20) of the Italian Group of International Institute for Conservation (IGIIC), 13–15 October 2022, at Campobasso, Italy (Table 4 and Figure 5) [40]. The scientific committee decided to collect the extended scientific contributions of the authors in traditional forms, such as an abstract book printed on A4 size paper (24 × 32 cm), all in black/white ink, and only the cover of the book in color. Each volume contains 472 pages, weighs 1.8 kg, and 200 copies were printed [41]. A copy of the book was inserted into a natural textile bag for each participant registered at the conference (more than 100 attended). Flyers on conference communications were printed in color, in A3 format.

Table 4. Main indicators suggested for calculating the environmental footprint originating from the conference titled "Lo Stato dell'Arte20" (The State of Art20), IGIIC, 13–15 October 2022, Campobasso, Italy [40]. DiBT, Università del Molise, Pesche, Italy.

Type Consumptions	**Evaluation of CO_2 eq. Emission**				
	Parameter	**Unit**	**Amount**	**K Conversion to CO_2**	**CO_2 Yield (kg)**
Energy (electric power by fossil)	Equipment (services)	kWh	40	0.22 [31]	8.80
	Toxic, hazard	kg	0	0.62 [32]	0
Wastes	Plastic	kg	5	1.74 [32]	8.70
	Paper b/w (abstract book: 200 copies, 1.8 kg each)	kg	360	2.42 [32]	871.20
	Colored paper (flyer, cover of books)	kg	10	13.60 [32]	136.00
	Effluents	m^3	2	0.29 [33]	0.58
CO_2 eq. total emissions (kg)		1009 (1.01 t)			
Social Costs (€)		40.50			
Mitigation Action [36]	N° of plants to be planted: 11 DiBT, Unimol, IT. 41°60′71.82″ N 14°26′46.28″ E	Which type (*Ostrya carpinifolia* Scop.); when (early winter); how (manually); who provides (expert gardeners); control and guarantee of the time (Green Service, DiBT).			

(**a**) USB key only for Poster Session IGIIC (**b**) *O. carpinifolia* and QR code Book Conference

Figure 5. (**a**) USB key including only posters presented at the conference *Lo Stato dell'Arte20* (IGIIC, 13–15 October 2022, Campobasso, Italy) [40]; (**b**) QR code traceability between environmental impact of abstract book (conference IGIIC) and mitigation action by *O. carpinifolia* plantation.

Authors that contributed a scientific poster to the conference were invited to send a digital version in advance; the poster discussion was carried out on a digital totem where authors were invited to summarize the aims and the results obtained to date.

In addition, information on local transport, what to see, what to eat, and where to sleep was furnished on separate sheets of paper.

Plant Mitigation Actions 3

Plants offer great biodiversity. Supported by botanic experts, we selected the following in case 3:

- *Ostrya carpinifolia* Scop. is a tree in the family Betulaceae. It is the only species of the genus *Ostrya* native to Europe. It is a medium-sized deciduous tree, which can reach up to 20–25 m tall, with a conical or irregular crown and scaly, rough bark. The wood is very heavy and hard and was historically used to fashion plane soles.
- K conversion kg CO_2 eq. to the number of plants, 100:1 [36].

3.3. Impact of This Manuscript

At the end of the writing of this manuscript, the method described above for how to assess, reduce, and mitigate environmental impacts was applied. Therefore, considering the notes in Tables 1 and 2, we estimated a value of less than 100 kg of CO_2 eq. emissions. An additional *T. baccata* tree was planted in an area adjacent to the previous plants (Section 3.1.2).

3.4. New Label

In Figure 6, a new label that schematizes the *S-Paper to T-Plant* action is reported.

