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Ph.D. thesis

NEW INSIGHTS INTO THE BIOLOGY AND ECOLOGY OF THE INSECT VECTORS OF APPLE PROLIFERATION FOR THE DEVELOPMENT OF SUSTAINABLE CONTROL STRATEGIES

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“Nella vita non c’è nulla da temere,

c’è solo da capire.”

(M. Curie)

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SUMMARY

Phytoplasmas are pleomorphic wall-less prokaryotes related to bacteria belonging to the *Mollicutes* class and are characterized by a very small genome. The lack of essential biosynthetic pathways makes them obligate parasites of plants and insect vectors. In plants, these pathogens are restricted to the phloem tissue and cause symptoms suggesting profound disorders in the normal balance of hormones. In insects, their effects on fitness can range from detrimental to beneficial, depending on the evolutionary relationships. Phytoplasmas are associated with diseases of hundreds of plant species worldwide, including many economically important crops, fruit trees, and ornamental plants. Insect vectors represent the principal means of phytoplasma transmission and interactions between phytoplasmas and insect hosts are, in some cases, very specific and involve a complex sequence of events. Only phloem-feeding insects like leafhoppers, planthoppers, and psyllids can potentially acquire and transmit these obligate parasites.

'*Candidatus* Phytoplasma mali' is the etiological agent of Apple proliferation (AP) disease, which represents one of the most severe problems in European apple orchards. In Trentino, one of the main apple producing regions in Italy, AP is the major threat for the production. The main symptoms are witches' brooms, enlarged stipules, and early leaf reddening in autumn. The fruits of infected trees, which are smaller and have altered organoleptic properties, cannot be commercialized. So far, two psyllid species, *Cacospylla picta* and *Cacopsylla melanoneura* (Homoptera: Psyllidae), are confirmed vectors, but their actual role in the epidemiology of AP is still debated, as studies conducted in different geographical regions show different transmission efficiencies.

This research is part of a project launched in Trentino after a serious outbreak of the disease reported in Valsugana (southeastern Trentino) in 2011. The main objectives of this work regarded the study of epidemiological traits of the disease and biological features of the insect vectors. The first aim was monitoring the disease spread and vector populations' dynamics, evaluating the natural infection level of psyllids and some transmission parameters, such as acquisition capacity, transmission efficiency, and the possibility of a transovarial phytoplasma transmission. The distribution of infected plants was mapped along Valsugana and the populations of the psyllid vectors were monitored in the period 2014-2016. After a three-year survey, the percentage of symptomatic apple plants drastically decreased. Regarding the psyllid vectors, *C. melanoneura* showed higher population levels compared to *C. picta* in both conventional and untreated orchards, but the percentages of infected individuals were higher in

the latter species. The transmission parameters were evaluated in psyllids during acquisition and transmission trials carried out with *C. picta* and *C. melanoneura*. Specific trials were conducted with *C. picta* to assess the vertical transmission of the phytoplasma. Experiments, conducted under semi-field and greenhouse conditions in spring and summer 2015 and 2016, involved overwintered adults of both species collected in Valsugana and nymphs and emigrant adults reared on infected apple plants. After each experiment, insects and test plants were analyzed by real-time PCR to assess the phytoplasma presence. Results confirm *C. picta* as a more competent '*Ca.* P. mali' vector in Trentino, but suggest the possibility of acquiring and transmitting the phytoplasma also for *C. melanoneura*, even though with a low efficiency. For both species, overwintered adults were able to transmit the disease only after an acquisition period spent on infected plants and the acquiring capacity of the stages developed on infected apple plants was very high. Moreover, for the first time was demonstrated that infected *C. picta* individuals are able to transmit '*Ca.* P. mali' to the progeny.

Another goal of this research was to unravel the tri-trophic relationship involving phytoplasma and its hosts by the study of the genetic diversity of phytoplasma isolates in plants and insect vectors and their geographical distribution. So, a genetic characterization of phytoplasma was conducted, and isolates obtained from insects and apple plants collected in different geographical areas were analyzed by a multilocus sequence typing method based on four phytoplasma genes. The results obtained are only partial, but the different '*Ca.* P. mali' genotypes observed so far indicate a higher genetic diversity in insects compared to host plants and suggest the hypothesis of specific relationships between phytoplasma genotypes and insect vectors.

New insights on psyllid ethology were achieved. In particular, a research was conducted to investigate the vibrational communication involved in reproductive behavior. For the two vector species, ethological observations and laser vibrometer recordings of the vibrational signals emitted during courtship were carried out. Signals appeared to be species-specific, but they did not seem to be a prerequisite for courtship and mating. Moreover, as already seen in other psyllid species, a scanning electron microscopy investigation showed the presence of a stridulatory mechanism on thorax and wings of both species.

Finally, as other homopteran species are known to be phytoplasma vectors, a goal of this research was to look for potential new vectors of AP phytoplasma, characterizing the leafhoppers' and planthoppers' communities in apple orchards of Valsugana and studying the effect of surrounding landscapes on their distribution.

Samplings were conducted in apple orchards surrounded by different landscapes and in different habitats inside the orchard. The results of this study indicate that landscapes influence

the species richness and, regarding habitats, that grasses are visited by higher numbers of species and individuals in all landscapes considered. All insect collected were tested by real-time PCR and results indicate that three samples belonging to three different species tested positive to '*Ca.* P. mali'.

Research conducted in this thesis drew a picture of AP disease spread in Valsugana. Results obtained in all the topics described above, from epidemiological studies to phytoplasma genetic variability, passing through vectors' courtship behavior and agroecosystem biodiversity, represent the theoretical background that helps advisors and growers to optimize the current disease management, and researchers to develop innovative and sustainable control strategies.

RIASSUNTO

I fitoplasmi sono procarioti pleomorfi, privi di parete cellulare, simili a batteri ed appartenenti alla classe *Mollicutes* e sono caratterizzati da un genoma molto ridotto. L'assenza di vie metaboliche essenziali li rende parassiti obbligati di piante ed insetti. Nelle prime, la presenza di questi patogeni è limitata ai tessuti floematici e causa sintomi associati a profondi disordini nel normale bilancio ormonale. Negli insetti, gli effetti dei fitoplasmi sulla *fitness* sono vari e, a seconda delle relazioni evolutive, possono essere deleteri o benefici. I fitoplasmi sono associati a centinaia di patologie di specie vegetali distribuite in tutto il mondo, comprese colture di elevate importanza economica, fruttiferi e anche piante ornamentali. Il principale mezzo di trasmissione di questi patogeni è costituito da insetti vettori e le interazioni tra fitoplasmi ed insetti sono, in alcuni casi, molto specifiche e coinvolgono una complessa catena di eventi. Solo fitomizi floematici, come cicadomorfi, fulgoromorfi e psille hanno il potenziale per acquisire e trasmettere questi parassiti obbligati.

'*Candidatus* Phytoplasma mali' è l'agente eziologico di Apple proliferation (AP), una malattina che rappresenta uno dei più gravi problemi nei meleti europei. In Trentino, una delle principali regioni produttrici di mele in Italia, AP è una minaccia per la produzione. I sintomi più evidenti sono la presenza di scopazzi sui rami, di stipole ingrandite nelle foglie e di arrossamenti anticipati delle chiome in autunno. I frutti degli alberi infetti, che hanno dimensioni inferiori e proprietà organolettiche alterate, non possono essere commercializzati. Finora due psille, *Cacospylla picta* e *Cacopsylla melanoneura* (Homoptera: Psyllidae), sono state confermate vettori della malattia, ma il loro reale ruolo nell'epidemiologia di AP è ancora oggetto di discussione, dal momento che studi condotti in aree geografiche diverse mostrano efficienze di trasmissione differenti.

Questa ricerca si inserisce all'interno di un progetto avviato in Trentino dopo che un'esplosione della fitoplasmosi è stata segnalata in Valsugana (Trentino sud-orientale) nel 2011. I principali obiettivi di questa ricerca hanno riguardato lo studio di alcuni aspetti dell'epidemiologia di AP alcune caratteristiche biologiche degli insetti vettori. Il primo scopo è stato quello di monitorare l'avanzamento della malattia e le dinamiche di popolazione dei vettori, valutando le percentuali di infezione naturale delle psille e alcuni parametri relativi al processo di trasmissione, come la capacità di acquisizione, l'efficienza di trasmissione e la possibilità di trasmissione transovarica del fitoplasma. La distribuzione delle piante infette è stata mappata lungo la Valsugana e le popolazioni delle psille vettrici sono state monitorate tra il 2014 e il 2016. Dopo tre anni di rilievi, si è ottenuta una drastica diminuzione della percentuale di piante sintomatiche. Riguardo

gli insetti vettori, *C. melanoneura* si è rivelata essere la specie con densità di popolazione più elevate sia in frutteti coltivati, sia in frutteti abbandonati, ma la specie con percentuali di infettività maggiori è risultata essere *C. picta*. I parametri di trasmissione per entrambe le specie di psilla sono stati valutati attraverso prove di acquisizione e trasmissione e prove specifiche sono state condotte per verificare se vi è trasmissione transovarica del fitoplasma in *C. picta*. Gli esperimenti condotti in condizioni di semi-campo e serra, durante la primavera e l'estate del 2015 e del 2016, hanno riguardato adulti svernanti di entrambe le specie (campionati in Valsugana) e ninfe e adulti di nuova generazione allevati su piante di melo infette. Alla fine di ogni esperimento, piante e insetti sono stati analizzati tramite real-time PCR per verificare la presenza di fitoplasma. I risultati confermano che *C. picta* è un vettore molto più efficiente in Trentino, ma suggeriscono la possibilità di acquisizione e trasmissione del fitoplamsa anche in *C. melanoneura*, anche se con una bassa efficienza. Per entrambe le specie, gli adulti svernanti sono stati in grado di trasmettere il fitoplasma solo dopo aver trascorso un periodo di acquisizione su piante infette e la capacità di acquisizione degli stadi che si sono sviluppati su piante infette è stata molto elevata. In più, è stata dimostrato per la prima volta che individui infetti di *C. picta* sono capaci di trasmettere '*Ca.* P. mali' alla progenie.

Uno degli obiettivi di questa ricerca era lo studio del rapporto tritrofico tra fitoplasma e i suoi ospiti, attraverso indagini sulla diversità genetica del fitoplasma in piante e insetti vettori e sulla loro distribuzione geografica. Quindi il fitoplasma è stato caratterizzato geneticamente e gli isolati ottenuti da piante di melo e insetti raccolti in differenti aree geografiche sono stati analizzati attraverso un metodo di *multilocus sequencing typing* basato su quattro geni del fitoplasma. I risultati ottenuti sono solo parziali, ma i differenti genotipi di '*Ca.* P. mali' ottenuti finora indicano la presenza di un'elevata variabilità genetica negli insetti rispetto alle piante, che suggerisce l'ipotesi dell'esistenza di relazioni specifiche tra genotipi di fitoplasma e insetti vettori.

Nuove informazioni sono state ottenute sull'etologia delle psille, in particolare per quanto riguarda il comportamento riproduttivo. Per questo, sono state condotte osservazioni etologiche e registrazioni dei segnali vibrazionali emessi durante il corteggiamento attraverso l'uso di un laser vibrometro. Il segnale sembra essere specie-specifico, ma non sembra essere un prerequisito per il corteggiamento e l'accoppiamento. Inoltre, come già visto in altri psillidi, indagini al microscopio elettronico a scansione hanno evidenziato la presenza di strutture stridulatorie su torace e ali di entrambe le specie.

Infine, poiché è noto che altre specie di omotteri sono vettori di fitoplasmosi, un obiettivo di questo studio è stato la ricerca di potenziali nuovi vettori di '*Ca.* P. mali', caratterizzando allo

stesso tempo le comunità di cicadomorfi e fulgoromorfi dei meleti della Valsugana e valutando l'effetto dei paesaggi circostanti sulla loro distribuzione.
I campionamento sono stati condotti in meleti circondati da diversi paesaggi e anche all'interno di diversi habitat nello stesso meleto. I risultati di questo studio indicano che i paesaggi influenzano la ricchezza in specie e che, riguardo gli habitat, il cotico erboso del frutteto è visitato da numeri più elevati di specie e individui in tutti i paesaggi considerati. Tutti gli individui raccolti sono stati analizzati tramite real-time PCR e i risultati indicano che almeno due specie di cicaline sono state capaci di acquisire '*Ca.* P. mali'.

Le ricerche condotte in questa tesi hanno permesso di tracciare un quadro diffusione di AP in Valsugana. I risultati ottenuti nei diversi ambiti di ricerca descritti sopra, dagli studi epidemiologici e di variabilità genetica, passando attraverso il comportamento sessuale dei vettori e la biodiversità nell'agroecosistema, rappresentano le basi teoriche per aiutare consulenti tecnici e agricoltori ad ottimizzare l'attuale gestione della malattia e i ricercatori a sviluppare strategie di controllo innovative e sostenibili.

INTRODUCTION

Phytoplasmas

Phytoplasmas are pathogens responsible of more than 700 plant diseases belonging to 98 families and cause different yellows, dwarf and witches' broom diseases (Bertaccini, 2007; Hogenhout *et al.*, 2008). Until their characterization in 1994, phytoplasmas were previously known as mycoplasma-like-organisms (MLO) because of the resemblance to mycoplasmas associated to human and animal diseases (Welliver, 1999). These organisms are distributed worldwide and, as host, include ornamental plants and many important food, vegetable and fruit crops (Lee *et al.*, 2000; Garnier *et al.*, 2001; Seemüller *et al.*, 2002; Bertaccini and Duduk, 2009).

Taxonomy- Phytoplasmas represent a monophyletic group within the *Mollicutes* class and belong to the *Acholeplasmataceae* family (Lee *et al.*, 2000; Hogenhout *et al.*, 2008). Currently, there is no formal taxonomy of phytoplasmas. In *Mollicutes*, formal recognition of species and assignment of binomial Latin names to species require description of biological properties of the species, such as growing in pure culture. As this is difficult for phytoplasmas, the convention of '*Candidatus* Phytoplasma' species has been adopted as a means to refer to distinct phytoplasma lineages and putative species (IRPCM, 2004). In the last decades, the identification and classification of phytoplasmas were based on biological properties as the symptoms induced in infected plants, plant host range, and relationships with insect vectors (Lee *et al.*, 2000; Duduk, 2009).

Recent molecular data on phytoplasmas have provided considerable insights into their diversity and genetic interrelationships that are the basis for several studies on phytoplasma phylogeny and taxonomy (Hogenhout *et al.*, 2008). RFLP (restriction fragment length polymorphism) and sequence analysis of 16S rDNA have shown that phytoplasmas constitute a coherent taxon and allowed dividing the phytoplasma clade into groups and subgroups. The first complete phytoplasma classification scheme based on of PCR-amplified 16S rDNA provides a reliable means for the differentiation of a broad array of phytoplasmas and has become the most comprehensive and widely accepted phytoplasma classification system. This approach provides a simple, reliable and rapid tool for differentiation and identification of known phytoplasmas (Duduk and Bertaccini, 2011). Based on RFLP analysis, it was possible to identify 28 phytoplasma groups (Figure 1), while the sequence analysis allowed the determination of 37 '*Candidatus* Phytoplasma' species so far (Aryan *et al.*, 2016).

'*Candidatus* Phytoplasma' (disease, acronym)	16Sr subgroup[1]	GenBank Acc. no.	Reference
'*Candidatus* Phytoplasma asteris' (aster yellows, AY)	**16SrI-B**	M30790	Lee *et al.* (2004a)
'*Ca.* P. aurantifolia' (witches' broom of lime, WBDL)	**16SrII-B**	U15442	Zreik *et al.* (1995)
'*Ca.* P. ulmi' (elm yellows, EY)	**16SrV-A**	AY197655	Lee *et al.* (2004b)
'*Ca.* P. ziziphi' (Jujube witches' broom, JWB-G1)	**16SrV-B**	AB052876	Jung *et al.* (2003a)
'*Ca.* P. rubi' (Rubus stunt, RuS)	**16SrV-E**	AY197648	Malembic-Maher *et al.* (2010)
'*Ca.* P. trifolii' (clover proliferation, CP)	**16SrVI-A**	AY390261	Hiruki and Wang (2004)
'*Ca.* P. fraxini' (ash yellows, AshY)	**16SrVII-A**	AF092209	Griffiths *et al.* (1999)
'*Ca.* P. phoenicium' (almond witches' broom, ALWB)	**16SrIX-B**	AF515636 AF515637	Verdin *et al.* (2002)
'*Ca.* P. mali' (apple proliferation, AP)	**16SrX-A**	AJ542541	Seemüller and Schneider (2004)
'*Ca.* P. prunorum' (European stone fruit yellows, ESFY)	**16SrX-B**	AJ542544	Seemüller and Schneider (2004)
'*Ca.* P. pyri' (pear decline, PD)	**16SrX-C**	AJ542543	Seemüller and Schneider (2004)
'*Ca.* P. spartii' (spartium witches' broom, SpaWB)	**16SrX-D**	X92869	Marcone *et al.* (2004a)
'*Ca.* P. oryzae' (rice yellow dwarf, RYD)	**16SrXI-A**	AB052873	Jung *et al.* (2003b)
'*Ca.* P. australiense' (Australian grapevine yellows, AUSGY)	**16SrXII-B**	L76865	Davis *et al.* (1997)
'*Ca.* P. cynodontis' (bermudagrass white leaf, BGWL)	**16SrXIV-A**	AJ550984	Marcone *et al.* (2004b)
'*Ca.* P. brasiliense' (hibiscus witches' broom, HiWB)	**16SrXV-A**	AF147708	Montano *et al.* (2001)
'*Ca.* P. graminis' (sugarcane yellow leaf, SYL)	**16SrXVI-A**	AY725228	Arocha *et al.* (2005)
'*Ca.* P. caricae' (papaya bunchy top, PBT)	**16SrXVII-A**	AY725234	Arocha *et al.* (2005)
'*Ca.* P. americanum' (American potato purple top wilt, APPTW)	**16SrXVIII-A**	DQ174122	Lee *et al.* (2006a)
'*Ca.* P. omanense' (cassia witches' broom, CaWB)	**16SXIX-A**	EF666051	Al-Saady *et al.* (2008)
'*Ca.* P. japonicum' hydrangea phyllody		AB010425	Sawayanagi *et al.* (1999)
'*Ca.* P. castaneae' chestnut witches' broom	***16SrXIX***	AB054986	Jung *et al.* (2002)
'*Ca.* P. rhamni' (Rhamnus witches' broom, RaWB)	***16SrXX***	AJ583009	Marcone *et al.* (2004a)
'*Ca.* P. pini' (*Pinus sylvestris* yellows, PinY)	***16SrXXI***	AJ310849	Schneider *et al.* (2005)
'*Ca.* P. allocasuarinae' (allocasuarina yellows, AllocY)		AY135523 AY135524	Marcone *et al.* (2004a)
'*Ca.* P. fragariae' (strawberry yellows, StrawY)		DQ086423	Valiunas *et al.* (2006)
'*Ca.* P. lycopersici' ('Brote grande' tomato, TBG)		EF199549	Arocha *et al.* (2007)
'*Ca.* P. tamaricis' (salt cedar witches' broom, SaltCWB)		FJ432664	Zhao *et al.* (2009)
'*Ca.* P. costaricanum' (soybean decline, SoyD)		HQ225630	Lee *et al.* (2011)

[1], in Italics groups designated by Wei *et al.* (2007).

Figure 1. '*Candidatus* Phytoplasma' nomenclature validity published compared with 16S rRNA classification based on Lee *et al.* (1998) when possible (modified from Duduk and Bertaccini, 2011).

Morphology- Phytoplasmas are single-celled sub-microscopic microorganisms surrounded by a single trilaminar membrane similar to bacteria, with smaller dimensions. Since they lack cell wall, they can change shape (Doi *et al.*, 1967). Morphologically, by microscopic observations of phloem tissue sections, they appear as rounded, pleiomorphic bodies with an average diameter ranging from 200 to 800 µm (Figure 2) (Bertaccini *et al.*, 2010). These organisms are obligate parasite that cannot survive apart from a host and grow and reproduce in the cytoplasm of host cell, both plants and insect vectors. Phytoplasmas reside in the phloem tissue of the

plants they infect, invading primarily phloem sieve tube element (Ploaie, 1981; Welliver, 1999). Since phytoplasmas' cultivation *in vitro* is very problematic, the study of this group of microorganisms is complicated. Recently, a method for cultivation of various phytoplasma strains has been reported (Contaldo *et al.*, 2012), but plant infections by cultures have not been reported yet (Aryan *et al.*, 2016).

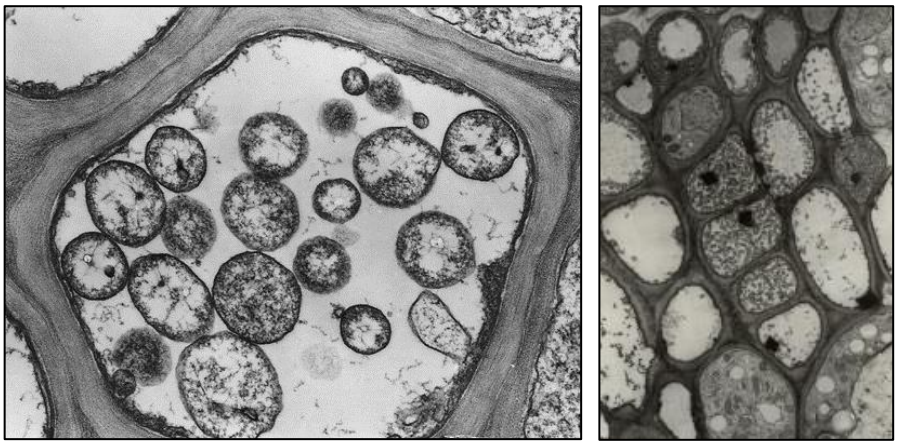

Figure 2. Electron micrograph of phytoplasmas in sections of sieve tubes.
(A. Bertaccini, http://www.costphytoplasma.ipwgnet.org/WG1/WG1_photogallery.htm)

Symptoms- The presence of phytoplasmas is associated to plants with typical symptoms, due to disturbances in the normal balance of plant hormones (Chang and Lee, 1995; Chang, 1998; Lee and Davis, 1992). Most common symptoms regard different apparatuses and include the proliferation of axillary shoots, phenomenon known as witches' broom, leaf yellowing, the loss of normal flower color to green flowers or virescence, and the development of floral parts similar to leaf-like structures or phyllody. Moreover, sterility of flowers and phloem necrosis could occur (Mc Coy *et al.*, 1989). All the symptoms produce a severe decline (Chang, 1998; Chang and Lee, 1995) and have a general negative impact on production (Figure 3). The symptoms induced in diseased plants vary with the phytoplasma and with the stage of infection (Lee *et al.*, 2000).

Transmission and spread- Phytoplasmas responsible of ornamental and fruit tree diseases are spread by vegetative propagation by means of cutting, storage tubers, rhizomes, or bulbs (Lee and Davis 1992). Phytoplasmas can be even transmitted through grafts, but not by inoculation with phytoplasma-containing sap or by mechanical actions. Plant viruses, mycoplasma-like and rickettsia-like organisms rely on the spreading function performed by several insect vectors, as most of them are not stable and require multiplication in the diverse plant and insect hosts (Hogenhout *et al.*, 2008). . In particular, the ecological niche of this bacterial group is localized

in the sieve elements of plant hosts and some organs of sap-sucking insect vectors, belonging to the Cicadellidae and Fulgoridae families (leafhoppers and planthoppers, respectively), and Psyllidae (Bosco and Marzachì, 2016). In this three-way interaction, insect vectors appear to play an active role; their feeding behavior and preference for certain host plants probably are, in most cases, the primary factors that determine the final niches for each phytoplasma (Lee *et al.*, 2000; Pedrazzoli, 2009).

Phytoplasmas may exhibit an overwintering behavior in insect vector or in perennial plants. Notwithstanding, no evidences of seed transmission have been recorded yet (Christensen *et al.*, 2005), despite the revelation of phytoplasma DNA in embryo tissues, which leads to hypothesize the potential for seed transmission (Cordova *et al.*, 2003).

Figure 3. Symptoms associated with phytoplasma presence in. A. Flavescence dorée (B. Duduk); B. Myrtle witches' broom (V. Prota); C. Pear decline (M. Cielinska); D. Purple coneflower (B. Duduk). (http://www.costphytoplasma.ipwgnet.org/WG1/WG1_photogallery.htm)

Detection- Before the development of molecular diagnostic tools, the detection of phytoplasma diseases was based on the observation of symptoms, experimental transmission to host plants, and fluorescence or electron microscopy observation of ultra-thin sections of the phloem.
Serological diagnostic techniques for the detection of phytoplasmas began to emerge in the 1980's with ELISA (enzyme-linked immunosorbent assay) based methods (Duduk, 2009).
In the early 1990's, molecular methods were developed with PCR coupled with RFLP analysis, allowing the accurate identification of different strains and species of phytoplasma (Namba *et al.*, 1993; Lee *et al.*, 1993; Schneider *et al.*, 1993). From this time, diagnosis and differentiation of phytoplasma infections have therefore relied on molecular methods (Delić, 2012), targeting a wide variety of regions within the 16S-23S rRNA genes and other conserved and less-conserved non ribosomal genes or variable genes encoding surface proteins (Smart *et al.*, 1996). The 16S-23S rRNA gene sequences have been effectively used to differentiate and classify phytoplasma strains (Maejima *et al.*, 2014; Wei *et al.*, 2007), resulting in the identification of at least thirty groups of phytoplasmas. More recently, several quantitative PCR (qPCR) techniques, based on different probes and dyes, have been developed to detect the phytoplasma infection and to quantify the phytoplasma titer in plants and insects (Jawhari *et al.*, 2015; Monti *et al.*, 2013; Christensen *et al.*, 2013; Baric *et al.*, 2011; Jarausch *et al.*, 2004; Angelini *et al.*, 2007; Nikolić *et al.*, 1996).

Phytoplasma transmission by insect vectors

Sap-sucking insect vectors are the most important means of phytoplasma transmission in nature, as these microorganism are not sufficiently stable to spread on their own. Many arthropods are capable of vectoring diseases in an ideal (e.g., laboratory) environment, but fail to be effective vectors in the field. Some factors influencing vector competence include age of the vector and host, synchrony of vector and host, availability of alternative hosts, behavior/host preference of the vector, and the ability of the pathogen to maintain or increase its titer in the vector, environmental conditions, and host resistance.
Plant host range for a phytoplasma is dependent upon vector specificity and feeding habits. Albeit multiple hosts may increase the chance for genetic exchange and local adaptations, allowing different infection pathways (Christensen *et al.*, 2005), the range of plant species that can be infected by a given phytoplasma in nature is determined largely by the number of insect vector species that are capable of transmitting the phytoplasma and by the feeding behaviors (monophagous, oligophagous, and polyphagous) of these vectors (Lee and Davis, 1992; Lee *et al.*, 2000). Polyphagous vectors have the potential to inoculate a wider range of plant species,

depending on the resistance to infection of each host plant. Several studies (Bosco *et al.*, 1997; Marzachì *et al.*, 1998) have shown that even insects that normally do not feed on certain plant species can acquire and transmit phytoplasmas to those plants under laboratory conditions. Hence, in many cases, the host range of a vector, rather than lack of phytoplasma-specific cell membrane receptors, limits the spread of phytoplasmas by that species (Bosco *et al.*, 1997; Pedrazzoli, 2009; Weintraub and Beanland, 2006).

Moreover, the capability of the host plants to harbor more than one type (or strain) of phytoplasmas depends on the susceptibility to phytoplasma infection and on the vector-phytoplama-plant interaction (Lee *et al.*, 2000). On the other hand, the natural host ranges of phytoplasmas in insect vectors and plants vary with the phytoplasma strain (Brcák, 1979; Lee *et. al.*, 2000; Mc Coy *et al.*, 1989; Tsai, 1979).

Phytoplasma-vector relationship- Vector may or may not be essential for the completion of the life cycle of the pathogenic microorganism, but its function is harboring and protecting them and creating feeding wounds and entry points into susceptible plants.

Phytoplasmas are transferred with saliva into the pierced sieve element. From here, the microorganisms spread in plant through the continuous sieve tube system. An important feature displayed by vectors is the presence of a propagative and persistent relationship with phytoplasmas (Weintraub and Beanland, 2006) (Figure 4).

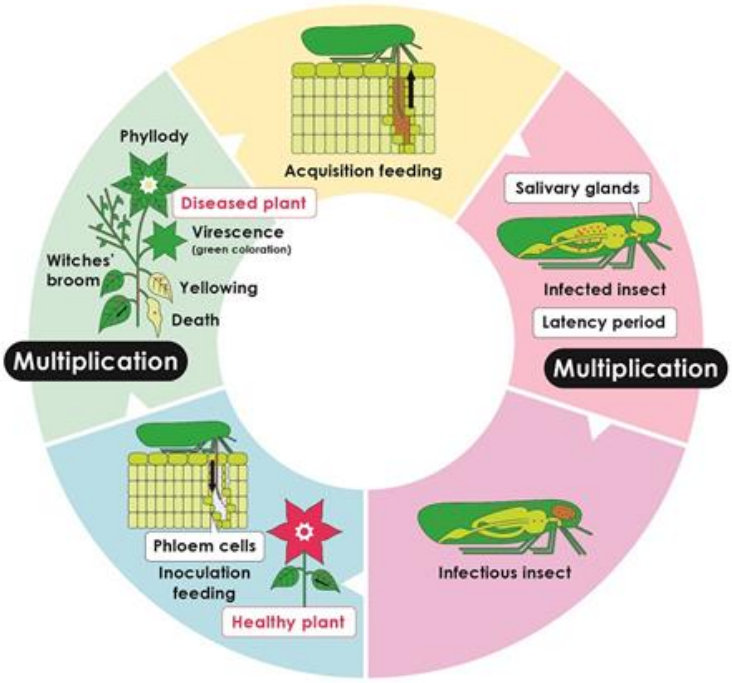

Figure 4. Phytoplasma life-cycle involves two different hosts: plants and insects. Phytoplasmas are acquired during insect feeding and multiply within the vector before being transmitted to another plant (http://www.u-tokyo.ac.jp/en/utokyo-research/feature-stories/elucidating-the-mystery-of-phytoplasmas-the-ultimate-idler-bacteria.html).

The term “propagative” means that the pathogen can multiply in insects; on the other hand, “persistent” means that the insect remains inoculative for life (Fletcher *et al.*, 1998).

A specific feeding duration by insect is required to acquire a sufficient titer of phytoplasma; this phase is a part of the phytoplasma reproductive cycle and is called acquisition access period (AAP). The longer is the duration of AAP, the greater will be the chance of acquisition (Purcell, 1982). This phenomenon may also depend on the titer of phytoplasmas in the plants, even though mechanisms are not clear yet (Weintraub and Beanland, 2006). AAP is followed by a latent period (LP), consisting in the time required from initial acquisition to the actual transmission of the phytoplasmas. During this period, which is temperature-dependent, phytoplasmas move through and multiply in the vector body (Murral *et al.*, 1996; Nagaich *et al.*, 1974). Phytoplasmas invade the midgut passing through the epithelial cells and, multiplying within a vesicle with intracellular movement or passing between two midgut cells (Lefol *et al.*, 1994), enter the hemocoel through the basement membrane. Through the hemolymph, they may infect other tissues such as the Malpighian tubules (Lett *et al.*, 2001), fat bodies and brain (Lefol *et al.*, 1994; Nakashima and Hayashi, 1995), or reproductive organs (Kawakita *et al.*, 2000); multiplication in these tissues suggest a longer co-evolutionary relationship between host and pathogen (Weintraub and Beanland, 2006). After reaching the salivary gland cells, phytoplasmas further multiply and are transmitted in the saliva (Hogenhout *et al.*, 2008; Kirkpatrick, 1991).

Homoptera as vectors of phytoplasmas- The order of Homoptera comprises insect groups with a specific piercing-sucking mouthparts, which conferred a relevant effect in their adaptive radiation (Goodchild, 1966). As phloem-limited, phytoplasmas can be acquired and transmitted only by phloem-feeding insects. Homoptera feeding habits range from phytophagy (the majority of species) to predation, including ectoparasitism and haematophagy. Phytoplasma vectors must feed specifically and selectively on this particular plant tissue, where pathogens reside, with a nondestructive way. Weintraub and Beanland (2006) reviewed the features required by an insect species to be a successful phytoplasma vector and, according to the authors, Homoptera are the main elicited insect group. Insects of this order are hemimetabolous and nymphs and adults besides feeding similarly, share the same physical location; often both immatures and adults can transmit phytoplasmas. They feed specifically and selectively on certain plant tissues, which makes them efficient vectors of pathogens residing in those tissues. Furthermore, their feeding is nondestructive, promoting successful inoculation of the plant vascular system without damaging conductive tissues and eliciting defensive responses. Moreover, they have a propagative and persistent relation-ship with phytoplasmas.

The Homoptera is the largest exopterygote group of insects with over 80.000 described species. Their specialized structure of mouthparts are modified into concentric stylets, the mandibular enclosing the maxillary ones and together forming the food and salivary channels. Homoptera is a very diverse group comprising scale insects, aphids, psyllids and whiteflies (Sternorrhyncha), true bugs (Heteroptera), and Auchenorrhyncha. The last one have been traditionally divided into two main suborders: Cicadomorpha (leafhoppers, treehoppers, spittlebugs, and cicadas) and Fulgoromorpha (the planthopper families) (Figure 5). Leafhoppers and planthoppers are among the most abundant groups of insects. Around 20.000 leafhopper (Cicadellidae) species have been described but estimates suggest 100.000 species may exist (Dietrich, 2005). In addition, there may be around 10.000 planthopper species of which the most significant pest species occur within the family Delphacidae. Around 200 vectors of phytoplasma are already known but many more are likely to be recognized because there are many more phytoplasma diseases characterized than there are known vectors of the diseases (Wilson and Turner, 2010). More than 75% of all confirmed phytoplasma vector species are found in the subfamily Deltocephalinae (Cicadellidae). The feeding habits of species within this subfamily range from monophagous to polyphagous, and members of this group can transmit one or more different phytoplasma taxa (Weintraub and Jones, 2010).

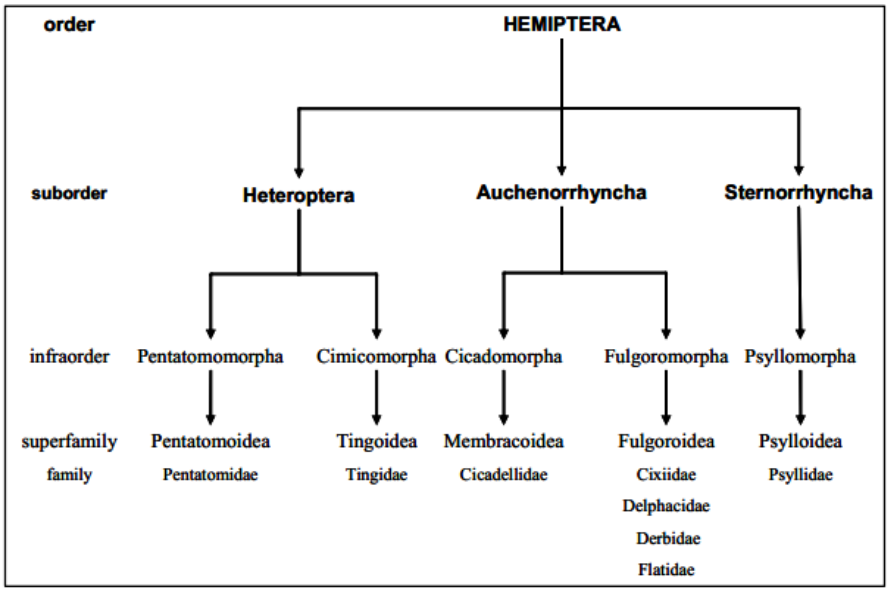

Figure 5. Homopteran families involved in the transmission of phytoplasmas (modified from Weintraub and Beanland, 2006).

'*Candidatus* Phytoplasma mali'

Three phytoplasma-associated diseases are known to cause serious damages to the fruit production of temperate areas: apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). Although the 16S rDNA sequences of strains of these pathogens are nearly identical, indicating a close phylogenetic relationship, other criteria considered, such as molecular markers, serological comparisons, vector transmission and host-range specificity, allowed the distinction of three different putative species: '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri', and '*Candidatus* Phytoplasma prunorum' (Seemüller and Schneider, 2004). The three pathogens form, together with the peach yellow leaf roll (PYLR) phytoplasma, a cluster designated the 'AP phytoplasma group' (Seemüller *et al.*, 1998) or 16SrX group (Lee *et al.*, 2000) within the AP subclade, which is one of the major branches of the phytoplasma clade.

AP is considered one of the most important diseases of apple (EPPO/CABI, 1997), particularly in the northern areas of southern Europe, where temperatures are the most conducive to symptom expression. Where cooler or warmer growing conditions occur, the disease appears to be less impacting (Seemüller *et al.*, 1998; Rui, 1950, Refatti and Ciferri, 1954).

Symptoms- '*Candidatus* Phytoplasma mali' causes symptoms associated with disturbance in the normal balance of growth regulators (Seemüller and Schneider, 2004). Late growth of terminal buds, with a delayed production of flowers in the autumn, is usually the first noticeable symptom. A rosette of terminal leaves, which often becomes infected with powdery mildew, sometimes develops late in the season in place of the normal dormant bud. However, the most reliable symptom is the premature development of axillary buds, during the first two or three years following infection, which gives rise to witches' brooms near the apex of the main shoot. Leaves of infected plants show abnormally long stipules and rather short petioles (Figure 6). In many cases, especially with trees on calcareous soils, there is a chlorosis and reddening of the leaves. Early defoliation often occurs. Depending on soil quality, fruits are markedly reduced in size, sometimes being only 25% of the weight of healthy fruit. In addition, flavor is poor, both sugar and acidity being reduced. The peduncles are longer and thinner and the fruit takes a flattened appearance (Blumer and Bovey, 1957; Schuch, 1962; Bovey, 1963; 1972).

In general, affected trees lack vigor, shoots are thin and necrotic areas appear on the bark. Diseased trees may die but, in mild infections, they may recover after the shock symptoms of the first 2-3 years and, subsequently, produce normal fruits again, especially if adequately fertilized (Schmid, 1965). The spontaneous remission of symptoms in AP-infected plants, called "recovery", is a natural phenomenon observed in the field in which phytoplasma

disappears from the aerial part of the trees and is confined to the roots (Carraro *et al.*, 2004; Musetti *et al.*, 2004). This phenomenon was studied in apple proliferation infected apple trees cv. Florina and in apomictic rootstocks deriving from crosses of *Malus sieboldii* and *Malus sargentii* with *Malus pumila*. The combination of low mortality with the elimination of the phytoplasma from the aerial part of trees opened new perspectives for the selection of resistant rootstocks suitable for controlling apple proliferation (Kartte and Seemüller, 1991).

Distribution in the tree- The individuation of AP presence in the orchard goes together with the distribution of phytoplasmas in the tree, which is not constant over the year (Pedrazzoli *et al.*, 2008). At the end of wintertime, the content of phytoplasmas declines in the tree due to sieve tube degeneration. They appear also to be more concentrated in the root system but, during April to May, reinvade the stem and the canopy from the roots, reaching a peak in late summer or early autumn (Baric *et al.* 2011; Bisognin *et al.*, 2008; Musetti *et al.*, 2010; Schaper and Seemüller, 1984). Normally, plants inoculated with infected buds show the first symptoms the following year, mostly on the inoculated branches. When carried in the rootstock, phytoplasmas produce symptoms on the first growth of the scion. AP phytoplasma has been observed in the phloem of leaf petioles, midribs and stipules and appears to be localized mainly in suckers and terminal shoots (Bovey, 1972; Seidl and Komarkova, 1974).

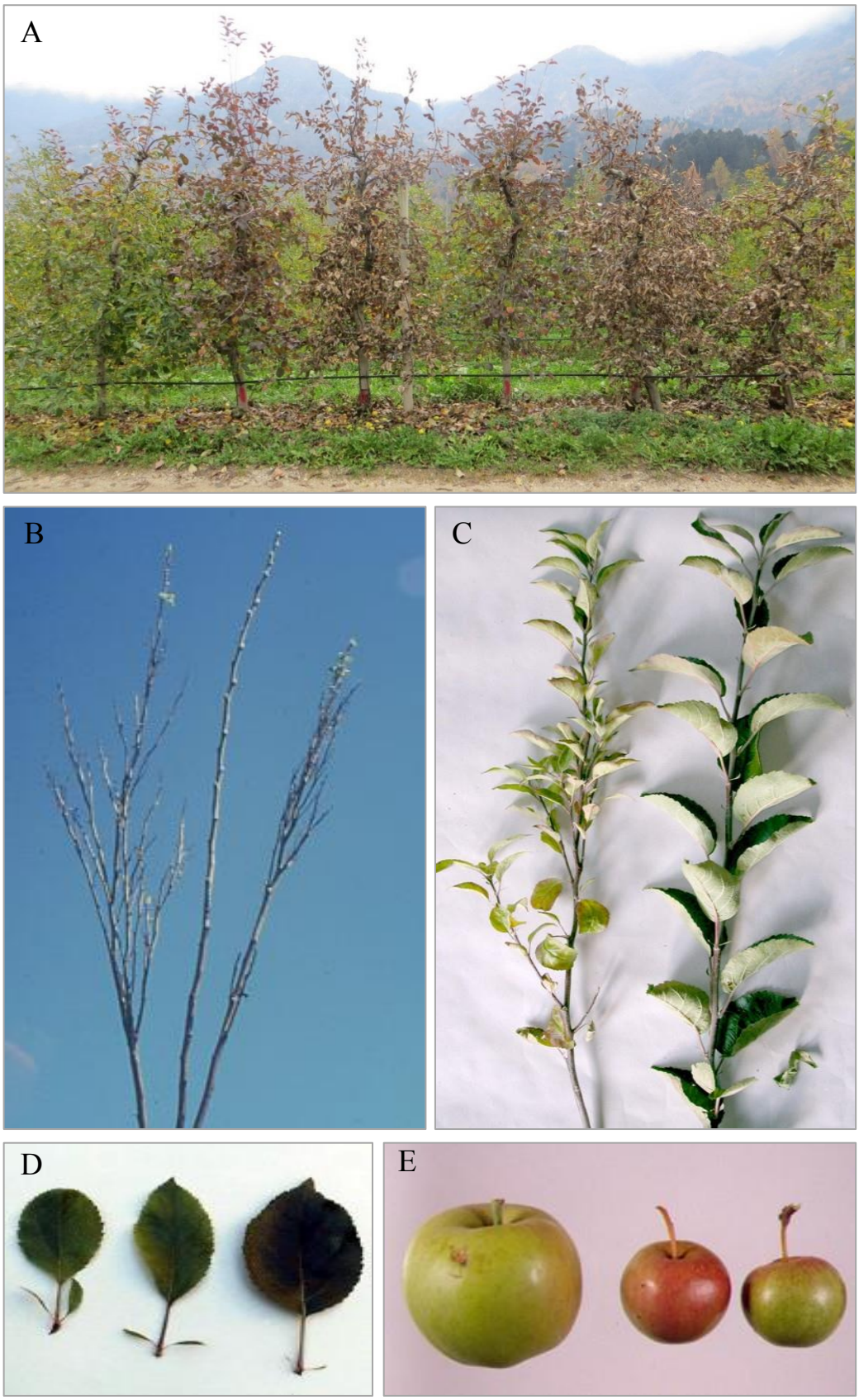

Figure 6. Different symptoms of AP: A. early reddening in autumn (M. Baldessari); B. branch with witches' brooms in winter (http://www.inspection.gc.ca). C. witches' brooms (left) compared to a healthy shoot (right) (J. Sucha); D. two leaves showing enlarged stipules (left) compared to a healthy one (right) (F. Bondaz) E. small-sized fruits with enlarged petioles (right) compared to a normal-sized fruit (left) (M. Cielinska).

Host plants- '*Ca.* P. mali' occurs in a wide range of species of the genus *Malus* (Kartte and Seemüller, 1991) and has been detected occasionally in plants such as *Pyrus* spp., *Prumus* spp., *Corylus avellana* L., *Crataegus monogyna* Jacq., *Quercus robur* L., *Quercus rubra* L., *Carpinus betulus* L., *Convolvolus arvensis* L. (Del Serrone *et al.*, 1998; Lee *et al.*, 1995; Marcone *et al.*, 1996; Mehle *et al.*, 2007; Schneider *et al.*, 1997; Seemüller, 2002; Seemüller and Schneider, 2004) and also in herbaceous plants, such as dahlia (*Dahlia cultorum* Thorsrud et Reisaeter) and oriental hybrids of *Lilium* plants (Kaminska and Śliwa, 2008a, 2008b).

Molecular characterization and diagnosis- '*Ca.* P. mali' has a linear chromosome (Kube *et al.*, 2008) and a very small genome averaging ~750 kb that differs from the other phytoplasmas for the law GC-content (Bai *et al.*, 2006). Analyses of a non-ribosomal DNA fragment, composed of three putative open reading frames (ORFs), proved the existence of at least three different AP phytoplasma subtypes named AT-1, AT-2, and AP-15 (Jarausch *et al.*, 1994, 2000). Molecular characterization of the genes coding the ribosomal proteins L22 and S3 revealed the presence of higher genetic heterogeneity within isolates of '*Ca.* P. mali' and led to the proposal of four subtypes rpX-A, rpX-B, rpX-C, and rpX-D (Martini *et al.*, 2008). Analyses of ribosomal and non-ribosomal DNA fragments of '*Ca.* P. mali' populations from northwestern Italy revealed the presence of the three AP phytoplasma subtypes (AT-1, AT-2 and AP-15) and reported the identification of at least two phytoplasmal genetic lineages, designated AT-1a and AT-1b, among the AP phytoplasma isolates of the AT-1 subtype (Casati *et al.*, 2010).

Several diagnostic tests have been developed to detect '*Ca.* P. mali' in both plants and insects. They range from the classic biological assays, in which the tested vegetal material is grafted onto indicator plants, to serological assays using specific antibodies against AP phytoplasma, e.g. enzyme-linked immunosorbent assays (ELISA), or DAPI staining and immunofluorescence, in which the pathogen is directly detected in vegetal sections under a fluorescence microscope.

The development of molecular techniques based on the DNA amplification allowed the establishment of very sensitive and specific diagnostic tools. Nested PCR, a highly sensitive DNA amplification in which the sample undergoes two separated PCR runs, has been used for the detection of AP phytoplasma in plants and insects using universal primers (P1/P7 and F2n/R2) and 16SrX-group specific primers (P1/P7 and fO1/rO1) (Lee *et al,.* 1995; Lorenz *et al.*, 1995). Due to the genetic closeness between AP group phytoplasma, specific identification often requires further steps, such as amplicon digestion with different restriction enzymes and subsequent RFLP analysis or sequencing (Gundersen and Lee, 1996; Jarausch *et al.*, 2000;

Kison *et al.*, 1994; Lee *et al.*, 1995; Lorenz *et al.*, 1995; Schneider *et al.*, 1995; Smart *et al.*, 1996). In recent years, different quantitative real-time PCR assays have been developed to detect and quantify AP titer in plants and insects based on SYBR Green (Galetto *et al.*, 2005; Jarausch *et al.*, 2004; Torres *et al.*, 2005), TaqMan (Aldaghi *et al.*, 2007; Baric and Dalla Via, 2004) and EvaGreen technologies (Monti *et al.*, 2013).

Geographical distribution- Apple Proliferation has only been reported from the European and Mediterranean Plant Protection Organization (EPPO) region (EPPO/CABI, 1997). In Europe, it has been detected in the following countries: Albania, Austria, Balkans, Belgium, Bosnia-Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, France, Germany, Greece, Hungary, Italy, Moldova, Netherlands, Norway, Poland, Romania, Russia, Serbia, Slovakia, Slovenia, Spain, Switzerland, Ukraine and Yugoslavia (Figure 7). The disease has also been detected in Turkey and Syria. However, there are unconfirmed reports from India and South Africa (Seemüller, 1990).