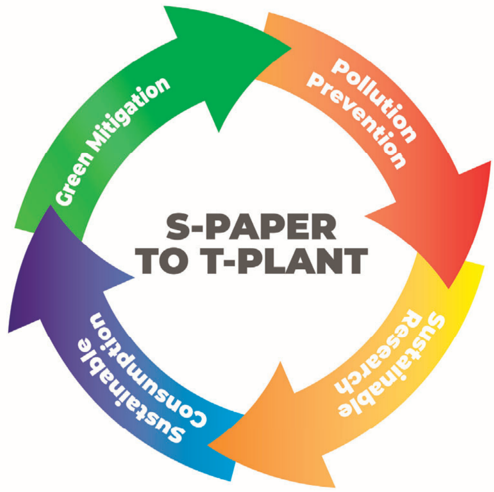


Figure 6. A new label that schematizes the Scientific Paper to Traceability Plant (*S-Paper to T-Plant*) action proposed.

The *S-Paper to T-Plant* action is a proposal to reduce the negative impacts and global greenhouse gas emissions caused by research activities in green, healthy, and environmentally friendly systems.

By putting our research activities on a sustainable path, the *S-Paper to T-Plant* action aims to involve new stakeholders and contribute to accelerating the global transition to a much-needed sustainable new lifestyle. We can contribute to having a neutral or positive environmental impact, helping to mitigate climate change, and adapting to its impacts. This can reverse the loss of biodiversity and preserve the natural ecosystem to ensure natural resources (soil and water) for food security, nutrition, and public health are protected.

Communication is currently an aspect of great importance; communicating clearly and correctly is an added value to communication. We believe that proposing an intuitive logo with text and keywords is a way to reinforce the value of the objectives set.

We hope that the proposed logo can meet at least part of the initial aspirations, and therefore, we believe it is realistic to start a patenting phase.

4. Discussion

In this paper, three examples of the application of the methodological frameworks are described. Two of these are the results of research published by the authors of this paper and relate to laboratory and in situ microbiological techniques applied to a case of dry biocleaning of artworks [1] and to the effect of EOs versus *E. coli* in natural water [39].

The third case was an evaluation of CO_2 emissions and mitigation actions after a conference on scientific activities (printed book) in cultural heritage sectors. This decision was made by a majority vote of the Scientific Committee of the conference, although it was objected to by one of the authors of this paper, who was a member of the same committee.

The results showed that, depending on the techniques used, emissions could vary greatly between sectors. However, in both cases of the research reported [1,31], energy consumption and the transportation of personnel and materials had the largest impacts (Tables 2 and 3). The presence of equipment in laboratories was among the predominant causes of electrical and thermal energy consumption and waste. Such consumption could be reduced by using equipment that is more efficient and by optimizing its use.

Regarding transportation, this referred mainly to field activities in which researchers collected useful information to calibrate, test, and validate methods developed in research.

For the conference, however, what impacted it the most was waste originating mainly from the printing of papers (Table 4). These emissions could be greatly reduced by publishing the conference proceedings online or on digital memory devices (USB keys). Reducing paper consumption could lead not only to a reduction in CO_2 emissions but also in NO_x, CO, and SO_x emissions (responsible for global warming), as well as lower water consumption.

According to the SSC method, the estimated economic costs were EUR 13.60 (USD 14.45) and EUR 17.00 (USD 18.10) for [1,21], respectively; furthermore, the estimated economic cost was EUR 40.50 (USD 43.0) for the IGIIC Conference [40].

To contribute to compensation for environmental damage produced by the emission of CO_2 into the atmosphere as a result of our work, we proposed the planting of new trees.

As cited above (Tables 1–3), the plant mitigation actions were based on the use of a coefficient (K) conversion of 100 kg CO_2 eq. to one plant (i.e., 100:1). This value was fixed, ranging between large interval values, including 80–150, as well as lower and higher values, and was influenced by several factors (type of soil, environmental temperature, rain, etc.), as reported in the literature [26]. In addition, a young (2–3-year-old) broadleaf plant was suggested; this is important because, at ten years, evidence of carbon sequestration equivalent to the amount of CO_2 in woody biomass is confirmed.

Moreover, these trees, which produce oxygen and absorb CO_2 when planted in urban areas, could contribute several further benefits: (i) they remove particulate matter (PM_{10}) emitted by anthropogenic activities; (ii) they regulate the local microclimate and mitigate the heat island effect that occurs on summer days with high temperatures; and (iii) the QR code labels on each tree informs people about the frontiers of knowledge being explored by research activities in several fields at local academic institutions.

A further advantage is contributing to sensitizing people to the environmental impact of our daily activities and increasing respect for environmental resources, nature preservation, and conservation.

This same model and path could then be followed by other public and private entities.

The costs of these mitigation and traceability activities need to be foreseen at an early stage of project drafting and budgeted and accounted for in the financial plan. Therefore, in the final phase, the budget can be accounted for as an expenditure item (expenditures).

The examples described above are not limited cases. In fact, at the request of the paper's authors, a new, structured initiative is being launched at the DiBT, University of Molise, with the identification of a green area (*Green Park*) in the neighboring area for the planting of additional plants to contribute to the mitigation of the impact of scientific works produced annually by researchers belonging to the same organization.

A new model of a departmental forest is being created to which researchers may adhere, initially on a voluntary basis and, subsequently, on a mandatory basis. For this proposal, the Council of the Department of Biosciences and Territory already gave a preliminary favorable opinion at its meeting in December 2022, followed by confirmation in February 2023.

Finally, it is important to remark that these environmental impact assessment activities must involve specialists from many disciplines (chemistry, biology, agronomy, botany, forestry, economics, and others).

5. Conclusions

In conclusion, in this study, we proposed schematic procedures and models for how to assess, reduce, and mitigate the greenhouse gas (GHG) emission impacts generated, for example, by three different previously published works (two scientific papers and one conference), which were well-known by the authors.

In addition, the analysis included a method of compensating for environmental damage through a conversion calculation for the number of trees to be planted to sequester the amount of CO_2 eq. emitted by a scientific work. This point of view should be considered as an initial and partial contribution to compensate for and mitigate the negative environmental impacts caused by conducted work.

The additional innovative content of this work is to help raise awareness of the impacts in terms of CO_2 emissions from the world of research and, in addition, to suggest mitigation actions with traceable plantings, not only locally but also globally. Examples of direct traceability between scientific papers and plants were given, with a new label (*S-Paper to T-Plant*) as sustainable environmental guidelines.

A new Green Park model linked to a department's scientific activities is being created to become a real reference for other communities.

We are aware that a comprehensive assessment of the environmental impacts caused by businesses and economic activities includes the analysis of more environmental parameters and areas.

The limitations of this research may be the quality and quantity of the input data on the types of consumption from research activities and conferences. A systematic collection of different types of consumption could allow a more precise estimation of CO_2 emissions and their mitigation.

We are truly convinced that the results presented here represent a small, new contribution to raising awareness of the need for greater respect for limited environmental resources, even on the part of those involved in research.

We hope that, even in the field of research, for both the upstream and downstream effects of experiments and publications, there will be a greater focus on environmental issues, with the inclusion of a final paragraph in the text of this manuscript regarding these aspects.

The proposal fits fully with the actions of the European Green Deal and Agenda 2030 for Sustainable Development, which suggest actions to reduce anthropogenic impacts on the environment.