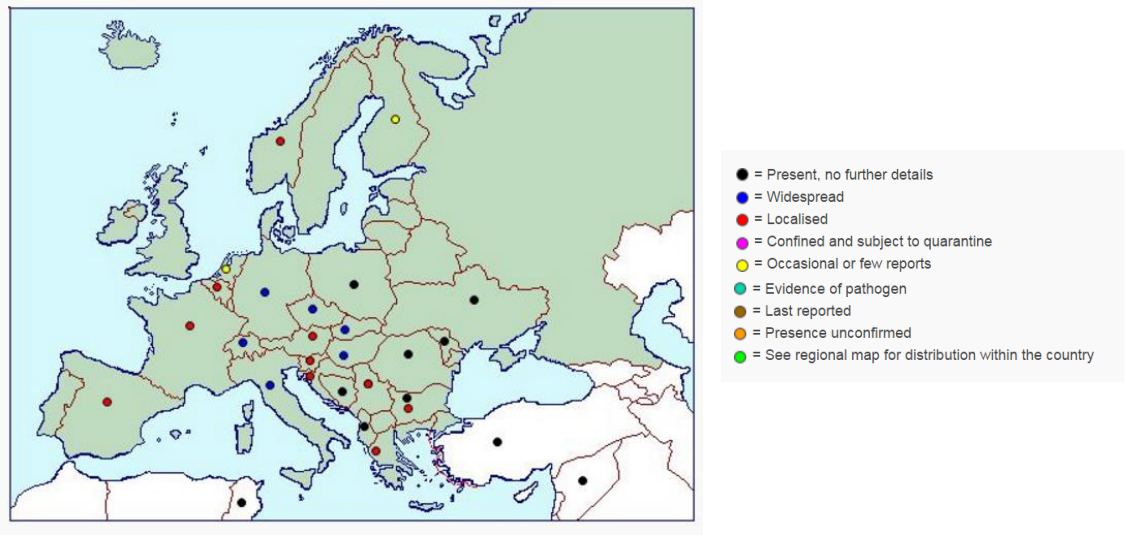

Figure 7. Geographical distribution of AP in Europe and surrounding countries (http://www.cabi.org/isc/datasheet/6502)

AP in Italy- Apple proliferation has been recorded in the main apple growing regions of Italy, especially in the North. Rui *et al.* (1950) described for the first time the presence of AP in Veneto. In Piemonte, the phytoplasma was reported for the first time at the end of the '90s in the provinces of Torino and Cuneo. However, the spread in these areas is not a big concern (Alma *et al.*, 2000; Minucci *et al.*, 1996; Pinna *et al.*, 2003; Spagnolo *et al.*, 2005). Differently, in Valle d'Aosta AP is widespread and represents a serious threat, especially in older orchards, reaching very high percentage of infection (Tedeschi *et al.*, 2002; Tedeschi *et al.*, 2003).

Figure 8. Apple proliferation is reported in northern Italian regions and in Basilicata.

In Lombardia, the presence of '*Ca.* P. mali' was investigated by Casati *et al.* in 2007. AP was recently described also in the South of Italy, in particular in Basilicata (Marcone and Seemüller, 2013) (Figure 8). In north-eastern Italy, Alto Adige represents an important apple-growing area, with more than 10% of the European apple production. Here, as in other regions, the first sporadic cases of AP were reported in the late 1950's, but a real outbreak of the disease was reported in 1998 in Valle d'Isarco. Afterwards, AP was monitored and a massive spread of the disease was recorded in the years 2005-2006, when symptomatic trees were found in about 75% of the monitored orchards and later, in 2011, when a new increase of infestations was observed in some districts (Oettl and Schlink, 2015). In Trentino, the presence of infected apple trees was reported in the early 1950's (Refatti and Ciferri, 1954), but the spread of the disease was rather sporadic until the beginning of the 1990's, when an outbreak started in Val di Non and caused significant economic damage (Vindimian and Delaiti, 1996; Vindimian *et al.*, 2002). In order to quantify the disease spread and to understand the predisposing factors, a survey of infected trees was started in Val di Non in 1999 (Figure 9). In general, higher percentages of infected trees were observed at higher altitudes and in older orchards, with more vigorous rootstocks. Surprisingly, infection levels of about 5-10% were reported in two-year-old orchards and up to 20% in some three-year-old orchards (Springhetti *et al.*, 2002).

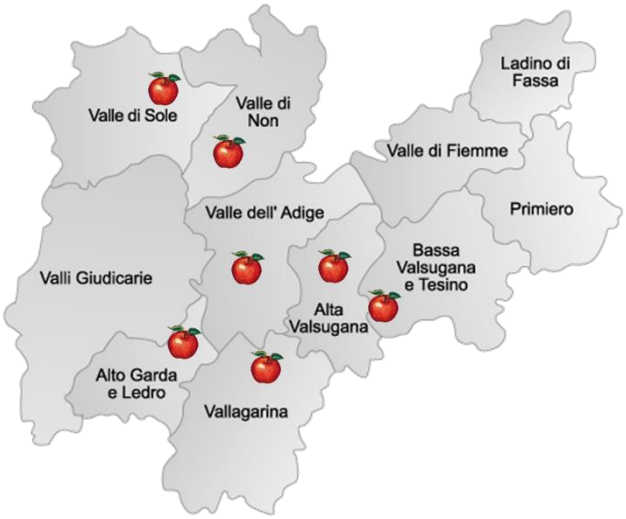

Figure 9. Representation of Trentino valleys; apples are positioned in the main areas of AP presence.

Starting from 2001, the Phytosanitary Service of Province of Trento is conducting an official monitoring, which covers the whole apple growing area of the province. A surface corresponding to 4% of the total apple growing area was chosen to evaluate the effect of differential agronomic measures, cultivars or altitudes on the disease spread (Vindimian, 2002). The infection rate rapidly decreased starting from 2006, when uprooting of infected trees became mandatory and strict chemical control measures against the insect vectors were applied. The adoption of these actions was enhanced by a subsidy for uprooting orchards older than 20 years or with more than 20% of infected trees. AP prevalence constantly decreased during the subsidized uprooting program from 2006 to 2010, when it reached the level of 0.27%. Unfortunately, the infection rate started increasing again from 2012, more significantly in the Val d'Adige and Valsugana. In these two apple districts, the average infection rate rose up to 6% in 2014, pushing up again the average infection rate of the Trentino province to 2% (Dallago, 2016).

Transmission of AP- '*Ca.* P. mali' can be commonly transmitted by grafting: the phytoplasma is often disseminated in scion wood and trees may yield a high proportion of apparently healthy but infected buds. According to the seasonal colonization of host plants by the phytoplasma, Pedrazzoli *et al.* (2008) obtained the highest percentages transmissions by grafting between June and August (12 to 30%), while grafts conducted from March to May were not very successful (0-0.08%). The authors concluded that the most suitable period for collecting scions is springtime, when the probability of AP transmission was at the lowest. Grafting of stem scions removed during dormancy prevented transmission or yielded only a low transmission

rate. Transmission by root grafting in winter is generally successful and for this reason it has become an established method for indexing trees (Seemuller *et al.*, 1984). AP has been reported to spread also via natural root bridges in middle-aged and old apple orchards (Baric *et al.*, 2008; Bliefernicht and Krczal, 1995; Ciccotti *et al.*, 2008; Vindimian *et al.*, 2002). There are also reports of experimental transmission to *Catharanthus roseus* (Madagascar periwinkle) using the parasitic plant *Cuscuta* spp. (dodder) (Heintz, 1986; Marwitz *et al.*, 1974), while the transmission via seed or pollen has not been reported (Seidl and Komarkova, 1974).

Insect species such as psyllids and leafhoppers have been investigated for their ability in spreading the disease (Seemuller, 1990). So far, two psyllid species, *Cacopsylla picta* Förster and *C. melanoneura* Förster, and the leafhopper *Fieberiella florii* Stål were demonstrated to be vectors of AP phytoplasma (Frisinghelli *et al.*, 2000; Krczal *et al.*, 1989; Tedeschi and Alma, 2004; Tedeschi and Alma, 2006). Regarding Trentino, the experiments conducted so far confirmed the vectoring capability of *C. picta*, which is able to transmit the pathogen as neanid/nymph and new generation adult (Frisinghelli *et al.*, 2000; Mattedi *et al.*, 2007; Pedrazzoli, 2009). On the other hand, the role of *C. melanoneura* is still unclear: a very low transmission efficiency was found by Mattedi *et al.* (2007), even though important percentages of infected individuals have been found in the orchards of Trentino and in Alto Adige (Malagnini *et al.*, 2010; Poggi Pollini *et al.*, 2002).

Psyllid vectors of '*Ca.* P. mali'- Psyllids are known in agriculture as important pests of cultivated crops but also as vectors of plant diseases (Hodkinson, 1974). The Psylloidea (Homoptera: Sternorryncha) superfamily is distributed worldwide and comprises eight families with about 3.850 species (Burckhardt and Ouvrard, 2012; Eben *et al.*, 2015). All species of these families live on plant sap and are phloem-feeders, both as nymphs and as adults.

During their life, they are generally narrowly host-specific and are restrict almost exclusively to perennial dicotyledonous plants (Eastop, 1972). Plants may play different roles in hosting psyllid species (Conci *et al.*, 1995). The "host plants" are the species on which insects spend time to lay eggs and develop. The "shelter plants" are the species to which adults compulsorily migrate in autumn for spending winter, reducing trophic activity. "Occasional plants" are species where insects may be accidentally transported by wind or other causes, but that normally have no importance for their biology. In relation to the host-plant range, psyllids have been divided into four categories: (1) monophagous species, where nymphs can develop exclusively on one plant species; (2) strictly oligophagous species that live on some congeneric plants; (3) widely oligophagous species that live on plants belonging to kindred genera of the same family and (4) polyphagous species that live on plants of different families (Conci *et al.*,

1995). Life-cycle in psyllids is often highly synchronized with host plant phenology (Pedrazzoli, 2009).

Cacopsylla picta Förster (1848) has a palaearctic distribution and is associated on *Malus* spp. (Lauterer, 1999; Jarausch *et al.*, 2011). Previously known as *C. costalis* Flor (1861), it has been synonymised with *C. picta* by Lauterer and Burckhardt in 1997. This psyllid is a univoltine species, completing one generation per year, and overwintering as an adult on conifers (Čermák and Lauterer, 2008; Mayer *et al.*, 2010). At the end of winter (March or April), *C. picta* remigrants move from their overwintering sites to their main hosts for oviposition. Larval development takes four to five weeks; the newly hatched imagines (emigrants) remain in the orchards for about two weeks before migrating to their overwintering sites in June or July. *C. picta* young adults are light green, with a mesothorax yellowish banded. Later their color is dirty yellow or orange-colored with more or less extensive dark brown or black markings. The abdomen is black with red segment borders (Ossiannilsson, 1992). During hibernation the body coloration changes to black-brown (Lauterer, 1999). Forewings are colorless, veins in old specimens are dark brown or black, pterostigma is fuscous. The overall length of males is 2.86-3.24 mm, of females 3.14-3.43 mm (Ossiannilsson, 1992). Fifth instar nymphs are light green, wing pads with a pale violet tinge. Abdominal margin has three pairs of sectasetae. The ocular seta is more or less simple, 0.03-0.04 mm in length. The length of the body is 1.57-2.19 mm (Figures 10 and 11) (Ossiannilsson, 1992).

The species is narrowly oligophagous on *Malus domestica* Borkh., *Malus sylvestris* Mill., *Malus* cv. and *Prunus armeniaca* L. (Conci *et al.*, 1992; Lauterer, 1999; Ossiannilsson, 1992). According to Harisanow (1966), who studied the biology of *C. picta* in Bulgaria, this species overwinters as adult on *Pyrus communis* L., *Prunus domestica* L., *Persica vulgaris* Mill., *Amygdalus communis* L., *Ulmus campestris* L. and other plants (Lauterer, 1999; Ossiannilsson, 1992). A female may lay approximately 160 eggs and ten-fourteen days after becoming adult, the new generation moves on first to annual herbs, e.g. *Brassica*, *Mentha*, *Vicia*, *Phaseolus*, *Pisum*, as well as grasses, e.g. *Avena*; later to perennial shelter plants (Lauterer, 1999; Ossiannilsson, 1992). According to Conci *et al.* (1992), *C. picta* overwinters on conifers. These data are confirmed by Flor (1861), who collected specimens on *Pinus abies* L. in August. Ossiannilsson (1992) in Uppland found one male on *Picea abies* (L.) H. Karst. at the end of November. Recently, the complete life-cycle of *C. picta* was described in a permanent rearing under controlled conditions by Jarausch and Jarausch (2014), who successfully reproduced overwintered sites on pine and spruce. The host location and the migration behavior of *C. picta* seems to be mediated by the chemical cues emitted by plants, and the preference of the insects

switches between the volatiles of the host and the shelter plants during the course of the year (Gross and Mekonen, 2005).

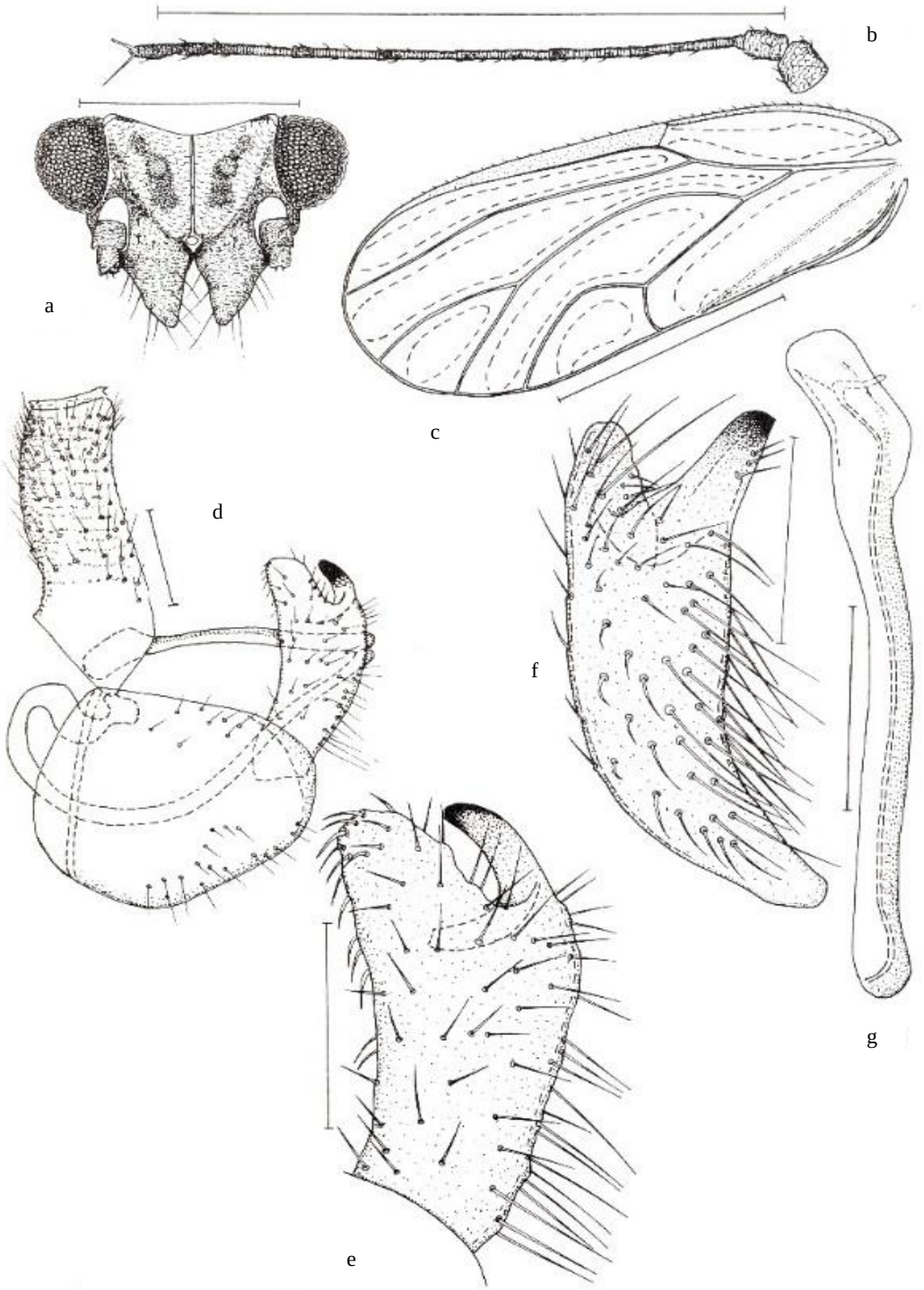

Figure 10. *Cacopsylla picta*. Female: (a) head in frontal aspect; (b) left antenna in dorsal aspect. Male: (c) left forewing, (d) terminalia from the left; (e) left paramere from the left; (f) same from behind; (g) terminal part of aedeagus from the left. Scale: 0.1 mm (modified from Ossiannilsson, 1992).

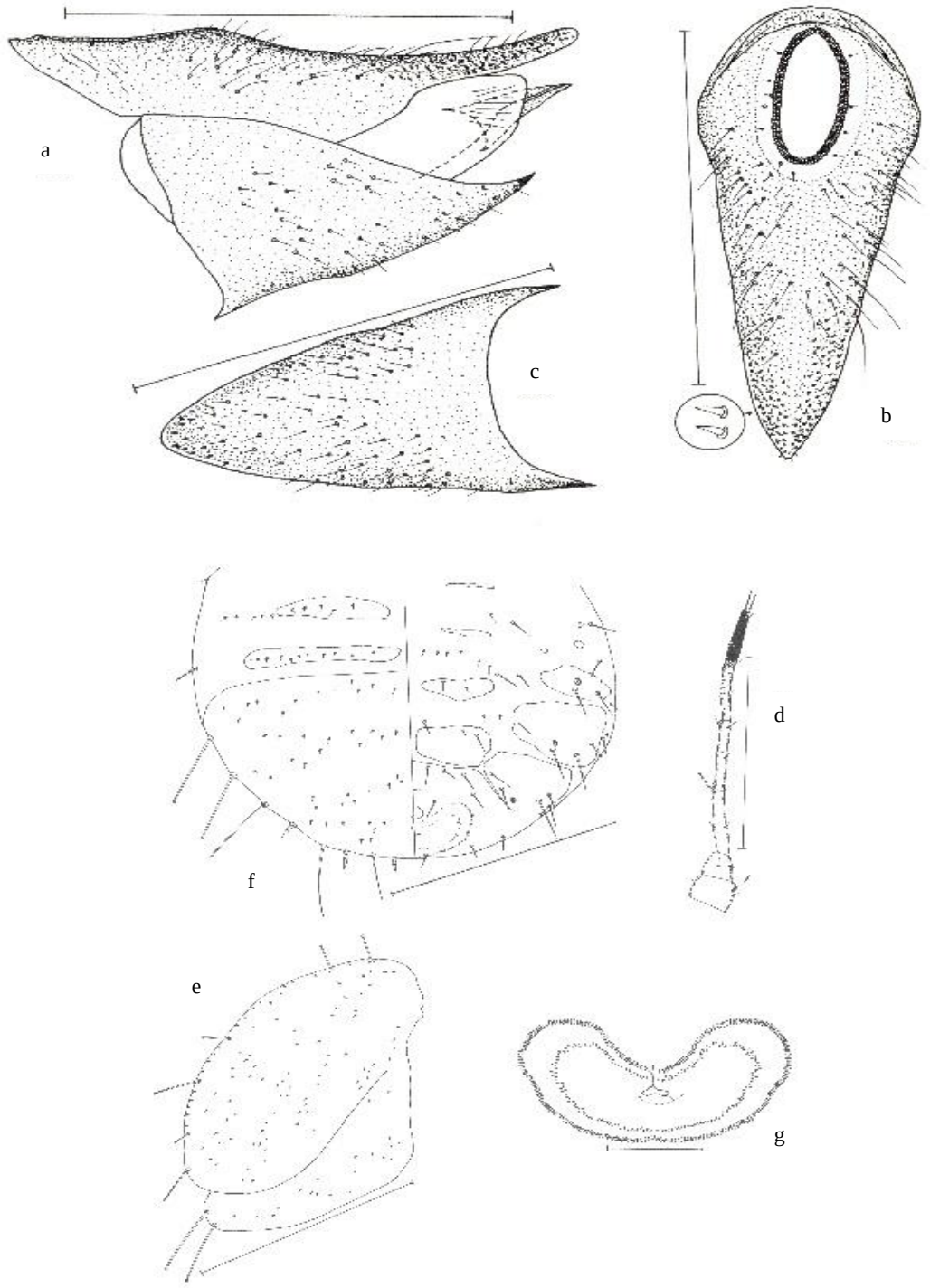

Figure 11. *Cacopsylla picta*. Female: (a) terminalia from the left; (b) proctiger from above; (c) subgenital plate from below. 5th instar nymph: (d) left antenna from above; (e) left wingpads from above; (f) abdominal dorsum (left) and venter (right); (g) circumanal pore rings from below. Scale: 0.1 mm for (g); 0.5 mm for the rest (modified from Ossiannilsson, 1992).

Cacopsylla melanoneura Förster (1848) is a holopaleartic species distributed everywhere with its host plants. Young adult specimens are orange-colored, pronotum and genal cones are whitish, forewing veins are yellow. Later, they are largely dark brown with a reddish tinge, head and pronotum are partly lighter, mesonotum with pale spots and bands, forewing veins are dark brown or black. Forewings alone veins have broad spinule-free bands becoming broader apically. Overall length of males is 2.52-3.10 mm, of females is 2.95-3.30 mm (Ossiannilsson, 1992). Fifth instar nymphs are entirely light green, or green to dirty green with yellow brownish sclerites. Wing pads are often whitish. The number of marginal setae on forewing-pads is variable. On abdominal margin there are three pairs of sectasetae. The body length is 1.33-2.00 mm. Ocular seta is more or less rod-like or spine-like, length is 0.011-0.017 mm (Figures 12 and 13) (Ossiannilsson, 1992).

This species is widely oligophagous on *Rosaceae Maloideae* such as *Crataegus* spp. (*Crataegus monogyna* Jacq., *Crataegus oxyacantha* L., *Crataegus maximowiczii* C.K.Schneid), *Malus* spp. and *Pyrus communis* L. (Conci *et al.*, 1992; Ossiannilsson, 1992). It is reported also on conifers and many other shelter and occasional plants of different families (Conci *et al.*, 1992; Lauterer *et al.*, 1999). Overwintering adults live for 9-10 months long on *Pinus* spp. at higher altitudes (250-1400 m asl), performing long-distance migrations between stands of pines and apple trees (Lazarev, 1974). The migrations to orchards take place during budding of the host plant. Each female lays about 200 eggs. Embryonic development lasts 7-20 days and larvae hatch at the time of maximum flowering of apple trees. The larvae develop over one month and then the new generation adults appear. After complete sclerotisation (i.e. about 5 days after their last skinning) the adults migrate to mountain elevations onto pine trees. Overwintering behavior and shelter plants of *C. melanoneura* were studied in Italy by Pizzinat *et al.* (2011). The altitudinal distribution and overwintering habitats were investigated following the direction of warm ascending currents, as proposed by Cěrmák and Lauterer (2008), and the suitability of different conifer species as shelter plants during aestivation and overwintering periods was assessed by insect collections and observation of insect survival in outdoor trials. The results indicate that this species can potentially survive on many coniferous species.

Ossiannilsson (1992) described the life cycle of *C. melanoneura* on hawthorn in Sweden, where the stages are slightly delayed in time and the migration of the new generation adults to conifers does not begin before July. In Czech Republic, in *Querco-Carpinetum* associations and particularly in floodplain forests, however, in absence of conifers, most of the population may hibernate on other broadleaved trees, hiding under bark scales and on sprouts (Lauterer, 1999). Apparently, the long-distance seasonal migrations of young adults to mountain elevations

shortly after having completed sclerotisation are limited to the warmer southern parts of Europe. In the conditions of central Europe the migrations are apparently shorter (Lauterer, 1999). Mass occurrence of new generation adults in Czech Republic was observed by the author in the first decade of June, whereupon their number dropped abruptly. This early emigration from the host plants to other plants agrees with the observation of Lazarev (1974), but in Czech Republic the migration to the shelter plants seems to be gradual, and the species first migrates on occasional plants and then to conifers. Thus, for this species, three migration phases can be distinguished (Lauterer, 1999). The role of chemical signals in the migration behavior and the orientation of *C. melanoneura* was studied with psyllids collected from both apple and hawthorn by Gross and Mekonen (2005) and by Mayer and Gross (2007). The behavioral responses of the insects corresponded with the different phases of the migratory behavior, the overwintered adults showing strong positive responses for apple or hawthorn odors, while the newly emerged adults showing strong responses for spruce volatiles. Attempts at copulation and copulating adults were observed already during June but, apparently, fertilization does not take place until copulations after hibernation between March and May (Lauterer, 1999). About one week after the last skinning and completed sclerotisation, the adults enter dormancy of the parapause type with aestivation, later passing into a diapause during hibernation. Reactivation and development of sexual glands only occur after the cold phase in winter (Lauterer, 1999). The altitude of hibernation and aestivation places differs according to the latitude: in Moravia the majority of individuals can be found between 160 and 450 m asl, while at higher altitudes the occurrence is only sparse; the populations of the southern European regions most often hibernate and aestivate on dwarf pines in high mountain altitudes. These results are confirmed by the study on the altitudinal distribution of *C. melanoneura* conducted by Pizzinat *et al.* (2011), where the best climate conditions for aestivation and overwintering were observed between 1350 and 1650 m asl. The distribution of this species seems to be partly conditioned by its thermophily, but first of all by the composition of vegetation (especially the presence of hawthorn, which is more present in warmer biogeographical units) (Lauterer, 1999). *C. melanoneura* frequently hibernates together with the salicicolous psyllids (the so-called *C. saliceti* group) and with *C. affinis* Löw. In its host plants it occurs together with *C. affinis*, *C. peregrina* Förster, and the phenologically delayed *C. crataegi* Schrank (Lauterer, 1999). In the Crimea, members of the population which lives on apple trees will not develop if transferred to hawthorn and die within several days (Lazarev, 1974). More recently, studies on the different *C. melanoneura* populations collected on hawthorn and on apple were carried out to investigate the exchanges in insect populations between the two host plants and the role of hawthorn as reservoir of AP

phytoplasma (Tedeschi *et al.*, 2009). Moreover, ecological trials and genetic analyses carried out by Malagnini *et al.* (2013) confirmed the existence of differentiated populations associated with the two host plants.

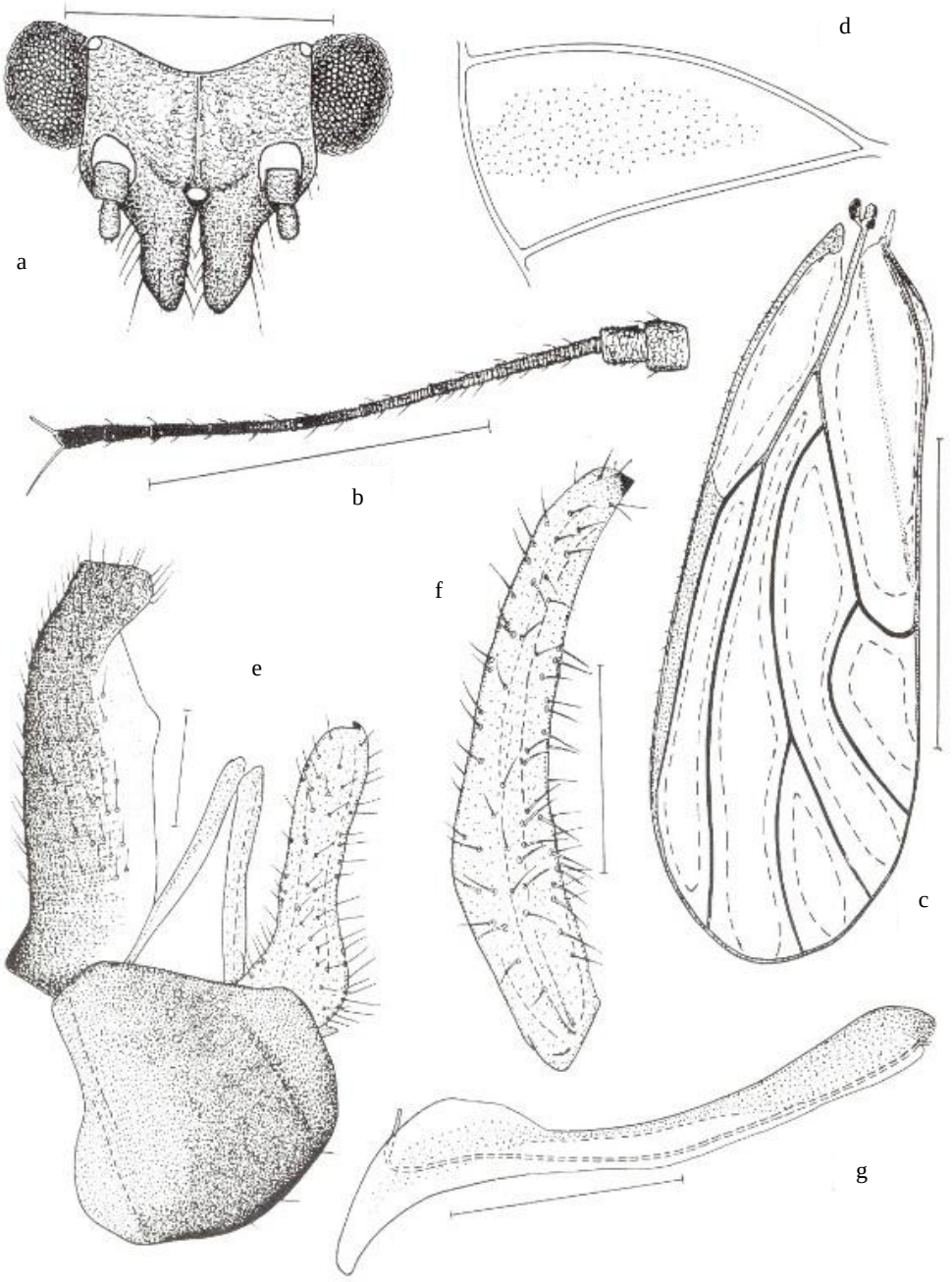

Figure 12**.** *Cacopsylla melanoneura*. Male: (a) head in frontal aspect; (b) left antenna in dorsal aspect; (c) left forewing; (d) cell m1 of forewing; (e) terminalia from the left; (f) left paramere from behind; (g) terminal part of aedeagus from the left. Scale: 1 mm for (c); 0.5 mm for (a) and (b); 0.1 mm for (e), (f) and (g) (modified from Ossiannilsson, 1992).

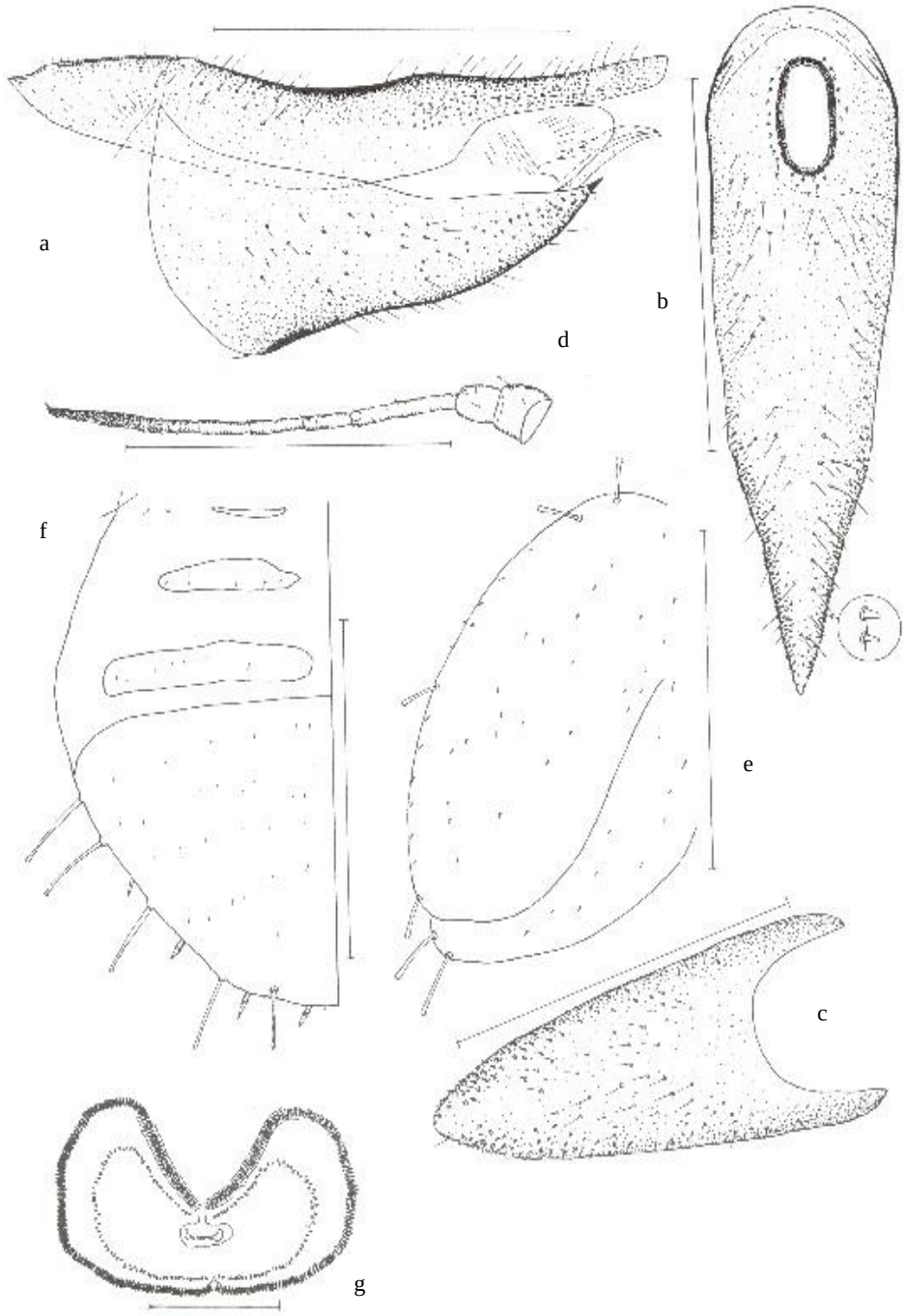

Figure 13. *Cacopsylla melanoneura*. Female: (a) terminalia from the left; (b) proctiger from above, (c) subgenital plate from below. 5th instar nymph: (d) left antenna from above; (e) left wing-pads from above; (f) left half of caudal part of abdominal dorsum; (g) circumanal pore rings from below. Scale: 0.1 mm for (g); 0.5 mm for the rest (modified from Ossiannilsson, 1992).

Other known vectors- Apart from the two psyllids, *Fieberiella florii* Stål (1864), a holarctic leafhopper widely distributed in Europe and introduced also in the USA and Canada, was demonstrated to be able of transmitting AP. This univoltine leafhopper is already known in North America as one of the most important vectors of X-disease (Gold and Silvester; 1982; Van Steenwyk *et al.*, 1990) and was successfully used in transmission trials conducted in Germany and in north-western Italy (Bliefernicht and Krczal, 1995; Krczal *et al.*, 1989; Tedeschi and Alma, 2006). Its presence in apple orchards of Trentino is only occasional (Ioriatti and Jarausch, 2008).

Disease control- All AP symptoms cause a strong economic impact of the disease. The loss of earnings calculated in Italy in 2001, due to lack of production of marketable apple fruits, was of about 100 million euro (Strauss, 2009). As the direct control of the phytoplasma is still unreliable unless using antibiotics, it is very important to adopt preventive strategies to fight AP spread. For instance, propagating material must be carefully selected from sources known to be free of the disease (the certification of plant material through the application of effective indexing procedures is required for new plantations). Moreover, the eradication of newly diseased trees as soon as symptoms appear in the orchards is very effective to reduce sources of inoculum. Trees must be uprooted and all the roots completely removed from the ground and destroyed. Moreover, a monitoring of insect vectors must be carried out and, in occurrence of established populations, the application of insecticide programs must be evaluated.

After a Ministry decree issued mandatory control measures against AP in 2006, a sanitation program was implemented. Trees are regularly inspected for the presence of typical symptoms or tested when no symptoms are found; infected trees are destroyed, and in case that a proportion of diseased trees higher than 20% is observed, the whole orchard has to be uprooted. As a consequence of these measures, the spread of AP has declined, but surveys have to be constantly carried out and treatments against the vectors are prescribed.

Aims of the research

Despite years of systematic control and the consequent strong reduction of psyllids population density, apple proliferation is still a major threat for apple production, especially in some apple growing areas of Trentino. *C. picta* and *C. melanoneura* are ordinarily controlled with chemicals in orchards by means of multiple treatments during springtime. After almost 20 years of research on apple proliferation disease in Trentino, some questions still remain open; in particular, the role of *C. melanoneura* as vector in Trentino is still unclear.

So, aim of this research was to deepen epidemiological, biological and ecological knowledge of the three-way system represented by the phytoplasma and its two hosts (plants and insect vectors). Valsugana (southeastern Trentino) was chosen as study area because of the sudden outbreak observed in the previous years.

The main objectives of the research activities carried out during the years 2014-2016 were:

- monitoring of disease spread and vectors population dynamics, with evaluation of the infectivity of psyllids;
- evaluation of transmission parameters, such as acquisition capacity and transmission efficiency, in different stages of *C. picta* and *C. melanoneura*;
- study of the genetic diversity of phytoplasma strains and geographical distribution in apple plants and psyllid vectors;
- investigations on psyllids' courtship and mating behavior, with special regard to vibrational communication as basic knowledge to develop new control strategies with low impact;
- research of potential new vectors of AP phytoplasma, with characterization of leafhoppers and planthoppers communities in apple orchards and effect of surrounding landscapes on their distribution.

References

Aldaghi M., Massart S., Steyer S., Lateur M., Jijakli M. H. (2007). Study on diverse grafting techniques for their capability in rapid and efficient transmission of apple proliferation disease to different host plants. Bulletin of Insectology, 60: 381-382.

Alma A., Navone P., Visentin C., Arzone A., Bosco D. (2000). Detection of "Apple proliferation" phytoplasmas in *Cacopsylla melanoneura* (Förster) (Homoptera Psyllidae). Petria, 10: 141-142.

Angelini E., Bianchi G. L., Filippin L., Morassutti C., Borgo M. (2007). A new TaqMan method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. Journal of Microbiological Methods, 68: 613-22.

Aryan A., Musetti R., Riedle-Bauer M., Brader G. (2016). Phytoplasma transmission by heterologous grafting influences viability of the scion and results in early symptom development in periwinkle rootstock. Journal of Phytopathology 164(9), DOI: 10.1111/jph.12486.

Bai X., Zhang J., Ewing A., Miller S. A., Jancso Radek A., Shevchenko D. V., Tsukerman K., Walunas T., Lapidus A., Campbell J. W., Hogenhout S. A. (2006). Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. Journal of Bacteriology, 188: 3682-3696.

Baric S., Berger J., Cainelli C., Kerschbamer C., Letschka T., Dalla Via J. (2011). Seasonal colonisation of apple trees by '*Candidatus* Phytoplasma mali' revealed by a new quantitative TaqMan real-time PCR approach. European Journal of Plant Pathology, 129: 455-467.

Baric S., Dalla-Via J. (2004). A new approach to apple proliferation detection: a highly sensitive real-time PCR assay. Journal of Microbiological Methods, 57(1): 135-145.

Baric S., Kerschbamer C., Vigl J., Dalla Via J. (2008). Translocation of apple proliferation phytoplasma via natural root grafts – a case study. European Journal of Plant Pathology, 121: 207-211.

Bertaccini A. (2007). Phytoplasmas: diversity, taxonomy, and epidemiology. Frontiers in Bioscience, 12: 673-89.

Bertaccini A., Contaldo N., Calari A., Paltrinieri S., Windsor H. M., Windsor D. (2010). Preliminary results of axenic growth of phytoplasmas from micropropagated infected periwinkle shoots. 18th IOM 2010, Chianciano Terme, Italy, July 11-16: 147-153.

Bertaccini A., Duduk B. (2009). Phytoplasma and phytoplasma diseases: a new review of recent research. Phytopathologia Mediterranea, 48: 355-378.

Bisognin C., Schneider B., Salm H., Grando M. S., Jarausch W., Moll E., Seemüller E. (2008). Apple proliferation resistance in apomictic rootstocks and its relationship to phytoplasma concentration and simple sequence repeat genotypes. Phytopathology, 98: 153–158.

Bliefernicht K., Krczal, G. (1995). Epidemiological studies on apple proliferation disease in Southern Germany. Acta Horticulturae, 386: 444-447.

Blumer S., Bovey, R. (1957). On apple proliferation virus. Phytopathologische Zeitschrift, 30: 237-258.

Bosco D., Minucci C., Boccardo G., Conti M. (1997). Differential acquisition of chrysanthemum yellows phytoplasma by three leafhopper species. Entomologia Experimentalis et Applicata, 83(2): 219-224.

Bosco D., Marzachì C. (2016). Insect transmission of phytoplasmas. In: Vector-mediated transmission of plant pathogens. Ed. J. Brown, APS Press, Chapter 22: 319-327.

Bovey R. (1963) Apple proliferation. In: Virus diseases of apples and pears, pp. 63-67. Technical Communication, Commonwealth Bureau of Horticulture and Plantation Crops No. 30.

Bovey, R. (1972). Maladie des proliférations. In: *La défense des plantes cultivées*, pp. 227-231. Ed. R. Bovey *et al*. (6th edition). Payot, Switzerland.

Brcák J. (1979) Leafhopper and planthopper vectors of plant disease agents in central and by the aster leafhopper (Homoptera: Cicadellidae). Journal of Economic Entomology, 89: 1223-1232.

Burckhardt D., Ouvrard D. (2012) A revised classification of the jumping plant-lice (Hemiptera: Psylloidea). Zootaxa, 3509: 1-34.

Carraro L., Ermacora P., Loi N., Osler R. (2004). The recovery phenomenon in apple proliferation-infected apple trees. Journal of Plant Pathology, 86(2): 141-146.

Casati P., Quaglino F., Tedeschi R., Spiga M. F., Alma A., Spadone P., Bianco P. A. (2010). Identification and molecular characterization of '*Candidatus* Phytoplasma mali' isolates in North-western Italy. Journal of Phytopathology, 158: 81-87.

Casati P., Stern A., Spadone P., Calvi M., Bulgari D., Bianco P. A. (2007). Molecular diversity in "*Candidatus* Phytoplasma mali" in Lombardia. Bulletin of Insectology, 60: 359-360.

Čermák V., Lauterer P. (2008). Overwintering of psyllids in South Moravia (Czech Republic) with respect to the vectors of the apple proliferation cluster phytoplasmas. Bulletin of Insectology, 61(1): 147-148.

Chang C. J. (1998). Pathogenicity of aster yellows phytoplasma and *Spiroplasma citri* on periwinkle. Phytopathology, 88: 1347-1350.

Chang, C. J., Lee I.-M. (1995). Pathogenesis of diseases associated with mycoplasma-like organisms. Pathogenesis and Host Specificity in Plant Diseases, 1: 237-246.

Christensen N. M., Axelsen K. B., Nicolaisen M., Schulz A. (2005). Phytoplasmas and their interactions with hosts. Trends in Plant Science, 10 (11): 526-535.

Christensen, N. M., Nyskjold, H., Nicolaisen, M. (2013). Real-time PCR for universal phytoplasma detection and quantification. In: *Phytoplasma: Methods and protocols. Methods in molecular biology series*, Vol. 938, pp. 245-252. M. Dickinson & J. Hodgetts, eds. New York, NY, Humana Press. 421 pp.

Ciccotti A. M., Bianchedi P. L., Bragagna P., Deromedi M., Filippi M., Forno F., Mattedi L. (2008). Natural and experimental transmission of *Candidatus* Phytoplasma mali by root bridges. Acta Horticulturae, 781: 459-464.

Conci C., Rapisarda C., Tamanini L. (1992). Annotated catalogue of the Italian *Psylloidea*. First Part (*Insecta Homoptera*). *Atti Accademia Roveretana degli Agiati*, a. 242, ser. VII, vol. II, B: 33-135.

Conci C., Rapisarda C., Tamanini L. (1995). Annotated catalogue of the Italian *Psylloidea*. Second Part (*Insecta Homoptera*). *Atti Accademia Roveretana degli Agiati*, a. 245, ser. VII, vol. V, B: 5-207.

Contaldo N., Bertaccini A., Paltrinieri S., Windsor H. M., Windsor G. D. (2012). Axenic culture of plant pathogenic phytoplasmas. Phytopathologia Mediterranea, 51:607-617.

Cordova I., Jones P., Harrison N.A. and Oropeza C. (2003). *In situ* PCR detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease. Molecular Plant Pathology, 4(2): 99-108.

Dallago G. (2016). Scopazzi del melo: siamo sulla strada giusta. L'Informatore Agrario, 41: 36-39.

Del Serrone P., La Starza S., Krystai L., Kolber M., Barba M. (1998). Occurrence of apple proliferation and pear decline phytoplasmas in diseased pear trees in Hungary. Journal of Plant Pathology, 80: 53-58.

Delić D. (2012). Polymerase chain reaction for phytoplasmas detection. In: *Polymerase Chain Reaction.* D. P. Hernandez-Rodriguez ed. InTech, Shanghai.

Dietrich C. (2005). Key to the families of Cicadomorpha and subfamilies and tribes of Cicadellidae (Hemiptera: Auchenorrhyncha). Florida Entomologist, 88: 502-517.

Doi Y., Terenaka M., Yora K., Asuyama H. (1967). Mycoplasma of PTL group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or Pawlonia witches' broom. Annals of the Phytopathological Society of Japan, 33: 259-266.

Duduk B. (2009). Molecular characterization of phytoplasmas detected in agronomically relevant crops in Serbia. Ph.D. thesis, University of Bologna, Italy.

Duduk B., Bertaccini A. (2011). Phytoplasma classification: Taxonomy based on 16S ribosomal gene, is it enough? Phytopathogenic Mollicutes, 1(1): 3-13.

Duduk B., Calari A., Paltrinieri S., Duduk N., Bertaccini A. (2009). Multigene analysis for differentiation of aster yellows phytoplasmas infecting carrots in Serbia. Annals of Applied Biology, 154(2): 219-229.

Eastop V. F. (1972). Deductions from the present day host plants of aphids and related insects. Symposia of the Royal Entomological Society of London, 6: 157-178.

Eben A., Mühlethaler R., Gross J., Hoch H. (2015). First evidence of acoustic communication in the pear psyllid *Cacopsylla pyri* L. (Hemiptera: Psyllidae). Journal of Pest Science, 88: 87-95.

EPPO/CABI (1997). Apple proliferation phytoplasma. In: *Quarantine pests for Europe*, pp. 959-962. I. M. Smith, D. G. McNamara, P. R. Scott, M. Holderness eds., 2nd ed. CAB International, Wallingford, UK.

Fletcher J., Wayadande A., Melcher U., Ye F. (1998). The phytopathogenic mollicute insect vector interface: a closer look. Phytopathology, 88: 1351-1358.

Flor G. (1861). Zur Kenntnis der Rhynchoten. Beschreibung neuer Arten aus der Familie *Psyllodea* Burm. Bulletin de la Société Impériale des Naturalistes de Moscou, 34: 331-422.

Frisinghelli C., Delaiti L., Grando M. S., Forti D, Vindimian M. E. (2000). *Cacopsylla costalis* (Flor 1861) as a vector of apple proliferation in Trentino. Journal of Phytopathology, 148: 425-431.

Galetto L., Bosco D., Marzachì C. (2005). Universal and group-specific real-time PCR diagnosis of flavescence dorée (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insect vectors. Annals of applied biology, 147(2): 191-201.

Garnier M., Foissac X., Gaurivaud P., Laigret F., Renaudin J., Saillard, Bové J. M. (2001). Mycoplasmas, plants, insect vectors: a matrimonial triangle. Comptes Rendus de l'Académie des Sciences, 324: 923-928.

Gold R. E., Silvester E. S. (1982). Pathogen strains and leafhopper species as factors in the transmission of western X-disease agent under varying light and temperature conditions. Hilgardia, 50 (3): 1-43.

Goodchild A. (1966). Evolution of the alimentary canal in the Hemiptera. Biological Reviews, 41: 97-139.

Gross J., Mekonen N. (2005). Plant odours influence the host finding behaviour of apple psyllids (*Cacopsylla picta*; *C. melanoneura*). IOBC/wprs Bulletin, 28: 351-355.

Gundersen D. E., Lee I. M. (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea, 35: 114-151.