Author Contributions: Conceptualization, M.P. and G.R.; methodology, investigation, and data curation, M.P., D.M., B.L. and G.R.; writing—original draft preparation, writing—review and editing, M.P., B.L., L.Q., M.R. and G.R.; supervision, D.M. and G.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received internal funding from the University of Molise, titled *Nature, environment and forest: from theory to practice*, years 2021–2022. DiBT 27/22. Furthermore, it was partially supported by *Safety materials, technologies and procedures for bio-conservation of Cultural Heritage*—Safebiotech project, Prin-Mur, 3/2022, Italy.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to acknowledge P. Fortini for supporting the selection of trees for mitigation action (Garden of the Apennine Flora of Capracotta—*Museo Giardino della Flora Appenninica*, Capracotta, Italy). Furthermore, we would like to thank M. Calvitto, M. Varone, and L. Sallustio for kindly providing support with the digital QR Codes. We also thank D. Rullo, E. Spoldi, and L. Appolonia (IGIIC) for kindly providing information on the "State of Art20" conference. We sincerely thank I. Doganieri and S. Ciccone for their support with the administration of the funding and procurement management.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ranalli, G.; Bosch-Roig, P.; Crudele, S.; Rampazzi, L.; Corti, C.; Zanardini, E. Dry Biocleaning of Artwork: An Innovative Methodology for Cultural Heritage Recovery? *Microb. Cell* **2021**, *8*, 91–105. [CrossRef] [PubMed]
2. Greever, C.; Ramirez-Aguilar, K.; Connelly, J. Connections between Laboratory Research and Climate Change: What Scientists and Policy Makers Can Do to Reduce Environmental Impacts. *FEBS Lett.* **2020**, *594*, 3079–3085. [CrossRef] [PubMed]
3. Achten, W.M.J.; Almeida, J.; Muys, B. Carbon Footprint of Science: More than Flying. *Ecol. Indic.* **2013**, *34*, 352–355. [CrossRef]
4. Moss, R.H.; Edmonds, J.A.; Hibbard, K.A.; Manning, M.R.; Rose, S.K.; Van Vuuren, D.P.; Carter, T.R.; Emori, S.; Kainuma, M.; Kram, T.; et al. The next Generation of Scenarios for Climate Change Research and Assessment. *Nature* **2010**, *463*, 747–756. [CrossRef] [PubMed]
5. Rockström, J.; Steffen, W.; Noone, K.; Persson, Å.; Chapin, F.S.; Lambin, E.F.; Lenton, T.M.; Scheffer, M.; Folke, C.; Schellnhuber, H.J.; et al. A Safe Operating Space for Humanity. *Nature* **2009**, *461*, 472–475. [CrossRef]
6. 2019 Refinement to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories—IPCC. Available online: https://www.ipcc.ch/report/2019-refinement-to-the-2006-ipcc-guidelines-for-national-greenhouse-gas-inventories/ (accessed on 2 March 2023).
7. Cluzel, F.; Vallet, F.; Leroy, Y.; Rebours, P. Reflecting on the Environmental Impact of Research Activities: An Exploratory Study. *Procedia CIRP* **2020**, *90*, 754–758. [CrossRef]
8. Babu, S.; Lamano, A.; Pawar, P. Sustainability Assessment of a Laboratory Building: Case Study of Highest Rated Laboratory Building in Singapore Using Green Mark Rating System. *Energy Procedia* **2017**, *122*, 751–756. [CrossRef]
9. Ni, K.; Hu, Y.; Ye, X.; AlZubi, H.S.; Goddard, P.; Alkahtani, M. Carbon Footprint Modeling of a Clinical Lab. *Energies* **2018**, *11*, 3105. [CrossRef]
10. Urbina, M.A.; Watts, A.J.R.; Reardon, E.E. Labs Should Cut Plastic Waste Too. *Nature* **2015**, *528*, 479. [CrossRef]
11. Belkhir, L.; Elmeligi, A. Carbon Footprint of the Global Pharmaceutical Industry and Relative Impact of Its Major Players. *J. Clean. Prod.* **2019**, *214*, 185–194. [CrossRef]
12. Karliner, J.; Slotterback, S.; Boyd, R.; Ashby, B.; Steele, K.; Karliner, J.; Slotterback, S.; Boyd, R.; Ashby, B.; Steele, K. *Health Care's Climate Footprint*; Health Care Without Harm: Reston, VA, USA, 2019; pp. 1–48.
13. Lopez, J.B.; Badrick, T. Proposals for the Mitigation of the Environmental Impact of Clinical Laboratories. *Clin. Chem. Lab. Med. (CCLM)* **2012**, *50*, 1559–1564. [CrossRef] [PubMed]
14. Joseph, E. *Microorganisms in the Deterioration and Preservation of Cultural Heritage*; Springer: Cham, Switzerland, 2021; ISBN 9783030694104.
15. De Silva, M.; Henderson, J. Sustainability in Conservation Practice. *J. Inst. Conserv.* **2011**, *34*, 5–15. [CrossRef]
16. The Strategic Innovation and Research Agenda (SIRA). Available online: https://biconsortium.eu/about/our-vision-strategy/sira (accessed on 17 January 2023).
17. Silence, P. How Are US Conservators Going Green? *Results of Polling AIC Members. Stud. Conserv.* **2010**, *55*, 159–163. [CrossRef]
18. Mariette, J.; Blanchard, O.; Berné, O.; Aumont, O.; Carrey, J.; Ligozat, A.; Lellouch, E.; Roche, P.-E.; Guennebaud, G.; Thanwerdas, J.; et al. An Open-Source Tool to Assess the Carbon Footprint of Research. *Environ. Res. Infrastruct. Sustain.* **2022**, *2*, 035008. [CrossRef]
19. Kroft, S.H. A Different Kind of Laboratory Stewardship. *Am. J. Clin. Pathol.* **2021**, *156*, 493–494. [CrossRef]
20. Güereca, L.P.; Torres, N.; Noyola, A. Carbon Footprint as a Basis for a Cleaner Research Institute in Mexico. *J. Clean. Prod.* **2013**, *47*, 396–403. [CrossRef]
21. Wynes, S.; Donner, S.D.; Tannason, S.; Nabors, N. Academic Air Travel Has a Limited Influence on Professional Success. *J. Clean. Prod.* **2019**, *226*, 959–967. [CrossRef]
22. Spinellis, D.; Louridas, P. The Carbon Footprint of Conference Papers. *PLoS ONE* **2013**, *8*, e66508. [CrossRef]
23. Desiere, S. The Carbon Footprint of Academic Conferences: Evidence from the 14th EAAE Congress in Slovenia. *EuroChoices* **2016**, *15*, 56–61. [CrossRef]
24. Stroud, J.T.; Feeley, K.J. Responsible Academia: Optimizing Conference Locations to Minimize Greenhouse Gas Emissions. *Ecography* **2015**, *38*, 402–404. [CrossRef]
25. Yusuf, E.; Luijendijk, A.; Roo-Brand, G.; Friedrich, A.W. The Unintended Contribution of Clinical Microbiology Laboratories to Climate Change and Mitigation Strategies: A Combination of Descriptive Study, Short Survey, Literature Review and Opinion. *Clin. Microbiol. Infect.* **2022**, *28*, 1245–1250. [CrossRef] [PubMed]
26. Chen, L.; Msigwa, G.; Yang, M.; Osman, A.I.; Fawzy, S.; Rooney, D.W.; Yap, P.-S. Strategies to Achieve a Carbon Neutral Society: A Review. *Environ. Chem. Lett.* **2022**, *20*, 2277–2310. [CrossRef] [PubMed]
27. United States Government. *Technical Support Document: Technical Update of the Social Cost of Carbon for Regulatory Impact Analysis under Executive Order 12866*; Environmental Protection Agency: Washington, DC, USA, 2015; pp. 65–88.
28. Ricke, K.; Drouet, L.; Caldeira, K.; Tavoni, M. Country-Level Social Cost of Carbon. *Nat. Clim Chang.* **2018**, *8*, 895–900. [CrossRef]
29. *The Greenhouse Gas Protocol: A Corporate Accounting and Reporting Standard*; WBCSD; WRI: Washington, DC, USA, 2012; ISBN 1-56973-568-9.
30. IWG. *Technical Support Document: Social Cost of Carbon, Methane, and Nitrous Oxide; Interim Estimates under Executive Order 13990*; The White House: Singapore, 2021; Volume 48.
31. Material Flow Accounts Statistics—Material Footprints. Available online: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Material_flow_accounts_statistics_-_material_footprints (accessed on 17 January 2023).