Harisanow A. (1966). Biologische Untersuchungen über den südlichen Apfelbatfloh - *Psylla costalis* Flor. Nauchni Trudove. Vissh Selskostopanski Institut "Vasil Kolarov", 15: 261-270. Heintz, 1986.

Heintz W. (1986) *Cuscuta odorata* - an effective vector for mycoplasma-like organisms (MLO).Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 38:138-141.

Hodkinson I. D. (1974). The biology of the Psylloidea (Homoptera): a review. Bulletin of Entomological Research, 64: 325-339.

Hogenhout S. A., Oshima K., Ammar E. D., Kakizawa S., Kingdom H. N., Namba S. (2008). Phytoplasmas: bacteria that manipulate plants and insects. Molecular Plant Pathology, 51: 91-111.

Ioriatti C., Jarausch W. (2008). Scopazzi del melo. 252 pp. Fondazione Edmund Mach, Trento, Italy.

IRPCM Phytoplasma/Spiroplasma Working Team--Phytoplasma Taxonomy Group (2004). '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. International Journal of Systematic and Evolutionary Microbiology, 54(4): 1243-1255.

Jarausch B., Jarausch W. (2014). Establishment of a permanent rearing of *Cacopsylla picta* (Hemiptera: Psylloidea), the main vector of '*Candidatus* Phytoplasma mali'in Germany. Journal of Pest Science, 87(3): 459-467.

Jarausch B., Schwind N., Fuchs A., Jarausch W. (2011). Characteristics of the spread of apple proliferation by its vector *Cacopsylla picta*. Phytopathology, 101(12): 1471-1480.

Jarausch W., Peccerella T., Schwind N., Jarausch B., Krczal G. (2004). Establishment of a quantitative real-time PCR assay for the quantification of apple proliferation phytoplasmas in plants and insects. Acta Horticulturae, 657: 415-420.

Jarausch W., Saillard C., Dosba F., Bové J. M. (1994). Differentiation of mycoplasmalike organisms (MLOs) in European fruit trees by PCR using specific primers derived from the sequence of a chromosomal fragment of apple proliferation, MLO. Applied and Environmental Microbiology, 60: 2916-2923.

Jarausch W., Saillard C., Helliot B., Garnier M., Dosba F. (2000). Genetic variability of apple proliferation phytoplasmas as determined by PCR-RFLP and sequencing of a non-ribosomal fragment. Molecular and Cellular Probes, 14: 17-24.

Jawhari M., Abrahamian P., Sater A., Sobh H., Tawidian P., Abou-Jawdah Y. (2015). Specific PCR and real-time PCR assays for detection and quantitation of '*Candidatus* Phytoplasma phoenicium'. Molecular and cellular probes, 29(1): 63-70.

Kaminska M., Śliwa H. (2008a). First report of '*Candidatus* Phytoplasma mali' in oriental lilies and its association with leaf scorch in Poland. Plant Pathology, 57: 363.

Kaminska M., Śliwa H. (2008b). Mixed infection of dahlia plants in Poland with apple proliferation and aster yellows phytoplasmas. Plant Pathology, 57: 363.

Kartte S., Seemüller E. (1991). Susceptibility of grafted *Malus* taxa and hybrids to apple proliferation disease. Journal of Phytopathology 131, 137-48.

Kawakita H., Saiki T., Wei W., Mitsuhashi W., Watanabe K., Sato M. (2000). Identification of mulberry dwarf phytoplasmas in the genital organs and eggs of leafhopper *Hishimonoides sellatiformis*. Phytopathology, 90: 909-914.

Kirkpatrick B. C. (1991). Mycoplasma-like organisms: plant and invertebrate pathogens. In: *The Prokaryotes*, pp. 4050-4067. A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.H. Schliefer (eds.). Springer-Verlag Press, New York (USA).

Kison H., Schneider B., Seemüller, E. (1994). Restriction fragment length polymorphisms within the apple proliferation mycoplamsalike organism. Journal of Phytopathology, 141: 395-401.

Krczal G., Krczal H., Kunze L. (1989). *Fieberiella florii* (Stål), a vector of apple proliferation agent. Acta Horticulturae, 235: 99-104.

Kube M., Schneider B., Kuhl H., Dandekar T., Heitmann K., Migdoll A. M., Reinhardt R, Seemüller E. (2008). The linear chromosome of the plant-pathogenic mycoplasma '*Candidatus* Phytoplasma mali'. BMC Genomics, 9: 306.

Lauterer P. (1999). Results of the investigation on Hemiptera in Moravia, made by the Moravian museum (Psylloidea 2). Acta Musei Moraviae, Scientiae Biologicae, 84: 71-151.

Lauterer P., Burckhardt D. (1997). Central and West European willow feeding jumping plant-lice of the genus *Cacopsylla* (Hemiptera: Psylloidea). Entomological Problems, 28: 81-94.

Lazarev M. A. (1974). Leaf-bugs (Homoptera: Psyllidae) of the apple and pear in the orchards of the Crimea. (Morphology, biology, control). Published degree dissertation. Academy of Sciences of the Moldavian SSR, Kishinyov.

Lee I. M., Bertaccini A., Vibio M., Gundersen-Rindal D. E. (1995). Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology, 85: 728-735.

Lee I. M., Davis R. E. (1992). Mycoplasmas which infect plants and insects. In: *Mycoplasmas: Molecular biology and Pathogenesis*, pp. 379-390. J. Maniloff, R. N. McElhansey, L. R. Finch and J. B. Baseman eds. American Society of Microbiology, Washington, DC (USA).

Lee I. M., Davis R. E., Gundersen-Rindal D. E. (2000). Phytoplasma: phytopathogenic mollicutes. Annual Review of Microbiology, 54: 221-255.

Lee I. M., Gundersen-Rindal D., Davis R. E., Bartoszyk I. M. (1998). Revised classification scheme of phytoplasmas based on RFLP analysis of 16S rRNA and ribosomal protein gene sequences. International Journal of Systematic Bacteriology, 48: 1153-1169.

Lee I. M., Hammond R. W., Davis R. E., Gundersen D. E. (1993). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology, 83: 834-842.

Lefol C., Lherminier J., Boudon-Padieu E., Larrue J., Louis C., Caudwell A. (1994). Propagation of Flavescence dorée MLO (mycoplasma-like organism) in the leafhopper vector *Euscelidius variegatus* Kbm. Journal Invertebrate Pathology, 63: 285-93.

Lett J. M., Granier M., Grondin M., Turpin P., Molinaro F., Chiroleu F., Peterschmitt M., Reynaud B. (2001). Electrical penetration graphs from *Cicadulina mbila* on maize, the fine structure of its stylet pathways and consequences for virus transmission efficiency. Entomologia Experimentalis et Applicata, 101: 93-109.

Lorenz K. H., Schneider B., Ahrens U., Seemüller E. (1995). Detection of apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and non ribosomal DNA. Phytopathology, 85: 771-776.

Maejima K., Oshima K., Namba S. (2014). Exploring the phytoplasmas, plant pathogenic bacteria. Journal of General Plant Pathology, 80(3): 210-221.

Malagnini V., Pedrazzoli F., Gualandri V., Zasso R., Bozza E., Fiamingo F., Pozzebon A., Mori N., Ioriatti C. (2010). Detection of '*Candidatus* Phytoplasma mali' in different populations of *Cacopsylla melanoneura* in Italy. Bulletin of Insectology, 63: 59-63.

Malagnini, V., Pedrazzoli F., Papetti C., Cainelli C., Zasso R., Gualandri V., Pozzebon A., Ioriatti C. (2013). Ecological and genetic differences between *Cacopsylla melanoneura* (Hemiptera, Psyllidae) populations reveal species host plant preference. PLoS ONE 8: e69663.

Marcone C., Ragozzino A., Seemüller E. (1996). Association of phytoplasmas with the decline of European hazel in southern Italy. Plant Pathology, 45: 857-863.

Marcone C., Seemüller E. (2013). Widespread occurrence of apple proliferation disease in low-intensity orchards of Basilicata. Journal of Plant Pathology, 95(4S): S4.50-51.

Martini M. Ermacora, P. Falginella, L. Loi, N. Carraro, L. (2008). Molecular differentiation of '*Candidatus* Phytoplasma mali' and its spreading in Friuli Venezia Giulia region (North-East Italy). Acta Horticulturae, 781: 395-402.

Marwitz R., Petzold H., Kunze L. (1974). [Investigation on the transfer of the possible causal agent of apple proliferation to an herbaceous host. Phytopathologische Zeitschrift, 81: 85-91.

Marzachì C., Veratti F., Bosco D. (1998). Direct PCR detection of phytoplasmas in experimentally infected insects. Annals of Applied Biology, 133(1): 45-54.

Mattedi L., Forno F., Varner M. (2007). Scopazzi del melo. Conoscenze ed osservazioni di campo. 144 pp. Arti Grafiche La Commerciale-Borgogno, Bolzano (Italy).

Mayer C. J., Gross J., (2007). Different host plant odours influence migration behaviour of *Cacopsylla melanoneura* (Förster), an insect vector of the apple proliferation phytoplasma. IOBC/WPRS Bulletin, 30: 177-184.

Mayer C. J., Vilcinskas A., Gross J. (2010). Chemically mediated multitrophic interactions in a plant–insect vector-phytoplasma system compared with a partially nonvector species. Agricultural and Forest Entomology, 13 (1): 25-35.

McCoy R. E., Caudwell A., Chang C. J., Chen T. A., Chiykowski L. N., Cousin M. T., Dale De Leeuw G. T. N., Golino D. A., Hackett K. J., Kirkpatrick B. C., Marwitz R., Petzolt H., Sinha R. H., Sugiura M., Whitcomb R. F., Yang I. L., Zhu B. M., Seemuller E. (1989). Plant diseases associated with mycoplasmalike organisms. In: *The Mycoplasmas*, 5: 545- 640. R. F. Whitcomb, J. G. Tully eds. Academic Press, New York (USA).

Mehle N., Brzin J., Boben J., Hren M., Frank J., Petrovič N., Gruden K., Dreo T., Žežlina I., Seljak G., Ravnikar M. (2007). First report of *'Candidatus* Phytoplasma mali' in *Prunus avium, P. armeniaca* and *P. domestica*. Plant Pathology, 56(4): 721.

Minucci C., Navone P., Boccardo G. (1996). Presenza di scopazzi del melo (apple proliferation) in frutteti del Piemonte. Informatore Fitopatologico, 46 (6): 47–49.

Monti M., Martini M., Tedeschi R. (2013). EvaGreen Real-time PCR protocol for specific '*Candidatus* Phytoplasma mali' detection and quantification in insects. Molecular and Cellular Probes, 27(3-4): 129-136.

Murral D. J., Nault L. R., Hoy C. W., Madden L. V., Miller S. A. (1996). Effects of temperature and vector age on transmission of two Ohio strains of aster yellows phytoplasma by the aster leafhopper (Homoptera: Cicadellidae). Journal of Economic Entomology, 89: 1223-1232.

Musetti R., di Toppi L. S., Ermacora P., Favali M. A. (2004). Recovery in apple trees infected with the apple proliferation phytoplasma: an ultrastructural and biochemical study. Phytopathology, 94(2): 203-208.

Musetti R., Paolacci A., Ciaffi M., Tanzarella O. A., Polizzotto R., Tubaro, F., Mizzau M., Ermacora P., Badiani M., Osler R. (2010). Phloem cytochemical modification and gene expression following the recovery of apple plants from apple proliferation disease. Phytopathology, 100(4): 390-399.

Nagaich B. B., Puri B. K., Sinha R. C., Dhingra M. K., Bhardwaj V. P. (1974). Mycoplasma-like organisms in plants affected with purple top-roll, marginal flavescence and witches' broom diseases of potatoes. Phytopathologische Zeitschrift, 81: 273-379.

Nakashima K., Hayashi T. (1995). Multiplication and distribution of rice yellow dwarf phytoplasma in infected tissues of rice and green rice leafhopper *Nephotettix cincticeps*. Annals of the Phytopathological Society of Japan, 61: 451-455.

Namba S., Kato S., Iwanami S., Oyaizu H., Shiozawa H., Tsuchizaki T. (1993). Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. Phytopathology, 83: 786-791.

Nikolić P., Mehle N., Gruden K., Ravnikar M., Dermastia M. (2010). A panel of real-time PCR assays for specific detection of three phytoplasmas from the apple proliferation group. Molecular and cellular probes, 24(5): 303-309.

Oettl S., Schlink K. (2015). Molecular identification of two vector species, *Cacopsylla melanoneura* and *Cacopsylla picta* (Hemiptera: Psyllidae), of apple proliferation disease and further common psyllids of Northern Italy. Journal of economic entomology, 108 (5): 2174-2183.

Ossiannilsson F. (1992). The Psylloidea (Homoptera) of Fennoscandia and Denmark. 346 pp., Fauna Entomologica Scandinavica, 26, E. J. Brill, Leiden (The Netherlands).

Pedrazzoli F. (2009). Investigations on the psyllid (Hemiptera: Psyllidae) vectors of '*Candidatus* Phytoplasma mali' in Trentino. 192 pp. Ph.D. Thesis, University of Padova, Italy.

Pedrazzoli F., Ciccotti A., Bianchedi P. L., Salvadori A., Zorer R. (2008). Seasonal colonisation behaviour of '*Candidatus* Phytoplasma mali' in apple trees in Trentino. Acta Horticulturae, 781: 483–489.

Pinna M., Gamba U., Spagnolo S., Zaccara P., Tedeschi R., Gallo S. (2003). Monitoraggio dei meleti del Canavese colpiti da fitoplasmi agenti causali di AP (Apple Proliferation). Bollettino di Agricoltura Biologica. Edited by CRAB (Reference Centre for Organic Farming, Turin Province), (1): 9-20.

Pizzinat A., Tedeschi R., Alma A. (2011). *Cacopsylla melanoneura* (Foerster): aestivation and overwintering habitats in Northwest Italy. Bulletin of Insectology, 64: S135-136.

Ploaie P. G. (1981). Mycoplasmalike organisms and plant diseases in Europe, pp. 61-104. In: *Plant Diseases and Vectors: Ecology and Epidemiology* K. Maramorosch, K. F. Harris, eds. New York, NY, USA: Academic Press.

Poggi Pollini C., Zelger R., Wolf M., Bissani R., Giunchedi L. (2002). Indagine sulla presenza di psillidi infetti dal fitoplasma degli scopazzi del melo (AP=apple proliferation) in provincia di Bolzano. ATTI Giornate Fitopatologiche, 2: 607-612.

Purcell A. H. (1982). Insect vector relationships with prokaryotic plant pathogens. Annual Review of Phytopathology, 20: 397-417.

Refatti E., Ciferri R. (1954). La virosi del tipo a scopazzi in vivai di melo. Annali della Sperimentazione Agraria, 8: 1543-1556.

Rui D. (1950). Una malattia inedita: la virosi a scopazzi del melo. Humus, 6 (11): 7-10.

Schaper U., Seemüller E. (1984). Recolonization of the stem of apple proliferation and pear decline-diseased trees by the causal organisms in spring. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz/Journal of Plant Diseases and Protection, 91(6): 608-613.

Schmid G. (1965). Five and more years of observations on the proliferation virus of apples in the field. Zastita Bilja, 16: 285-291.

Schneider B., Ahrens U., Kirkpatrick B. C., Seemüller E. (1993). Classification of plant pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. Journal of General Microbiology, 139: 519-527.

Schneider B., Marcone C., Kampmann M., Ragozzino A., Lederer W., Cousin M. T., Seemüller E. (1997). Characterization and classification of phytoplasmas from wild and cultivated plants by RFLP and sequence analysis of ribosomal DNA. European Journal of Plant Pathology, 193: 675-686.

Schneider B., Seemüller E., Smart C. D., Kirkpatrick B. C. (1995). Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology*, vol. 1, pp. 369-380. S. Razin and J. G. Tully eds. San Diego: Academic Press.

Schuch K. (1962). Importance diagnostique des stipules pour la prolifération du pommier. Nachrichtenblatt für den Deutschen Pflanzenschutzdienst (Braunschweig), 14: 85-88.

Seemüller E. (1990). Apple proliferation. In: *Compendium of apple and pear diseases*, pp. 67-68. American Phytopathological Society, St. Paul, Minnesota (USA).

Seemüller E. (2002). Apple proliferation: etiology, epidemiology and detection. In: *Atti Giornate Fitopatologiche*, vol. I, pp. 3-6. A. Brunelli A. and A. Canova eds. Baselga di Piné (Trento), 7-11 April 2002.

Seemüller E., Marcone C., Lauer U., Ragozzino A., Göschl M. (1998). Current status of molecular classification of the Phytoplasmas. Journal of Plant Pathology, 80: 3-26.

Seemüller E., Schaper U., Zimbelmann F. (1984). Seasonal variation in the colonization patterns of mycoplasma-like organisms associated with apple proliferation and pear decline. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz, 91: 371-382.

Seemüller E., Schneider B. (2004). '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. International Journal of Systematic and Evolutionary Microbiology, 54: 1217-1226.

Seidl V., Komárková (1974). Studies on natural spread of proliferation disease of apple. Phytopathologische Zeitschrift, 81: 301-313.

Smart C. D., Schneider B., Blomquist C. L., Guerra L. J., Harrison N. A., Ahrens U., Lorenz K. H. , Seemüller E., Kirkpatrick B. C. (1996). Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. Applied and Environmental Microbiology, 62(8): 2988-2993.

Spagnolo S., Gamba U., Pinna M., Zaccara P., Tedeschi R., Gallo S. (2005). Monitoraggio dei meleti del Pinerolese colpiti da Apple Proliferation. Bollettino di Agricoltura Biologica edited by CRAB (Reference Centre for Organic Farming, Turin Province), (2): 21-36.

Springhetti M., Ianes P. G., Dallago G. (2002). Diffusione degli scopazzi (AP) nel melo nelle valli del noce. In: *Atti Giornate Fitopatologiche*, vol. II, pp. 599-606. A. Brunelli A. and A. Canova eds. Baselga di Piné (Trento), 7-11 April 2002.

Strauss E. (2009). Phytoplasma research begins to bloom. Science, 325(5939): 388-390.

Tedeschi R., Alma A. (2004). Transmission of apple proliferation phytoplasma by *Cacopsylla melanoneura* (Homoptera: Psyllidae). Journal of Economic Entomology, 97(1): 8-13.

Tedeschi R., Alma A. (2006). *Fieberiella florii* (Homoptera: Auchenorrhyncha) as a vector of "*Candidatus* phytoplasma mali". Plant Disease, 90 (3): 284-290.

Tedeschi R., Bosco D., Alma A. (2002). Population dynamics of *Cacopsylla melanoneura* (Homoptera: Psyllidae), a vector of apple proliferation phytoplasma in northwestern Italy. Journal of Economic Entomolgy, 95 (3): 544-551.

Tedeschi R., Lauterer P., Brusetti L., Tota F., Alma A. (2009). Composition, abundance and phytoplasma infection in the hawthorn psyllid fauna of northwestern Italy. European Journal of Plant Pathology, 123(3): 301-310.

Tedeschi R., Visentin C., Alma A., Bosco D. (2003). Epidemiology of apple proliferation (AP) in northwestern Italy: evaluation of the frequency of AP-positive psyllids in naturally infected populations of *Cacopsylla melanoneura* (Homoptera: Psyllidae). Annals of Applied Biology, 142: 285-290.

Torres E., Bertolini E., Cambra M., Montón C., Martín M. P. (2005). Real-time PCR for simultaneous and quantitative detection of quarantine phytoplasmas from apple proliferation (16SrX) group. Molecular and Cellular Probes, 19(5): 334-340.

Tsai J. H. (1979). Vector transmission of mycoplasmal agents of plant diseases. In: *The Mycoplasmas*, 3: 265-307. R. F. Whitcomb and J. G. Tully eds. Academic Press, New York (USA).

Van Steenwyk R. A., Havens D. M., Freeman R. (1990). Evaluation of trap types for two vectors of western X-Disease: *Colladonus montanus* and *Fieberiella florii* (Homoptera: Cicadellidae). Journal of Economic Entomology, 83: 2279-2283.

Vindimian M. E. (2002). Scopazzi del melo: diffusione in Trentino e ricerche in atto. In: *Atti Giornate Fitopatologiche*, vol. I, pp. 7-12. A. Brunelli A. and A. Canova eds. Baselga di Piné (Trento), 7-11 April 2002.

Vindimian M. E., Delaiti L. (1996). Indagine sistematica sugli scopazzi del melo. Terra Trentina, 11: 30-33.

Wei W., Davis R. E., Lee I. M., Zhao Y. (2007). Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma group. International Journal of Systematic and Evolutionary Microbiology, 57(8): 1855-1867.

Weintraub P. G., Beanland L. (2006). Insect vectors of phytoplasmas. Annual Review of Entomology, 51: 91-111.

Weintraub P. G., Jones P. (Eds.). (2010). Phytoplasmas: genomes, plant hosts and vectors. 331 pp. CABI International, Oxfordshire, UK.

Welliver R. (1999). Diseases caused by phytoplasmas. Regulatory horticulture. Plant Pathology Circular, 42: 17-22.

Web pages:

Wilson M. R., Turner, J. A. (2010). Leafhopper, Planthopper and Psyllid Vectors of Plant Disease. Amgueddfa Cymru - National Museum Wales. Available online at http://naturalhistory.museumwales.ac.uk/Vectors

CHAPTER 1

Apple proliferation in Valsugana: three years of disease and psyllid vectors' monitoring

Abstract

Apple proliferation (AP) is a phytoplasma-caused disease widespread in many European regions, and represents a serious problem in Italian apple orchards. The etiological agent, '*Candidatus* Phytoplasma mali', causes symptoms suggesting profound disorders in the normal balance of hormones in infected plants: witches' brooms, enlarged stipules, early leaf reddening in autumn. The fruits of infected trees, which are smaller and have altered organoleptic properties, cannot be commercialized. Valsugana (Trentino, Italy), a representative apple-growing area with high incidence of AP, was chosen as model region to study and to monitor the evolution and spread of AP during three years. To reach this goal, the distribution of infected plants was mapped along the valley and the populations of the psyllid vectors (*Cacopsylla picta* and *Cacopsylla melanoneura*) were monitored. The final aims were drawing a picture of the disease spread to help advisors to optimize the current control strategies and to have the information necessary to correlate the presence of psyllid vectors and symptomatic trees, building new knowledge on their biology and ecology. To limit AP spread, a national strategy was established, in which uprooting of diseased trees and control of vector populations are compulsory.

After a three-year survey, thanks to the phytosanitary measures adopted in the area, the percentage of apple plants drastically decreased. Regarding the psyllid vectors, *C. melanoneura* showed higher population levels compared to *C. picta* in both conventional and untreated orchards, but the percentages of infected individuals were higher in the latter species.

Key words: apple proliferation, psyllid vectors, control strategies.

Introduction

Apple proliferation (AP) is a phytoplasma-caused disease affecting many apple-growing areas in Europe. The etiological agent is '*Candidatus* Phytoplasma mali', which is transmitted mainly by sap-sucking insects (Tedeschi and Alma, 2004, Frisinghelli *et al.*, 2000, Jarausch *et al.*, 2004), by grafting and by root bridges (Ciccotti *et al.*, 2007; Mattedi *et al.*, 2007).

Cacopsylla picta Förster was proved to be the most important vector of '*Ca.* P. mali' in Germany and in north-eastern Italy (Jarausch *et al.*, 2007), whereas *Cacopsylla melanoneu*ra Förster was reported to play an important role in transmitting AP in northwestern Italy (Tedeschi *et al.*, 2002). Overwintered adults (remigrants) of both psyllid species reach the orchards in springtime (*C. melanoneur*a around the beginning of February and *C. picta* in March) to reproduce on apple trees. *C. melanoneura* can survive and reproduce also on hawthorn (*Crataegus* spp.), even if the presence of two different host races, one reproducing preferentially on apple and the other on hawthorn, was hypothesized by Malagnini *et al.* (2013). After nymph development, the springtime generation (emigrants) of the two species leaves the orchards (*C. melanoneura* around the middle of June; *C. picta* in July) to aestivate and overwinter on shelter plants (probably conifers at high altitudes) (Mattedi *et al.*, 2007; Ossiannilsson, 1992; Lauterer, 1999; Tedeschi *et al.*, 2012).

In Trentino, AP was reported for the first time in 1954 by Refatti and Ciferri and, until the early '90s, its presence was sporadic. The first impacting outbreak of the disease was reported in Val di Non and Val di Sole, two hilly areas in the northwestern part of Trento province, in the early 2000s (Vindimian and Delaiti, 1996; Vindimian *et al.*, 2002). It was supposed that this sudden outbreak had to be attributed to several causes, such as rising temperatures, the presence of new plantations with more sensitive and weaker plants, the increase of virulence of the phytoplasma strains and even the exponential increase of the insect vector populations due to the significant reduction of insecticides (Dallago, 2016).

After this outbreak, a monitoring activity started in all the valleys of the region in which the disease was recorded. All varieties of commercial rootstocks are susceptible to the infection of AP phytoplasma and there are no therapies that can cure infected plants. The current management strategy to control the spread of AP is based on the application of insecticides against the known vectors and on uprooting the symptomatic plants and replacing them with healthy material. These phytosanitary measures, which are aimed at reducing the inoculum source of '*Ca.* P. mali', resulted in a strong reduction of symptomatic trees, which reached an average level of 0.38% in Trentino in 2010 (Figure 1.1). Unfortunately, the infection rate re-increased again starting from 2011, more significantly in Valsugana, which is an important apple growing area of the province (Dallago, 2016).

A systematic monitoring of the psyllid populations and a survey of the symptom evolution is being carried out in a representative number of apple orchards of Trento province, providing the basic information for the technical advisory service of Fondazione Edmund Mach (FEM) to recommend the correct control strategies to the producers. In parallel, in order to maximize

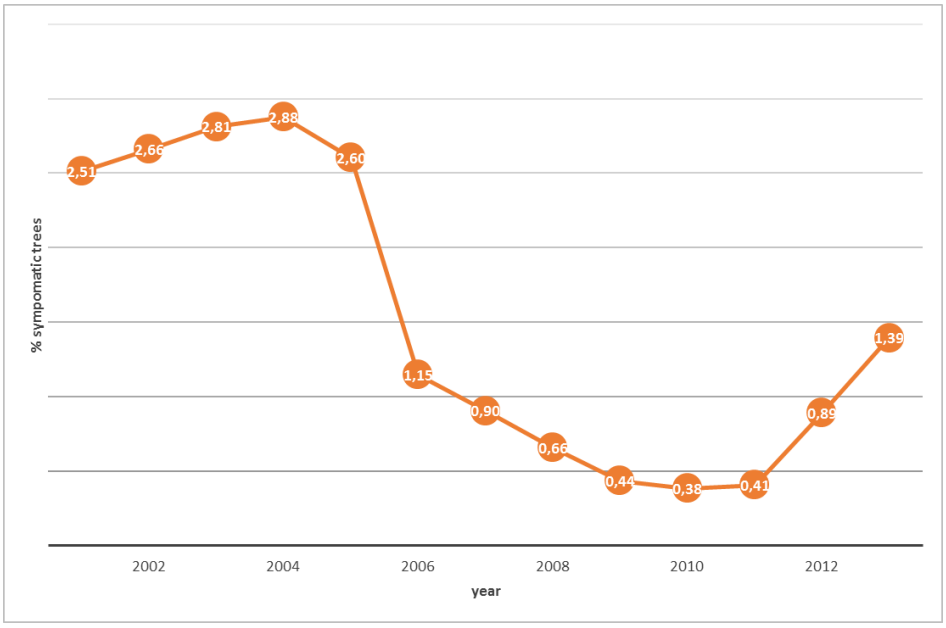

Figure 1.1. Trend of symptomatic trees resulting from the monitoring activity in Trentino during the years 2001-2013 (modified from Dallago, 2016).

the effectiveness of insect vectors control by minimizing the negative effect of insecticide treatments, new formulations, for which a lower impact on the health of local citizenship and, in general, on the environment can be assumed, are tested at FEM. These activities are completed by the assessment of the risk of resistance to insecticides in the vector populations due to their increased use (Baldessari *et al.*, 2017).

Territorial observations collected in a systematic way are essential to improve the existing measures of disease control and implement new information. These activities, linked to basic research, aim at obtaining a shared knowledge useful for taking operational decisions and interpretation of research results, and provide continuity and interrelation between research and applications in field.

The last 15 years of monitoring revealed a significant presence of AP in some areas of the valley, especially in Alta Valsugana. Control strategies are oriented to eradication of the phytoplasma inoculum source, which is the basic precondition to control the disease.

Regarding phytoplasma vectors, knowledge about psyllids' dynamics in the orchards is important to manage insecticide applications. Tedeschi *et al.* (2012) correlated immigration dynamics to the temperatures registered in the apple orchards, and defined an immigration index to predict the progressive arrival of overwintered adults of *C. melanoneura* from winter sites. Later, thanks to the data collected in Valsugana, Baldessari *et al.* (2015) validated this immigration index. They improved the index to ascertain whether it is feasible to make a quantitative prediction of the *C. melanoneura* migration.

This index follows the formula:

$$Ii = [(T_{7n} - T_{th}) + dd_n]$$

where T_{7n} is the average of maximum temperatures of the seven days preceding any sampling date and T_{th} the thermal threshold of 9.5°C; dd_n is the number of hours above the threshold per week. Remigration starts when Ii >0, but first captures in the orchard happen when Ii > 2. The migration process is scalar, as the population does not move massively in one or few episodes, but gradually colonizes the orchards. Migration and immigration processes into the orchards seem to be driven by climatic parameters such as updraft and temperature (Figure 1.2).

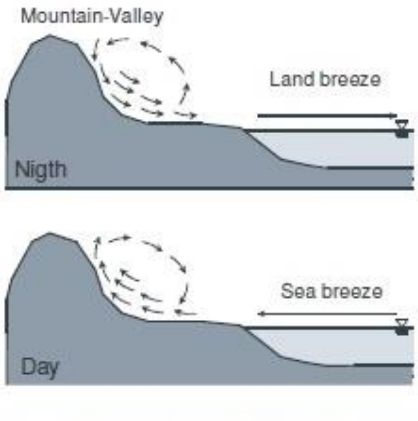

Figure 1.2. The mountain- valley winds configuration (from Truccolo, 2011).

This information is useful to plan the control strategies as well as to avoid an uncorrected and untimely use of insecticides, thus reducing the number of treatments (Baldessari *et al.*, 2010). The proportion of AP-infected trees observed in Valsugana was 5.04% in 2013, resulting the highest recorded in Trentino in that year (Dallago, 2014). Therefore, a working group composed of researchers and technicians from FEM, in collaboration with representatives of the Valsugana fruit cooperatives, was involved in regular monitoring activities. The aim of this three years-work was to study AP spread in Valsugana through monitoring both symptomatic plants and psyllid population dynamic and infectivity to draw a picture of AP epidemiology, build new knowledges about psyllids immigration and help advisors to optimize current control strategies.

Material and methods

Study area

Valsugana, one of the most important apple producing valleys in the province of Trento, is divided into two geographical districts (Alta Valsugana and Bassa Valsugana) and extends for approximately 970 km^2 in the southeastern Trentino (Figure 1.3). It is enclosed by two mountain ranges and is characterized by the occurrence of an enormous variability of habitats. The area of apple orchards is almost 800 ha and every year there is a mean fruit production of 450 q/ha. Besides apples, the other main products of the valley are corn and soft fruits.

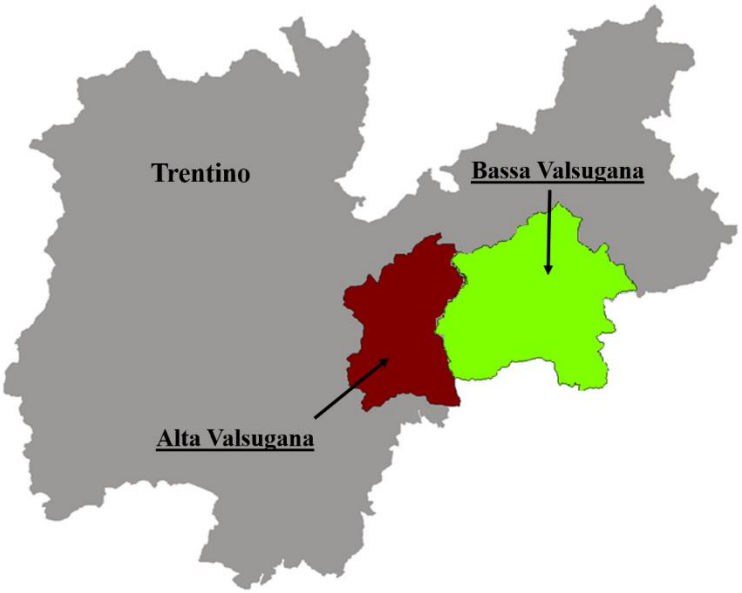

Figure 1.3. Map of Trentino, representing the two districts of Valsugana.

Disease monitoring

AP monitoring, a mandatory surveillance activity conducted by the phytosanitary service of the Autonomous Province of Trento (PAT), was carried out in post-harvest time (end of September, October and November) for three years: 2014, 2015 and 2016. The sampling was conducted by dividing the orchards into fruit subunits. It was carried out by choosing a minimum number of plots to be statistically significant and following the "rotational sample" method. In this way, each year 30% of the surveyed plots was randomly replaced with an equal number of new plots. AP affected plants were identified through the detection of characteristic symptoms: witches' brooms and enlarged and serrated stipules together with reddening, small apples, altered apical rosettes and autumn flowers. More or less intense chlorotic phenomena only represent an alert and need more accurate controls, so plants with only this symptom were not considered symptomatic.

The plots monitored protocol consisted of: each plant was controlled by both sides simultaneously by two technicians and symptomatic plants were marked at the base of the trunk with two longitudinal bands. The data collected and additional observations were recorded. Different colors were chosen every year mark symptomatic trees to verify whether the plants previously labeled had been eradicated (Figure 1.4).

Figure 1.4. Symptomatic apple plants marked with different colors during the monitoring in the years (M. Baldessari).

In the already monitored orchards, symptomatic plants were reported and divided into three categories, respectively denominated new-, old- and latent- symptomatic plants. The sum of the three categories represents the total number of symptomatic plants for each fruit subunit. The data referring to Alta Valsugana district were clustered in six groups representing the rural areas where AP caused the most serious damages in the past years: Perginese, Tenna, Vigolana, Caldonazzo, Levico Terme. Regarding Bassa Valsugana, characterized by a lower presence of the disease, data were merged in a unique group.

Psyllid vectors monitoring

To monitor the vector populations during the years, weekly samplings were carried out in 17 conventional orchards and in one control untreated orchard. Data collected in the untreated orchard are useful to define the species phenology, avoiding stop given by multiple treatments that occur in conventional orchards. All the operators involved in the monitoring respected the same meteorological conditions. In particular, all samplings were carried out without wind and rain, on dry canopy and avoiding cloudy days.

A map and a list of the sampling sites are reported in Figure 1.5 and Table 1.1, respectively. As for symptomatic plant monitoring, data clustered per area are shown.

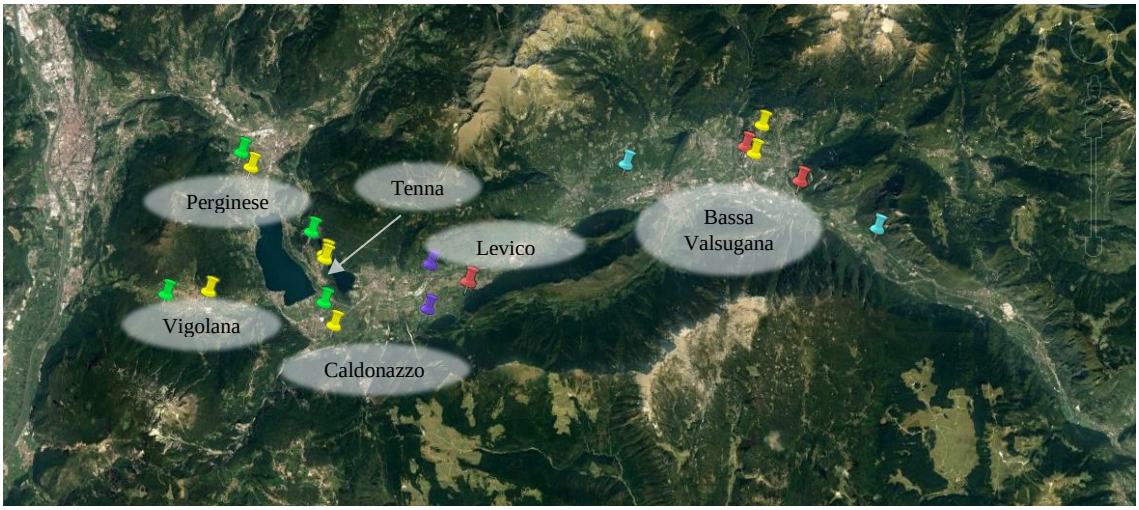

Figure 1.5. Aerial vision of the 18 points chosen for psyllid monitoring; different colors represent the different operators who did surveys.

Insects were collected using the beating tray method (Müther and Vogt, 2003): the branches of trees were hit with padded sticks and the insects falling down were collected in a rectangular funnel (60 x 40 x 35 cm), with a plastic box at the base. For each site, 50 branches (25 per each side of a row) were beaten twice, for a total of 100 beatings (Figure 1.6).

Figure 1.6. Insect sampling: A. both sides of a row were beaten in each orchard; B. *Frappage* was used to collect insects.

After collection, insects were stored at -20°C prior to taxonomic classification and molecular analysis to detect AP infection. Psyllid species were identified at the stereomicroscope using the identification keys in the book "The Psylloidea (Homoptera) of Fennoscandia and Denmark" (Ossiannilsson, 1992) and in the web page http://www.psyllidkey.com.

Table 1.1. List of the sites monitored for the psyllid population dynamics in Valsugana.

	Site	Position	Altitude (msl)
Alta Valsugana	Susà (Pergine Valsugana)	46° 3'0.13"N 11°13'28.09"E	530
	Susà (Pergine Valsugana)	46° 3'20.19"N 11°13'6.89"E	525
	Vigolo Vattaro	46° 0'0.23"N 11°10'51.54"E	690
	Vigolo Vattaro	46° 0'5.37"N 11°12'17.60"E	684
	Caldonazzo	45°59'24.30"N 11°16'27.47"E	482
	Caldonazzo	45°59'55.83"N 11°16'5.51"E	456
	Tenna	46° 1'2.20"N 11°16'3.00"E	610
	Tenna	46° 1'32.37"N 11°15'35.28"E	616
	Levico Terme	46° 0'53.30"N 11°19'34.20"E	501
	Barco (Lavico Terme)	46° 0'31.37"N 11°20'50.90"E	438
	Santa Giuliana (Levico Terme)	45°59'51.78"N 11°19'32.70"E	450
Bassa Valsugana	Borgo Valsugana	46° 3'21.60"N 11°25'58.26"E	402
	Scurelle	46° 3'51.97"N 11°29'58.88"E	396
	Scurelle	46° 3'42.80"N 11°30'16.55"E	378
	Villa Agnedo	46° 3'6.33"N 11°31'49.93"E	360
	Spera	46° 4'22.18"N 11°30'29.91"E	601
	Ospedaletto	46° 2'2.80"N 11°34'25.98"E	294
	Untreated orchard (Tenna)	46° 0'56.07"N 11°16'4.00"E	587

'Ca. P. mali' detection in psyllids

The specimens were subjected to molecular analysis in order to assess the presence of AP phytoplasma. After lyophilization and mechanical disruption of the samples, the total DNA was

extracted using the commercial kit NucleoSpin® Tissue (Macherey-Nagel) and the samples were analyzed by real-time PCR following the method developed by Baric and Dalla Via (2004). Real-time PCR analyses of the insects were performed in duplicates in 20 µl-reactions, containing 10 µl Kapa Probe Fast qPCR Master Mix (2X) Universal (Kapa Biosystems Roche), 900 nM of primers qAP-16S-F (CGA ACG GGT GAG TAA CAC GTA A) and qAP-16S-R (CCA GTC TTA GCA GTC GTT TCC A), 200 nM of qAP-16S probe (FAM-TAA CCT GCC TCT TAG ACG) and 2 µl template DNA, normalized to 10 ng/µl. Reactions were performed in a Roche LightCycler® 480 and cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

To evaluate the infection of samples, amplification curves and C_p values were considered. C_p, the crossing point-PCR-cycle, corresponds to the cycle at which fluorescence achieves a defined threshold. The defined threshold is based on the "baseline fluorescence" that represents the background signal, which is more evident before a significant accumulation of target amplicon. After this stage, the exponential phase, when amplification is most efficient, starts. The number of cycles needed for the amplification-associated fluorescence to reach this threshold level of detection is inversely correlated to the amount of nucleic acid that was in the original sample (Rodriguez-Lazaro and Hernandez, 2013). Therefore, samples showing a sigmoidal amplification curve and a C_p value lower than 30 cycles in both replicates were considered highly positive; samples with a sigmoidal amplification curve but C_p values above 30 in at least one replication were judged weakly positive; samples without sigmoidal amplification curve were considered negative for the presence of '*Ca.* P. mali'.

All samples were extracted and analyzed singularly, apart from *C. melanoneura* individuals collected in 2016, due to the high numbers. In this case, following Tedeschi *et al.* (2003), all specimens corresponding to the same date and sampling site were grouped in pools of five individuals. The proportion of infected insects was estimated with the maximum likelihood estimator, p^, calculated according to Swallow (1985):

$$p\hat{} = 1\text{-}H^{1/k}$$

where H is the observed fraction of uninfected insects and k is the number of insects per group.

Overwintering sites and shelter plants

To search for the overwintering sites of psyllids in Valsugana, and to further validate the immigration index developed by Tedeschi *et al.* (2012), some samplings were conducted during

January 2015, before the arrival of overwintered psyllids into the apple orchards. The sampled areas were identified by considering the updraft of winds between mountains and valley. Furthermore, the maps of wind currents were overlapped with the maps of forest roads, in order to be able to reach the sites (Figure 1.7). Ten sites were chosen at different altitudes (between 720 and 1480 m asl) on the mountains of Valsugana. To collect insects, a modified net (2-3 m long) was used (Figure 1.8). The monitored plant species were conifers, as with special regards to those reported in literature (Ossiannilsson, 1992; Lauterer, 1999). The insects collected were stored at -20°C prior to taxonomic classification, performed as described above.

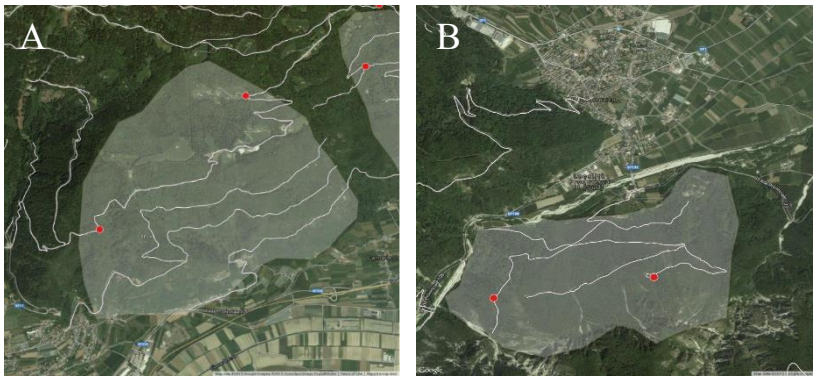

Figure 1.7. Examples of the areas surveyed to look for overwintering sites.
A. Levico Terme; B. Centa di Caldonazzo.

Figure 1.8. Insect collection from conifers with a modified net.

Results

Disease monitoring

The numbers of plants monitored in Valsugana during the three years of survey are summarized in Table 1.2, which reports the surface of the sampled area, the total number of monitored plants and the number of symptomatic trees.

Table 1.2. Summary of the data collected during the three-year monitoring in Valsugana.

year	surface (ha)	monitored plants	sympotomatic plants	percetage
2014	75.3	210.307	12.208	5.80
2015	32.0	96.056	1.012	1.05
2016	55.3	159.403	1.911	1.20

The orchards surveyed are representative of the whole valley. The results of the monitoring performed in 2016 are shown in Figures 1.9, 1.10 and 1.11, which represent a picture of the actual situation. Valsugana is divided into three areas: Alta Valsugana, Caldonazzo-Levico (the central part of Valsugana), and Bassa Valsugana. The different colors show the ranges of symptomatic tree percentages recorded in each monitored subunit. The percentages are grouped in classes on the basis of the historical data, where the majority of orchards were characterized by an AP prevalence between 0 and 1%.

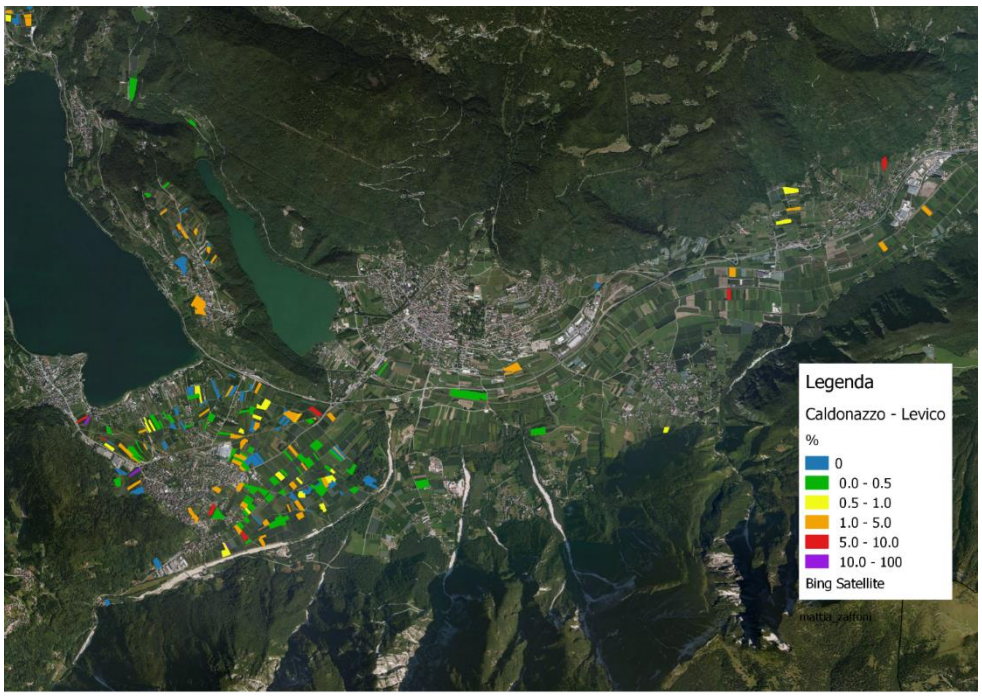

Figure 1.9. AP prevalence in Alta Valsugana (2016).

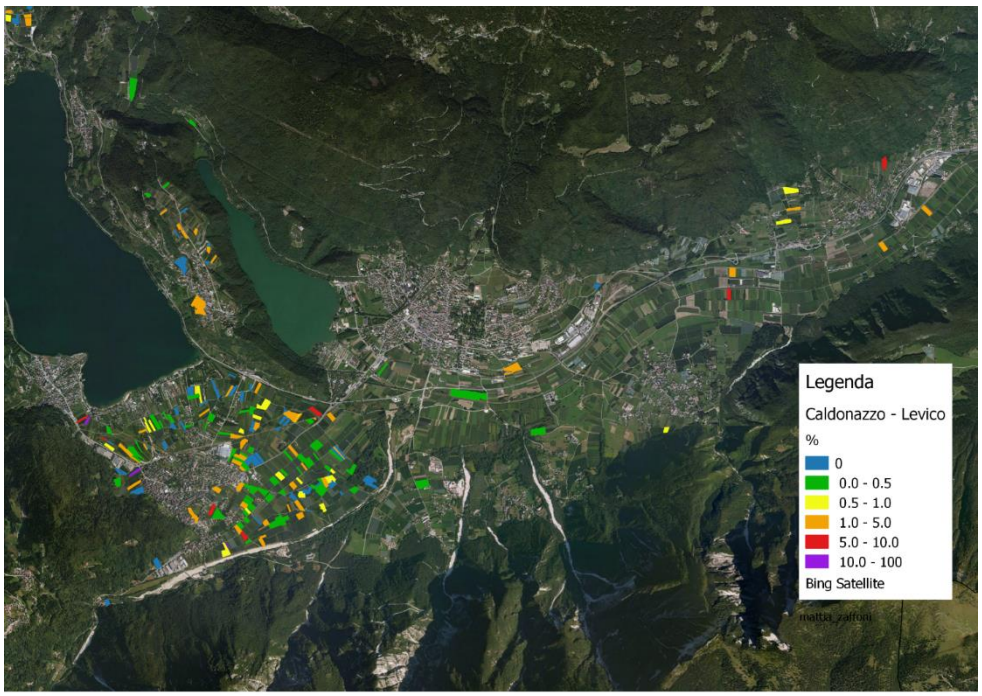

Figure 1.10. AP prevalence in Caldonazzo-Levico (2016).