32. Emission Factors in Kg CO_2-Equivalent per Unit. Search–City of Winnipeg. 2012. Available online: https://legacy.winnipeg.ca/search/default.stm?q=682+-+2012#gsc.tab=0&gsc.q=Emission%20factors%20in%20kg%20CO2-equivalent%20per%20unit%20682%20-%202012&gsc.sort= (accessed on 17 January 2023).
33. Wang, J.; Karliner, J.; Slotterback, S.; Boyd, R.; Ashby, B.; Steele, K.; Wang, J. Contribution and Opportunities for Action 17. In *E Workshop: Returning Disease Prevention and Health Promotion Back to the Table: From the 1980ies to the 2050ies*; Oxford University Press: Oxford, UK, 2020; Volume 2020.
34. ENEA. *Rapporto Energia e Ambiente 2005*; ENEA: Stockholm, Sweden, 2005; ISBN 9788882862138.
35. CO_2 Performance of New Passenger Cars in Europe. Available online: https://www.eea.europa.eu/ims/co2-performance-of-new-passenger (accessed on 17 January 2023).
36. Forestazione Urbana: Alberi a Campobasso (CB). Available online: https://www.reteclima.it/sink/forestazione-urbana-alberi-a-campobasso-cb/ (accessed on 17 January 2023).
37. Hoppe, H.A. *Angiospermen*; De Gruyter: Berlin, Germany, 1975; ISBN 978-3-11-003849-1.
38. Wren, R.C. *Potter's Cyclopedia of Botanical Drugs and Preparations*; Potter & Clarke: London, UK, 1988; pp. 1–256.
39. Aquilano, C.; Baccari, L.; Caprari, C.; Divino, F.; Fantasma, F.; Saviano, G.; Ranalli, G. Effects of EOs vs. Antibiotics on *E. coli* Strains Isolated from Drinking Waters of Grazing Animals in the Upper Molise Region, Italy. *Molecules* **2022**, *27*, 8177. [CrossRef] [PubMed]
40. Lo Stato dell'Arte 20—Atti del XX Congresso Nazionale IGIIC. Available online: https://www.nardinieditore.it/prodotto/lo-stato-arte-20-congresso-nazionale-igiic/ (accessed on 17 January 2023).
41. Gioventù, E.; Ranalli, G.; Vittorini Orgeas, E. *Il Biorestauro. Batteri per La Conservazione Delle Opere d'Arte. Biopulitura e Bioconsolidamento*; Nardini Press srl: Firenze, Italy, 2020; ISBN 88-404-0136-9.

ACKNOWLEDGEMENTS

I would like to extend my heartfelt thanks to the CNR-IRET of Porano (TR) in the person of the director Dr. Carlo Calfapietra and in the person of Dr. Claudia Mattioni, for giving me the opportunity to carry out part of the activities related to my doctorate at the Genetics laboratory. I would also like to thank Dr. Marcello Cherubini, for her support during the laboratory analysis and Dr Claudia Mattioni for the interpretation and discussion of the results. I would also like to thank the Senkenberg Institute of Frankfurt in the person of Prof. Thomas Hickler, for hosting me during my abroad period in which I carried out statistical analyses related to the study of traits. Finally I would like to heartfelt thanks the Professor Gabriella Stefania Scippa for giving me the opportunity to cooperate with her laboratory in the NRRP project and supporting my research analysis.