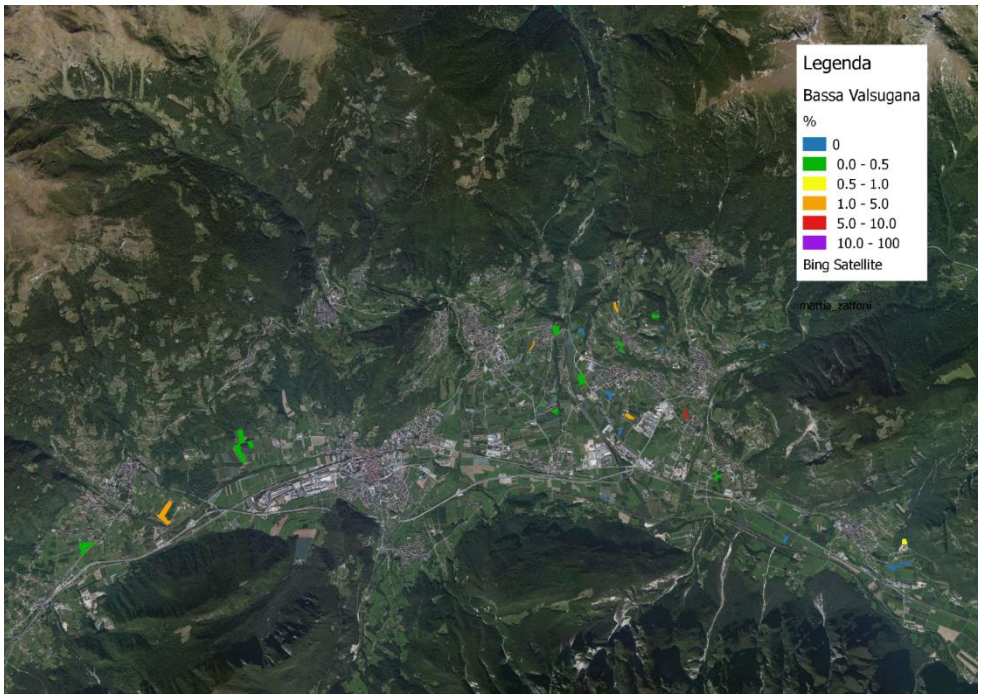

Figure 1.11. AP prevalence in Bassa Valsugana (2016).

The results of the three-year survey show an increase of the percentages of symptomatic trees in 2014 compared to 2013 (5.04%), while in 2015 and 2016 the level of AP-infected trees decreased in all the considered areas, apart from the areas of Levico and Bassa Valsugana, where a slight increase was recorded. Probably, this trend is a consequence of the awareness campaign promoted by PAT to eradicate and substitute symptomatic trees, which is in force since 2015 (Figure 1.12).

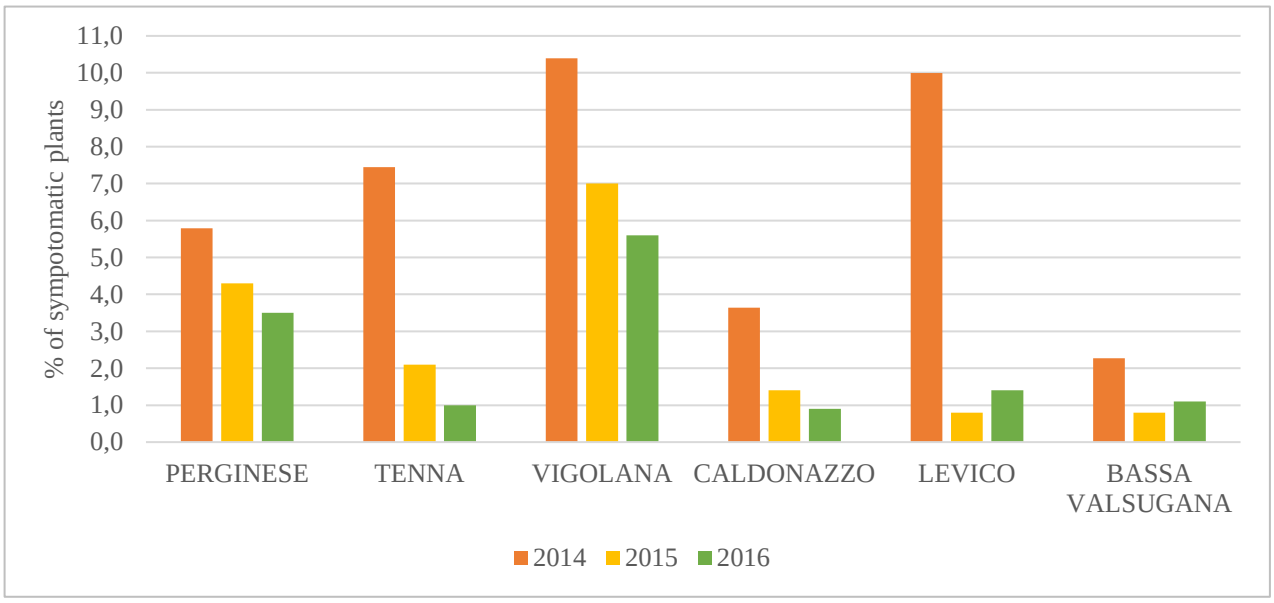

Figure 1.12. Percentages of symptomatic trees observed in the six monitored areas of Valsugana.

Moreover, the age of apple plants was recorded during surveys. Figure 1.13 represents the average percentage of symptomatic plants in relation to the ages, which are cluster in five-year ranges. The data represent the results of the whole monitoring in Valsugana, independently of the area, and show that the high proportion of symptomatic plants was recorded in older apple trees.

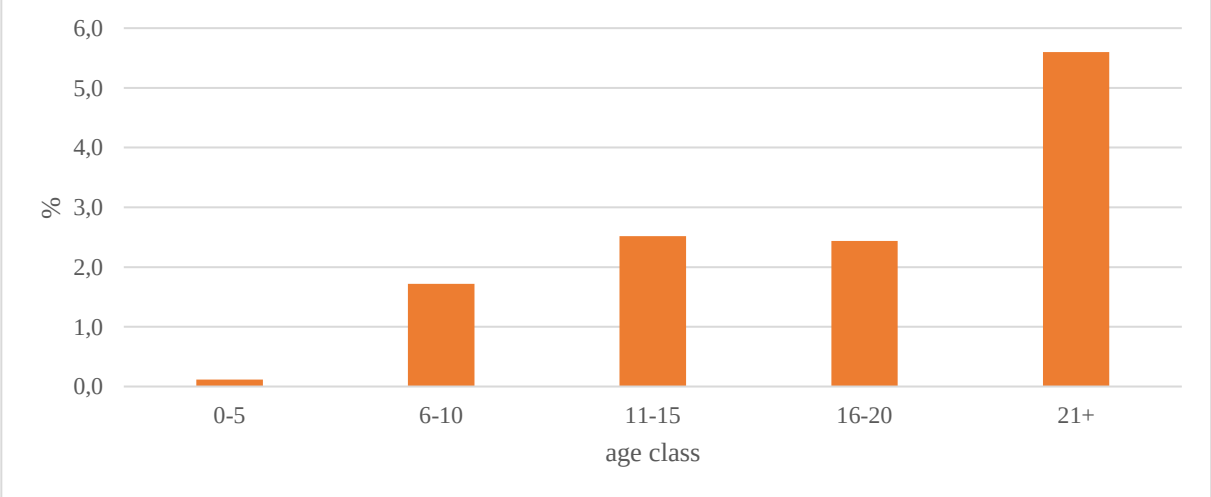

Figure 1.13. Percentages of symptomatic trees within the different age classes.

Psyllid vectors monitoring

Psyllid populations were regularly monitored in 18 orchards (17 conventional and one untreated control) of Valsugana from the last week of January to the first week of November. The

untreated orchard showed higher levels of both species, allowing description of species phenology. Figures 1.14 and 1.15 represent the population dynamics of *C. melanoneura* and *C. picta* in 2014, 2015, and 2016 based on the insect captures in the untreated orchard. In 2014, no *C. picta* individuals were observed, whereas the presence of this species was registered from 2015.

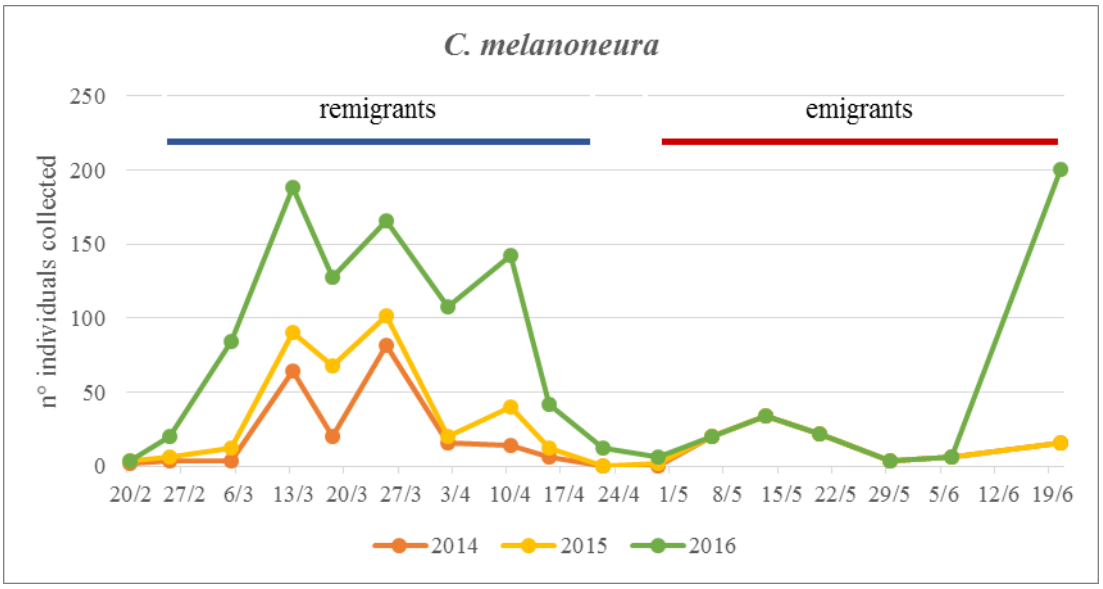

Figure 1.14. Population dynamics of *C. melanoneura* in the untreated control of Valsugana.

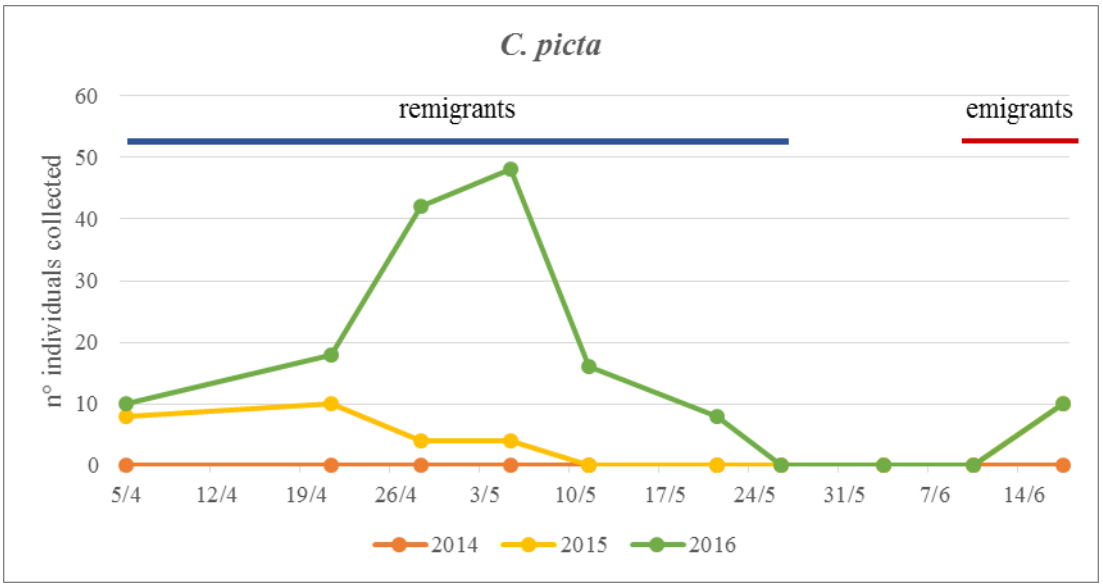

Figure 1.15. Population dynamics of *C. picta* in the untreated control in Valsugana.

The population dynamics observed in the three years confirmed higher presence of *C. melanoneura* compared to *C. picta*. For both species, increasing numbers of individuals were

collected from 2014 to 2016: captures of *C. melanoneura* raised from 799 to 1142 individuals, while *C. picta* captures increased from 0 to 128 individuals (Table 1.3).

Table 1.3 Psyllids collected in the survey of Valsugana in the 17 treated orchards and in the untreated control.

species	management	2014	2015	2016
C. melanoneura	treated*	477	218	398
	untreated	322	156	744
C. picta	treated*	0	16	12
	untreated	0	36	116

* numbers are the sum of the individuals collected in the 17 orchards monitored.

The total numbers of individuals collected in the six surveyed areas of Valsugana in the three years are represented in Figure 1.16 for *C. melanoneura* and in Figure 1.17 for *C. picta*, respectively.

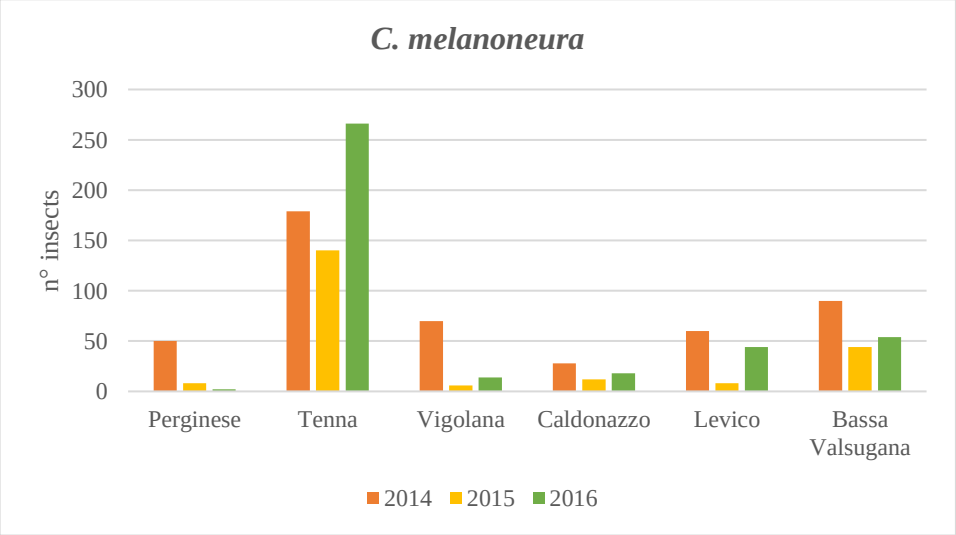

Figure 1.16. Total number of *C. melanoneura* individuals collected in Valsugana.

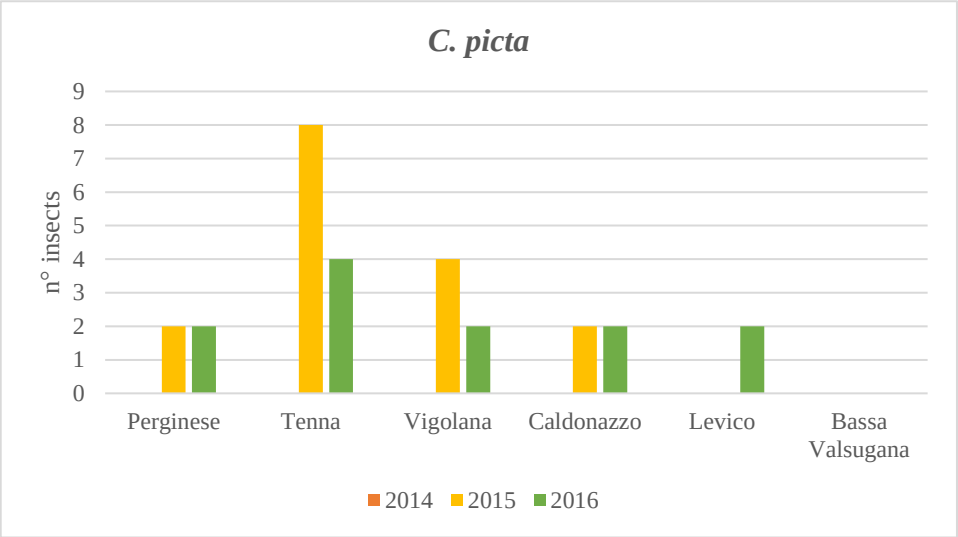

Figure 1.17. Total number of *C. picta* individuals collected in Valsugana.

Evaluation of psyllid infectivity

After classification, the individuals were lyophilized and crushed prior to DNA extraction. Then, insects were analyzed by real-time PCR. Generally, analyses were performed on single individuals and the real-time-PCR allowed determining the infection level of samples (highly or slightly positive and negative). In 2016, due to the high number of *C. melanoneura* collected (N= 1142), individuals of this species were grouped into pools of five, as mentioned before, and the estimated proportion of infected individuals was calculated.

Regarding *C. melanoneura*, 11.76% of the analyzed insects tested positive in 2014 and 20.25% in 2015 (Figure 1.18).

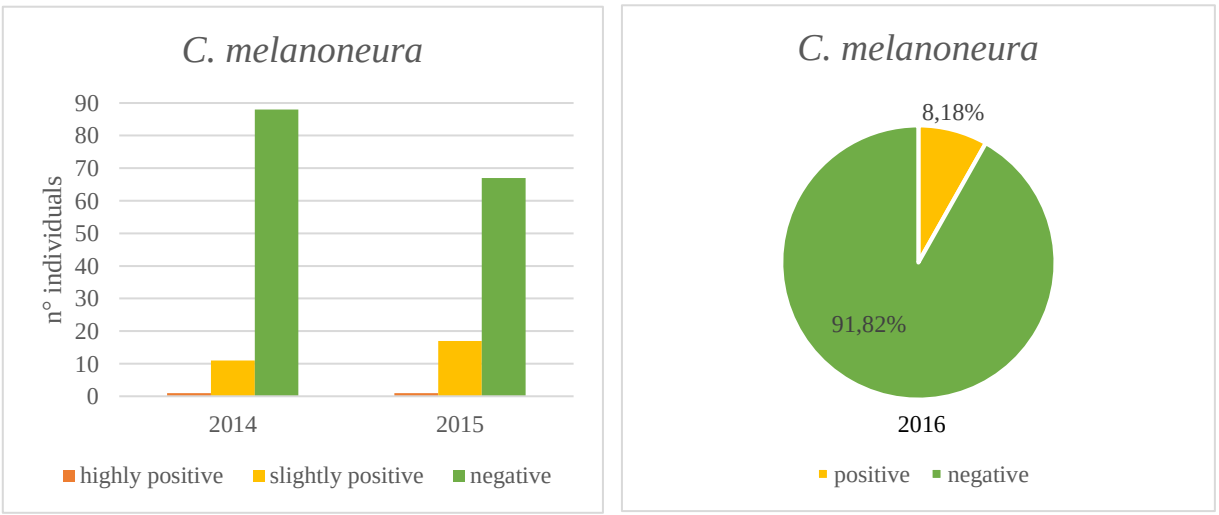

Figure 1.18. Infectivity rates in *C. melanoneura*. For 2014 and 2015, the numbers of highly positive, slightly positive and negative individuals are shown; in 2016, as the individuals were analyzed in pools of five, the proportion of positive individuals is represented according to Tedeschi *et al.* (2003).

Among these individuals, only 8.82% and 12.12% resulted highly positive in in the two years, respectively. The estimated proportion of infected individuals calculated for 2016 corresponds to 8.18% of the population.

The numbers of *C. picta* collected and analyzed are much lower. Anyway, in this species 54.55% of the individuals tested positive in 2014 and 42.86% in 2015. Among these individuals, 66.67% and 70.37% resulted highly infected by the phytoplasma in the two years (Figure 1.19).

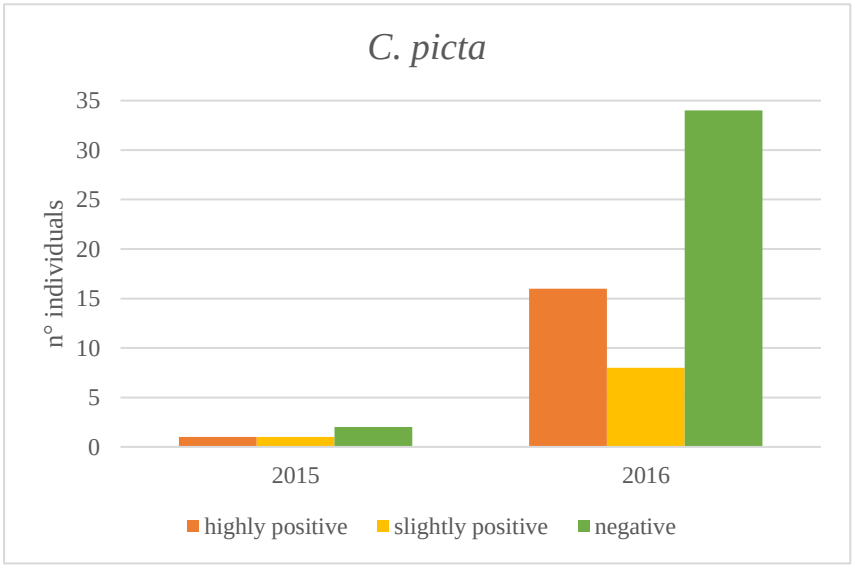

Fig. 1.19. Infectivity rates in *C. picta*.

Other psyllid species

During vector monitoring, other psyllid species were sporadically found in apple orchards: *Cacopsylla albipes* Flor, *Cacopsylla breviantenna* Flor, *Cacopsylla corcontum* Šulc, *Cacopsylla crataegi* Schrank, *Cacopsylla moscovita* Andrianova, *Cacopsylla pruni* Scopoli, *Cacopsylla pulchrella* Löw, *Cacopsylla pyri* L., *Cacopsylla pyricola* Förster, *Cacopsylla saliceti* Förster, *Homotoma ficus* L., *Trioza* Spp. None of these was analyzed to detect phytoplasma infection.

Overwintering sites and shelter plants

During January 2015, two surveys were conducted on mountains enclosing Valsugana to search for overwintering sites of psyllids. Table 1.4 shows the location predicted by the previous study of wind updrafts and the altitude ranged from 720 to 1480 m. In some case, two different altitudes for the same location were considered. The conifer species considered for sample collections were *Pinus mugo* Turra, *Pinus cembra* L., *Juniperus communis* L., *Picea abies* Karst. and *Abies alba* Mill. The sampling indicates the presence of several psyllids (Table 1.5);

in particular, *Cacopsylla breviantennata* Flor was the most abundant species. Whereas no individuals of *C. picta* were found, we collected two individuals of *C. melanoneura*: one on *P. cembra* at 900 m asl in the area of Caldonazzo and one on *P. mugo* at 940 m asl in the area of Borgo Valsugana.

Table 1.4. List of mountain locations and monitored conifer species.

location	altitude (m asl)	plant species
Caldonazzo-Centa	720	*Pinus mugo*, *Pinus* spp
	900	*Pinus cembra*, *Juniperus communis*, *Picea abies*
Levico Terme	1.300	*Picea abies*
	1.320	*Picea abies*, *Abies alba*
Novaledo	1.140	*Picea abies*
Levico-Vetriolo	920	*Picea abies*
Val di Sella	1.100	*Picea abies*, *Abies alba*
Telve-Musiera di sopra	1.480	*Picea abies*
Borgo Valsugana	940	*Pinus mugo*
	1.100	*Picea abies*

Table 1.5. List psyllid species and related number of specimens collected on shelter plants.

sampling site	altitude (m asl)	conifer species	psyllid species	n. individuals collected
Caldonazzo-Centa	720	*Pinus mugo*	*Cacopsylla breviantennata*	10
			Trioza sp.	1
			Cacopsylla moscovita	3
			Cacopsylla saliceti group	3
		Pinus spp.	*C.breviantennata*	16
			C. saliceti group	2
	900	*Pinus cembra*	*C. breviantennata*	11
			Trioza sp.	1
			Cacopsylla melanoneura	1
			C. saliceti group	1
			Bactericera albiventris	1
		Picea abies	*C.breviantennata*	8
			C.breviantennata	12
		Juniperus communis	*C. breviantennata*	5
Levico Terme	1.300	*Picea abies*	*C. saliceti* group	1
			C. saliceti group	1
			Cacopsylla elegantula	1
Novaledo	1.140	*Picea abies*	*Trioza* spp.	3
Levico-Vetriolo	920	*Picea abies*	*Trioza* sp.	1
Telve-Musiera di sopra	1.480	*Picea abies*	*Trioza* sp.	1
			Trioza sp.	1
Borgo Valsugana	940	*Pinus mugo*	*C. breviantennata*	2
			C. melanoneura	1

Discussion and conclusions

Apple proliferation (AP) was reported for the first time in 1954 by Refatti and Ciferri in Trentino and, until the early '90s, its presence was sporadic. The first impacting outbreak of the disease was reported in Val di Non and Val di Sole in the early 2000s (Vindimian *et al.*, 2002). The emergence was faced with the implementation of a public founded sanitation program, following a Ministry decree that established mandatory control measures against AP. As a consequence of these measures, AP spread declined in both areas. After several years, and despite the mandatory control strategies, the disease reappeared, reaching the level of 5.08% symptomatic apple trees in Valsugana in 2013. This reason brought researchers and producers to join the forces into a new project to deepen the knowledges about disease spread. A scientific operative group was created to face the issue directly in Valsugana, in cooperation with producers and with the contribution of the Autonomous Province of Trento.

After three years of epidemiological studies through disease and insect vectors' monitoring, the plant health status has improved, as the reduced percentages of symptomatic plants can demonstrate.

The present study revealed an irregular distribution of the disease in the Valsugana valley: Alta Valsugana still shows the highest AP infection rate compared to Bassa Valsugana. In particular, in 2014 Tenna, Vigolana and Levico Terme were the most affected rural areas. In 2016, after the start of the project which encouraged the implementation of the eradication of the symptomatic trees, Perginese and Vigolana remained the areas with the highest percentages, while in Tenna and Caldonazzo the percentage decreased. Conversely, a small increase was recorded in Levico and Bassa Valsugana compared to 2015, but a decreasing trend is evident in both cultivated areas compared to 2014.

During the disease monitoring, among the information collected, also the age of plants was recorded. These data clearly show that highest percentages of the infected plants can be found in trees older than 20 years. This suggests the possibility that plants were infected in the previous years, before the beginning of the project, underlining the importance of the mandatory control measures for eradication and renewal of diseased plantations, thus reducing the phytoplasma inoculum source.

The population dynamics of the two psyllid vectors, *C. picta* and *C. melanoneura*, were regularly monitored for three years. Results suggest that *C. melanoneura* is the most abundant AP vector present in Valsugana and also the one that spends more time in the orchards (from February to June). No individuals of *C. picta* were collected in 2014, but after several years this species reappeared again in apple orchards in a low numbers in 2015 and 2016. A possible

explanation for the differences in the numbers of individuals collected during the three years might lie in the climatic conditions: in particular, the higher numbers of individuals collected in 2016 can be a consequence of the mild winter of 2015-2016.

The rate of infected individuals was evaluated in the two psyllids. The results show that both species acquire '*Ca.* P. mali', but *C. melanoneura* samples were only slightly infected compared to *C. picta*, even if its presence is lower than *C. melanoneura*.

Data collected in Valsugana in the years 2005-2007, showed that percentage of infected individuals of *C. melanoneura* (*C. picta* presence was only sporadic) was around 11% (Malagnini *et al.*, 2010), which is in agreement with the values observed in this work.

Regarding *C. picta*, which seem to be the most important vector in Trentino (Mattedi *et al.*, 2008), the low number of individuals collected doesn't allow such comparisons.

Data collected in forests are useful to deepen the knowledge of psyllid life cycle, in particular about the overwintering sites. Overwintering shelter plants have been reported in the conifers for both psyllids (Conci *et al.*, 1992; Ossiannilsson, 1992; Lauterer, 1999). Only two of *C. melanoneura* were collected at 900 and 940 m asl on *P. cembra* and *P. mugo*, respectively. The presence of *C. melanoneura* on conifers confirms previous observation conducted both in Trentino (Val d'Adige) and Val d'Aosta and Piemonte (northwestern Italy), even if this species was collected mainly at higher altitudes (Pedrazzoli *et al.*, 2005; Pizzinat *et al.*, 2011). On the other hand, overwintering sites of *C. picta* found in Germany by Jarausch and Jarausch (2014) and in Czech Republic by Čermák and Lauterer (2008), were not found in this research. This data collected in forest, help to further validate the immigration index proposed for *C. melanoneura* (Tedeschi *et al.*, 2012; Baldessari *et al.*, 2015), in fact, in the week in which psyllids were found in overwintering sites, no psyllid were recorded in orchard.

In conclusion, all the information obtained provided a description of the present spread of AP in Valsugana and insect vector presence. This data, shared within the working group, gave the opportunity to optimize the current control strategies and to improve the timing of treatments. This project was also an opportunity to involve producers in an applied research activity through periodic technical communications (see Attachment 1).

References

Baldessari M., Angeli G., Oppedisano T. (2017). Nuove strategie contro le psille vettori degli scopazzi del melo. L'informatore Agrario, 9: 47-51.

Baldessari M., Corradini S., Ioriatti C., Mazzoni V. (2015). Validation of an immigration index for the apple jumping louse *Cacopsylla melanoneura* in trentino. Atti del XVIII Convegno Nazionale Di

Agrometeorologia (AIAM). Agrometeorology to feed the planet: water, air, soil, plants, animals. San Michele all'Adige (TN), 9-11 giugno 2015 Fondazione Edmund Mach.

Baldessari M., Trona F., Angeli G., Ioriatti C. (2010). Effectiveness of five insecticides for the control of adults and young stages of *Cacopsylla melanoneura* (Forster) (Hemiptera: Psyllidae) in a semi-field trial. Pest Management Science, 66: 308-312.

Baric S., Dalla Via .J. (2004). A new approach to apple proliferation detection: a highly sensitive real-time PCR assay. Journal of Virological Methods, 57: 135-45.

Čermák V., Lauterer P., (2008). Overwintering of psyllids in South Moravia (Czech Republic) with respect to the vectors of the apple proliferation cluster phytoplasmas. Bulletin of Insectology, 61(1): 147-148.

Ciccotti A. M., Bianchedi P. L., Bragagna P., Deromedi M., Filippi M., Forno F., Mattedi L. (2007). Transmission of '*Candidatus* Phytoplasma mali' by root bridges under natural and experimental conditions. Bulletin of Insectology, 60(2): 387-388.

Conci C., Rapisarda C., Tamanini L. (1992). Annotated catalogue of the Italian Psylloidea. First Part (Insecta Homoptera). Atti Accademia Roveretana degli Agiati, 242: 33-135.

Dallago G. (2014). Relazione annuale sul monitoraggio di Apple proliferation (*Ca.* Phytoplasma mali) in provincia di Trento. A cura di: Unità Frutticultura- Centro di Trasferimento Tecnologico, Fondazione Edmund Mach- Istituto Agrario San Michele all'Adige (TN).

Dallago G. (2016). Scopazzi del melo: siamo sulla strada giusta. L'Informatore Agrario, 41: 36-39.

Truccolo E. C. (2011). Assessment of the wind behavior in the northern coast of Santa Catarina. Revista Brasileira de Meteorologia, 26 (3): 451-460.

Frisinghelli C., Delaiti L., Grando M. S., Forti D., Vindimian M. E. (2000). *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. Journal of Phytopathology, 148 (7-8): 425-431.

Jarausch B., Schwind N., Jarausch W., Krczal G. (2004). Overwintering adults and springtime generation of *Cacopsylla picta* (synonym *C. costalis*) can transmit apple proliferation phytoplasmas. Acta Horticulturae, 657: 409-413.

Jarausch B., Fuchs A., Schwind N., Krczal G., Jarausch W. (2007). *Cacopsylla picta* as most important vector for '*Candidatus* Phytoplasma mali' in Germany and neighbouring regions. Bulletin of Insectology, 60: 189-190.

Jarausch B., Jarausch W. (2014). Establishment of a permanent rearing of *Cacopsylla picta* (Hemiptera: Psylloidea), the main vector of '*Candidatus* Phytoplasma mali' in Germany. Journal of Pest Science, 87: 459.

Lauterer P. (1999). Results of the investigation on Hemiptera in Moravia, made by the Moravian museum (Psylloidea 2). Acta Musei Moraviae, Scientiae Biologicae, 84: 71-151.

Malagnini V., Pedrazzoli F., Papetti C., Cainelli C., Zasso R., Gualandri V., Pozzebon A., Ioriatti C. (2013). Ecological and genetic differences between *Cacopsylla melanoneura* (Hemiptera, Psyllidae) populations reveal species host plant preference. PLoS ONE, 8 (7): e69663.

Malagnini V., Pedrazzoli F., Gualandri V., Zasso R., Bozza E., Fiamingo F., Pozzebon A., Mori N., Ioriatti C. (2010). Detection of '*Candidatus* Phytoplasma mali' in different populations of *Cacopsylla melanoneura* in Italy. Bulletin of Insectology, 63: 59-63.

Mattedi L., Forno F., Varner M. (2007). Scopazzi del melo, p. 144. In: Conoscenze ed osservazioni di campo- Arti Grafiche La Commerciale, Borgogno, Bolzano, Italy.

Mattedi L., Forno F., Cainelli C., Grando M. S., Jarausch W. (2008). Research on *Candidatus* Phytoplasma mali transmission by insect vectors in Trentino. Acta Horticulturae, 781: 369-374.

Müther J., Vogt H. (2003). Sampling methods in orchard trials: a comparison between beating and inventory sampling. IOBC/wprs Bulletin, 26 (5): 67-72.

Ossiannilsson F. (1992). The Psylloidea (Homoptera) of Fennoscandia and Denmark, p. 346. In: Fauna Entomologica Scandinavica, vol. 26. E.J. Brill, Leiden, The Netherlands.

Pedrazzoli F., Forno F., Malagnini V., Mattedi L. (2005). Indagini bioecologiche su *Cacopsylla melanoneura* (Förster) (Homoptera: Psyllidae), p. 251. In: Proceedings XX Congresso Nazionale Italiano di Entomologia, Perugia, Assisi, Italy, 13-18 June 2005.

Pizzinat A., Tedeschi, R., Alma A. (2011). *Cacopsylla melanoneura* (Foerster): aestivation and overwintering habitats in Northwest Italy. Bulletin of Insectology, 64: 135-136.

Refatti E., Ciferri R. (1954). La virosi del tipo 'scopazzi' in vivai di melo. Annali Sperimentazione Agraria, 8: 1543- 1556.

Rodríguez-Lázaro D., Hernández M. (2013). Introduction to the Real-time PCR. In Real-Time PCR in Food Science: Current Technology and Applications. D. Rodriguez-Lazaro, ed. (Norfolk, UK: Caister Academic Press). ISBN: 978-1-908230-15-7.

Swallow W. H. (1985). Group-testing for estimating infection-rates and probabilities of disease 403 transmission. Phytopathology, 75: 882-889.

Tedeschi R., Bosco D., Alma A. (2002). Population dynamics of *Cacopsylla melanoneura* (Homoptera: Psyllidae), a vector of apple proliferation phytoplasma in Northwestern Italy. Journal of Economic Entomology, 95 (3): 544-551.

Tedeschi R., Visentin C., Alma A., Bosco D. (2003). Epidemiology of apple proliferation (AP) in northwestern Italy: evaluation of the frequency of AP-positive psyllids in naturally infected populations of *Cacopsylla melanoneura* (Homoptera: Psyllidae). Annals of Applied Biology, 142 (3): 285-290.

Tedeschi R., Alma A. (2004). Transmission of apple proliferation phytoplasma by *Cacopsylla melanoneura* (Homoptera: Psyllidae). Journal of Economic Entomology. 97 (1): 8-13.

Tedeschi R., Baldessari M., Mazzoni V., Trona F., Angeli G. (2012). *Cacopsylla melanoneura* in northeast Italy: its role in the Apple proliferation epidemiology and dynamics of orchard colonization. Journal of Economic Entomology, 105 (2): 322-328.

Vindimian E., Delaiti L. (1996). Indagine sistematica sugli scopazzi del melo. Terra trentina, 13 (11): 30-33.

Vindimian M. E., Ciccotti A. M., Filippi M., Springhetti M., Deromedi M. (2002). Trasmissione di Apple Proliferation (AP) tramite anastomosi radicale. Atti del workshop II Incontro nazionale sulle malattie da fitoplasmi. Roma: Istituto sperimentale per la patologia vegetale: 375.

Web page: http://www.psyllidkey.com/

Attachment 1

FONDAZIONE EDMUND MACH

ISTITUTO AGRARIO
DI SAN MICHELE ALL'ADIGE

CENTRO TRASFERIMENTO TECNOLOGICO
SEDE DI VIGALZANO

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COMUNICATO TECNICO FRU VIG01

N° 32 del 02.05.2015

SCOPAZZI DEL MELO

Nelle ultime settimane sono sempre state catturate psille negli incolti e si è iniziata a rilevare anche la presenza di ***Cacopsilla picta***, specie molto pericolosa ed altamente infettiva. **Considerata la presenza di queste psille e che il territorio frutticolo della Valsugana è ad elevato rischio per quanto riguarda la diffusione degli scopazzi, si ritiene necessario intervenire nuovamente contro le psille** a partire da lunedì 4 maggio, impiegando:

Clorpirifos (es. Dursban 75WG alla dose di **1.050 g/ettaro** pari a 70 g/hl)
oppure
Abamectina (es. Vertimec EC alla dose max di 1,5 l/ha, consigliati 70 ml/hl)
da impiegare in vicinanza delle aree sensibili

NEI FRUTTETI BIOLOGICI O IN CONVERSIONE IMPIEGARE:
piretrine alle dosi di etichetta ripetendo l'intervento dopo 7 – 8 giorni

La lotta contro i vettori degli scopazzi del melo è obbligatoria e deve essere effettuata in tutti i frutteti (delibera della G.P. n. 1545 del 28 luglio 2006).

Le proposte di intervento contenute in questo comunicato, sono state concordate con il gruppo di lavoro "Progetto scopazzi Valsugana" e con le cooperative APASO, COBAV, COFAV e LEVICOFRUTTA.

Avvertenze:

- Preferire l'utilizzo di Abamectina negli appezzamenti confinanti con zone sensibili (scuole, asili, abitazioni, parchi, ecc.) e vicino a corsi d'acqua
- Ad abamectina aggiungere come bagnante Break-Thru alla dose di 200 ml/ha
- Abamectina si degrada con la luce per cui è preferibile eseguire l'intervento nelle ore serali
- Clorpirifos e Abamectina sono miscibili con i prodotti impiegabili per la ticchiolatura e con Imidacloprid consigliato per la difesa dagli afidi
- rispettare le dosi massime ammesse in etichetta e i tempi di rientro (48 ore)
- sfalciare il prato interfilare prima di effettuare l'intervento insetticida se presenti erbe in fiore
- attenzione agli impianti messi a dimora quest'anno, in fioritura in questo periodo

Riduzione della deriva: vicino a centri abitati, piste ciclabili, corsi d'acqua, ecc. è importante adottare alcuni accorgimenti, quali:

- trattare in assenza di vento, utilizzare un corretto volume d'aria, impiegare ugelli antideriva, deflettori o paratie per l'esclusione dell'aria
- trattare la fila di confine dall'esterno verso l'interno dell'appezzamento
- maggiori informazioni riguardo a questo argomento si trovano sulla circolare IASMA Notizie frutticoltura n.2 del 23 marzo 2015, pagine 3 e 4

G. DALLACQUA, C. DEFANT, L. DELAITI, C. PANIZZA, M. ZAFFONI

RISPETTARE QUANTO PREVISTO DAL PAN ART. A.5.6: NELLE AREE AGRICOLE ADIACENTI AD AREE PUBBLICHE È VIETATO L'UTILIZZO DI P.F. CON LE FRASI DI RISCHIO R40, R42, R43, R60, R61, R62, R63 E R68 (ED EQUIVALENTI CLASSIFICAZIONE CLP) A DISTANZA INFERIORE A 30 m, RIDOTTI A 10 m IN CASO DI IMPIEGO DI DISPOSITIVI ANTIDERIVA.

CHAPTER 2

Evaluation of the current vectoring efficiency of *Cacopsylla melanoneura* and *Cacopsylla picta* in Trentino

Abstract

Phytoplasmas are microorganisms associated with severe plant diseases affecting many agricultural crops worldwide. Only phloem-feeding insects can potentially acquire and transmit these obligate parasites. Some species of the psyllid genus *Cacopsylla* (Homoptera: Psyllidae) have been demonstrated to be involved in the transmission of important fruit tree phytoplasmas in Europe, such as '*Candidatus* Phytoplasma mali', associated with apple proliferation disease (AP). Two psyllids, *Cacospylla picta* and *Cacopsylla melanoneura*, are confirmed vectors, but the studies conducted in different geographical regions show a different transmission efficiency for the two species. In this work, acquisition and transmission trials were carried out with *C. picta* and *C. melanoneura*, to better understand the epidemiology of AP disease in Trentino (northern Italy). Experiments were conducted under semi-field and greenhouse conditions in spring and summer 2015 and 2016, respectively. The trials involved overwintered adults of both species collected in Valsugana and nymphs and emigrant adults reared on infected apple plants. Results confirm *C. picta* as a more competent '*Ca.* P. mali' vector in Trentino, but suggest also the possibility of acquiring and transmitting the phytoplasma for *C. melanoneura*, even though with a low efficiency. For both species, the acquiring capacity is largely increased for the stages developed on infected apple plants: nymphs and emigrant adults. This confirms the importance of the control measures adopted aimed at reducing the inoculum source in the field.

Key words: psyllid vectors, apple proliferation phytoplasma, acquisition and transmission efficiency

Introduction

Phytoplasmas are obligate parasites of plants and insects and their life cycle depends by the close association with their hosts. Study the relationships between phytoplasma, vector and plant host are important to understand the epidemiology of the diseases associated with these pathogens (Bosco *et al.*, 2007). Phytoplasmas are transmitted by phloem-sucking insects in the families Cicadellidae, Cixiidae, Cercopidae, Delphacidae and Psyllidae in a persistent-

propagative manner. After being passively acquired by the insect vector during feeding in the phloem of an infected host plant, they pass through the intestinal barrier, penetrate cells of different tissues, such as the Malpighian tubules (Lherminier *et al.*, 1990), fat bodies and brain (Lefol *et al.*, 1994; Nakashima and Hayashi, 1995), or reproductive organs (Kawakita *et al.*, 2000). To be transmitted to plants, phytoplasmas must penetrate specific cells of the salivary glands where they multiply and accumulate to high levels before being ejected into a new plant with the saliva (Kirkpatrick, 1992; Weintraub and Beanland, 2006). The effects of phytoplasmas on insect hosts are contrasting, from beneficial to harmful: reduced fitness in insect infected by phytoplasmas was reported (Marzachì *et al*., 2004; Weintraub and Beanland, 2006; Bressan *et al*., 2005; Malagnini *et al.*, 2010), whereas some infected insects show improved overwintering and increased fertility and longevity (D'Amelio *et al.*, 2012; Beanland *et al*., 2000; Ebbert and Nault, 2001).

'*Candidatus* Phytoplasma mali', the etiological agent of apple proliferation (AP) disease, is transmitted by the psyllids *Cacopsylla picta* Förster and *Cacopsylla melanoneura* Förster and by the leafhopper *Fieberiella florii* Stål (Frisingelli *et al.*, 2000; Tedeschi and Alma, 2004; Jarausch *et al*., 2003; Krczal *et al.*, 1988). Their distribution, natural infection rate and transmission capacity are heterogeneous among different geographic regions (Jarausch *et al*., 2007). In most of the areas affected by AP, both psyllid species are present (Miñarro *et al*., 2016; Baric *et al*., 2010; Carraro *et al*., 2001; Jarausch-Wehrheim *et al*., 2005; Mattedi *et al*., 2008), with the exception of Val d'Aosta, where only *C. melanoneura* has been found in high amounts (Tedeschi *et al*., 2007). '*Ca.* P. mali' has been detected also in other species of the genus *Cacopsylla* and in two exotic eucalypt psyllids, but their potential to transmit the disease still has to be investigated (Rosa García *et al*., 2014; Tedeschi *et al.*, 2009; Baric *et al.*, 2010).
In Trentino, *C. melanoneura* is present mainly in bottom valley environments, while *C. picta* is typically diffused on hills (Tomasi *et al.*, 2000). These species spend only few months in the orchard, accomplishing one generation per year, and then the new generation migrates on forest shelter plants (Pedrazzoli *et al.*, 2007). *C. melanoneura*, which has been demonstrated to be vector only in Italy, shows a variable transmission efficiency in the different Italian areas. Whereas it is the main vector of '*Ca.* P. mali' in Val d'Aosta and Piemonte (Tedeschi *et al.*, 2002; Tedeschi and Alma, 2004), a very low transmission efficiency was found in Trentino in the past years (Mattedi *et al.*, 2007; Pedrazzoli, 2009). Tedeschi *et al.* (2003) demonstrated that only a low percentage of the individuals appeared infected at the beginning of the migration, while this number can increase with the time spent on infected trees. So, sensitive and accurate detection of phytoplasmas in all vector life stages is a prerequisite for the management of phytoplasma-associated diseases (Duduk and Bertaccini, 2011).

After a new outbreak of the disease in 2011, which was observed especially in Valsugana, a project on AP epidemiology was launched. Particular attention was put to deepen the knowledge on the disease vectors. The aim of this work was to investigate the acquisition and transmission dynamics of both species. Nevertheless, as historical monitoring reports a low presence of *C. picta* (the main AP vector in Trentino) in Valsugana, this study focuses especially on *C. melanoneura*, whose role in the transmission of the AP phytoplasma in Trentino is still object of much debate (Malagnini *et al.*, 2010). Specifically, in the years 2015 and 2016 trials under semi-field and greenhouse conditions were conducted to investigate the differences in acquisition capacity and transmission efficiency between (1) *C. melanoneura* and *C. picta*; and (2) different developmental stages of both species. Knowledges on the period in which the two vectors are mainly able to acquire and transmit the phytoplasma to apple plants, as well as their potential infectivity, is fundamental for the management of disease control strategies.

Materials and Methods

Insect sampling area

Insects were collected from branches by beating tray method (Müther and Vogt, 2003). Two abandoned and untreated orchards in Alta Valsugana were chosen as abundant source of psyllids. They are located in Bosentino-Vigolana area (46° 0'0.24" N; 11°13'0.45" E; 657 m asl) and in Tenna (46° 1'21.50" N; 11°15'50.29" E; 622 m asl) (Figure 2.1). 7

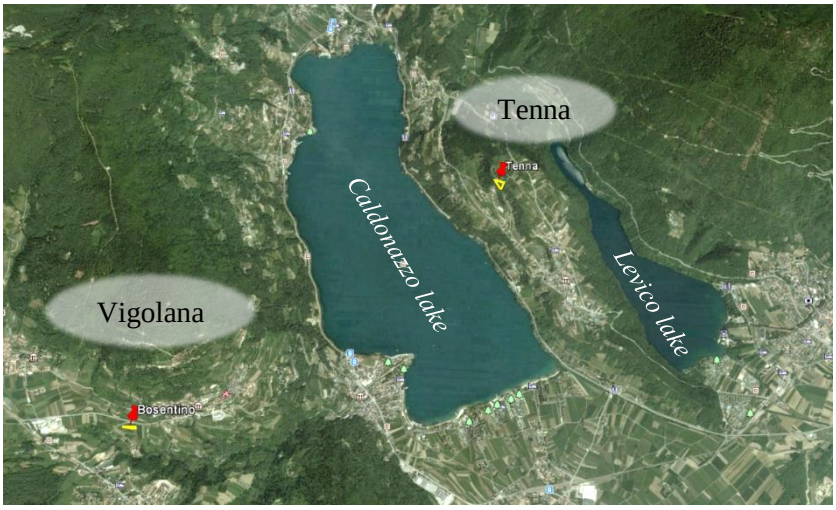

Figure 2.1. Map representing the two abandoned orchards where the insects used in the trials were collected (red pins).

The massive captures, essential to initiate the rearing, occurred according to populations dynamics observed for both species into the two years. So, *C. melanoneura* overwintered adults were collected during the week 9-13 March in 2015 and 28-31 March in 2016; while *C. picta* individuals during the weeks 13-18 April and 15-20 April in 2015 and 2016, respectively.

Taxonomic identification and psyllid rearing

Psyllid individuals were isolated from other species and collected in tubes, anaesthetized with carbon dioxide and identified by the stereomicroscope using dichotomous keys (Ossiannilsson, 1992). The numbers of *C. melanoneura* and *C. picta* males and females were recorded after each identification session. An outer psyllid rearing was started in a tunnel to reproduce semi-field conditions. Two big cages have been divided into two sub-cells, one with healthy apple plants and one with AP-infected plants. Before starting rearing, plants were tested to assess the presence/absence of AP phytoplasma.

Figure 2.2. Rearing of *C. melanoneura* and *C. picta* established in semi-field condition.

Plant material

Healthy Golden Delicious apple plants, produced by micro-propagation, were used to rear psyllids and for the trials. Five-year old healthy apple plants were used for the rearing and one-year old plantlets were used for the transmission experiments. For acquisition trials, five-year old micro-propagated Golden Delicious apple plants, infected with AT-2 strain of '*Ca*. P. mali', were used. The plants, which are regularly tested by real-time PCR, were stored in a screen-house covered with an insect-proof net.

Figure 2.3. *In vitro* Golden delicious apple plant produced in micro-propagation laboratory (A), maintained in *ex-vitro* conditions (B) and stored in screen-house at the end of the trials (C).

Experimental set up

Acquisition was studied during the outdoor insect rearing on infected plants, whereas for transmission trials cages (50 x 25 x 25 cm) were built with insect-proof net with single healthy plants. Phytoplasma transmission was investigated under semi-field conditions in 2015 and in an air-conditioned greenhouse, with natural photoperiod, 20 ± 2 °C and 10 ± 2 °C temperatures for day and night, respectively, and relative humidity around 60%- 65%, in 2016.

The two different methodological approaches followed in the two years were pursued because of the several difficulties arisen: the management of single-caged plants under the tunnel was arduous due to watering needs and high temperature. In addition, high temperature contributed to increase the insect mortality and to accelerate the degradation of dead individuals, especially in young and more delicate stages (nymphs and newly developed emigrants). On the opposite, working in the greenhouse in the following year allowed avoiding all these troubles.

During the experiment four kind of trials were set up in both years, corresponding to four psyllid categories. In particular, trials were conducted with (1) overwintered adults, (2) overwintered adults after acquisition access period (AAP), (3) 4^{th} -5^{th} nymph stages, (4) newly developed adults (emigrants). For the first category, individuals collected in the field where put directly onto healthy test plants to test their transmission efficiency. Regarding the other overwintered adults' category, insects were primarily confined on infected plants for one week to acquire phytoplasma. An AAP of one week was demonstrated to be sufficient for an efficient phytoplasma acquisition in both species (Pedrazzoli *et al.*, 2007). After this period, psyllids were moved to healthy plantlets (Figure 2.4). In 2015, insect were left on healthy plants until death, checking each single cage daily to recollect and store dead insects; in 2016, a transmission period of one week was chosen. Regarding nymphs and newly developed adults (third and fourth category), insects reared on infected apple plants were used in both years. In 2015, nymphs were left on healthy test plants until the appearance of new generation adults. When a new emigrant appeared, they were immediately collected and stored even if alive.

Regarding new generation adults, they were left on the test plants until death. In 2016, a specific transmission period of one week was established for nymphs and emigrants. Table 2.1 summarizes the number of insects used and the time schedule of the trials.

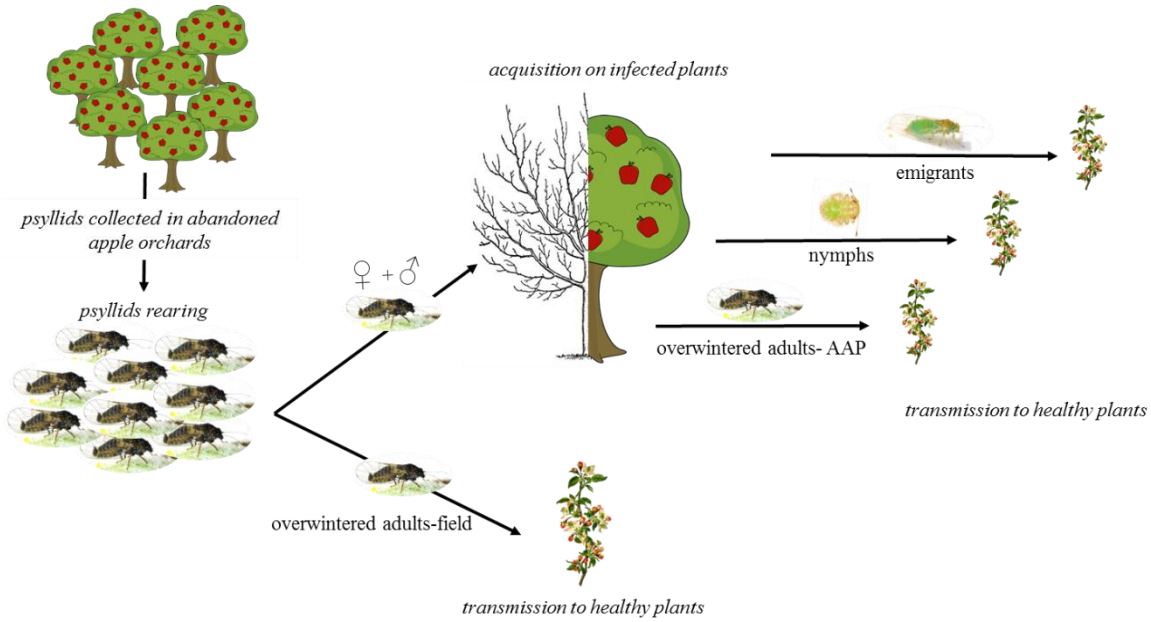

Figure 2.4. Scheme of the experimental set up for acquisition and transmission trials.

At the end of the trials, insects were collected and stored prior to molecular analysis; plantlets were treated with insecticides and recovered in screen-house until November, when the symptoms of the disease were surveyed and samples from each test plant were collected and analyzed for the presence of '*Ca.* P. mali'.

Table 2.1. Insect categories used in the trials; acquisition access periods (AAP) were the same in the two years, while transmission periods (TP) were modified.

		AAP	TP	insects/plant
2015	overwintered adults- field	-	2-4 weeks	10
	overwintered adults- AAP	7 days	2-3 weeks	10
	nymphs	from birth to 4th-5th instar	3-4 weeks	15
	emigrants	from birth	3-4 weeks	10
2016	overwintered adults- field	-	1 week	10
	overwintered adults- AAP	7 days	1 week	10
	nymphs	from birth to 4th-5th instar	1 week	20
	emigrants	from birth	1 week	10

Molecular analyses: insects

All specimens used in the experiments underwent a molecular analysis in order to assess the

presence of AP phytoplasma. After lyophilization and mechanical disruption of the samples, the total DNA was extracted using the commercial kit NucleoSpin® Tissue (Macherey-Nagel).
2015- Samples were analyzed by real-time PCR following the method developed by Baric and Dalla Via (2004). The analyses of the insects were performed in 20-µl reactions, containing 10 µl Kapa Probe Fast qPCR Master Mix (2X) Universal (Kapa Biosystems Roche), 900 nM of primers qAP-16S-F (CGA ACG GGT GAG TAA CAC GTA A) and qAP-16S-R (CCA GTC TTA GCA GTC GTT TCC A), 200 nM of qAP-16S probe (FAM-TAA CCT GCC TCT TAG ACG), and 2 µl template DNA normalized to 10 ng/µl. Reactions were performed in a Roche LightCycler® 480 and cycling conditions were as follows: 3 min at 95 °C, followed by 50 cycles of 3 s at 95 °C and 20 s at 60 °C. All insects were tested individually and each sample was tested in duplicates.

To evaluate the infection of samples, amplification curves and C_p values were considered. C_p, the crossing point-PCR-cycle, corresponds to the cycle at which fluorescence achieves a defined threshold. The threshold is defined based on the "baseline fluorescence" that represents the background signal, more evident before a significant accumulation of target amplicon. After this stage, the exponential phase, when amplification is most efficient, starts. The number of cycles needed for the amplification-associated fluorescence to reach this threshold level of detection is inversely correlated to the amount of nucleic acid that was in the original sample (Rodriguez-Lazaro and Hernandez, 2013). Therefore, samples showing a sigmoidal amplification curve and a C_p value lower than 30 cycles in both replicates were considered highly positive; samples with a sigmoidal amplification curve but C_p values above 30 in at least one replication were judged weakly positive; samples without sigmoidal amplification curve were considered negative for the presence of '*Ca.* P. mali'.

2016- '*Ca.* P. mali' titer was quantified in insects by SYBR Green real-time PCR with primers rpAP15f-mod and rpAP15r3 targeting the ribosomal protein gene *rpl22*, as described in Monti *et al.* (2013). A 2-µL sample of template DNA was mixed with 5 µL of 2 x SYBR® FAST qPCR Kit Master Mix (Kapa Biosystems), 2.5 µL nuclease-free water and 0.25 µL each of forward and reverse primer (10 µM). The cycling conditions were applied as follows: initial denaturation at 95 °C for 20 s, 35 cycles of amplification at 95 °C for 3 s and 60 °C for 30 s, and melting curve ramp from 65 to 95 °C at an increment of 0.5 °C for 5 s (CFX384 TouchTM Real-Time PCR Detection System, Bio-Rad Laboratories). All insects were tested individually and each sample was tested in triplicates in three independent PCR runs.

Phytoplasma PCR detection limits were carefully determined using a four-point tenfold dilution series (6.5×10^4 – 6.5×10^1 DNA copies / PCR reaction) of the plasmid pJET1.2-rpl22 containing the subcloned '*Ca*. P. mali' rpl22 PCR amplicon. For the dilution series, which was

included in every real-time PCR run, the plasmid was diluted in TE-elution buffer (10 mM Tris Cl, 0.5 mM EDTA, pH 9.0). Samples with a mean quantification cycle (C_q) value lower than 30 and a melting curve peak similar to the positive control were considered infected. Among these, samples with C_q values between 28 and 30 were considered weakly positive. On the other hand, samples with a mean C_q value above 30 were considered negative and detection limits could be verified to be comparable as described in Monti *et al.* (2013).

The phytoplasma titer was quantified based on the four-point plasmid standard curve analyzed in parallel with the samples in each PCR run. As a control of DNA integrity and to normalize phytoplasma amounts, in parallel to the amplification of rpl22 fragment, a region of the single-copy *wingless* (*wg*) gene (Brower and DeSalle, 1998) of psyllids was amplified with primers specific for psyllid species, qPSY-WG-F (TCA CGG GCG GCA ATG) and qPSY-WG-R (CCC ACA GCA CAT CAG ATC ACA). The PCR was performed as described above with 0.25 µM each of the wg-specific forward and reverse primer including in each PCR run dilution series ($7.3 \times 10^5 - 7.3 \times 10^2$ DNA copies/PCR reaction) of the plasmid pJET1.2-wg containing the subcloned *wg* gene PCR amplicon. Dilution series and standard curves were prepared as previously described for the pJET1.2-rpl22 plasmid. Phytoplasma concentration was calculated in relation to the *wg* gene. Phytoplasma concentration was quantified within the range of the four-point standard dilution series and samples out of the range were diluted in elution buffer and reanalyzed if necessary. Samples with a mean C_q value above 30 were considered negative and detection limits could be verified to be comparable as described in Monti *et al.* (2013).

Threshold calculation and data analysis was performed using the CFX ManagerTM software (Bio-Rad Laboratories), considering only runs with a PCR efficiency between 95 and 105% and a coefficient of determination (R^2) ≥ 0.99. Three non-template controls (NTC, nuclease free water) were performed together with each PCR run.

Molecular analyses: plants

Phloem tissue was isolated from three different branches of each plant, mixed and processed by lyophilization and mechanical disruption in order to obtain mixed powders. DNA extraction occurred using the NucleoSpin® Plant II (Macherey-Nagel) commercial kit. The presence of '*Ca.* P. mali' in apple tree samples was determined by real-time PCR, following the method developed by Baric *et al*. (2011). For the analysis, a multiplex TaqMan real-time PCR assay was used with primer/probe sets amplifying the 16S rRNA gene of '*Ca.* P. mali' (Baric and Dalla Via 2004) and the chloroplast DNA gene of *Malus domestica* 1-aminocyclopropane-1-carboxylate oxidase (*ACO*) as internal positive reference. The primers and probe for the *ACO* gene are as follows: forward primer qMd-ACO-F (CCA GAA TGT CGA TAG CCT CGT T),

reverse primer qMd-ACO-R (GGT GCT GGG CTG ATG AAT G), and the TaqMan probe qMd-ACO (TAC AAC CCA GGC AAC G). The 5' end of the '*Ca.* P. mali' probe qAP-16S was labelled with the reporter dye FAM, whereas the probe amplifying the apple gene *ACO* was 5'-labelled with VIC, thus allowing distinction of the two amplification products. Both probes were conjugated with a Minor Groove Binder (MGB) and a non-fluorescent quencher dye (NFQ) at their 3'-ends (Applied Biosystems).

Statistical analyses

Data collected during the two years for acquisition trials for *C. melanoneura* and *C. picta*, were statistically analyzed with the software SPSS Base ver. 15.0. Differences in the percentages of infected individuals of the insect categories were evaluated with Pearson's chi-squared and Fisher's exact tests. The Levene test for the homogeneity of variances was applied on rough or transformed data and, based on results, parametric or non-parametric tests were used to compare the phytoplasma levels in the different categories and species. As in both years the Levene test showed inhomogeneous variances, a Kruskal-Wallis test followed by pairwise comparisons with Mann-Whitney test were performed.

Results

Acquisition trials (2015)

The presence of AP phytoplasma in samples was evaluated by real-time PCR and the insects were grouped, according to the C_p values obtained, in three categories: slightly or highly positives and negatives. At the end of the trials, a total number of 149 specimens of *C. melanoneura* belonging to the four categories were collected and analyzed for '*Ca.* P. mali' detection. Among overwintered from field, 13 out 73 insects (17.8%) tested positive; among overwintered after acquisition access period (AAP), the infection rate was 25/46 (56.8%); among nymphs, 15/15 (100%); in emigrants 13/15 (86.6%) (Figure 2.5). The Pearson's chi-squared test performed on the proportion of infected individuals indicates highly significant differences between overwintered from field and overwintered after AAP ($\chi^2_{(1)}$= 17.313; p= 0.000). Regarding *C. picta*, a total number of 127 individuals were analyzed: in overwintered from field, 40 out of 47 insects resulted AP-infected (85.1%); among overwintered after AAP, 20/21 (95.2%); among nymphs the infection rate was 25/25 (100%) and among emigrants 34/34 (100%) (Figure 2.6). The Fisher's exact test, applied due to the low number of samples, indicates that the increase in the infected insects between overwintered from field and overwintered after AAP is not significant (p= 0.760).

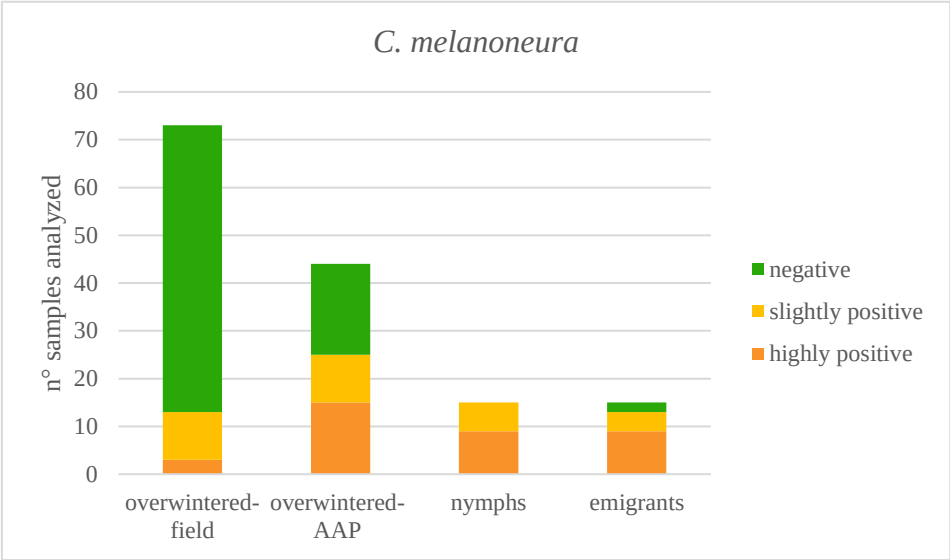

Figure 2.5. AP-infection rates of *C. melanoneura* in samples collected during acquisition trials in 2015.

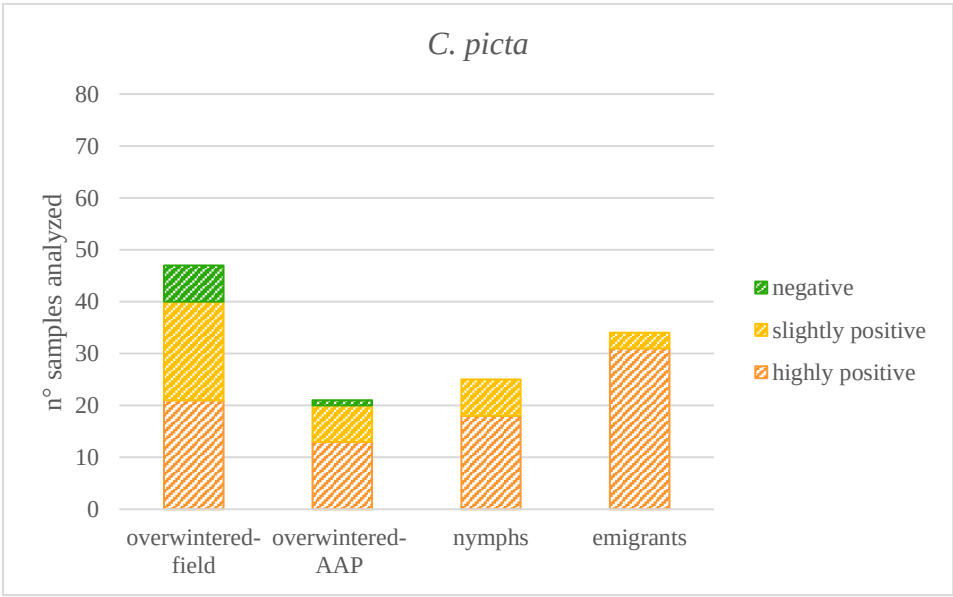

Figure 2.6. AP-infection rates of *C. picta* in samples collected during acquisition trials in 2015.

These results show the capacity of acquiring phytoplasma by both psyllid species, evidencing differences between species and between the stages of the same species. In particular, C_p values were used as indicators of the level of phytoplasma present in the samples (Baric *et al.*, 2010). Figures 2.7 and 2.8 represent, for each category, the mean C_p values and show an increase in the phytoplasma titer in overwintered adults of both species after the AAP.

Regarding *C. melanoneura*, overwintered adults showed a C_p value of 35.36 ± 1.81 (mean ± std. err.); overwintered adults after AAP a C_p value of 33.97 ± 0.91; nymphs a C_p value of 34.31

± 1.13; emigrants a C_p value of 30.18 ± 1.76. Regarding *C. picta*, overwintered adults showed a C_p value of 33.84 ± 0.83 (mean ± std. err.); overwintered adults after AAP a C_p value of 30.00 ± 1.66; nymphs a C_p value of 31.80 ± 1.27; emigrants a C_p value of 31.22 ± 0.79.

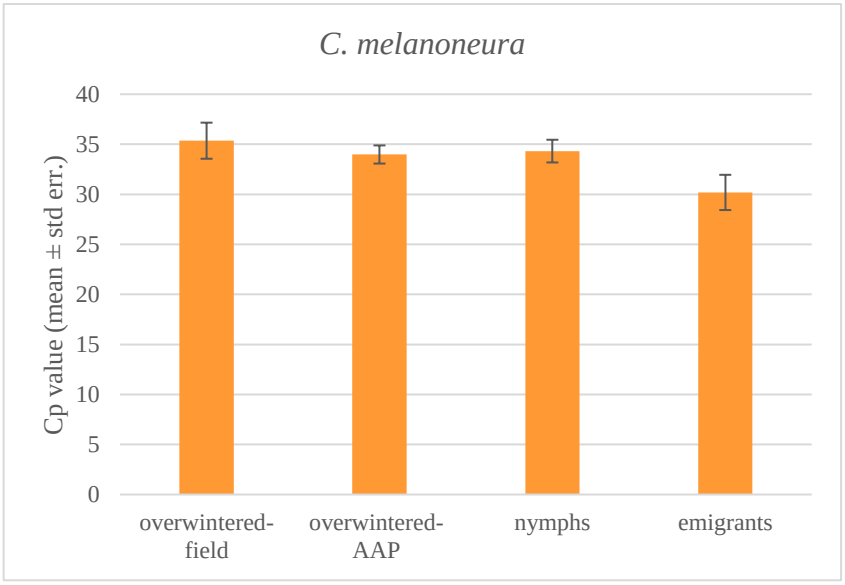

Figure 2.7. Phytoplasma titer in *C. melanoneura* estimated by C_p values.

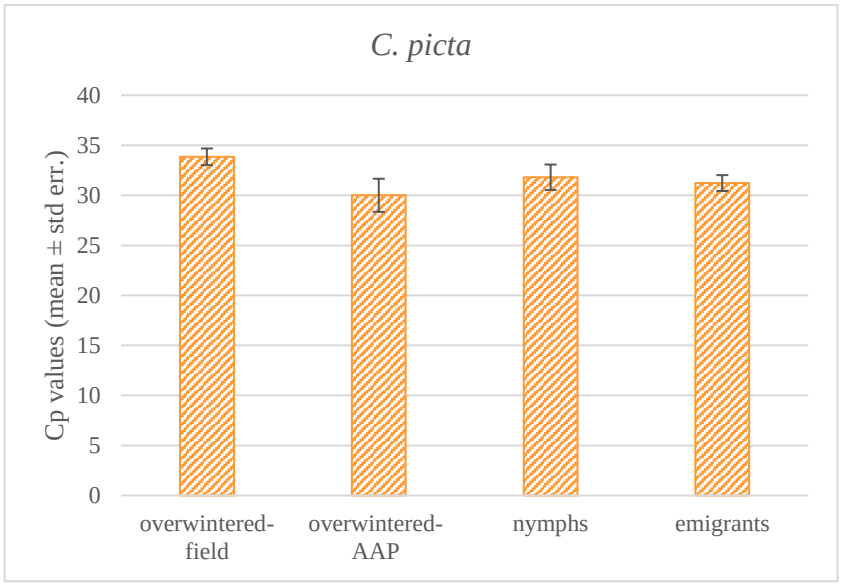

Figure 2.8. Phytoplasma titer in *C. picta* estimated by C_p values.

The Kruskal-Wallis test showed highly significant differences in the overall C_p values ($\chi^2_{(7)}$= 26.086; p= 0.000). Pairwise comparisons, performed with Mann-Whitney test on the results, indicate significant differences between overwintered *C. melanoneura* from field and after AAP (U= 96.000; p= 0.015) and between overwintered *C. picta* from field and after AAP (U= 263.500; p= 0.014). By comparing the same categories of the two species, significantly higher

phytoplasma levels emerged in overwintered adults of *C. picta* compared to *C. melanoneura* (U= 166.000; p= 0.028) (Figure 2.9).

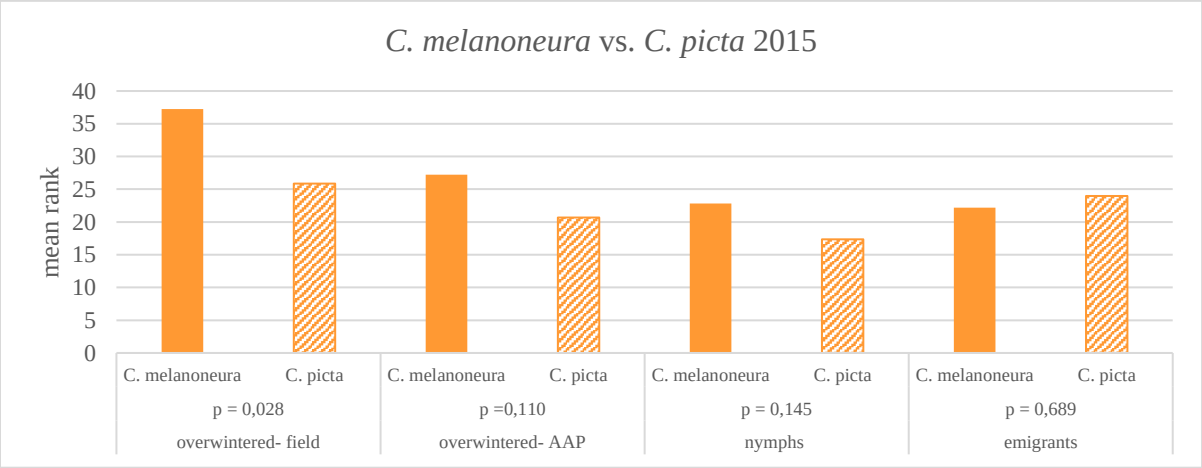

Figure 2.9. Mann-Whitney test for the comparison between categories in *C. melanoneura* and *C. picta*.

Transmission trials (2015)

All survived test plants used in transmission trials were monitored for specific symptoms of the disease between October and November and branches were sampled and analyzed for the presence of '*Ca.* P. mali' in the year of the experiment as well as in the year after. Out of 79 sampled plants, only one showed clear symptoms of AP, in this case witches' brooms, in both years. Table 2.1 reports the number of tested plants and the results of the analyses performed in the two years.

Table 2.1. Results of transmission trials conducted in 2015: molecular tests were performed in autumn 2015 and repeated in autumn 2016; (*) symptomatic plant.

		2015		2016	
species	**category**	**tot.**	**AP-infected**	**tot.**	**AP-infected**
C. melanoneura	overwintered- field	20	-	20	-
	overwintered- AAP	12	1(*)	12	2 (1*)
	nymphs	6	-	6	-
	emigrants	1	-	1	-
C. picta	overwintered- field	15	-	15	-
	overwintered- AAP	6	1	6	1
	nymphs	4	1	4	1
	emigrants	15	-	15	-
total		**79**	**3**	**79**	**4**

The analyses of 2016, repeated one year after the trials, confirmed that overwintered adults of the two species after AAP and nymphs of *C. picta* are able to transmit AP phytoplasma.

Acquisition trials (2016)

The total number of *C. melanoneura* recollected after trials and analyzed were 774 individuals. Among overwintered from field, two out 182 insects (1.1%) tested positive; among overwintered after acquisition access period (AAP), the infection rate was 4/165 (2.4%); among nymphs, 40/283 (14.1%); in emigrants 20/144 (13.9%) (Figure 2.10).

The Pearson's chi-squared test, performed on the proportion of infected individuals indicates no significant differences between overwintered from field and overwintered after AAP ($\chi^2_{(1)}$= 0.285; p= 0.594). Regarding *C. picta*, a total number of 753 individuals were analyzed: in overwintered from field, 27 out of 140 insects resulted AP-infected (19.3%); among overwintered after AAP, 13/83 (15.7%); among nymphs the infection rate was 80/338 (23.7%) and among emigrants 75/192 (39.1%) (Figure 2.11). The Pearson's chi-squared test, between overwintered from field and overwintered after AAP, indicates no significant differences ($\chi^2_{(1)}$= 0.251; p= 0.616). For both species, also in this case the two figures below show individuals testing highly- and slightly-positive.

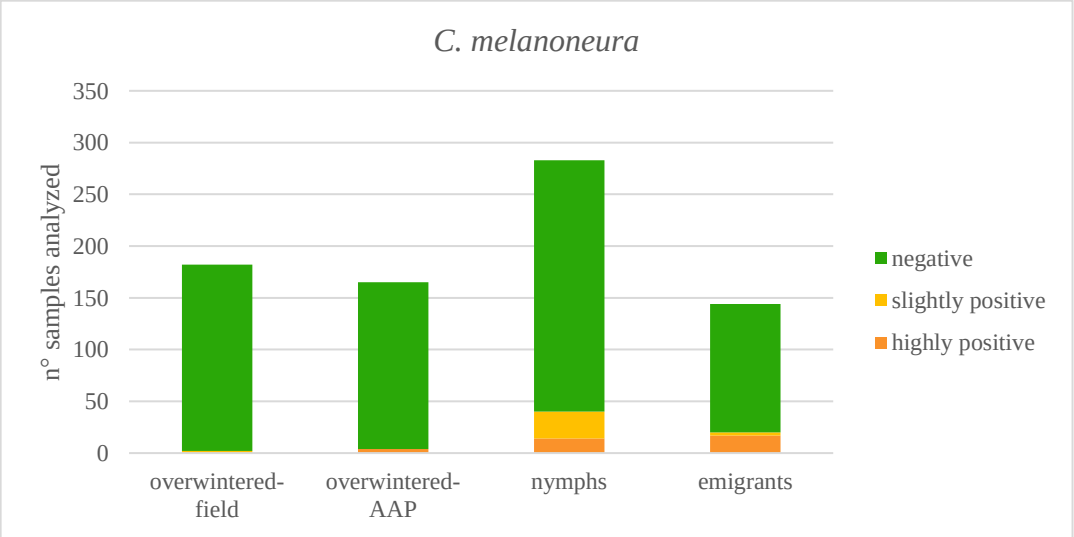

Figure 2.10. AP-infection rates of *C. melanoneura* in samples collected during acquisition trials in 2016.

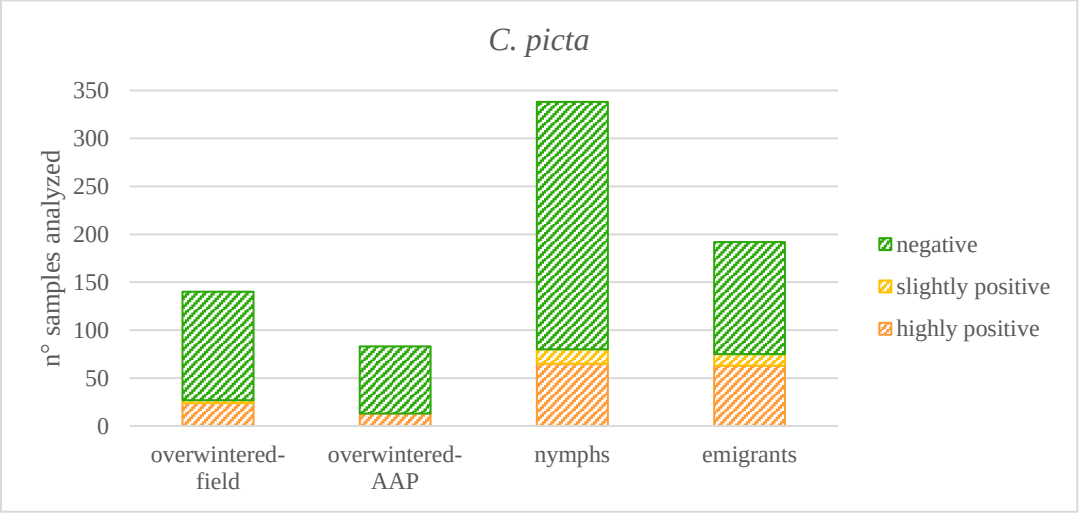

Figure 2.11. AP-infection rates of *C. picta* in samples collected during acquisition trials in 2016.

Data of the quantitative analyses for *C. melanoneura* and *C. picta* are represented in Figures 2.12 and 2.13, respectively. Regarding *C. melanoneura*, overwintered adults showed a titer of 0.066 ± 0.066 *rpl22*/*wg* gene copies (mean ± std. err.); overwintered adults after AAP a titer of 0.240 ± 0.091 *rpl22*/*wg* gene copies; nymphs a titer of 0.021 ± 0.010 *rpl22*/*wg* gene copies; emigrants 0.223 ± 0.055 *rpl22*/*wg* gene copies. Regarding *C. picta*, overwintered adults showed a titer of 0.335 ± 0.038 *rpl22*/*wg* gene copies (mean ± std. err.); overwintered adults after AAP a titer of 0.477 ± 0.102 *rpl22*/*wg* gene copies; nymphs a titer of 0.136 ± 0.031 *rpl22*/*wg* gene copies; emigrants 0.358 ± 0.059 *rpl22*/*wg* gene copies.

The Kruskal-Wallis test, applied to relative phytoplasma titers (*rpl22*/*wg* gene copies), showed highly significant differences ($\chi^2_{(7)}$= 48.775; p= 0.000). Pairwise comparisons, performed with Mann-Whitney test, indicates significant differences in *C. melanoneura* between overwintered adults after AAP and nymphs (U= 18.000; p= 0.011) and between nymphs and emigrants (U= 103.000; p= 0.000). Regarding *C. picta*, the statistical analysis evidenced significant differences between the phytoplasma titer of overwintered adults after AAP and nymphs (U= 272.500; p= 0.006).

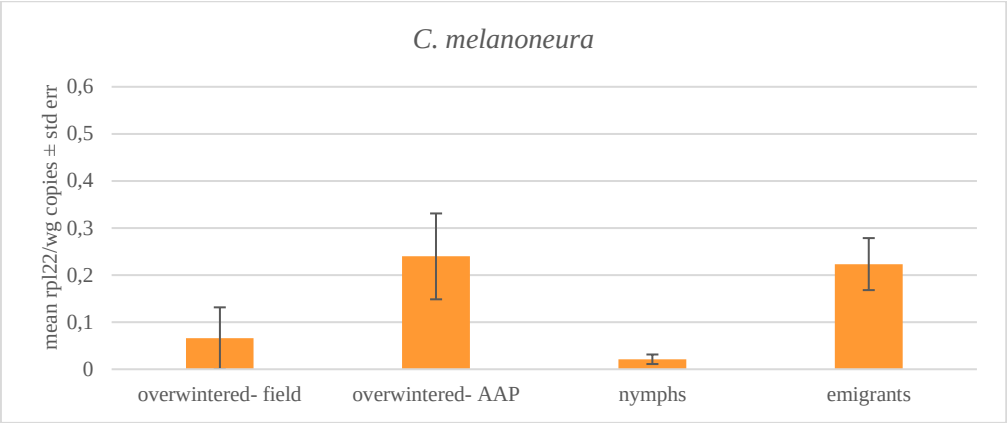

Figure 2.12. Titer of '*Ca.* P.mali' in the different categories of *C. melanoneura*, calculated as the ratio of *rpl22* gene copies ('*Ca.* P. mali'-specific) to *wingless* (*wg*) gene copies (psyllid-specific).

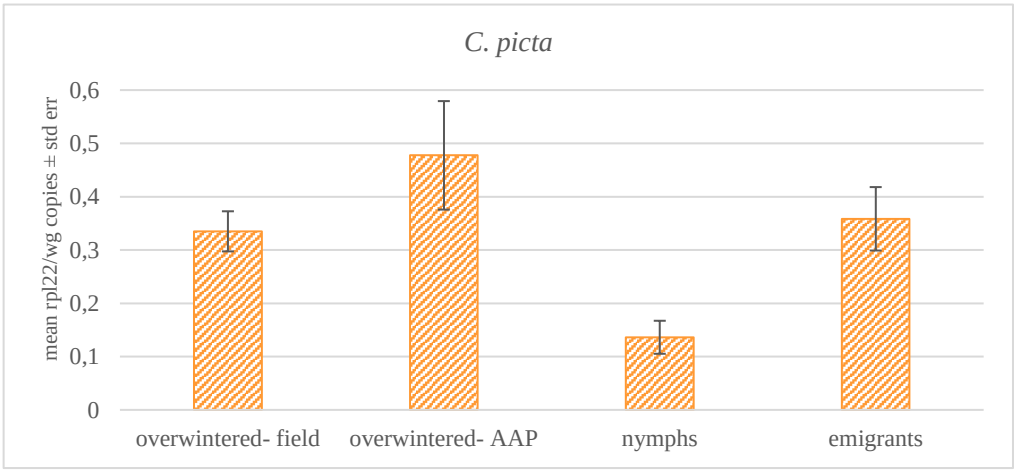

Figure 2.13. '*Ca.* P.mali' titer in different categories of *C. picta*, calculated as the ratio of *rpl22* gene copies ('*Ca.* P. mali'-specific) to *wingless* (*wg*) gene copies (psyllid-specific).

By comparing the same categories of the two species, significantly higher phytoplasma levels emerged in nymphs of *C. picta* compared to *C. melanoneura* (U= 904.500; p= 0.000) (Figure 2.14).

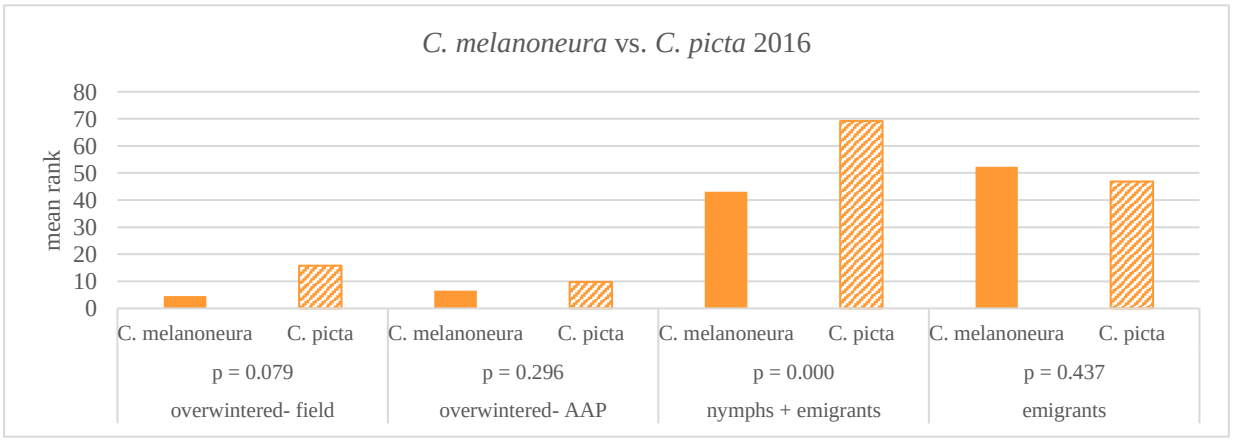

Figure 2.14. Mann-Whitney test for the comparison between categories in *C. melanoneura* and *C. picta*.

Transmission trials (2016)

The number of trials conducted in 2016 is summarized in Table 2.2. During autumn surveys in the screen-house, several symptomatic plants were recorded. Most of the symptoms were leaf reddening, apical rosettes and enlarged stipules and witches' brooms. Out of seven plants testing positive, only five showed symptoms. The real-time PCR analysis of apple plants showed that overwintered adults of *C. picta* after AAP, nymphs of *C. melanoneura* and *C. picta* transmitted AP phytoplasma.

Table 2.2. Results of transmission trials conducted in 2016. Molecular analyses were performed in autumn 2016; (*) symptomatic plant.

		2016	
species	**category**	**tot.**	**AP-infected**
C. melanoneura	overwintered- field	20	-
	overwintered- AAP	18	-
	nymphs	20	1 (1*)
	emigrants	20	-
C. picta	overwintered- field	18	-
	overwintered- AAP	10	1 (1*)
	nymphs	19	5 (3*)
	emigrants	20	-
total		145	7

Discussion

Apple proliferation (AP) disease is reported in many European regions, where it causes big losses in apple production. Unluckily, there are no direct measures to control this phytoplasmosis, and resistant plant propagative material is not available yet (Seemüller *et al.*, 2008). Therefore, actual strategies are based on preventive methods: in particular, several studies have been conducted on insect vectors of the disease to disentangle the three-way relationship among microorganism-insect-plant.

Two psyllids have been identified as vectors of '*Ca.* P. mali', the etiological agent of AP. Their biology and transmission parameters have been investigated in different geographical conditions, providing in some cases contrasting indications. In southwestern Germany, about 10% of overwintering *C. picta* and 0.2% of overwintering *C. melanoneura* were naturally infected with the pathogen (Jarausch-Wehrheim *et al*., 2005). Overwintered adults transmitted the phytoplasma at higher rates compared to emigrants, whereas the role of *C. melanoneura* in AP epidemiology was considered irrelevant (Mayer *et al.*, 2009). Regarding Italy, in Piemonte

and Valle d'Aosta regions, where *C. melanoneura* is the only widespread species, Tedeschi *et al*. (2003) found higher infection levels in overwintered adults (3.5%) compared to springtime generation (0.8%). The first category resulted also more effective in transmitting the disease (Tedeschi and Alma, 2004). On the other hand, in Friuli-Venezia Giulia, the natural infection rate in overwintered and emigrants of *C. picta* was found to be 9% and 13%, respectively, and springtime generation resulted more efficient in transmitting the disease (Carraro *et al.*, 2008). In Trentino-Alto Adige, both psyllids species are able to naturally acquire phytoplasma, with percentages of infected individuals around 4.6% for *C. melanoneura* and around 21.1% for *C. picta* in the years 2002-2005, when an outbreak of the disease was reported (Cainelli, 2007). Contrastingly, transmission trials reveled transmission efficiencies of 0.36% in *C. melanoneura* and 4.1% in *C. picta* in the same years (Mattedi *et al.*, 2008). Anyway, *C. melanoneura*, the most common species in Valsugana, showed infection levels between 10.4% and 11.5% during the years 2005-2007 (Malagnini *et al.*, 2010), but never transmitted in experiments under controlled conditions (Pedrazzoli, 2009).

The overwintered adults of natural populations collected for this study showed higher natural infection levels in *C. picta* compared to *C. melanoneura* in both years, even if 2015 was characterized by higher infection rates. Acquisition from infected trees seems to be an important process in overwintered adults, especially in *C. melanoneura*. This is confirmed by the significant increment observed in 2015 in the mean phytoplasma titer in insects and, in *C. melanoneura*, also in the percentages of infected individuals. In 2016, a similar trend was observed, even though differences are not significant. These data suggest a higher acquisition efficiency in *C. melanoneura* overwintered adults. On the other hand, the lower percentages of infected individuals collected in the field could indicate that this species overwinters with lower phytoplasma titers compared to *C. picta*. A possible hypothesis for this behavior could lay in the phenology of the two psyllid species: in particular, studies about *C. melanoneura* report that overwintering adults pass into a diapause during hibernation, in which sexual glands are deactivated and quiescent (Lauterer, 1999). So, it is possible that a decreased metabolic activity in this species reduces also the multiplication rate of '*Ca.* P. mali'. Unfortunately, the lack of further information about overwintering behavior and physiology on the two species limits a better understanding of the correlation between phytoplasma level and psyllids biology. Another factor influencing the phytoplasma acquisition in overwintered adults could be the behavior of the pathogen in plants. As reported by Pedrazzoli *et al.* (2008) and observed also in other phytoplasma-host plant systems (Garcia-Chapa *et al.*, 2003; Jarausch, 1999), '*Ca.* P. mali' follows a seasonal colonization dynamic, in which the presence in the aerial part can show high concentration levels during March, when *C. melanoneura* reaches the orchard, and is very

low and scattered in April, when *C. picta* re-migrates. Therefore, a high acquisition efficiency can have undergone a positive selection especially in *C. melanoneura* overwintered adults, whose phenology seems to be more synchronous with the phytoplasma cycle in apple trees reported by Pedrazzoli *et al.* (2008). However, in both psyllid species, only overwintered adults after the AAP were able to transmit the disease in the experiments, suggesting that a period on infected apple plants is required by vectors to acquire the phytoplasma, if not yet infected, or, if already infected, to allow its multiplication and circulation.

High percentages of infected nymphs and emigrants in both species resulted able to acquire AP phytoplasma, even if the mean phytoplasma titer did not result higher than in overwintered adults. In 2016, when the phytoplasma levels were quantified in relation to housekeeper gene, nymphs showed lower titers than overwintered adults of both species. Surprisingly, nymphs of *C. picta* were able to transmit the pathogen in one out of four and five out of 19 test plants in 2015 and 2016, respectively, and nymphs of *C. melanoneura* in one out of 20 test plants in 2016. In transmission trials with nymphs, 20 individuals were used for each plantlet instead of 10 (as in the experiments with the other stages). It can be hypothesized that many individuals with a low phytoplasma titer can be more efficient in transmitting than few insects with a high phytoplasma level, as already hypothesized by Frisinghelli *et al.* (2000) and reported for the transmission of pear decline by Davies *et al.* (1992). In addition, nymphal instars, which are developing into adults, are supposed to be characterized by an active metabolism and feeding behavior. Contrastingly, newly developed adults are characterized by high infection rates and levels, but never succeeded in transmitting phytoplasma under the experimental conditions chosen. Personal observations suggest that emigrant individuals, after completing development from 5^{th} nymph stage, spend less time in feeding if compared to the other stages, but move from plants to the walls of the cages, indicating their tendency to migrate to shelter plants.

Biological and ecological associations between phytoplasma and psyllid vectors open several questions on their evolutionary relationships. For instance, the report of the detrimental effects of the phytoplasma on *C. melanoneura* (Malagnini *et al.*, 2010), would indicate a recent co-evolution between the two organisms, but the presence of two alternative host plants, such as apple and hawthorn, among which an exchange of '*Ca.* P. mali' through the psyllid was suggested (Tedeschi *et al.*, 2009), seems to confirm an established role of vector in this species. On the other hand, *C. picta* populations of Trentino show a strong affinity with '*Ca.* P. mali', which is confirmed by a higher acquisition capacity in all life stages considered in the experiments and a higher transmission efficiency compared to *C. melanoneura*.

During the years 2015 and 2016, two different molecular methodologies were adopted to detect phytoplasma titer in insects. In the first year, C_p values were used to compare the different

categories; in the following year, a relative quantification of phytoplasma titer was done standardizing the phytoplasma amount with a house-keeper gene of the insects. Even if the results of the two methods are not comparable for phytoplasma level detection, similar trends were observed across the stages.

Initial purpose of this work was to investigate the relations between psyllid vectors and '*Ca.* P. mali' under outdoor conditions, in particular for *C. melanoneura*. The influence of environmental conditions, especially temperature, on phytoplasma transmission has already been reported (Maggi *et al.*, 2014). Therefore, to understand the actual role of the two psyllid species in the disease epidemiology, reproducing natural conditions was very important. To achieve this aim, in 2015 a tunnel was built in the open field. Trials with overwintered adults of both species and rearing were successful, whereas in trials with young stages some inconveniences occurred, probably due to the high temperatures. Plants suffered drought, despite regular watering and insects, especially stages not completely sclerotized, showed a high mortality. Trees in the orchards provide to insect populations a stable microclimate, which compensates peaks of temperature and humidity, especially during summertime. Reproducing natural conditions is not easy; after the experience of 2015, the trials were conducted in greenhouse to maintain the same experimental conditions for all categories.

In conclusion, the results obtained in this work confirm the main role of *C. picta* in spreading AP disease in Valsugana. However, the most important finding of these trials regard *C. melanoneura*, about whose vectoring behavior in Trentino has always been speculated. This species represents, under the experimental conditions adopted, a potential risk factor, especially at high population densities and in presence of a high inoculum source. Transmission rates found out leave open the possibility of the presence of other AP vectors or of different transmission ways. Moreover, only little information is available on the relationships between phytoplasma strains, insects and plant hosts. As only apple plants infected with '*Ca.* P. mali' AT-2 strain, which is reported as the most common in Valsugana, were used in acquisition trials (Cainelli, 2007), it will be an intriguing challenge to investigate the differences in virulence and vector affinity of the different '*Ca.* P. mali' strains.

References

Baric S., Öttl S., Dalla Via J. (2010). Infection rates of natural psyllid populations with '*Candidatus* Phytoplasma mali' in South Tyrol (Northern Italy). Julius-Kühn-Archiv, 427:189-192.

Beanland L., Hoy C. W., Miller S. A., Nault L. R. (2000). Influence of aster yellows phytoplasma on the fitness of aster leafhopper (Homoptera: Cicadellidae). Annals of the Entomological Society of America 93: 271-276.

Bosco D., Galetto L., Leoncini P., Saracco P., Raccah B., Marzachì C. (2007). Interrelationships between "*Candidatus* Phytoplasma asteris" and its leafhopper vectors (Homoptera: Cicadellidae). Journal of Economic Entomology, 100: 1504-1511.

Bressan A., Clair D., Sémétey O., Boudon-Padieu É. (2005). Effect of two strains of Flavescence dorée phytoplasma on the survival and fecundity of the experimental leafhopper vector *Euscelidius variegatus* Kirschbaum. Journal of Invertebrate Pathology, 89: 144-149.

Cainelli C., 2007. Population dynamics of apple proliferation in Trentino. 171 pp. Ph.D. Thesis, Università degli Studi di Verona, Italy.

Carraro L., Osler R., Loi N., Ermacora P., Refatti E. (2001). Fruit tree phytoplasma diseases diffused in nature by psyllids. Acta Horticulturae, 550: 345-350.

Carraro L., Ferrini F., Ermacora P., Loi N., Labonne G. (2008). Infectivity of *Cacopsylla picta* (Syn. *Cacopsylla costalis*), vector of '*Canditatus* Phytoplasma mali' in North East Italy. Acta Horticulturae, 781: 403-408.

Davies D. L., Guise C. M., Adams A. N. (1992). Parry's disease of pears is similar to pear decline and is associated with mycoplasma-like organisms transmitted by *Cacopsylla pyricola*. Plant Pathology, 41: 195-203.

D'Amelio R., Palermo S., Marzachì C., Bosco D. (2012). Influence of Chrysanthemum yellows phytoplasma on the fitness of two of its leafhopper vectors, *Macrosteles quadripunctulatus* and *Euscelidius variegatus*. Bulletin of Insectology 61(2): 349-354.

Duduk B., Bertaccini A. (2011). Phytoplasma classification: Taxonomy based on 16S ribosomal gene, is it enough? Phytopathogenic Mollicutes, 1(1): 3-13.

Ebbert M. A, Nault L. R. (2001). Survival in Dalbulus leafhopper vectors improves after exposure to maize stunting pathogens. Entomologia Experimentalis et Applicata, 100: 311-324.

Frisinghelli C., Delaiti L., Grando M. S., Forti D, Vindimian M. E. (2000). *Cacopsylla costalis* (Flor 1861) as a vector of apple proliferation in Trentino. Journal of Phytopathology, 148: 425-431.

Garcia-Chapa M., Medina V., Viruel M. A., Lavina A., Batlle A. (2003). Seasonal detection of pear decline phytoplasma by nested-PCR in different pear cultivars. Plant pathology, 52(4): 513-520.

Jarausch B., Fuchs A., Schwind N., Krczal G., Jarausch W. (2007). *Cacopsylla picta* as most important vector for '*Candidatus* Phytoplasma mali' in Germany and neighbouring regions. Bulletin of Insectology, 60: 189-190.

Jarausch B., Schwind N., Jarausch W., Krczal G. (2004). Overwintering adults and springtime generation of *Cacopsylla picta* (synonym *C. costalis*) can transmit apple proliferation phytoplasmas. Acta Horticulturae, 657: 409-413.

Jarausch B., Schwind N., Jarausch W., Krezal G., Seemüller E., Dickler E. (2003). First report of *Cacopsylla picta* as a vector for apple proliferation phytoplasma in Germany. Plant Disease, 87: 101.

Jarausch-Wehrheim B., Schwind N., Jarausch W., Peccerella T., Krczal G. (2005). Identificazione di *Cacopsylla picta* (syn. *Cacopsylla costalis*) come vettore del fitoplasma apple proliferation in Germania. Petria, 15 (1/2): 43-45.

Kawakita H., Saiki T., Wei W., Mitsuhashi W., Watanabe K., Sato M. (2000). Identification of mulberry

dwarf phytoplasmas in the genital organs and eggs of leafhopper *Hishimonoides sellatiformis*. Phytopathology, 90: 909-914.

Kirkpatrick B. C. (1992). Mycoplasma-like organisms: plant and invertebrate pathogens. In The Prokaryotes, pp. 4050- 4067. Edited by A. Balows, H. G. Trutper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

Krczal G., Krczal H., Kunze L. (1988). *Fieberiella florii* (Stål), a vector of apple proliferation agent. Acta Horticulturae, 235: 99-106.

Lefol C., Lherminier J., Boudon-Padieu E., Larrue J., Louis C., Caudwell A. (1994). Propagation of Flavescence dorée MLO (mycoplasma-like organism) in the leafhopper vector *Euscelidius variegatus* Kbm. Journal Invertebrate Pathology, 63: 285-93.

Lauterer P. (1999). Results of the investigation on Hemiptera in Moravia, made by the Moravian museum (Psylloidea 2). Acta Musei Moraviae, Scientiae Biologicae, 84: 71-151.

Lherminier J., Prensier, G., Boudon-Padieu, E. and Caudwell, A. (1990) Immunolabeling of grapevine flavescence doree MLO in salivary glands of *Euscelidius variegatus*: a light and electron microscopy study. Journal of Histochemistry and Cytochemistry, 38: 79-85.

Maggi F., Galetto L., Marzachì C., Bosco D. (2014). Temperature-dependent transmission of *Candidatus* Phytoplasma asteris by the vector leafhopper *Macrosteles quadripunctulatus* Kirschbaum. Entomologia, 2: 87-94.

Malagnini V., Pedrazzoli F., Gualandri V., Zasso R., Bozza E., Fiamingo F., Pozzebon A., Mori N., Ioriatti C. (2010). Detection of '*Candidatus* Phytoplasma mali' in different populations of *Cacopsylla melanoneura* in Italy. Bulletin of Insectology, 63: 59-63.

Marzachì C., Milne R. G., Bosco D. (2004). Phytoplasma plant-vector relationships, pp. 211-241. In: Recent Research Development in Plant Pathology, Vol. 3 (Pandalai S. G., Gayathri A., Eds). Research Signpost, Trivandrum, Kerala, India.

Mattedi L., Forno F., Varner M. (2007). Scopazzi del melo. Conoscenze ed osservazioni di campo. 144 pp. Arti Grafiche La Commerciale-Borgogno, Bolzano (Italy).

Mattedi L., Forno F., Cainelli C., Grando M. S., Jarausch W. (2008). Research on *Candidatus* Phytoplasma mali transmission by insect vectors in Trentino. Acta Horticulturae, 781: 369-374.

Mayer C. J., Jarausch B., Jarausch W., Jelkmann W., Vilcinskas A., Gross J. (2009). *Cacopsylla melanoneura* has no relevance as vector of apple proliferation in Germany. Phytopathology, 99: 729-738.

Miñarro M., Somoano A., Moreno A., Rosa García R. (2016). Candidate insect vectors of apple proliferation in Northwest Spain. Sringerplus, 5(1): 1240.

Monti M., Martini M., Tedeschi R. (2013). EvaGreen Real-time PCR protocol for specific '*Candidatus* Phytoplasma mali' detection and quantification in insects. Molecular and Cellular Probes, 27: 129-136.

Nakashima K., Hayashi T. (1995). Multiplication and distribution of rice yellow dwarf phytoplasma in infected tissues of rice and green rice leafhopper *Nephotettix cincticeps*. Annals of the Phytopathological Society of Japan, 61: 451-455.

Pedrazzoli F., Gualandri V., Forno F., Mattedi L., Malagnini V., Salvadori A., Stoppa G., Ioriatti C. (2007). Acquisition capacities of the overwintering adults of the psyllid vectors of '*Candidatus*

Phytoplasma mali'. Bulletin of Insectology, 60: 195-196.

Pedrazzoli F. (2009). Investigations on the psyllid (Hemiptera: Psyllidae) vectors of '*Candidatus* Phytoplasma mali' in Trentino. 192 pp. Ph.D. Thesis, Università degli Studi di Padova, Italy.

Rosa García R., Somoano A., Moreno A., Burckhardt D., de Queiroz D. L., Miñarro M. (2014). The occurrence and abundance of two alien eucalypt psyllids in apple orchards. Pest Management Science, 70(11): 1676-1683.

Seemüller E., Moll E., Schneider B. (2008). Apple proliferation resistance of *Malus sieboldii*- based rootstocks in comparison to rootstocks derived from other *Malus* species. European Journal of Plan Pathology, 121: 109-119.

Tedeschi R., Alma A. (2004). Transmission of apple proliferation phytoplasma by *Cacopsylla melanoneura* (Homoptera: Psyllidae). Journal of Economic Entomology, 97(1): 8-13.

Tedeschi R., Bertignolo L., Alma A. (2005). Role of the hawthorn psyllid fauna in relation to the apple proliferation disease. Petria, 15: 47-49.

Tedeschi R., Bosco D., Alma A. (2002). Population dynamics of *Cacopsylla melanoneura* (Homoptera: Psyllidae), a vector of apple proliferation phytoplasma in Northwestern Italy. Journal of Economic Entomology, 95(3): 544-551.

Tedeschi R., Lauterer P., Brusetti L., Tota F., Alma A. (2009). Composition, abundance and phytoplasma infection in the hawthorn psyllid fauna of northwestern Italy. European Journal of Plant Pathology, 123: 301-310.

Tedeschi R., Visentin C., Alma A., Bosco D. (2003). Epidemiology of apple proliferation (AP) in northwestern Italy: evaluation of the frequency of AP-positive psyllids in naturally infected populations of *Cacopsylla melanoneura* (Homoptera: Psyllidae). Annals of Applied Biology, 142(3): 285-290.

Tomasi F., Branz A., Grando M. S., Forno F., Vindimian M. E. (2000). Individuazione di fitoplasmi del gruppo AP nelle psille presenti nei frutteti. L'informatore Agrario, 38: 51-54.

Weintraub P. G., Beanland L. (2006). Insect vectors of phytoplasmas. Annual Review of Entomology, 51: 91-111.

CHAPTER 3

The insect vector *Cacopsylla picta* vertically transmits the bacterium '*Candidatus* Phytoplasma mali' to its progeny

Abstract

The phloem-sucking psyllid *Cacopsylla picta* Förster 1848 plays an important role in transmitting the bacterium '*Candidatus* Phytoplasma mali', the causative agent of apple proliferation disease. The psyllid can ingest '*Ca.* P. mali' from infected apple trees and spread the bacterium by subsequently feeding on uninfected trees. Until now, this is the most important way of '*Ca.* P. mali' transmission. The aim of this study was to investigate if infected *C. picta* are able to transmit '*Ca.* P. mali' directly to their progeny. This method of transmission would allow the bacteria to bypass a time-consuming reproductive cycle in the host plant. Furthermore, this would cause a high number of infected F1 individuals in the vector population. To address this question, eggs, nymphs and adults derived from infected overwintering adults of *C. picta* were reared on non-infected apple saplings and subsequently tested for the presence of '*Ca.* P. mali'. In this study it was shown for the first time that infected *C. picta* individuals transmit '*Ca.* P. mali' to their eggs, nymphs and F1 adults, thus providing the basis for a more detailed understanding of '*Ca.* P. mali' transmission by *C. picta*.

Keywords: apple proliferation, epidemiology, insect vectors, phytoplasma and relatives, psyllids, transovarial transmission

Introduction

'*Candidatus* Phytoplasma mali' is the causal agent of apple proliferation (AP), a major threat in several apple growing regions (Bertaccini *et al.*, 2014). The most important symptom is the production of small, tasteless and colorless fruit which leads to large monetary loss in affected apple growing areas (Tedeschi *et al.*, 2003; Bertamini *et al.*, 2002; Bertaccini *et al.*, 2014). Phytoplasmas are transmitted by insect vectors belonging to the taxonomic groups Cicadellidae, Fulgoromorpha and Psyllidae (Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2014), and can additionally be transmitted by natural root grafts (Baric *et al.*, 2008). In northern Italy the two phloem-feeding psyllids *Cacopsylla picta* Förster, 1848 (Hemiptera: Psyllidae) and *Cacopsylla melanoneura* Förster, 1848 (Hemiptera: Psyllidae) are vectors of '*Ca.* P. mali' (Carraro *et al.*, 2008; Frisinghelli *et al.*, 2000; Tedeschi *et al.*, 2002), while in Germany only *C. picta* was found

to be able to transmit the pathogen (Mayer *et al.*, 2009; Jarausch *et al.*, 2011). (Mayer *et al.*, 2008a, 2008b). Studies conducted in Trentino-South Tyrol (northern Italy) show a higher transmission efficiency for *C. picta* compared to *C. melanoneura* (Mattedi *et al.*, 2008). Remigrant individuals of *C. picta* migrate from end of March to April from their overwintering shelter plants into apple orchards for reproduction and feeding (Mattedi *et al.*, 2008). *C. picta* accomplishes one generation per year and the development from eggs to F1 adults involves five larval instars. *C. picta* offspring (emigrants) leave the apple orchards within July and migrate to the overwintering shelter plants (Mattedi *et al.*, 2008). The life cycle of the bacteria is strongly connected to its insect vector and the host plant (Mayer e*t al.*, 2008b, 2008a; Bertaccini *et al.*, 2014). The insect vectors acquire the AP phytoplasma by ingesting plant sap from the phloem of infected apple trees (Weintraub, 2007; Pedrazzoli *et al.*, 2007; Mattedi *et al.*, 2008). After ingestion, the phytoplasma move through the food canal of the stylet and invade different cellular tissues of the insects. The bacteria multiply in secretory salivary glands and are translocated into a new plant via the saliva when the infected insect is feeding (Hogenhout *et al.*, 2008b). Currently treatment against the spread of transmitting insects and uprooting of infected trees are the only ways to limit disease spread in affected regions (Baric *et al.*, 2010). Aside from the acquisition of infected phloem sap by ingestion, another putative way for phytoplasma spread would be a transovarial or 'vertical' pathogen transmission. This type of transmission is well known in plant virus insect vectors (Hogenhout *et al.*, 2008a). Transovarial transmission of plant viruses occurs only in the group of persistent and propagative viruses that persist and replicate inside their insect vectors (Hogenhout *et al.*, 2008a; Huo *et al.*, 2014). However, until now transovarial transmission was reported only in 4% of phytoplasma transmitting insect vectors (Arismendi *et al.*, 2015). Vertical transmission was found in the leafhopper species *Scaphoideus titanus* Ball, the vector of Aster yellows phytoplasma (Alma *et al.*, 1997), in *Hishimonoides sellatiformis* Ishihara, the vector of mulberry dwarf phytoplasma (Kawakita *et al.*, 2000), and in *Matsumuratettix hiroglyphicus* Matsumura, the vector of sugarcane white leaf phytoplasma (Hanboonsong *et al.*, 2002). Tedeschi *et al.* (2006) showed that females of *Cacopsylla pruni* Scopoli infected by '*Candidatus* Phytoplasma prunorum' are able to vertically transmit the pathogen and demonstrated infectivity of transovarially-infected F1 individuals. The authors of this study, however, could not show transovarial transmission of '*Ca.* P. mali' in *C. melanoneura*. The ability of *C. picta* to vertically transmit '*Ca.* P. mali' was hypothesized (Tedeschi *et al.*, 2006) but has never been experimentally addressed. Thus, the aim of this study was to test if '*Ca.* P. mali'-infected *C. picta* are able to vertically transmit the pathogen to their progeny. Specifically, the study aimed to answer the questions if the number of F1 individuals, deriving from infected or uninfected parental females differs and if

the phytoplasma titer does change between developmental stages of the insect. Additionally, a rearing-feeding-oviposition method was established to study the influence of a potential phytoplasma acquisition on the puncture site of parental insects.

Material and Methods

Insects

Overwintering adults of *C. picta* were collected in April 2016 using the beating tray method (Müther and Vogt, 2003) in two abandoned apple orchards in Valsugana Valley (Trentino, Italy). The collected living individuals were isolated in glass collection tubes, anesthetized on ice and morphologically characterized using identification keys of Ossiannilsson (1992). The collected winter generation (remigrants) of *C. picta* will hereon be referred to as 'parental generation', and the reared summer generation (emigrants) as 'F1 generation'.

Insect rearing

Insects from the natural populations were reared at Laimburg Research Centre (Laimburg) and at Fondazione Edmund Mach (FEM). A total number of 37 couples (27 at Laimburg and 10 at FEM) of parental females and males of *C. picta* were released into single net-cages under controlled conditions (15-25 °C, natural light and 70-100% relative humidity), on recently (same year) grown apple bud grafts (cv. Golden Delicious on M9 rootstock). Each couple was released on an individual plant. Additionally, 14 parental females (12 at Laimburg, 2 at FEM) were single-caged on grafts. Cages were monitored every day for vitality of parental individuals and the presence of eggs. After egg laying, parental insects were collected, frozen at -80 °C, and subsequently analyzed by PCR. Based on the PCR results of *C. picta* parental individuals, plants with eggs were selected and divided into three groups (Figure 3.1; Table 3.1). Plants with progeny from an infected parental female, plants with progeny from an uninfected female and an infected parental male (control group 1), plants with progeny from both uninfected female and male parental individuals (control group 2). One or two leaves with eggs were taken from the branches of each groups. Using titanium needles and a stereo-zoom microscope (Zeiss Axiozoom V16), all eggs were removed from leaves and pooled in batches of five. Developmental stages of *C. picta* were documented with ZEN PRO microscope software. In the following weeks, assorted samples of each instar-stage (first instars in batches of five,

from second instars to F1 adults as single insects) were collected and frozen at -80 °C.

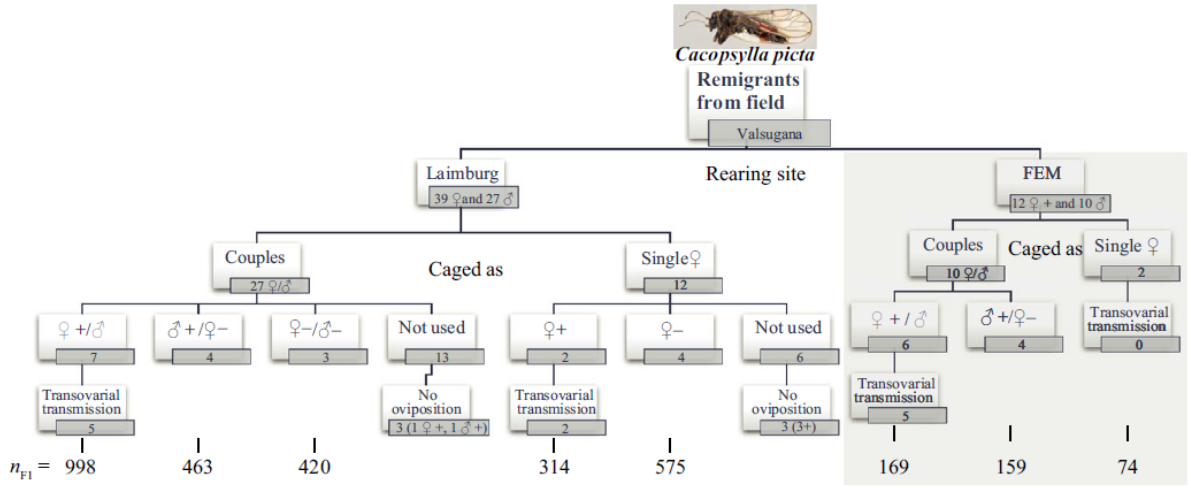

Figure 3.1. Schematic overview of experimental design. Remigrant *C.picta* individuals collected in orchards in Valsugana valley (Trentino Province, Italy) were caged as couples or single individuals (numbers are given in grey boxes). Rearing experiments were performed at two different sites: at Laimburg Research Centre (Laimburg) and at Fondazione E. Mach (FEM). Remigrants were sorted according to their infectious state as follows: infected females (♀ +), infected males (♂ +), uninfected females (♀ -) and uninfected males (♂ -). Numbers of analyzed F1 individuals (*n*F1) of the respective parental groups are mentioned underneath the chart.

Table 3.1. Total number of analyzed *Cacopsylla picta* parental and F1 individuals.

Infection state of remigrants	No. of parental females[a]	Developmental stage (F_1)[b]							Total (F_1)
		Egg	L1	L2	L3	L4	L5	Adult	
Infected parental female	17	389	428	131	109	57	268	173	1555
Control group 1, uninfected parental female, infected parental male	8	73	191	33	86	85	91	63	622
Control group 2, uninfected parental individuals	7	244	320	7	93	69	135	127	995
Total	32	706	939	171	288	211	494	363	3172

[a]Remigrant parental individuals were collected in apple orchards in Valsugana Valley and released onto single net-caged apple graftings for oviposition at Laimburg Research Centre or at Fondazione Edmund Mach, Italy.
[b]F_1 individuals were collected from the apple graftings. L1 to L5, nymphal instars.

Remaining insects were left on the branches to complete development until adult stage. Then, adults were collected and frozen at -80 °C. In total, the study group consisted of 17 infected parental females captured in orchards located in Valsugana Valley (Trentino) and reared in single-net cages (nine at Laimburg, eight at FEM) and a total of 1555 F1 individuals were analyzed. Control group 1 consisted of eight uninfected females (four at Laimburg, four at FEM) caged with an infected parental male. The resulting 622 F1 individuals were analyzed, as well, to study the importance of a potential passive impact of an infected individual to the transmission rate, by locally providing bacterial inoculum to the un infected host plant. Control group 2 comprised seven uninfected parental females and males, all reared at Laimburg, with 995 F1 individuals serving as a negative control (Figure 3.1; Table 3.1). At the end of the experiment,

phloem tissue was isolated from root samples of plants where eggs had developed and was stored at -80 °C for subsequent DNA extraction to assess the absence of '*Ca.* P. mali'.

DNA extraction and PCR analysis

DNA purification of insects was performed using the DNeasy Blood and Tissue extraction Kit from Qiagen. DNA from remigrants reared at FEM, was extracted with the Nucleo Spin® tissue kit from Macherey-Nagel. DNA of each insect, egg or first instar batch (five eggs or first instar nymphs were pooled prior to DNA purification) was eluted with 100 µL TE-elution buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0). Genomic DNA from plants was extracted according to the manufacturer's instructions of DNeasy Plant Mini Kit (Qiagen) with the following optimization step: plant material (100 mg) was disrupted by adding of 400 µL buffer AP1, for three minutes in a TissueLyser II (Qiagen). To the disrupted plant material 4 µL RNase A were added and the mix was incubated for at least 30 minutes in a water bath at 65°C. DNA was eluted two times with 50 µL TE buffer as mentioned above. '*Ca.* P. mali' DNA was detected by SYBR Green real-time PCR with primers rpAP15f-mod and rpAP15r3 as described in Monti *et al.* (2013) targeting the ribosomal protein gene *rpl22*. A 2 µL sample of template DNA was mixed with 5 µL of 2 x SYBR® FAST qPCR Kit Master Mix (Kapa Biosystems), 2.5 µL nuclease-free water and 0.25 µL each of forward and reverse primer (10 µM). The cycling conditions were applied as follows: initial denaturation at 95 °C for 20 s, 35 cycles of amplification at 95 °C for 3 s and 60 °C for 30 s, and melting curve ramp from 65 to 95 °C at an increment of 0.5 °C for 5 s (CFX384 Touch™ Real-Time PCR Detection System, Bio-Rad Laboratories). All insects were tested individually and each sample was tested in triplicates in three independent PCR runs. Phytoplasma PCR detection limits were carefully determined using a four-point tenfold dilution series ($6.5 \times 10^4 – 6.5 \times 10^1$ DNA copies / PCR reaction) of the plasmid pJET1.2-*rpl22* containing the subcloned '*Ca.* P. mali' *rpl22* PCR amplicon. For the dilution series, which was included in every real-time PCR run, the plasmid was diluted in TE-elution buffer (10 mM Tris- Cl, 0.5 mM EDTA, pH 9.0). Samples with a mean quantification cycle (Cq) value lower than 30 and a melting curve peak similar to the positive control were considered '*Ca.* P. mali' positive. The phytoplasma titer was quantified based on the four-point plasmid standard curve analyzed in parallel with the samples in each PCR run. As a control of DNA integrity and to normalize phytoplasma amounts, in parallel to the amplification of *rpl22* fragment, a region of the single-copy *wingless* (*wg*) gene (Brower and De Salle, 1998) of *C. picta* was amplified with primers specific for *C. picta* and other psyllid species, qPSY-WG-F (TCA CGG GCG GCA ATG) and qPSY-WG-R (CCC ACA GCA CAT CAG ATC ACA). The PCR was performed as described above with 0.25 µM each of the *wg*-

specific forward and reverse primer including in each PCR run dilution series (7.3×10^5 - 7.3×10^2 DNA copies/PCR reaction) of the plasmid pJET1.2-*wg* containing the subcloned *wg* gene PCR amplicon. Dilution series and standard curves were prepared as previously described for the pJET1.2-*rpl22* plasmid. Phytoplasma concentration was calculated in relation to the *wg* gene. Phytoplasma concentration was quantified within the range of the four-point standard dilution series and samples out of the range were diluted in elution buffer and reanalyzed if necessary. Samples with a mean Cq value above 30 were considered '*Ca.* P. mali'-negative and detection limits could be verified to be comparable as described in Monti *et al.* (2013). Because of batch analysis, quantification of '*Ca.* P. mali' in egg or first instar batches was not possible. Threshold calculation and data analysis was performed using the CFX Manager™ software (Bio-Rad Laboratories), considering only runs with a PCR efficiency between 95 and 105% and a coefficient of determination (R^2) ≥ 0.99. Three non-template controls NTC, nuclease free water) were performed together with each PCR run. Plant material was analyzed with the same *rpl22*-based SYBR approach as described above. DNA integrity from plant samples was verified by real-time PCR with a probe against a chloroplast gene as described in Baric and Dalla Via (2004).

Control of psyllid species identification by PCR-RFLP

Morphological identification of live psyllid specimens is cumbersome and less precise than identification of dead insects. Certain important morphological characteristics, such as wings or terminalia, cannot be properly inspected in detail without harming the insect. To verify the accuracy of morphological psyllid species identification of living parental insects after finalizing the experiments, DNA from all parental individuals was analyzed by restriction fragment length polymorphism (RFLP) according to Oettl and Schlink (2015). Only insects morphologically and genetically identified as *C. picta* were considered in this study.

Statistical analysis

For comparison of phytoplasma titer increase in instars, a one-way ANOVA with a Tukey post-hoc test was applied, while in all other comparisons, student's *t*-test was used. All data were statistically analyzed using GRAPHPAD PRISM 7.0.

Results

Infected C. picta females produce infected progeny

In 30.2% (average per parental female) of the tested egg batches it was possible to detect phytoplasma. This ratio increased with each developmental stage and finally 99.1 % of the F1adults (average rate per parental female) were found to contain phytoplasma (Figure 3.2).

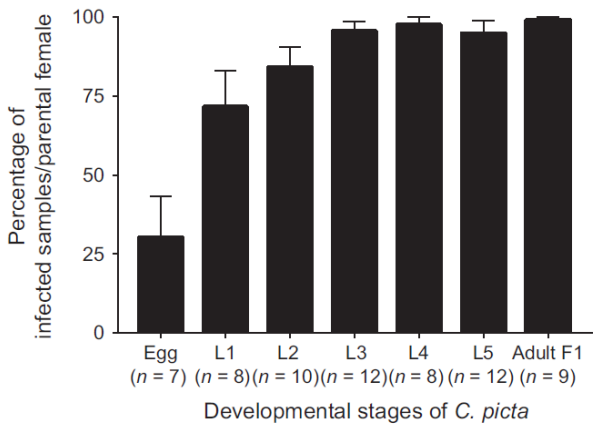

Figure 3.2. The percentage of positive tested *C. picta* increases during insect development. Columns show the average percentage of egg batches, L1 nymph batches and L2 nymph- F1 adults produced by infected parental females (*n* = total number of infected females), in which phytoplasma was detectable. Bars show the standard error of the mean.

Phytoplasma concentration in eggs and first instar batches was very low but unambiguous as characterized by a Cq < 30 and a specific amplicon melting peak. Phytoplasma concentration was determined as the ratio of *rpl22/wg* gene copies. The phytoplasma titer increased exponentially from second instar nymphs to the F1 adult stage. A significant increase was detected between the titer of fourth and fifth instar nymphs ($p = 0.0249$) and between fifth instar nymphs and F1 adults ($p < 0.0001$). F1 adults contained 100-fold more phytoplasma than second instars (Figures 3.3 and 3.4) and comparable concentrations to those of parental females (Figure 3.5).

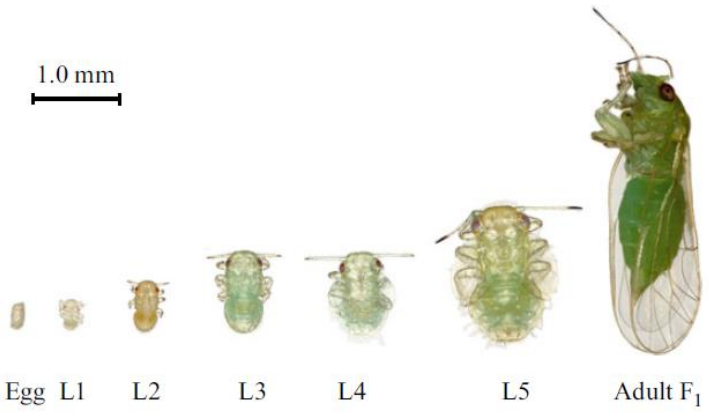

Figure 3.3. Developmental stages of *C. picta*: egg, nymphal instars (L1-L5) and F1 adult.

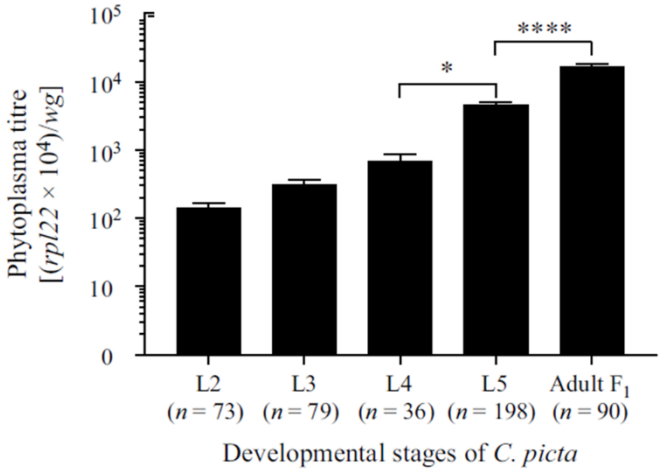

Figure 3.4. Exponential increase of phytoplasma titer during insect development in different stages of infected *C. picta* is depicted as a ratio of *rpl22* gene copies ('*Ca.* P. mali' specific) and *wingless* (*wg*) gene copies (*C. picta* specific). Vertical bars show the standard error of the mean. Statistical differences are indicated by * $P \leq 0.05$, **** $P \leq 0.0001$.

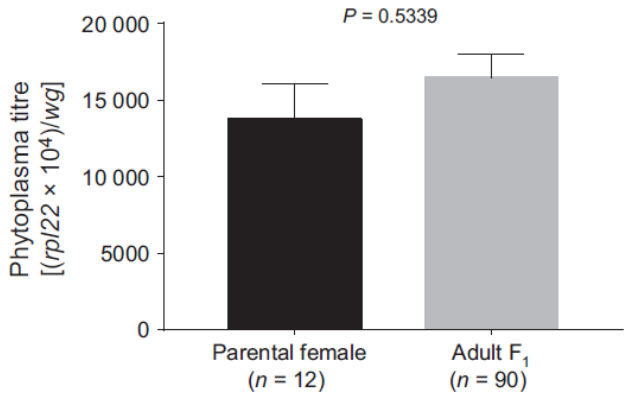

Figure 3.5. The titer in *C. picta* parental females and in F1 adults does not differ. The bars show the phytoplasma titer of *C. picta* parental females and F1 adults. Error bars indicate the standard error of the mean for each experimental group. There is no significant difference between the two groups (P = 0.5339 > 0.05, t = 0.6242, d.f. = 100).

Five out of 17 infected parental females of the study group did not transmit the phytoplasma to their progeny at all. Parental females that did not transmit phytoplasma to their progeny had a significantly lower phytoplasma titer (p = 0.0017, t = 3.805, d.f. = 15) than parental females that transmitted '*Ca.* P. mali' (Figure 3.6).

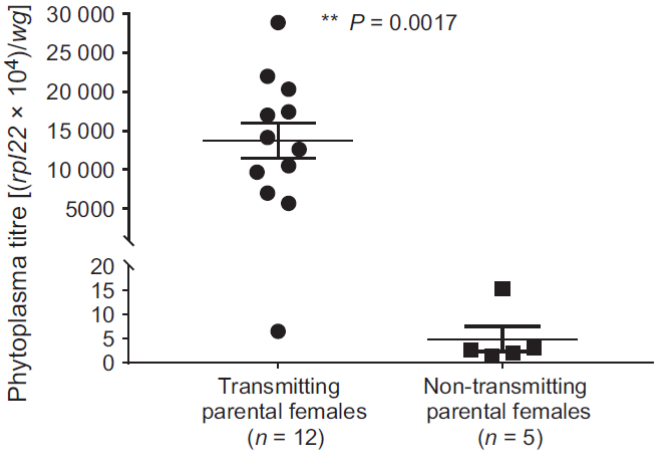

Figure 3.6. Infected *C. picta* parental females were clustered into two groups regarding their ability to transovarially transmit '*Ca* .P. mali'. Each dot represents the bacterial load of a *C. picta* individual that was able to transmit '*Ca.* P. mali' to its progeny. Each square shows the bacterial load of a *C. picta* female that did not transmit '*Ca.* P. mali' to its progeny. Horizontal bars show the mean, while vertical bars show the standard error of the mean of each group. Statistical differences are indicated by ** $P \leq 0.01$ ($t = 3.805$, d.f. = 15).

Transmitting parental females contained on average 13759 (*rpl22* x 10^4)/*wg* gene copies, while non-transmitting insects contained only 5 (*rpl22* x 10^4)/*wg* gene copies. There was no difference detectable between '*Ca.* P. mali' titer in parental females and males (Figure 3.7).

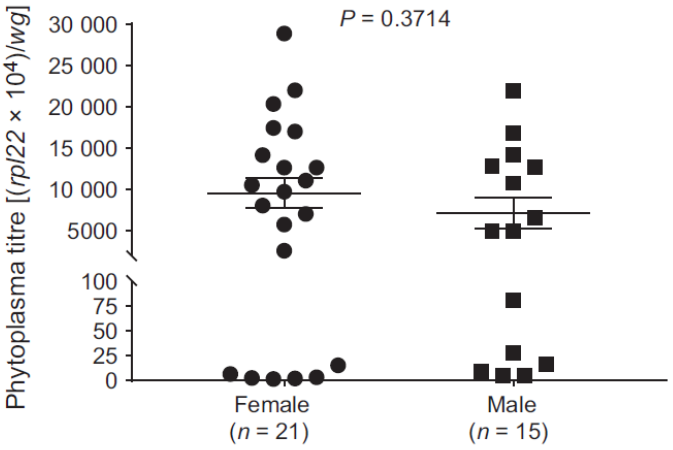

Figure 3.7. The titer of '*Ca.* P. mali' in infected *C. picta* remigrant males and females does not differ. The phytoplasma titer of remigrant *C. picta* females and males is represented by circles and squares, respectively. Horizontal bars show the mean, while vertical bars show the standard error of the mean for each experimental group. No significant difference between the two groups could be found ($P = 0.371 > 0.05$, $t = 0.9058$, d.f. = 34).

Potential phytoplasma acquisition on the bite spot of parental individuals

Only in one individual (fourth instar nymph) derived from control group 1 (uninfected female and infected male) was the detected phytoplasma titer at a very low concentration (4.7 (*rpl22* x 10^4)/ *wg* copies). All other 621 tested individuals tested negative for '*Ca.* P. mali'. In none of

the 995 tested F1 individuals deriving from uninfected *C. picta* females caged with uninfected male remigrants (control group 2) was '*Ca.* P. mali' detectable. '*Ca.* P. mali' infection does not affect oviposition rate and the number of produced eggs of *C. picta* A total of 13 out of the 39 parental females (reared at Laimburg) tested positive, which corresponds to an infection rate of 33.3%. In total, 32 out of 39 (82.1%) parental females laid eggs in the net cages. Oviposition rate of uninfected (84.2%) and infected females (87.5%) caged as couples was nearly the same. Parental females reared under controlled conditions (Figure 3.8) on average deposited similar egg numbers, independent of their infection status (p = 0.3731, t = 0.9201, d.f.= 14).

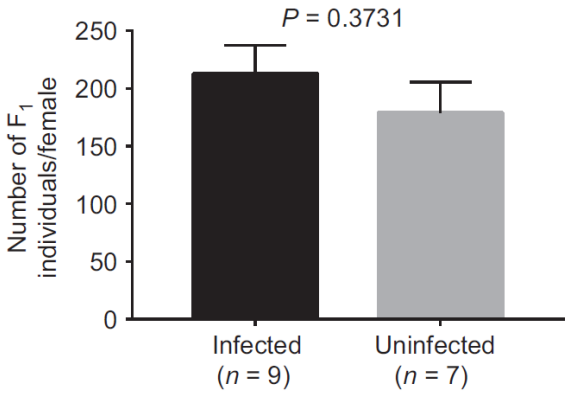

Figure3.8. The number of generated F1 individuals by a single *C. picta* parental female (n) was analyzed with respect to its infectious state. The black bar represents the number of F1 individuals derived from infected individuals and the grey bar from uninfected individuals. Each bar shows the mean with the respective standard error of the mean. No significant differences between the two groups could be found (P = 0.3731 > 0.05, *t* = 0.9201, d.f. = 14).

Infection status of plant material

All grafts used for the net cages tested negative for '*Ca.* P. mali'.

Discussion

The aim of this study was to test if '*Ca.* P. mali'-infected *C. picta* are able to transmit the bacterium to their progeny. To address the principal question of this study, naturally infected *C. picta* individuals were collected and tested for vertical transmission of '*Ca.* P. mali'. As '*Ca.* P. mali' was detected in 30.2% (average per parental female) of the egg batches and 99.1% of the F1 adults, a vertical transmission of the bacteria could be demonstrated. Interestingly, both the percentage of infected individuals and the phytoplasma titer gradually increased during the developmental stages, as was hypothesized by Tedeschi *et al.* (2006). Thus, it is reasonable that all eggs and early instars are actually infected, but contain very low phytoplasma

concentrations that are below the detection limit of the applied detection system. The results of this study show that the transmission ability is dependent on the phytoplasma load of the respective parental female, indicating that a critical phytoplasma concentration threshold determines if vertical transmission occurs. However, it remains unclear how phytoplasma distribution in parental females can influence the transmission ability. It is reasonable that '*Ca.* P. mali' must be present in the ovaries of *C. picta* to be vertically transmitted. However, further research is required in order to understand if the bacteria actively migrate to the reproductive organs or are distributed all over the insect's body, and are therefore also present in the insect ovaries. However, the infectious state of the parental female did not influence the average number of produced eggs. The possibility that nymphs acquire phytoplasma from the bite spot on the leaf where the parental individual was initially sucking has been discussed by Arismendi *et al.* (2015). Due to the fact that one infected nymph derived from an uninfected parental female caged with an infected male was found, the possibility of the phytoplasma acquisition from the insect puncture sites cannot be excluded. However, it can be assumed that this way of phytoplasma acquisition is quantitatively rather negligible in comparison to the demonstrated efficiency of transovarial transmission. In South Tyrol, the first dramatic outbreak of apple proliferation coincided with the appearance of *C. picta*, which was previously absent in the region (Baric *et al.*, 2011; Waldner, 2006). Different studies conducted in Trentino-South Tyrol show that *C. picta* is more efficient than *C. melanoneura* in transmitting the disease and that *C. picta* populations on average contain a much higher percentage of infected individuals than *C. melanoneura* (Baric *et al.*, 2010; Mattedi *et al.*, 2008; Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2007; Jarausch *et al.*, 2011; Mittelberger *et al.*, 2016). Interestingly, a correlation between certain '*Ca.* P. mali' strains and *C. picta* or *C. melanoneura* has been observed (Baric *et al.*, 2011), but no biological explanations for this correlation have been described so far. Strain differences might be responsible for a differential spread in insects and subsequent transovarial transmission, e.g. caused by bacterial adhesion and distribution properties as hypothesized by Arismendi *et al.* (2015). The observed ability of *C. picta* to transmit the pathogen to its progeny could thus be a reason why *C. picta* is a more efficient *'Ca.* P. mali' vector than *C. melanoneura*. Phytoplasma titre in newly emerged F1 adults is similar to that in infected parental individuals. Thus, it can be assumed that transovarially infected F1 adults are as infective as remigrants (Jarausch *et al.*, 2011; Mattedi *et al.*, 2008). However, transmission trials with transovarially infected *C. picta* would be necessary to determine their transmission efficiency. Infected remigrants of *C. picta* are able to transmit the phytoplasma directly to their progeny. These findings are very important for orchard management, since they emphasize the necessity of reducing remigrant and emigrant individuals to avoid oviposition of

infected eggs and the fast, exponential reproduction and spread of highly infectious insect vectors. To our knowledge, the results of this study are the first that clearly show vertical transmission of '*Ca.* P. mali' in its insect vector *C. picta* and thus pave the way for further elucidating the molecular processes of transovarial transmission of '*Ca.* P. mali' in *C. picta*.

References

Alma A., Bosco D., Danielli A., Bertaccini A., Vibio M., Arzone A. (1997). Identification of phytoplasmas in eggs, nymphs and adults of *Scaphoideus titanus* Ball reared on healthy plants. Insect Molecular Biology, 6: 115-121.

Arismendi N. L., Fiore N., Carrillo R. (2015). Absence of transovarial transmission of '*Candidatus* Phytoplasma ulmi' in the vector *Amplicephalus curtulus* Linnavuori & DeLong (Hemiptera: Cicadellidae): is it a rule more than an exception? Neotropical Entomology, 44: 1-9.

Baric S., Berger J., Cainelli C., Kerschbamer C., Dalla Via J. (2011). Molecular typing of '*Candidatus* Phytoplasma mali'- and epidemic history tracing by a combined T- RFLP/VNTR analysis approach. European Journal of Plant Pathology, 131: 573-584.

Baric S., Dalla Via J. (2004). A new approach to apple proliferation detection: a highly sensitive real-time PCR assay. Journal of Microbiological Methods, 57: 135-145.

Baric S., Kerschbamer C., Vigl J., Dalla Via J. (2008). Translocation of apple proliferation phytoplasma via natural root grafts – a case study. European Journal of Plant Pathology, 121: 207-211.

Baric S., Oettl S., Dalla Via J. (2010). Infection rates of natural psyllid populations with '*Candidatus* Phytoplasma mali' in South Tyrol (Northern Italy). Julius-Kühn-Archiv, 189-192.

Bertaccini A., Duduk B., Paltrinieri S., Contaldo N. (2014). Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. American Journal of Plant Sciences, 5: 1763-1788.

Bertamini M., Muthuchelian K., Grando M. S., Nedunchezhian N. (2002). Effects of phytoplasma infection on growth and photosynthesis in leaves of field grown apple (*Malus pumila* Mill.cv. Golden Delicious). Photosynthetica 40: 157-160.

Brower A. V. Z., De Salle R. (1998). Patterns of mitochondrial versus nuclear DNA sequence divergence among nymphalid butterflies: the utility of *wingless* as a source of characters for phylogenetic inference. Insect Molecular Biology, 7: 73-82.

Bustin S. A., Benes V., Garson J. A., Hellemans J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffl M. V., Shipley G. L., Vandesompele J., Wittwe C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry, 55: 611-622.

Carraro L., Ferrini F., Ermacora P., Loi N., Labonne G. (2008). Infectivity of *Cacopsylla picta* (Syn. *Cacopsylla costalis*), Vector of '*Candidatus* Phytoplasma mali' in North East Italy. Acta Horticulturae, 781: 403-408.

Frisinghelli C., Delaiti L., Grando M. S., Forti D., Vindimian M. E. (2000). *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. Journal of Phytopathology 148: 425-431.

Hanboonsong Y., Choosai C., Panyim S., Damak S. (2002). Transovarial transmission of sugarcane

white leaf phytoplasma in the insect vector *Matsumuratettix hiroglyphicus* (Matsumura). Insect Molecular Biology, 11: 97-103.

Hogenhout S. A., Ammar E., Whitfield A. E., Redinbaugh M. G. (2008a). Insect vector interactions with persistently transmitted viruses. Annual Review of Phytopathology, 46: 327-359.

Hogenhout S. A., Oshima K., Ammar E., Kakizawa S., Kingdom H. N., Namba S. (2008b). Phytoplasmas: bacteria that manipulate plants and insects. Molecular Plant Pathology, 9: 403-423.

Huo Y., Liu W., Zhang F., Chen X., Li L., Liu Q., Zhou Y., Wei T., Fang R., Wang X. (2014). Transovarial transmission of a plant virus is mediated by vitellogenin of its insect vector. PLoS Pathogens, 10: e1003949.

Jarausch B., Fuchs A., Schwind N., Krczal G., Jarausch W. (2007). *Cacopsylla picta* as most important vector for '*Candidatus* Phytoplasma mali' in Germany and neighbouring regions. Bulletin of Insectology, 60: 189-190.

Jarausch B., Schwind N., Fuchs A., Jarausch W. (2011). Characteristics of the spread of apple proliferation by its vector *Cacopsylla picta*. Phytopathology, 101: 1471-1480.

Jarausch B., Weintraub P. G., Sauvion N., Maixner M., Foissac X. (2014). Diseases and insect vectors, in Bertaccini, A. (Ed.), *Phytoplasma and Phytoplasma Disease Management: How to Reduce their Economic Impact*. International Phytoplasmologist Working Group pp. 111-121.

Kawakita H., Saiki T., Wei W., Mitsuhashi W., Watanabe K., Sato M. (2000). Identification of mulberry dwarf phytoplasmas in the genital organs and eggs of leafhopper *Hishimonoides sellatiformis*. Phytopathology, 90: 909-914.

Mattedi L., Forno F., Cainelli C., Grando M. S., Jarausch W. (2008). Research on *Candidatus* Phytoplasma mali transmission by insect vectors in Trentino. Acta Horticulturae, 781: 369-374.

Mayer C. J., Jarausch B., Jarausch W., Jelkmann W., Vilcinskas A., Gross J. (2009). *Cacopsylla melanoneura* has no relevance as vector of apple proliferation in Germany. Phytopathology, 99: 729-738.

Mayer C. J., Vilcinskas A., Gross J. (2008a). Pathogen-induced release of plant allomone manipulates vector insect behavior. Journal of Chemical Ecology, 34: 1518-1522.

Mayer C.J., Vilcinskas A., Gross J. (2008b). Phytopathogen lures its insect vector by altering host plant odor. Journal of Chemical Ecology, 34: 1045-1049.

Mittelberger C., Mitterrutzner E., Fischnaller S., Kerschbamer C., Janik K. (2016). Populationsdichten der Apfeltriebsuchtvektoren 2012 - 2014 im Burggrafenamt. Obstbau- Weinbau, 53: 17-20.

Monti M., Martini M., Tedeschi R. (2013). EvaGreen Real-time PCR protocol for specific '*Candidatus* Phytoplasma mali' detection and quantification in insects. Molecular and Cellular Probes, 27: 129-136.

Müther J., Vogt H. (2003). Sampling methods in orchards trials: a comparison between beating and inventory sampling. IOBC WPRS Bulletin, 26: 67-72.

Oettl S., Schlink K. (2015). Molecular identification of two vector species, *Cacopsylla melanoneura* and *Cacopsylla picta* (Hemiptera: Psyllidae), of apple proliferation disease and further common psyllids of Northern Italy. Journal of Economic Entomology 1-10.

Ossiannilsson F. (1992). The Psylloidea (Homoptera) of Fennoscandia and Demark, Fauna Entomologica Scandinavica. Leiden, New York: E.J. Brill.

Pedrazzoli F., Gualandri V., Forno F., Mattedi L., Malagnini V., Salvadori A., Stoppa G., Ioriatti C. (2007). Acquisition capacities of the overwintering adults of the psyllid vectors of '*Candidatus* Phytoplasma mali'. Bulletin of Insectology, 60: 195-196

Tedeschi R., Bosco D., Alma A. (2002). Population dynamics of *Cacopsylla melanoneura* (Homoptera: Psyllidae), a vector of apple proliferation phytoplasma in northwestern Italy. Journal of Economic Entomology, 95: 544-551.

Tedeschi R., Ferrato V., Rossi J., Alma A. (2006). Possible phytoplasma transovarial transmission in the psyllids *Cacopsylla melanoneura* and *Cacopsylla pruni*. Plant Pathology, 55: 18-24.

Tedeschi R., Visentin C., Alma A., Bosco D. (2003). Epidemiology of apple proliferation (AP) in northwestern Italy: evaluation of the frequency of AP-positive psyllids in naturally infected populations of *Cacopsylla melanoneura* (Homoptera: Psyllidae). Annals of Applied Biology, 142: 285-290.

Waldner W. (2006). Überwachung und Abwehr der Blattsauger im kommenden Jahr. Obstbau-Weinbau, 43: 358-361.

Weintraub P. G. (2007). Insect vectors of phytoplasmas and their control- an update. Bulletin of Insectology, 60: 169-173.

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Mittelberger C., Obkircher L., Oettl S., Oppedisano T., Pedrazzoli F., Panassiti B., Kerschbamer C., Anfora G., Janik K. (2017). The insect vector *Cacopsylla picta* vertically transmits the bacterium '*Candidatus* Phytoplasma mali' to its progeny. Plant Patholgy, doi: 10.1111/ppa.12653.

My contribution regards planning and execution of the experiments (insect rearing and molecular analyses) conducted at Fondazione Edmund Mach.

Mobility

During this Ph.D. experience, I had the opportunity to spend six months abroad. The next two chapters (Chapter 4 and Chapter 5) deal with the results obtained in research activities conducted during my attendance at two different international host institutes. In particular:

- from February to May 2016, I worked at the Department of Organisms and Ecosystems Research of the National Institute of Biology in Ljubljana (Slovenia). The short project: "Studies on substrate-borne vibrational communication in the vectors of apple proliferation *Cacopsylla picta* and *Cacopsylla melanoneura*" was carried out under the supervision of Dr. Meta Virant-Doberlet;

- from October to December 2016, I worked at the Department of Sustainable Plant Protection of IRTA (Research and Tecnology Food and Agriculture) in Cabrils (Spain). The short project: "Analysis of the relationships among '*Candidatus* Phytoplasma mali' strains, psyllid vectors and host plants" was carried out under the supervision of Dr. Assumpciò Batlle-Durany.

CHAPTER 4

'*Candidatus* Phytoplasma mali' in Trentino: study of the distribution of isolates in plants and psyllid vectors using a multilocus sequence approach

Abstract

Apple proliferation (AP) is a phytoplasma-caused disease representing one of the most severe problems in European apple orchards. In Trentino, the main apple producing region in Italy, AP is the major threat for production. The etiological agent is '*Candidatus* Phytoplasma mali' and infected plants show symptoms like witches' brooms and small fruits. Genetic characterization led to the identification of different strains of '*Ca*. P. mali' that show different degrees of symptom expression. Genetic background does not only determine the virulence in the host plants, but likely shapes also the relationship with the insects that mainly vector '*Ca*. P. mali': the psyllids *Cacopsylla picta* and *Cacopsylla melanoneura.* Disentangle the ecological relationships that underlie the triangle '*Ca*. P. mali' strain - host plant - insect vector to better understand the evolution and spreading of AP as well, will contribute to design more effective control strategies. In this research a multilocus analysis were adopted to study the genetic variability of phytoplasma isolated from insects and host plants collected in different areas. Preliminary results show a higher phytoplasma variability in genotypes isolated from psyllids compared to apple plants.

Key words: phytoplasma, apple proliferation, MLST, genetic variability, geographical distribution

Introduction

Phytoplasmas are plant-pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less microorganisms phylogenetically related to Gram-positive bacteria with a low G+C-content (Weisburg *et al.*, 1989). These pathogens cause hundreds of crop diseases distributed worldwide (Lee *et al.,* 2000) and are vectored by sap-sucking hemipteran insects (Weintraub and Beanland, 2006). Three phytoplasmoses are known to cause serious damages to the fruit production of temperate areas: apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). Even though the 16S rDNA sequences of strains of these pathogens

indicate a close phylogenetic relationship, it was possible to identify three different putative species: '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri', and '*Candidatus* Phytoplasma prunorum' (Seemüller and Schneider, 2004). The three pathogens form, a cluster designated the 'AP phytoplasma group' (Seemüller *et al.*, 1998) or 16SrX group (Lee *et al.*, 2000) within the AP subclade.

AP is considered one of the most important diseases of apple (EPPO/CABI, 1997), particularly in the northern areas of southern Europe, causing symptoms associated with disturbance in the normal balance of growth regulators (Seemüller and Schneider, 2004), such as delayed production of flowers in the autumn, the development of rosettes of terminal leaves late in the season, abnormally long stipules on leaves and rather short petioles. However, the most reliable symptom is the premature development of axillary buds, which gives rise to witches' brooms on branches. Fruits of infected trees show a reduced size and a poor flavor, both sugar and acidity being reduced. The peduncles are longer and thinner and the fruit takes a flattened appearance (Blumer and Bovey, 1957; Schuch, 1962; Bovey, 1972).

Several genes and techniques have been used over the years to differentiate '*Ca.* P. mali' strains on a molecular basis to investigate genetic variability (Jarausch *et al.*, 2000; Kison *et al.*, 1994; Seemüller and Schneider, 2007; Danet *et al.*, 2008; Martini *et al.*, 2008). From a phytopathological point of view, the molecular characterization of '*Ca.* P. mali' for the identification of markers linked to virulence would be a useful tool in epidemiological studies and would greatly facilitate plant breeding and resistance tests (Schneider and Seemüller, 2009). The completed genome sequence of '*Ca.* P. mali' strain AT offered new opportunities thanks to the availability of the complete set of the genetic endowment (Kube *et al.*, 2008). While the 16S rRNA sequences are almost identical and unsuitable for strain differentiation (Seemüller and Schneider, 2004), other non-ribosomal DNA fragments display higher sequence variability. Currently, the multilocus sequence typing (MLST) is the most appropriate and complete method for genotyping prokaryotes and is widely used in bacterial epidemiology and population genetics (Maiden *et al.*, 1998; Urwin and Maiden, 2003). However, it requires the identification of gene sequences. For this purpose, Danet *et al.* (2011) designed a multilocus sequence analysis for phytoplasmas of the group 16SrX, initially targeting two genes involved in sugar and nucleotide metabolism (*aceF* and *pnp*). Later, to obtain a more accurate genotyping, two genes encoding an immunodominant surface protein (*imp*) and a component of the protein secretion machinery (*secY*) were added in the analysis (Danet *et al.*, 2007). This strategy was already applied in studies about '*Ca.* P. prunorum' genetic variability, providing useful information about the geographical distribution of isolates and the relationships between

genotype and phenotypic features, such as hypo-virulence (Danet *et al*., 2011). Regarding '*Ca.* P. mali', the sequence of *secY* was described by the '*Ca.* P. mali' genome sequence consortium (Kube *et al.*, 2008). Sequence analysis of the *imp* gene was employed to compare different strains from apple and periwinkle originating from Germany, Italy, Austria and France which were differentiated into five groups (Danet *et al.*, 2007, 2008). Moreover, ribosomal protein genes and the *pnp* gene were used to type pathogen strains and to determine the frequency of '*Ca.* P. mali' subtype combinations present in the field (Danet *et al.*, 2008; Martini *et al.*, 2008). Aim of this work was to apply a multilocus sequence analysis to evaluate the relationships among genetic polymorphism of '*Ca.* P. mali', plant and psyllid hosts, and the geographical distribution of genotypes.

Materials and methods

Infected psyllids collection

Infected *C. picta* and *C. melanoneura*, collected during the disease monitoring in conventional and abandoned orchards in Valsugana in the years 2014-2015, were used for this work. After insects' classification by dichotomous keys (Ossiannilsson, 1992) the total DNA was extracted using the commercial kit NucleoSpin® Tissue (Macherey-Nagel) and the samples were analyzed by real-time PCR following the method developed by Baric and Dalla Via (2004), as described in Chapter 1, to assess the presence of AP phytoplasma. AP-infected samples were selected and used for further analyses, performed at the Plant Pathology laboratory of IRTA in Cabrils (Spain), where DNA of infected psyllid vectors collected in Pais Basque and Asturias regions was provided for comparative studies.

Plants material selection

Branches of symptomatic trees were collected in October 2015 during surveys in Valsugana, covering the areas of Alta Valsugana, where a high presence of symptomatic plants is reported, and Bassa Valsugana, characterized by low presence of AP (Dallago, 2016). To have a more complete picture of '*Ca.* P. mali' distribution in Trentino, samples of symptomatic plants were also collected in Val di Non and Alto Garda and Ledro area at the end September in 2016. As for psyllids, DNA from symptomatic apple samples collected in Pais Basque and Asturias regions were provided by colleagues of IRTA (Figure 4.1).

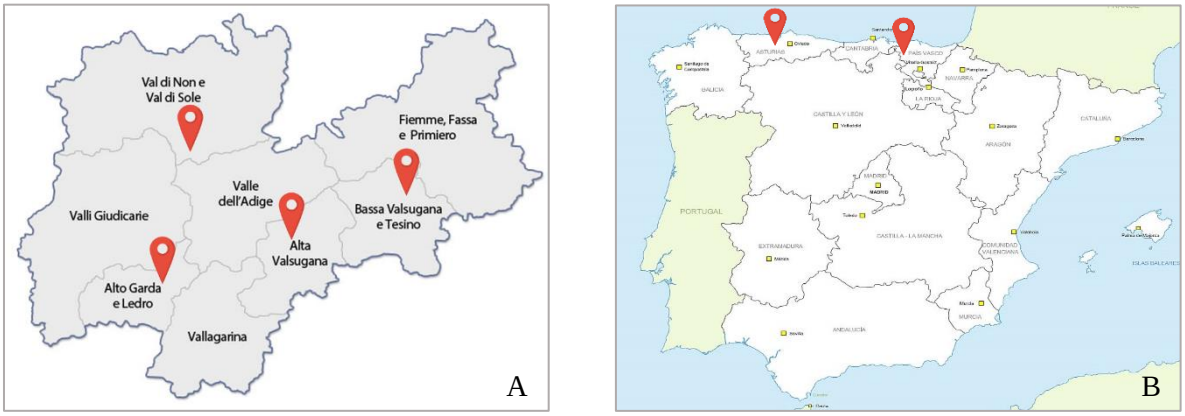


Figure 4.1. Geographical region were symptomatic plants were collected to study the genetic variability of '*Ca.* P. mali' in (A) Trentino and (B) Spain.

For each symptomatic plants, three branches were collected, possibly in presence of evident symptoms such as witches' brooms and enlarged stipules. Phloem tissue was isolated from three branches and mixed together (Figure 4.2). After lyophilization and mechanical disruption of the samples, the total DNA was extracted with the NucleoSpin® Plant II (Macherey-Nagel) commercial kit.

Figure 4.2. Phloem tissues were isolated from symptomatic apple branches, in this case witches' broom are evident.

PCR amplification

To study the variability of the four genes *aceF*, *pnp*, *secY* and *imp*, the target DNA in insect and plant samples was amplified by nested PCR using the primers described in Figure 4.3. According to Danet *et al.* (2011), first amplifications were performed using an initial denaturation step at 95° C for 3 min, followed by 20 cycles consisting of 94° C for 30 s, 50° C for 30 s and 66° C for 45 s, and by a final extension step at 66° C for 7 min. Nested amplification was carried out using 1 µl of the first amplification product with an initial denaturation step at

95° C for 3 min followed by 35 cycles consisting of 94° C for 30 s, 50° C for 30 s and 66° C for 45 s, and by a final extension step at 66° C for 7 min. Nested-PCR products were separated on a 1,5 % agarose gel; DNA was stained with Gel Red and visualized under UV light.
PCR products were purified using the commercial kit NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel).

Gene	Length (bp)	Purpose	Primer	Sequence (5′–3′)
aceF	797	PCR	AceFf1	TAAAATTCGCTGATGTTGGCG
			AceFr1	CATCTTTCAATTCATTAAAACTAG
		Nested-PCR	AceFf2	AGGTATTGAAGAAGGAACTG
			AceFr2	CAACCGCTTTCATAATAAAAG
pnp	549	PCR	Pnpf1	GAAGTTGGTATTACTGCTTTAC
			Pnpr1	GATAAATCTATTTGACCGCG
		Nested-PCR	Pnpf2	TACAATTAGATATTAAAGTTAAAGG
			Pnpr2	ATTAATTTTAATACATTTCGC
secY	664	PCR	SecYMalF1	TTAGGACGTAGTATACAAATCCCNTT
			SecYMalR1	ACAATAATTAAAAATCCTGTNCC
		Nested-PCR	SecYMalF2	AAGAATGGCGTGAACARGGNGA
			SecYMalR2	GCATCTTGTTTAGATAAATGTTC
imp	670	PCR	IMPF1	CAAATGATAAAGCTGATCAA
			IMPR1bis	CAAGACCTTTAAGGCCACATC
		Nested-PCR	IMPF3	GTTTTATGTTATAATAAACAGTG
			IMPR3mal	CAAACTATAGTTAAAATTAAAGC

Figure 4.3. Oligonucleotides used as primers for PCR and nested PCR (modified from Danet *et al.*, 2011).

Sequencing

Purified PCR products, added with the specific primers (AceFf2, PnpF2, SecYMalF2, respectively), were sent to a sequencing platform (Sanger sequencing) and the analysis of obtained sequences was carried out using the software MEGA v. 6.0. Sequences of the PCR products were compared by multiple alignments using CLUSTAL W (Tamura *et al.*, 2013) and visualized by GENEDOC v. 2.6 (www.psc.edu). The subsequent phylogenetic analyses were conducted using MEGA 6.0 and the phylogenetic relationships of the unrooted tree were based on the Maximum Likelihood method with a 500 replicate bootstraps search. The BOOTSTRAP option in the MEGA program was used for the bootstrap analysis.
When differences between samples nucleotide sequences and deposited genotypes emerged, the alteration of amino acid sequence was assessed by "Translate tool" provided by the website http://web.expasy.org.

Preliminary results

PCR and electrophoresis

The nested PCR protocol developed for the four primer pairs was applied in all analyses. In most of the cases, the first PCR did not yield an appreciable amount of amplicon, which was much more visible on the agarose gel after the nested PCR. Among the primer sets tested, only the three amplifying the genes *pnp*, *aceF* and *secY* were finally used for the analyses, as the *imp* gene was not amplified successfully in all samples (Figures 4.4 and 4.5). The products of nested PCR using IMPF3 and IMPR3mal, indeed, revealed to be nonspecific, as in most samples up to five bands were visible on agarose gel. The other primer pairs used worked well with samples from both plants and insects.

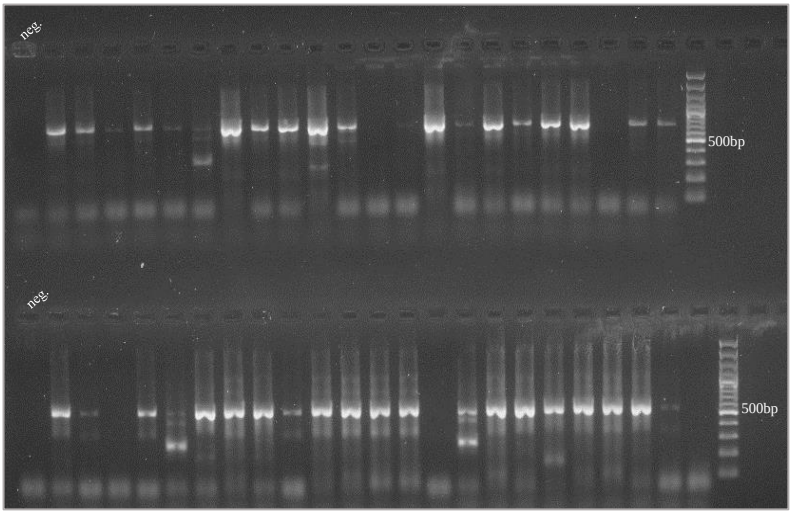


Figure 4.4. Agarose gel showing the results of the nested PCR performed on samples with the primer pairs SecYMalF1/R1 and SecYMalF2/R2 (above) and Pnpf1/r1 and Pnpf2/r2 (below).

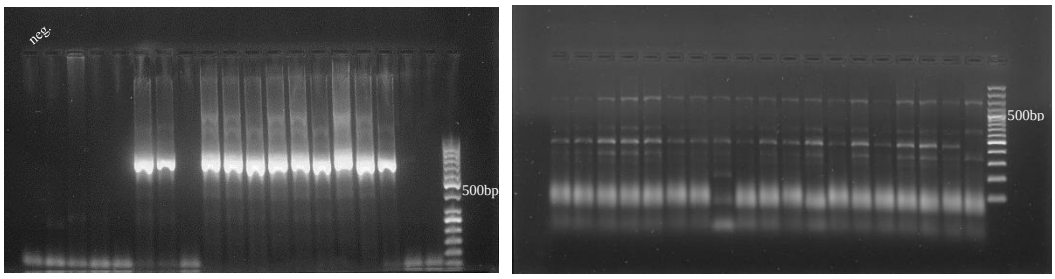


Figure 4.5. Agarose gel showing the results of the nested PCR performed on samples with the primer pairs AceFf2/AceFr2 (left) and IMPF3/IMPR3mal (right).

After nested PCR amplification and electrophoresis, only samples showing amplicons for all three primer pairs were used for further analysis. Moreover, samples whose nested PCR

produced more than one band were discarded. Table 4.1 shows the number of insects and plants analyzed for the different areas. Especially with insect samples, the PCR with primers specific for *secY* and *aceF* genes were not always successful. Other experiments need to be carried out with these samples to optimize the protocol.

Table 4.1. List of the plant and insect samples used for the amplification of the genes *aceF*, *pnp* and *secY*.

	Italy						**Spain**			
	Valsugana		Val di Non		Alto Garda and Ledro		Pais Basque		Asturias	
	tested	amplified	tested	amplified	tested	amplified	tested	amplified	tested	amplified
apple plants	150	118	22	14	22	22	8	7	33	27
C. picta	35	25	-	-	-	-	27	14	18	12
C. melanoneura	73	3	-	-	-	-	7	1	4	1
tot.	258	146	22	14	22	22	42	22	55	40

So far, the three genes were sequenced in 32 apple plants (24 from Valsugana, four from Val di Non, and four from Alto Garda and Ledro) and in 27 insects (25 *C. picta* and two *C. melanoneura* from Alta Valsugana). The analyses of the remaining insects and plants are still in progress.

The sequences obtained for each phytoplasma gene were aligned and then pooled in homogeneous groups, which were compared to the sequences described and characterized by Danet *et al.* (2011) and deposited in GeneBank (https://www.ncbi.nlm.nih.gov/). The groups obtained with each gene analysis were compared. Results of identification of phytoplasma haplotypes are summarized in the Table 4.2, where also the different sampling sites representative of Valsugana, Val di Non and Alto Garda and Ledro are listed. Among the haplotypes described by Danet *et al.* (2011), four were obtained for the *aceF* gene, four for *pnp* and four for *secY*. Interestingly, a new haplotype that had not been described before resulted from the analyses. Some sequences were only partially readable and therefore they were removed from the alignment analyses. Regarding plants, 29 sequences were obtained for *aceF*: nine samples are associated with A13 genotype, even if one sample shows a substitution in one nucleotide and can be considered as a variant of the described genotype, and 20 samples are associated with A15 genotype. All samples show the same sequence, but differ from the deposited reference for the insertion of one nucleotide (adenine) in the position 61. Interestingly, all samples analyzed and the other reference sequences contain this nucleotide. The other genotypes described in Danet *et al.* (2011) for '*Ca.* P. mali' (A14, A16, A22, A23) are not represented in this group. For *pnp*, 26 sequences were aligned: 23 correspond to P9 genotype, three to P12 genotype, while none to P10, P11 and P13. For *secY*, 24 sequences were

obtained: three are associated with S10 genotype, eight with S11 genotype and 13 with S12 genotype, even if one sample represents a variant showing a substitution in one nucleotide. About insects, all 27 sequences were readable for all genes. Regarding *C. picta*, for *aceF*, 15 isolates are associated with A13 genotype, eight isolates with A15 genotype and two with A23 genotype. For *pnp*, 19 correspond to P9 genotype (five of which are variants differing in one nucleotide), three are associated with P11 (one of which is a variant differing in one nucleotide), three insects with P12 genotype. For *secY*, three samples are associated with S9 genotype, three with S10 genotype, five with S11 genotype, and 12 with S12 genotype. Interestingly, one undescribed genotype was found in two *C. picta* individuals. This genotype shows an intermediate sequence between the described genotypes, being identical to S10 between the position 281 and 622, while showing homologies with the other ones (S9, S11 and S12) between the positions 1 and 280 (Supplement 2). Regarding *C. melanoneura*, only two individuals have been analyzed so far. Surprisingly, both of them grouped apart from *C. picta* for two of the three genes: *aceF* (A16 genotype) and *pnp* (P13). For *secY*, instead, the two individuals shared the genotype S12 with *C. picta*.

Table 4.2. Samples clustered for phytoplasma haplotypes of the genes *aceF*, *pnp* and *sec*Y; (*) indicates genotypes different from the deposited sequences, considered as variants; (**) indicates that all sequences obtained are identical and differ from the deposited reference in one nucleotide (see supplementary materials).

Gene	Reference isolate	EMBL accession n°	Genotype	n° of plants	Alta Valsugana	Bassa Valsugana	Riva del Garda	Val di Non	n° of insects	Alta Valsugana	
										C. melanoneura	*C. picta*
aceF	AP-AT	FN598184	A13	**8 + 1***	3	4 + 1*	0	1	**15**	0	15
	AP13	FN598185	A14	**0**	0	0	0	0	**0**	0	0
	AP032-10	FN598186	A15	**20****	11	2	4	3	**8****	0	8
	AP1Luca	FN598187	A16	**0**	0	0	0	0	**2**	2	0
	TN/1	FN598188	A22	**0**	0	0	0	0	**0**	0	0
	NW/2	FN598189	A23	**0**	0	0	0	0	**2**	0	2
pnp	AP-AT	FN598200	P9	**23**	13	7	1	2	**14 + 5***	0	14 + 5*
	AP15	FN598201	P10	**0**	0	0	0	0	**0**	0	0
	AP28	FN598202	P11	**0**	0	0	0	0	**2 + 1***	0	2 + 1*
	AP032-10	FN598203	P12	**3**	2	0	1	0	**3**	0	3
	AP1Luca	FN598204	P13	**0**	0	0	0	0	**2**	2	0
secY	AP-AT	FN598213	S9	**0**	0	0	0	0	**3**	0	3
	AP4Luca	FN598214	S10	**3**	2	0	1	0	**3**	0	3
	AP032-10	FN598215	S11	**8**	5	2	1	0	**5**	0	5
	AP15	FN598216	S12	**12 + 1***	5	4	0	3 + 1*	**14**	2	12
			new	**0**	0	0	0	0	**2**	0	2

The isolates showing variations from the described sequences were checked for the corresponding amino acid sequences. The online translate tool showed no modifications in the primary structure of the protein codified in any case.

The multiple alignments of reference nucleotide sequences deposited for the three considered genes are shown in the supplementary materials (Supplement 1 for plants and Supplement 2 for insects). The unrooted trees obtained by phylogenetic analyses, showing how the samples group together are reported in the supplementary materials (Supplement 3 for plants and Supplements 4 for insects).

Discussion

Interactions between pathogens, insect vectors and plant hosts are receiving increasing interest among agricultural scientists, who look at them with special regards to develop alternative and more sustainable pest control strategies. The aim of this work was to study the three-way interaction between '*Ca.* P. mali', psyllid vectors and apple plants with a molecular approach. After the genome sequencing of AP etiological agent (Kube *et al.*, 2008), besides more known approaches, new tools became available to investigate its biodiversity, such as single-strand conformation polymorphism and sequence analyses of the *hflB* gene (Schneider and Seemüller, 2009). Janik *et al.* (2015) described the local distribution of '*Ca.* P. mali' genetic variants in Alto-Adige based on multi locus sequence typing studies (MLST), revealing the presence of up to 17 different genotypes per gene fragment, then clustered into two groups. Danet *et al.* (2011) proposed a MLST method to genotype temperate fruit tree phytoplasmas of the phylogenetic group 16SrX, tracing their variability through four phytoplasma genes: *aceF*, *imp*, *pnp* and *secY*.

In this research, the last method was applied to deepen the knowledge of '*Ca.* P mali' epidemiology and distribution in Trentino region. To achieve this goal, plant and insect vector samples, collected in three different areas of the region, were analyzed. Moreover, samples collected in the North of Spain were analyzed to try to compare the spreading dynamics of AP phytoplasma in different geographic regions. The areas of the two countries, northeastern Italy and northern Spain, are characterized by an important production of apple fruits, but, at the same time, they share the problematic of AP presence in the orchards (Vindimian, 2002; Dallago, 2016; Batlle *et al.*, 2012; Miñarro *et al.*, 2016).

As a gene variant (*imp*) was discarded, and the sequencing analyses of PCR products are still going on, the results presented here have to be considered very preliminary. So far, sequences from 32 infected plants collected in different areas of Trentino and from 27 insects from Valsugana were aligned and clustered into groups, suggesting intriguing information. Unlikely, not all sequences obtained could be analyzed. The presence of overlapping peaks suggests the hypothesis of multiple infections.

The results show a higher phytoplasma variability in genotypes isolated from psyllids compared to apple plants: the sequences obtained from plant samples can be associated with two out of six genotypes described for *aceF*, two out of five genotypes described for *pnp*, and three out of four genotypes described for *secY*. Regarding insects, all genotypes described by Danet *et al.* (2011) are represented in the population considered. Moreover, for *C. picta*, the main AP vector in Trentino, which is more represented than *C. melanoneura* in this sample due to its higher natural infection levels, an undescribed genotype (observed in two individuals) was isolated. Contrarily, for *C. melanoneura*, whose role as AP vector in Trentino has always been debatable, only two individuals were analyzed. Interestingly, both specimens share the same genotypes, which have not been observed in *C. picta* yet. If these '*Ca.* P. mali' isolates found only in *C. melanoneura* were demonstrated to be species-specific, different hypotheses could be advanced. First, this phenomenon could be explained by the different ecological niches that this species occupies if compared to *C. picta*, in particular by the presence of another host plant, such as hawthorn. Moreover, these results could suggest a different degree of co-evolution with the pathogen, but further information is needed to confirm this hypothesis, for example on the correlation between phytoplasma isolates and their virulence on apple plants or their effects on vectors' fitness. The presence of fewer genotypes described in plants compared to insects could suggest that only few isolates of '*Ca.* P. mali' can be efficiently transmitted by the vectors, or, once inoculated, successfully colonize the plant host. As the insects characterized so far belong only to populations collected in Trentino, it will be interesting to compare these results to other insect populations and plants. Future investigations should be focused on specific issues, as the complete identification of the phytoplasma haplotypes in both psyllid species and the assessment of associations between phytoplasma strains and psyllid vectors. Further analyses could give information on geographical distribution of phytoplasma haplotypes in insects and plants. Study of the relationships between phytoplasma genotype and phenotype (e.g. symptom expression in plants) could help to clarify the role of *C. picta* and *C. melanoneura* in AP spreading, thus contributing to design more effective control strategies too.

References

Baric S., Dalla Via J. (2004). A new approach to apple proliferation detection: a highly sensitive real-time PCR assay. Journal of Virological Methods, 57: 135-45.

Batlle A., Sabaté J., Berra D., Laviña A. (2012). Epidemiological study of apple proliferation in Spain. Petria, 22(3): 176-180.

Blumer S., Bovey R. (1957). On apple proliferation virus. Phytopathologische Zeitschrift 30: 237-258.

Bovey R. (1972). Maladie des proliférations. In: La défense des plantes cultivées (Ed. by Bovey, R. et al.) (6th edition), pp. 227-231. Payot, Switzerland.

Dallago G. (2016). Scopazzi del melo: siamo sulla strada giusta. L'Informatore Agrario, 41: 36-39.

Danet J. L., Bonnet P., Jarausch W., Carraro L., Skoric D., Labonne G., Foissac X. (2007). Imp and secY, two new markers for MLST (multilocus sequence typing) in the 16SrX phytoplasma taxonomic group. Bulletin of Insectology, 60: 339-340.

Danet J. L., Bahriz H., Cimerman A., Foissac X. (2008). New molecular typing tools to monitor fruit tree phytoplasma variability in the 16SrX taxonomic group. XXth International symposium on virus and virus-like diseases of temperate fruit crops. Acta Horticolurae, 781: 343-349.

Danet J. L., Balakishiyeva G., Cimerman A., Sauvion N., Marie-Jeanne V., Labonne G., Laviňa A., Batlle A., Križanac I., Škorić D., Ermacora P., Serçe C.U., Çağlayan K., Jarausch W., Foissac X. (2011). Multilocus sequence analysis reveals the genetic diversity of European fruit tree phytoplasmas and supports the existence of inter-species recombination. Microbiology, 157: 438-450.

EPPO/CABI (European and Mediterranean Plant Protection Organization/ Centre for Agriculture and Biosciences International). 1997. Quarantine Pests for Europe. 2nd edition. Edited by Smith IM, McNamara DG, Scott PR, Holderness M. CABI International, Wallingford, UK, 1425 pp.

Janik J., Oettl S., Schlink K. (2015). Local distribution of '*Candidatus* Phytoplasma mali' genetic variants in South Tyrol (Italy) based on a MLST study. Phytopathogenic Mollicutes, 5(1): 29-30.

Jarausch W., Saillard C., Helliot B., Garnier M., Dosba F. (2000). Genetic variability of apple proliferation phytoplasmas as determined by PCR-RFLP and sequencing of a non-ribosomal fragment. Molecular and Cellular Probes, 14: 17-24.

Kison H., Schneider B., Seemüller E. (1994). Restriction fragment length polymorphism within the apple proliferation mycoplasmalike organism. Journal of Phytopathology, 141: 395-401.

Kube M., Schneider B., Kuhl H., Dandekar T., Heitmann K., Migdoll A. M., Reinhardt R., Seemüller E. (2008). The linear chromosome of the plant-pathogenic mycoplasma '*Candidatus* Phytoplasma mali'. BMC Genomics 9: 306.

Lee I. M., Davis R. E., Gundersen-Rindal D. E. (2000). Phytoplasma: phytopathogenic mollicutes. Annual Review of Microbiology, 54: 221-255.

Maiden M. C. J., Bygraves J. A., Feil E., Morelli G., Russell J. E., Urwin R., Zhang Q., Zhou J., Zurth K., Caugant D. A., Feavers I. M., Achtman M., Spratt B.G. (1998). Multilocus sequence

typing: a portable approach to the identification of clones within populations of pathogenic microorganisms Proceedings of the National Academy of Sciences USA, 95: 3140-3145.

Martini M., Ermacora P., Falginella L., Loi N., Carraro L. (2008). Molecular differentiation of '*Candidatus* Phytoplasma mali' and its spreading in Friuli Venezia Giulia region (North-East Italy). Acta Horticulturae, 781: 395-402.

Miñarro M., Somoano A., Moreno A., Rosa García R. (2016). Candidate insect vectors of apple proliferation in Northwest Spain. Springerplus. 5(1): 1240.

Ossiannilsson F. (1992). The Psylloidea (Homoptera) of Fennoscandia and Denmark. 346 pp., Fauna Entomologica Scandinavica, 26, E.J. Brill, Leiden (The Netherlands).

Schuch K. (1962). Diagnostic importance of stipules in apple proliferation. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes (Braunschweig) 14: 85-88.

Seemüller E., Schneider B. (2004). '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. International Journal of Systematic and Evolutionary Microbiology, 54: 1217-1226.

Seemüller E., Schneider B. (2007). Differences in virulence and genomic features of strains of *Candidatus* Phytoplasma mali, the apple proliferation agent. Phytopathology, 97: 964-970.

Seemüller E., Marcone C., Lauer U., Ragozzino A., Göschl M. (1998). Current status of molecular classification of the Phytoplasmas. Journal of Plant Pathology, 80: 3-26.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution, 30(12): 2725-2729.

Urwin R., Maiden M. C. J. (2003). Multi-locus sequence typing: a tool for global epidemiology. Trends in Microbiology, 11: 479-487.

Vindimian M.E. (2002). Scopazzi del melo: diffusione in Trentino e ricerche in atto. ATTI Giornate fitopatologiche, 1: 7-12.

Weintraub P. G., Beanland L. (2006). Insect vectors of phytoplasmas. Annual Review of Entomology, 51: 91-111.

Weisburg W. G., Tully J. G., Rose D. L., Petzel J. P., Oyaizu H., Yang D., Mandelco L., Sechrest J., Lawrence T. G., Van Etten J., Maniloff J., Woese C.R. (1989). A phylogenetic analysis of the mycoplasmas:basis for their classification. Journal of Bacteriology. 171: 6455-6467.

Web pages:

https://www.ncbi.nlm.nih.gov

https://www.psc.edu

http://web.expasy.org

Relationships among sequences of '*Ca*. P. mali' fragment genes isolated from plant and insect samples in Trentino (Italy) and the reference genotypes described by Danet *et al*. (2011).

Supplement 1: Multiple alignment of reference nucleotide sequences deposited for the genes *aceF* (**A**), *pnp* (**B**), *secY* (**C**) - related gene sequences obtained for the Italian plant samples.

Supplement 2: Multiple alignment of reference nucleotide sequences deposited for the genes *aceF* (**A**), *pnp* (**B**), *secY* (**C**) - related gene sequences obtained for the Italian insect samples.

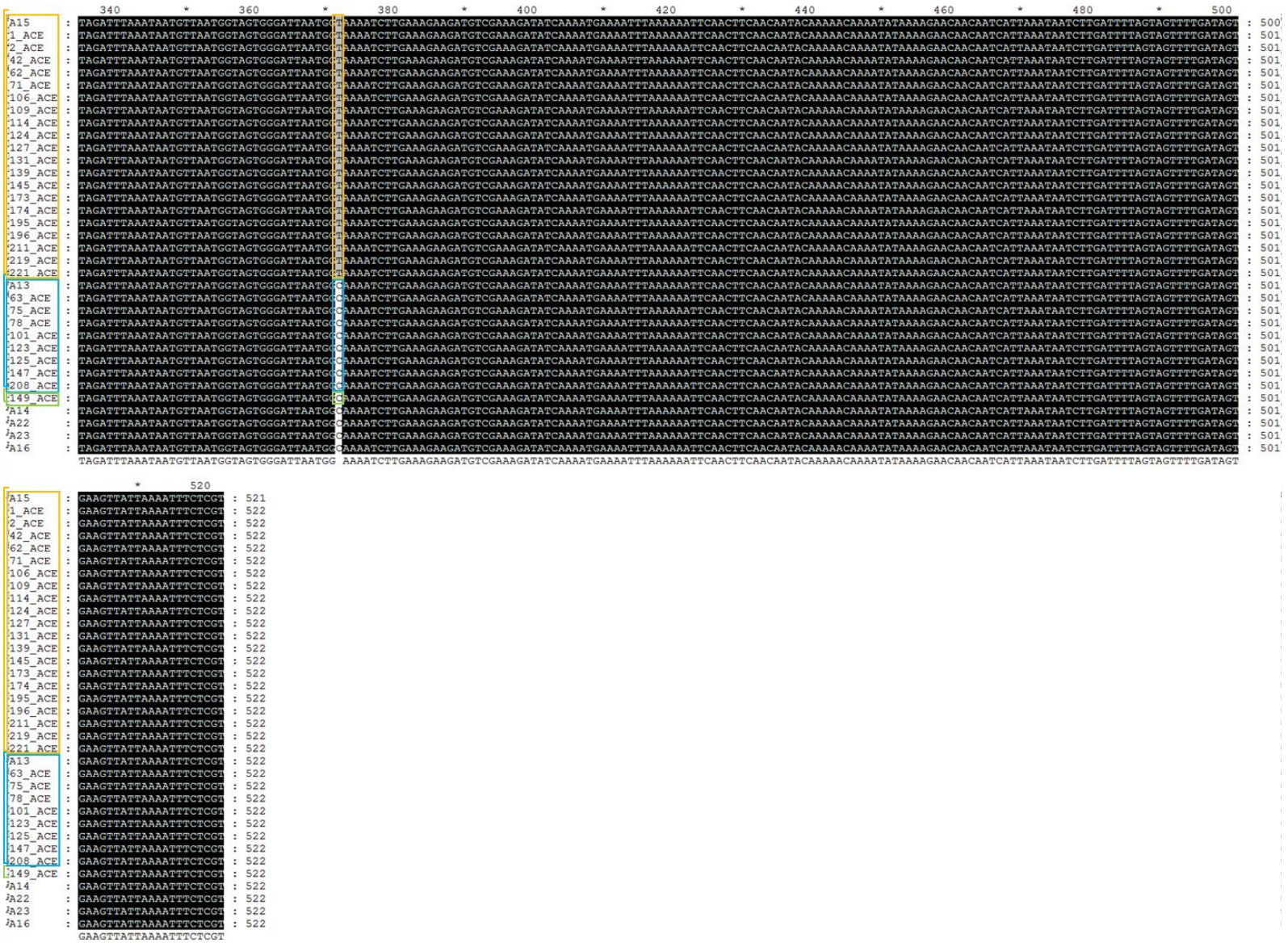
Nucleotides conserved in all sequences are in black background, whereas those in gray background are conserved in the majority of the sequences. White background indicates nucleotides present only in few sequences.
In the consensus sequences on the bottom, nucleotides conserved in all sequences appear with capital letters, while the most common ones are indicated with lower case letters.

Supplement 3: Unrooted phylogenetic trees of the genotypes isolated from plants collected in Trentino for the three genes *aceF* (**A**), *pnp* (**B**), *secY* (**C**).

Supplement 4: Unrooted phylogenetic trees of the genotypes isolated from insects collected in Valsugana for the three genes *aceF* (**A**), *pnp* (**B**), *secY* (**C**).

The relationships were inferred by using the Maximum-Likelihood method. The percentage of replicate trees in which the associated samples and references genotypes clustered together in the bootstrap test (500 replicates) is shown next to the branches. The trees are drown to scale, branch lengths were calculated using the average pathway method and represents the number of nucleotide changes over the whole sequences.

Supplement 1.A.



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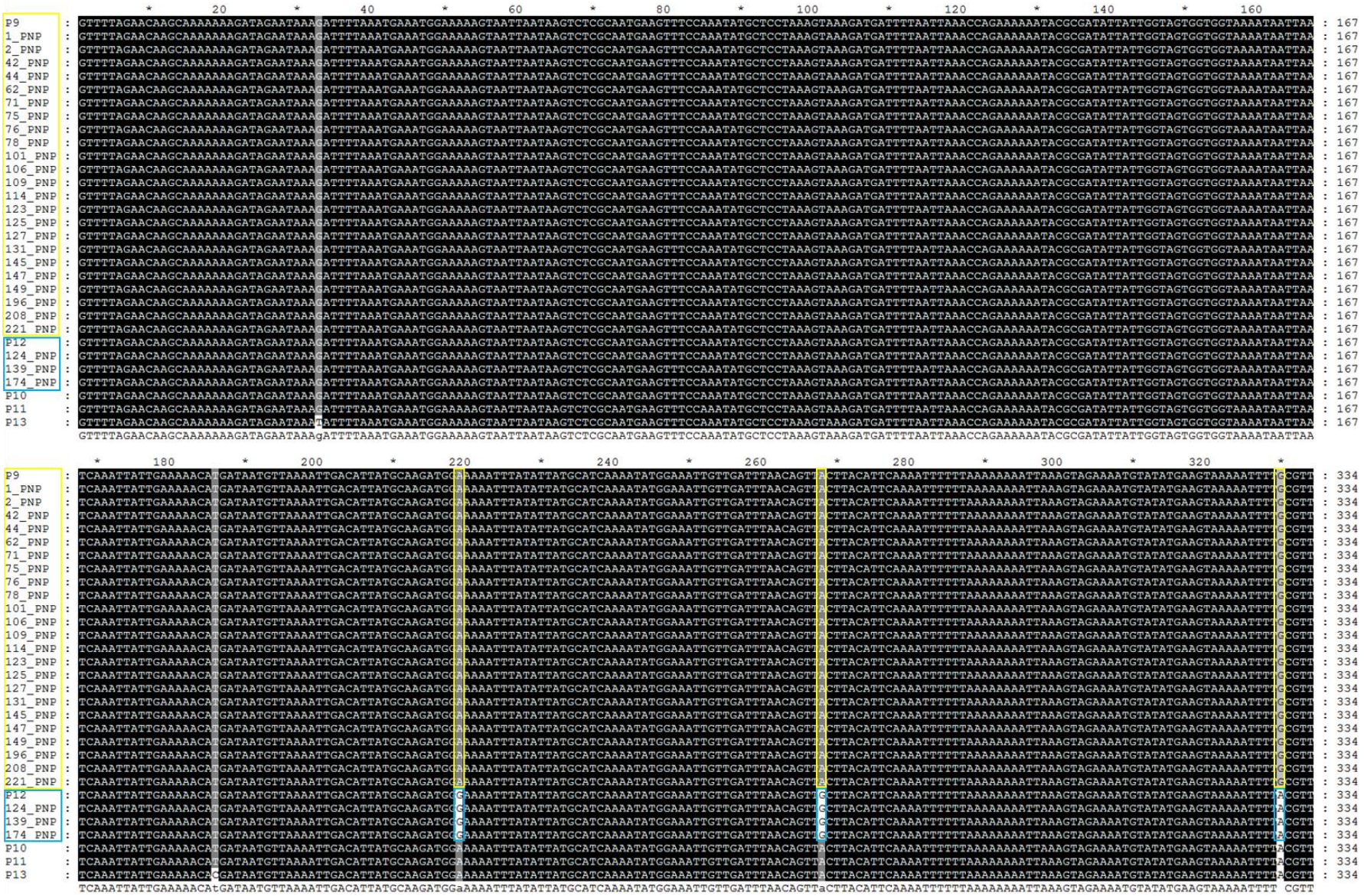

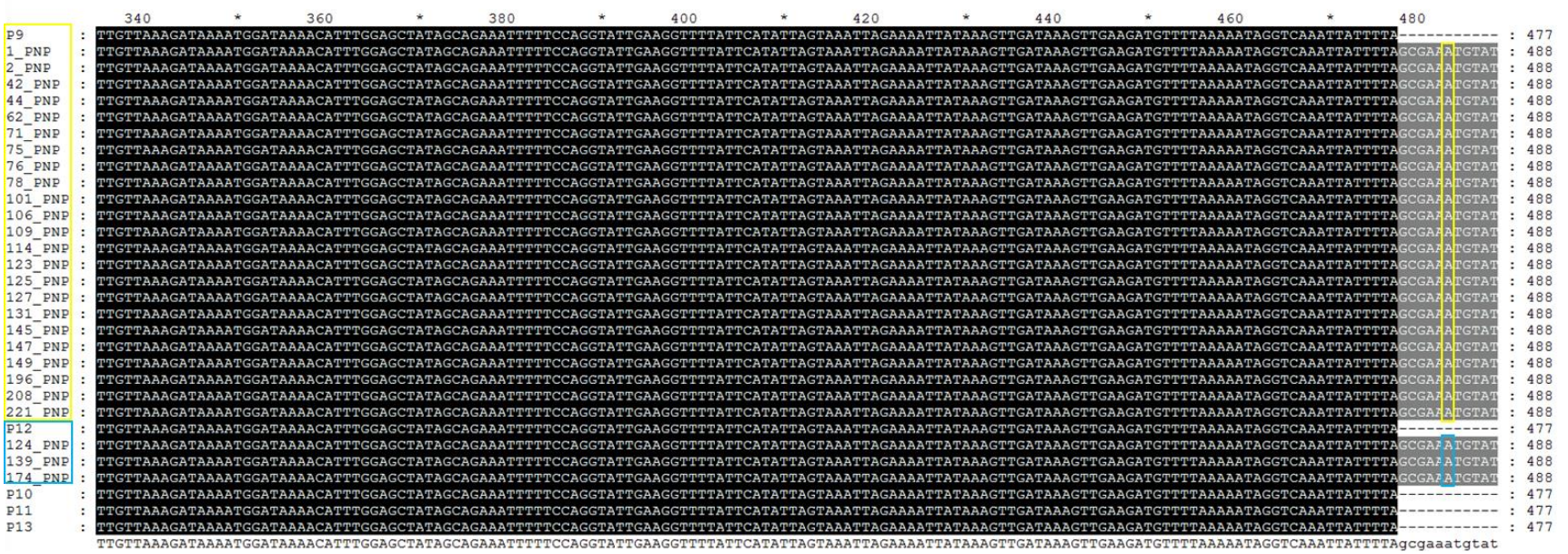
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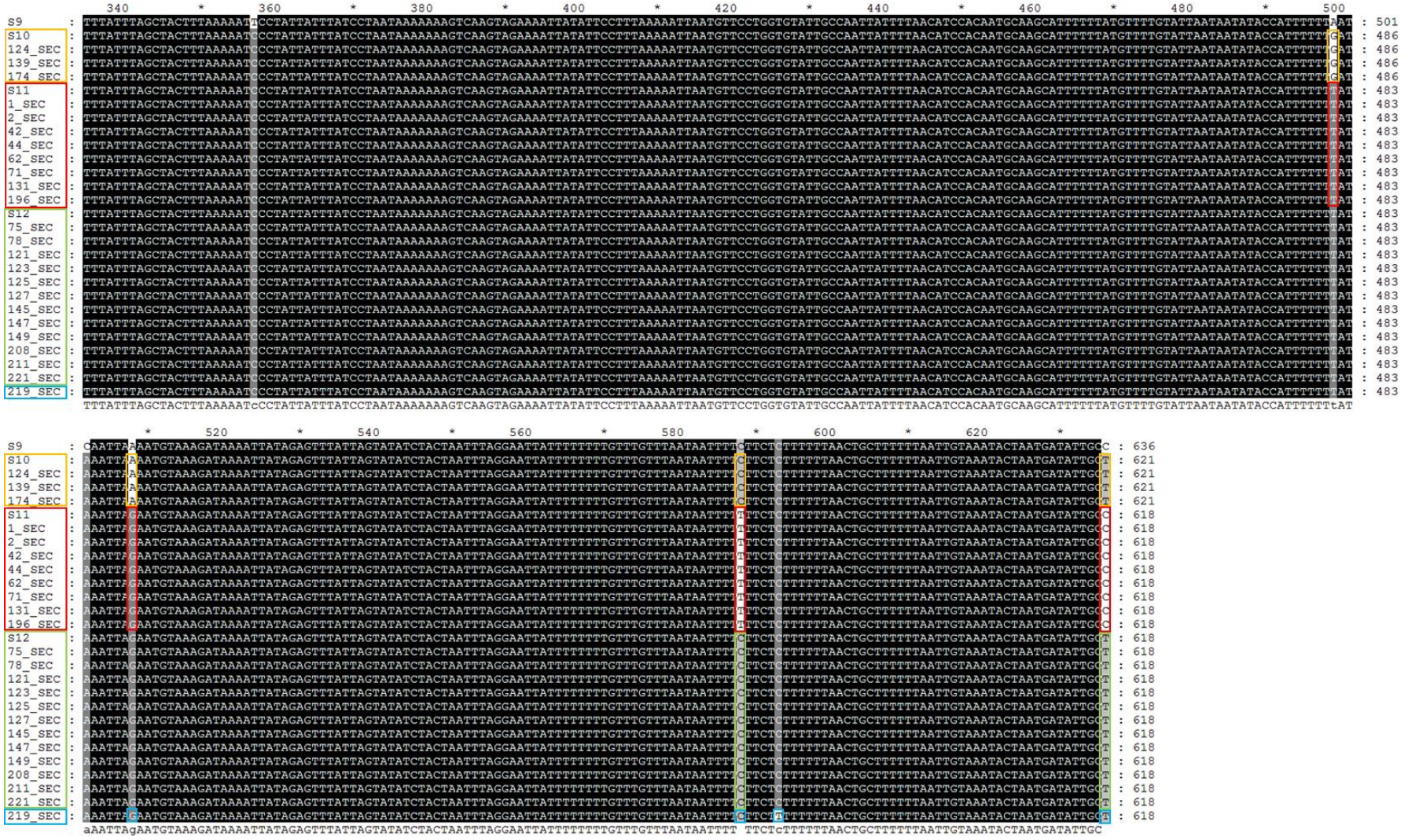

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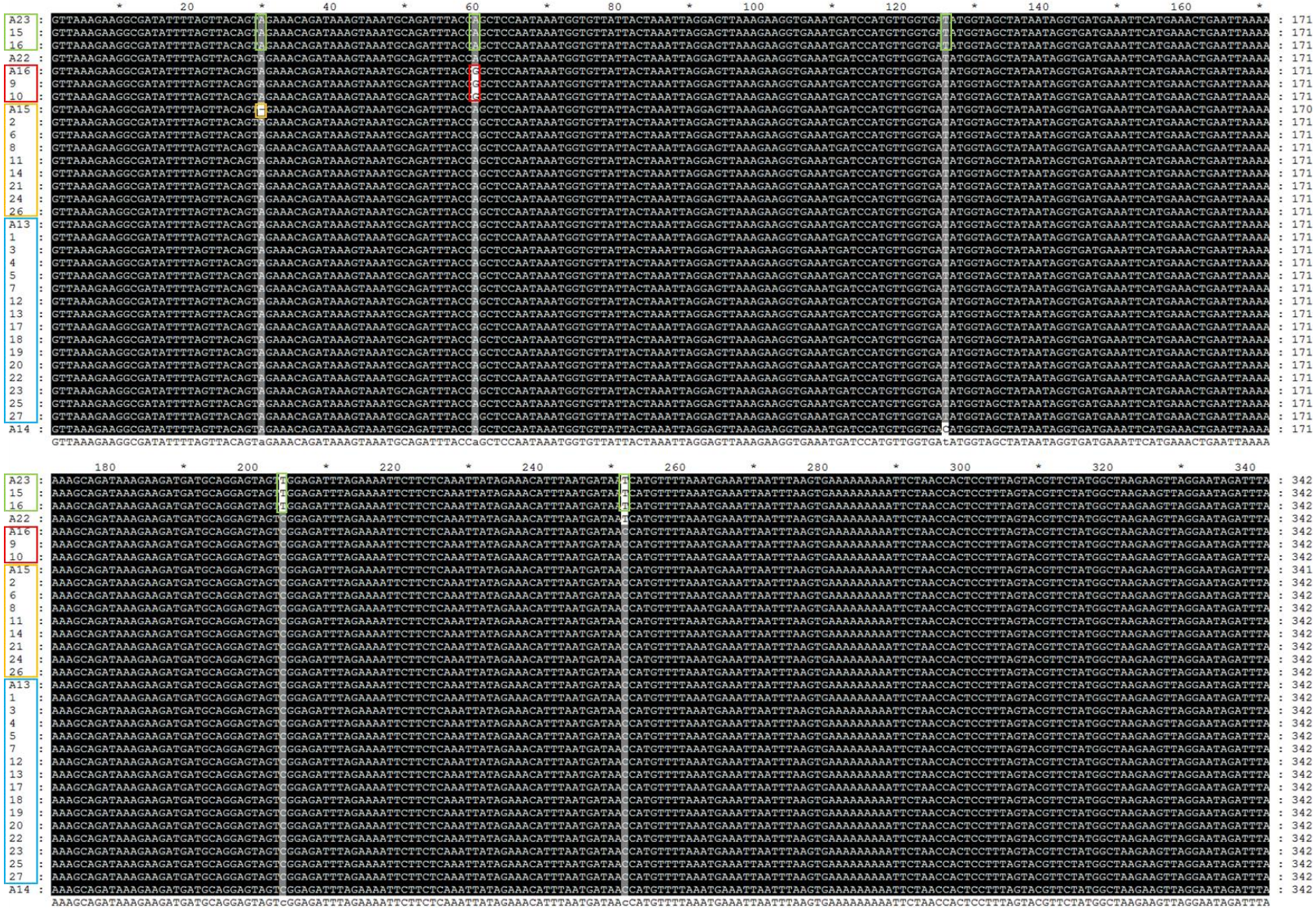

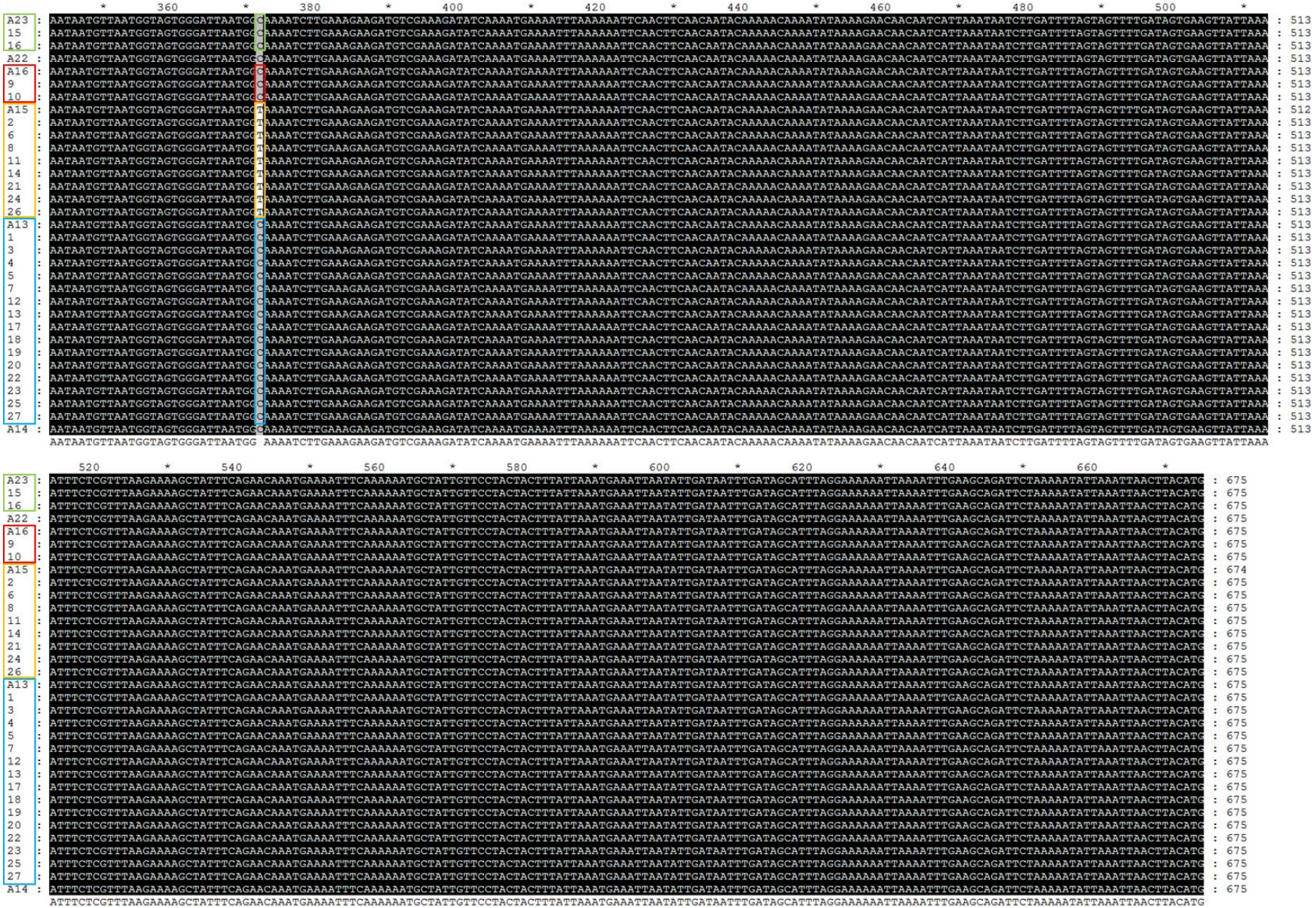

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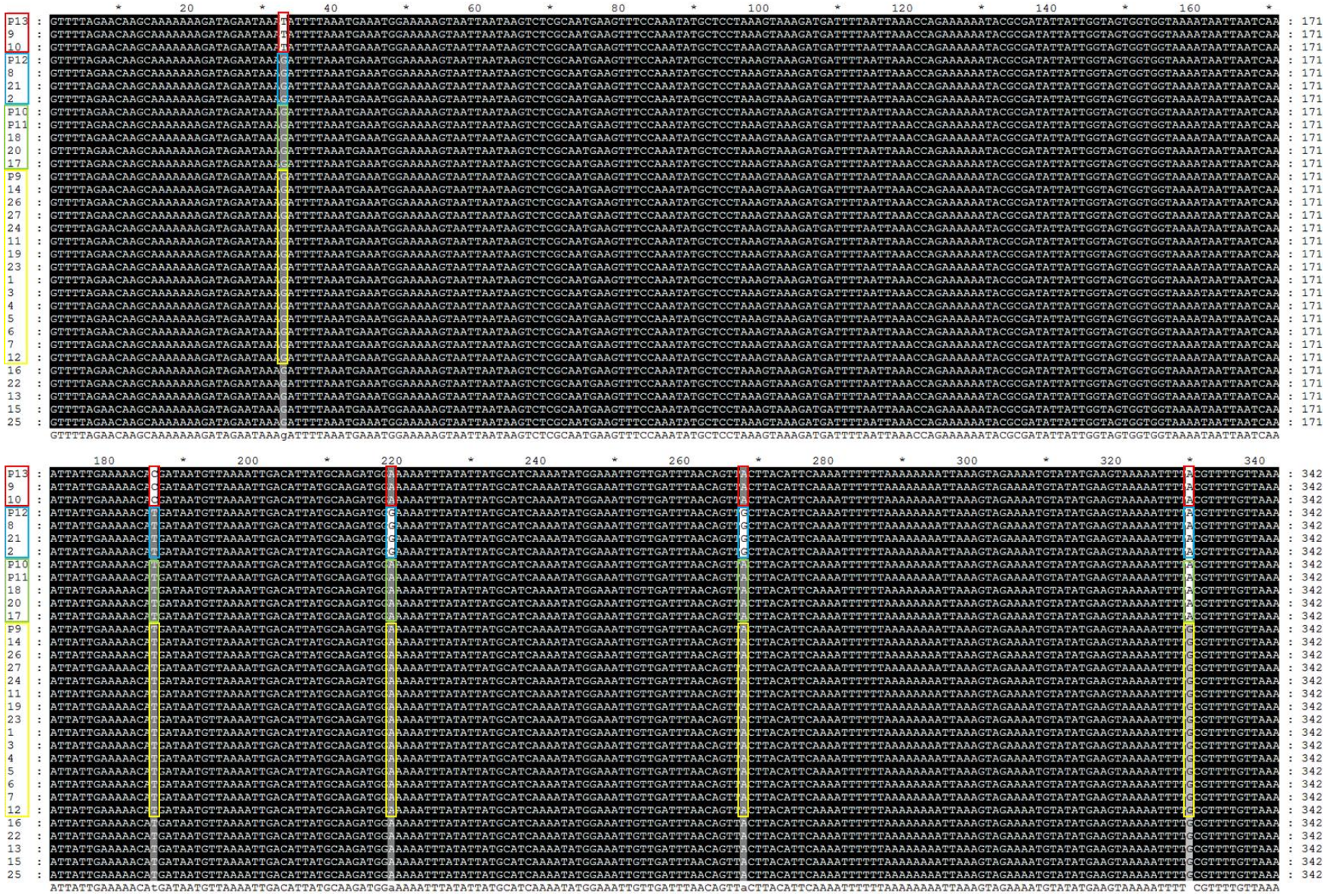

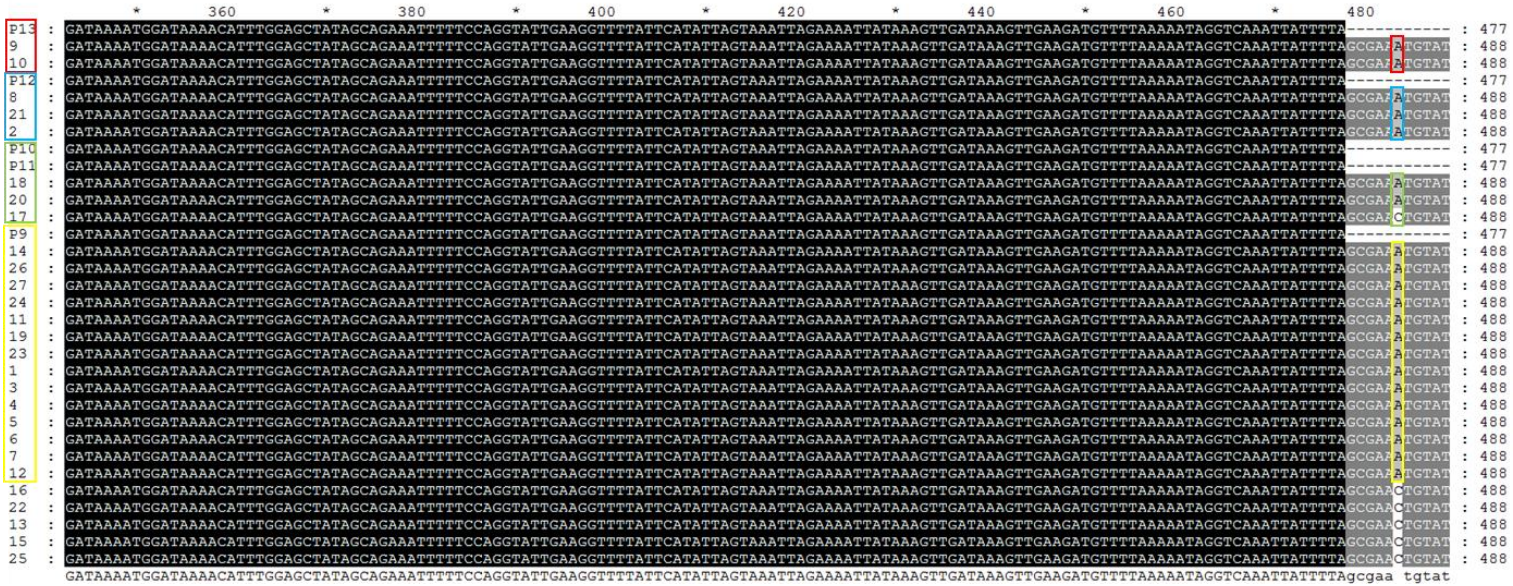
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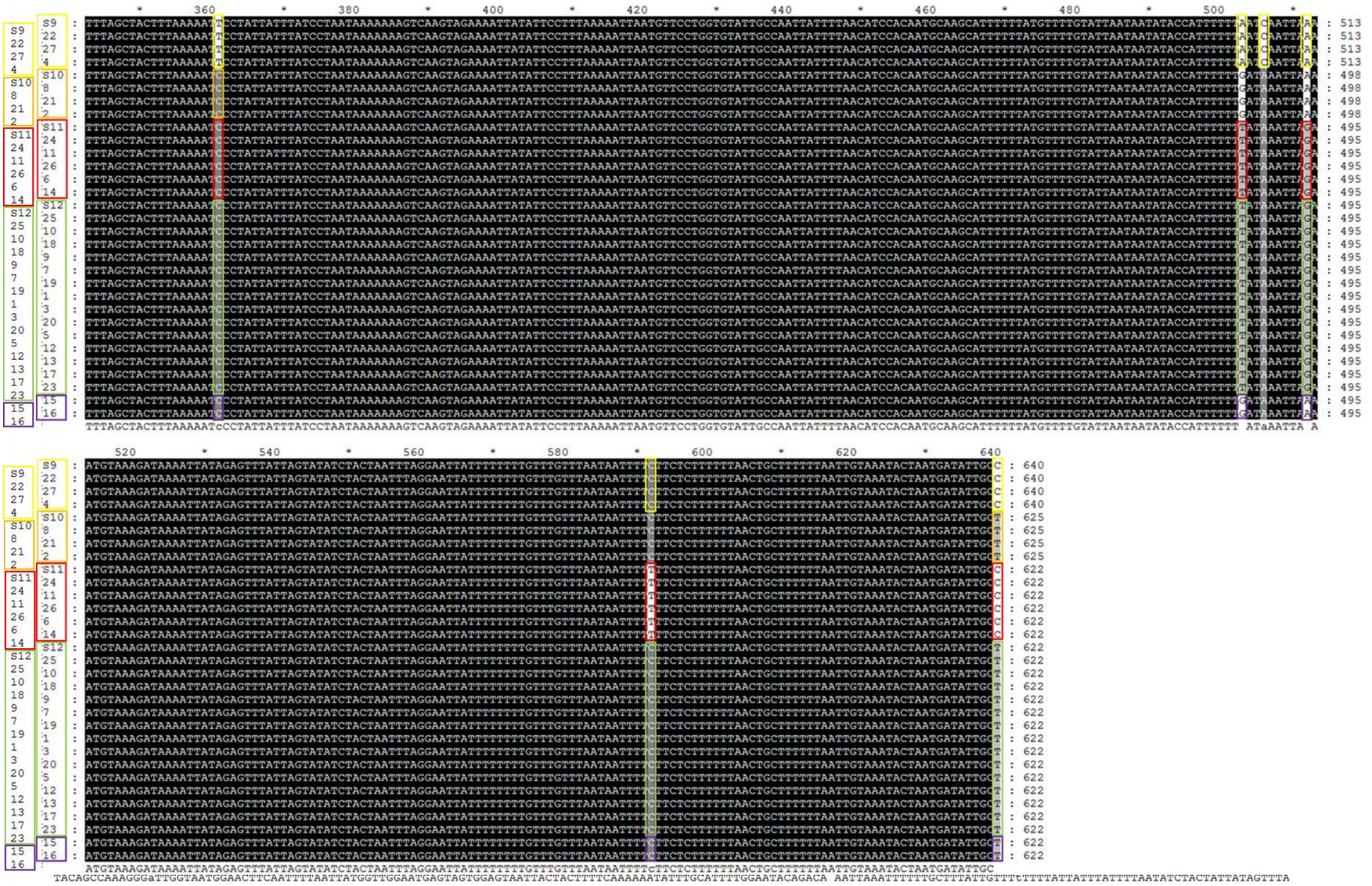
Supplement 2.C.



Supplement 3

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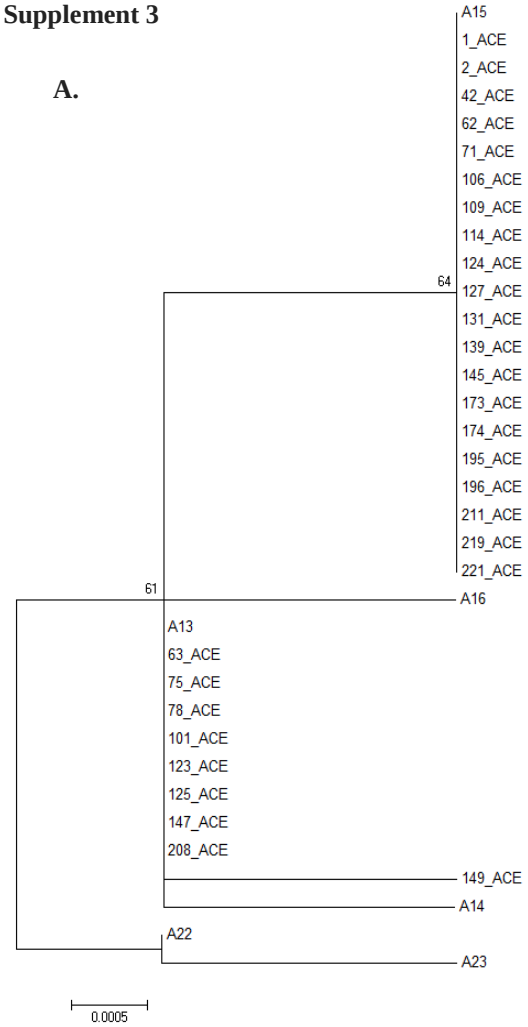

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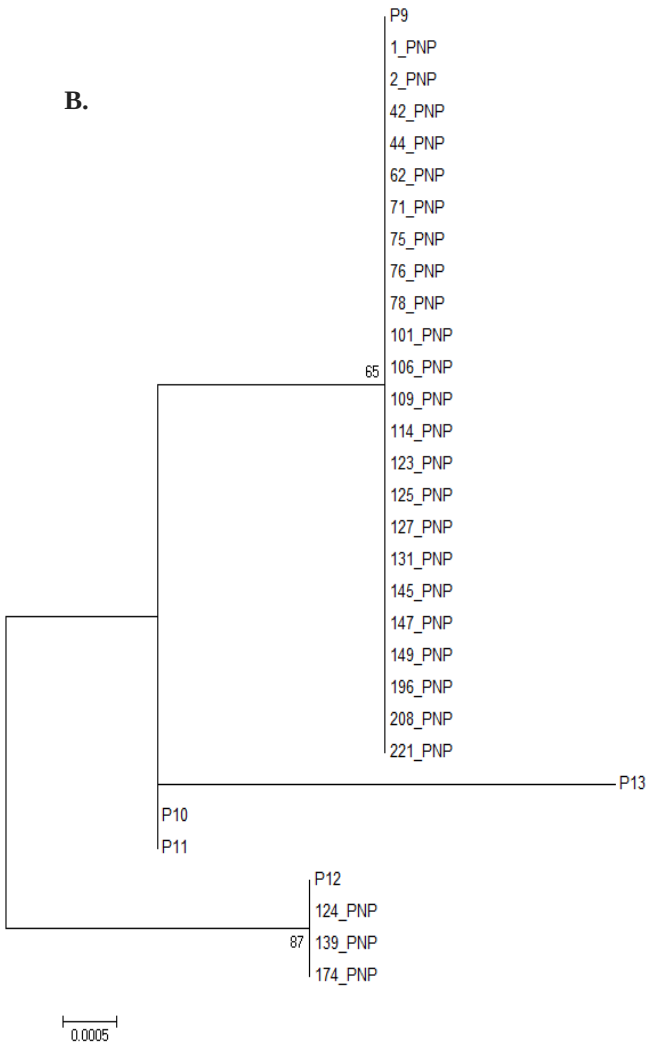

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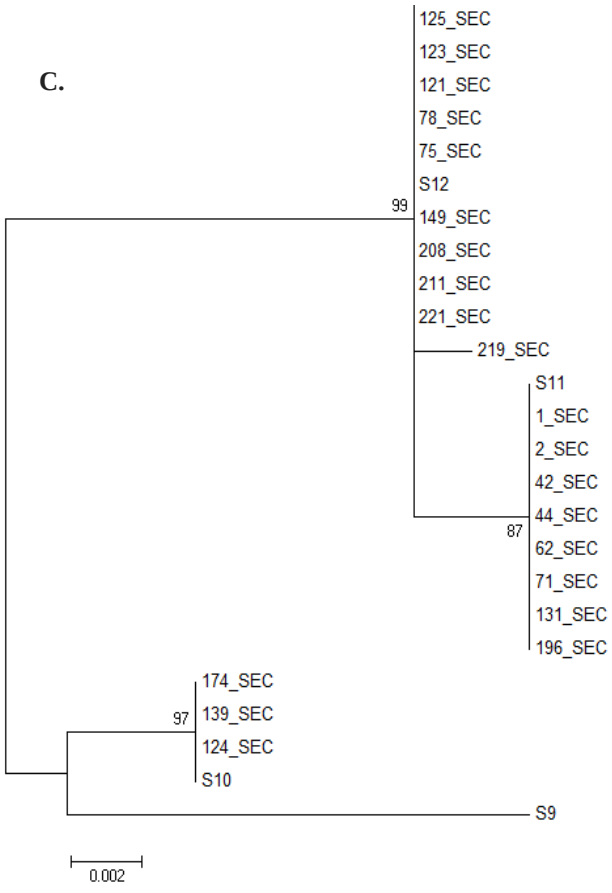

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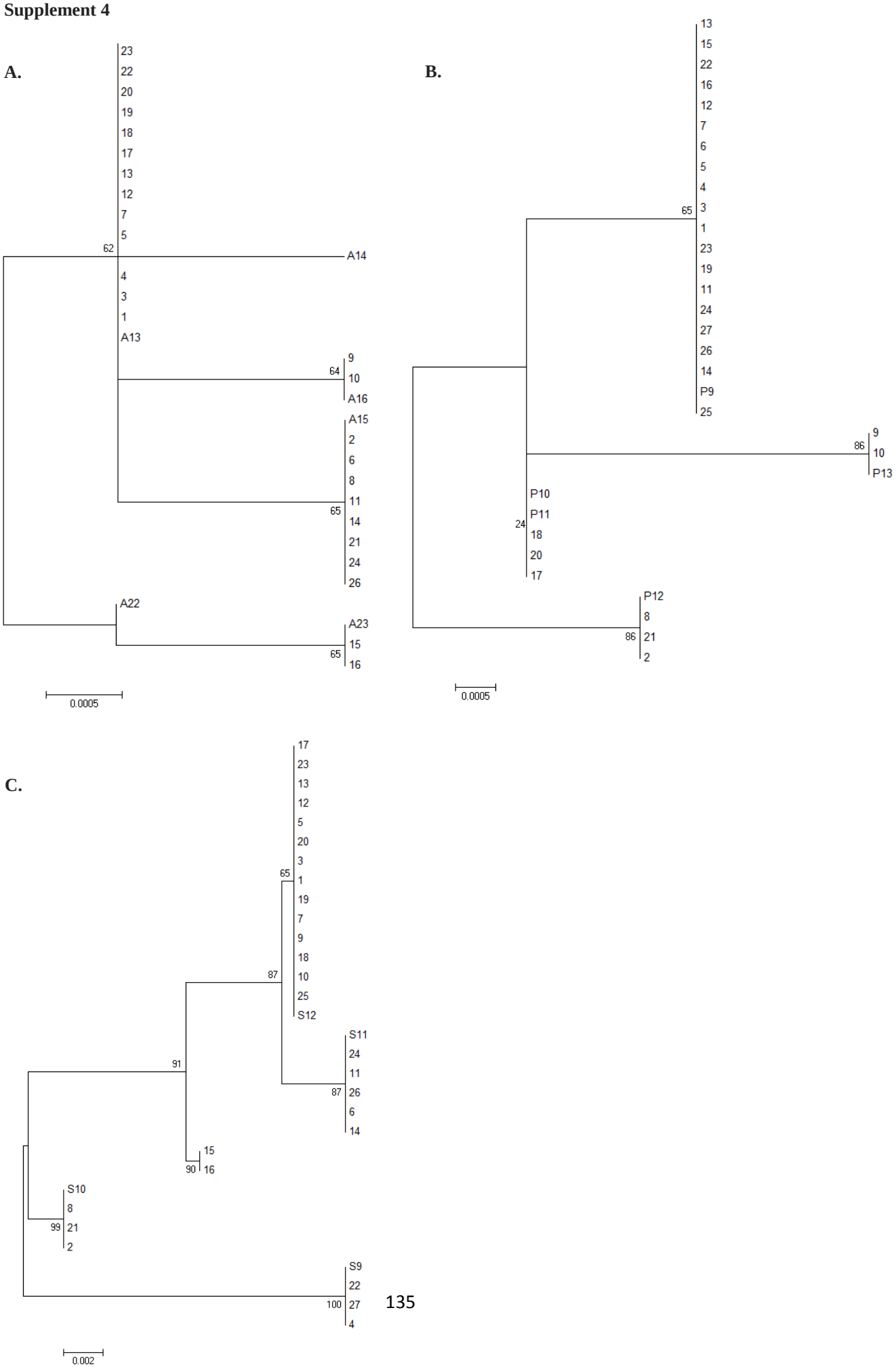


CHAPTER 5

Substrate-borne vibrational communication in the psyllids *Cacopsylla picta* and *Cacopsylla melanoneura*

Abstract

Cacopsylla picta and *Cacopsylla melanoneura* are the main known vectors of apple proliferation, a phytoplasma-caused disease known in many fruit-growing European regions that represents one of the most severe problems in apple orchards. These psyllids are vectors of apple proliferation, a serious phytoplasma-associated disease reported in apple orchards of different European areas. Therefore, there is a mandatory requirement to treat against them in many affected regions. Sexual communication using substrate-borne vibrations was demonstrated in several psyllid species and this practice was applied in the field as a mating disruption strategy for other insect groups, obtaining good results. Here, it is reported the result of the first laser vibrometer recordings of the vibrational signals emitted by the two vectors during courtship behavior. The signals appear to be species-specific, but they are not a prerequisite for courtship and mating. Even if data obtained were not sufficient to describe the signaling sequence of *C. melanoneura*, several male calls were recorded. Moreover, as already seen in other psyllid species, a scanning electron microscopy investigation showed the presence of a stridulatory mechanism on thorax and wings of both species. These preliminary results provide new information about the biology of these phytoplasma vectors and could suggest an innovative approach for a low impact pest management strategy.

Keywords: psyllids, apple proliferation phytoplasma, vibrational communication, sustainable control strategy

Introduction

Insects can use chemical, visual, and acoustic modalities to exchange information and coordinate complex courtship behaviors (Lubanga, 2016). Acoustic signals among insects can be divided into two categories based on the medium of transmission: air-borne signals (e.g. Cicadoidea, Grylloidea, Tettigoniidae) and substrate-borne signals (e.g. Psylloidea, Chrysopidae) (Liao and Yang, 2015). Psylloidea (Homoptera: Sternorrhyncha) is a superfamily comprising eight families with about 3,850 species (Burckhardt and Ouvrard, 2012). Psyllids are exclusively phytophagous, and some species are known in agricultural as economically

important vectors of pathogenic microorganisms (Weintraub and Beanland 2006). Ossiannilsson reported sound production in psyllids for the first time in 1950. Few details are available regarding how reproductively adult individuals attract or locate mates (Wenninger *et al.*, 2009). Sex pheromones play a role in the pear psyllid *Cacopsylla bidens* (Soroker *et al.*, 2004) and *Cacopsylla pyricola* (Horton *et al.*, 2007) and in the lemon psyllid *Diaphorina citri* (Wenninger *et al.*, 2008). As mating rates in some psyllid species are reduced when held in darkness (Krysan, 1990, Wenninger and Hall 2007), visual and chemicals cues may also be important for orientation to mate, at least at short distances (Wenninger *et al.*, 2009).

Vibrational signals play a crucial role in communication in many insect groups (Virant-Doberlet and Cokl, 2004). These kind of signals might be related to mating behavior, alarm, aggregation, maternal care, and defense in insects (Cocroft 1999, Cocroft and Rodrìguez, 2005). In the Psylloidea, the substrate-borne signals have a function only in mating and specific recognition. The male and female psyllids usually perform reciprocal duets during courtship (Tishechkin 2005, Percy *et al.*, 2006, Eben *et al.*, 2014, Liao and Yang, 2015).

In agriculture, knowledge on vibrational communication could give new solutions for potential use in arthropod pest control, as already shown in the case of *Scaphoideus titanus*, vector of flavescence dorèe phytoplasma (Eriksson *et al.*, 2012; Polajnar *et al.*, 2016), where mating disruption methods were tested to manage populations.

So far, acoustic signals of 37 psyllid species have been recorded and described (Liao and Yang, 2015). The univoltine psyllids *Cacopsylla picta* (Förster, 1848) and *Cacopsylla melanoneura* (Förster, 1848) are vectors of '*Candidatus* Phytoplasma mali', the etiological agent of apple proliferation disease (Frisinghelli *et al.*, 2000; Tedeschi and Alma, 2004). Their control is mandatory in the European Union and, for this reason, the populations are ordinarily controlled with chemicals by means of multiple treatments during springtime. To pursue a low environmental impact, it is important to develop non-chemical methods to manage pests. Manipulation of insect behavior in field, like mating disruption based on substrate-borne vibrations, is a good candidate to reach this goal. The main objective of this study was to describe the courtship and mating behavior of *C. picta* and *C. melanoneura*, with the characterization of their vibrational communication. Furthermore, scanning electron microscope observations were conducted to detect the presence of possible stridulatory organs in males and females of this species. The final aim was to build the scientific background to develop an innovative approach for a low impact pest management strategy.

Materials and methods

Insect collection and rearing

Overwintered insect were collected at the occurrence during the end of winter and the springtime in 2016 in two areas of Slovenia. The first one is a forest situated in the Northeast of the city of Nova Gorica (45°58'20.52"N; 13°40'39.18"E) (Figure 5.1A). Here, most of the samples of *C. melanoneura* used in the experiments were captured on hawthorn (*Crateagus* spp.), a secondary host plant for this psyllid. In the second site, a conventional apple orchard situated at Brdo pri Lukovici (46°10'02.6" N; 14°40'51.8" E) in province of Ljubljana, samples of both psyllid species were captured (Figure 5.1B). Insects were collected using the beating tray method (Müther and Vogt, 2003), as described in Chapter 1.

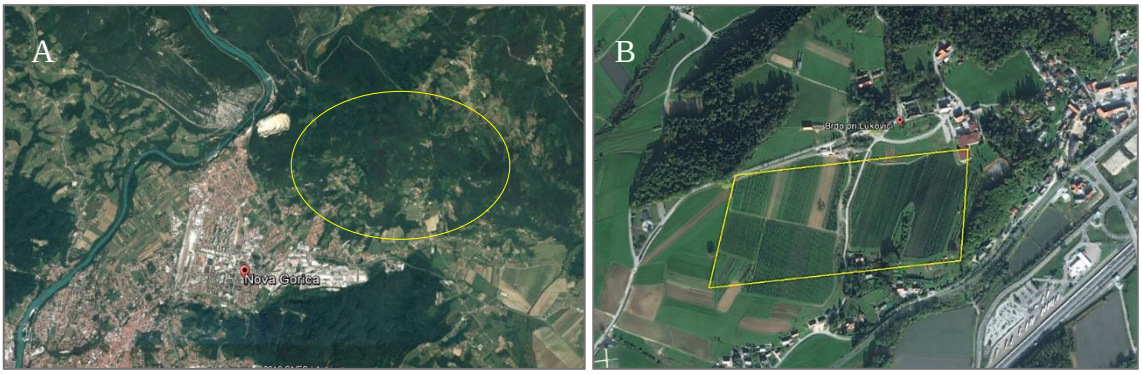


Figure 5.1. Psyllid were captured in two areas of Slovenia. A. Forest surrounding Nova Gorica; B. Conventional apple orchard in province of Ljubljana.

Individuals were isolated in collection tubes, anaesthetized with carbon dioxide and identified at the stereomicroscope using dichotomous keys (Ossiannilsson, 1992).

To avoid mating, male and female of both species were separated and released into single net-cages (25 x 25 x 50 cm). For *C. picta*, a rearing was established on one-year micropropagated apple plants (cv. Golden Delicious) at room conditions (15-21°C, natural light) (Figure 5.2A). The same rearing conditions were used for *C. melanoneura* with the addition of some hawthorn branches as feeding source (figure 5.2B). For all rearing, the number of the psyllids into the cages were regularly monitored, trying to keep it constant by adding individuals when a conspicuous number of them died.

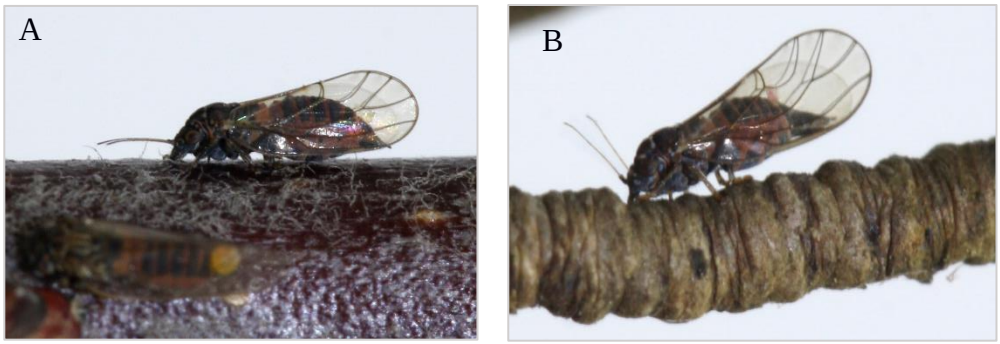


Figure 5.2. *C. picta* individuals on apple branch (A) and *C. melanoneura* on *Crataegus* sp. (B), (J. Polajnar).

Experimental setup

All experiments took place at the Bioacoustics Laboratory of National Institute of Biology of Ljubljana. Insects were placed onto a branch of apple or (for *C. melanoneura*) hawthorn plant and enclosed into a plastic box. Signals were recorded in three control groups: couples (one male and one female), groups of mixed sexes and groups of separated sex. Moreover, after recorded the first signals, playback experiments were established in all the above mentioned groups using an electromagnetic shaker to vibrate the branch (Figure 5.3).

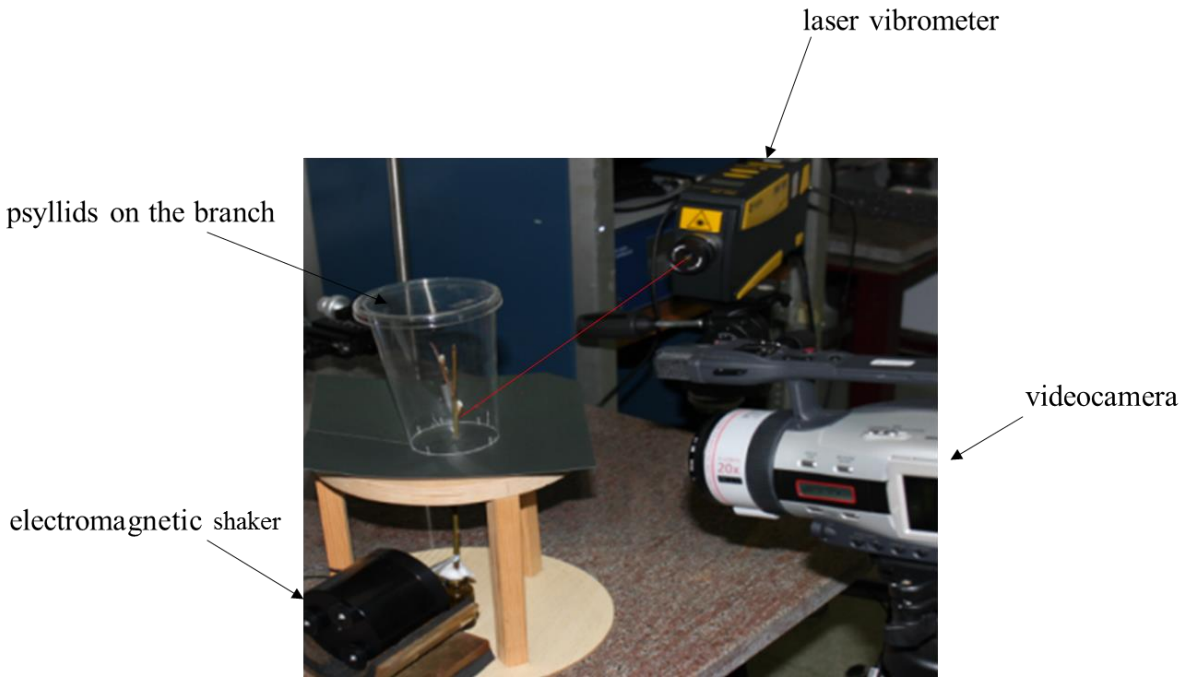


Figure 5.3. Setup of equipment used for monitoring and producing vibrational signals.

Recording and analysis of signals

The vibrations were recorded by a laser vibrometer (PDV-100, Polytec, Waldbronn, Germany) and a video camera (Canon®) was used to record on a videotape the psyllid courtship and mating behavior. SAMURAI (*SINOUS Acoustic Multi- channel Universal Real-time Analysis Instrument*) software package was used to measure the real-time signal spectral. At last, analyses of recorded signals were carried out using the bioacoustics software Raven Pro 1.4.

Morph-functional investigation

To detect the presence of stridulatory organs, specimens were air-dried in desiccator and mounted on aluminum stubs. Prior to observation with field emission scanning electron microscope (JSM 7500F, Jeol, Japan), the samples were coated with platinum using sputter coater (SCD 050, BAL-TEC, Germany).

Results

Signal emission

C. picta- A total number of 65 recordings were obtained. *C. picta* females emitted signals in 17 out of 65 cases and - among these- the males replied only four times. In these last cases, the two emitting psyllids formed a duet and in three out of four duets they mated. Mating also occurred in seven case without any preceding duet.

C. melanoneura- A total number of 22 recordings of signals were obtained, always in the case of groups composed by males, but six mating events without any signal emission were observed.

Table 5.1 summarizes the total number of recordings obtained in all experiments for both species, detailing the number of records for signal type and the occurrence or not of duets and mating events.

Table 5.1. Summary of the dataset obtained for both psyllid vectors.

dataset	***C. picta***	***C. melanoneura***
recordings	65	22
signals emitted	17	4
male signals	3	4
female signals	17	0
duets	4	0
duets + mating	3	0
mating without duet	7	6

Description of courtship behavior and vibrational signal

Data obtained were not sufficient to characterize the behavior of *C. melanoneura*, so the description below regards mainly the observation of courtship and mating behavior of *C. picta*. For this species, the couple formation process before mating seems to be separated into two main phases: identification and courtship with duets. The identification phase is characterized by a stationary female that initiates the communication on host plant by emitting trains of vibrational pulses. On the other hand, during courtship, males replay to the female call by emitting a signal consisting in a series of pre-pulses and a buzz. Male signal is followed by a duet consisting of male call and female reply: while she is stationary, the female replies again, generating an alternated signal emissions with male, which walks to approach the female. In all observations recorded, the female remained stationary on the plant (Figure 5.4).

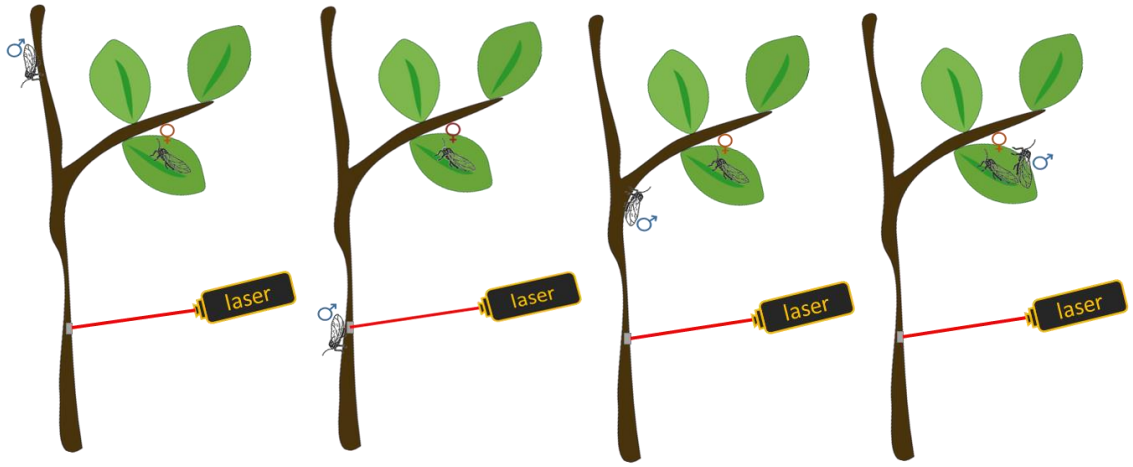


Figure 5.4. Representation of the courtship behavior in *C. picta*. Female initiates the communication and remains stationary on plant; male replies and a duet between both sexes is generated. Mating appears few seconds after the male has approached the female.

The oscillograms and the spectrograms of *C. picta* vibrational signals recorded during courtship behavior show that the female signal consists in a sequence of pulses. The number of pulses is variable, from few to several dozens. Male signal consists in a series of pre-pulses, from zero up to five, and a buzz (Figure 5.5). The mean amplitude of the signal is 40-60 µm/s in males and around 80 µm/s in females, although one female reached 200 µm/s (Figure 5.6). The time interval between the end of the female call and the male response was not of constant duration. Finally, some trials by playback were conducted using an electromagnetic shaker. Signal of both species were recorded and then sent to males or females of the same and of the other species. This "crossing method" gave no response, neither from male nor from female of both

species, suggesting the hypothesis that the signal could be species-specific.

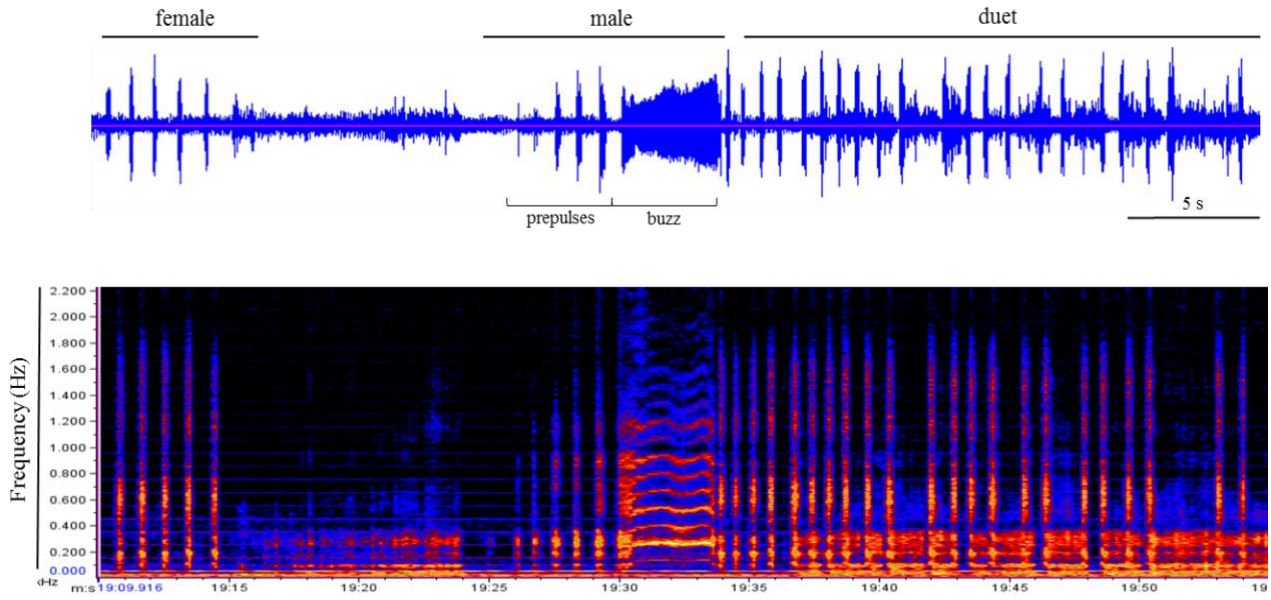


Figure 5.5. Oscillograms (above) and the spectrogram (below) of *C. picta* vibrational signals recorded during courtship behavior. Female signal is a sequence of pulses; male signal consists in a series of pre-pulses and a buzz.

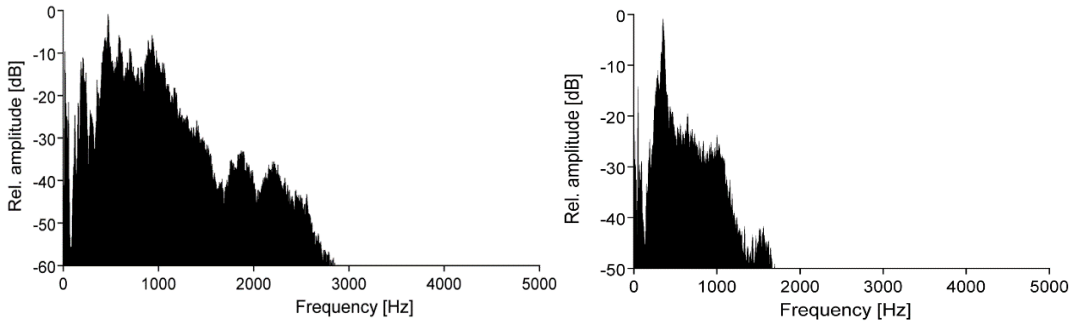


Figure 5.6. For *C. picta*, the mean amplitude is 40-60 µm/s in males (spectrum on left) and around 80 µm/s in females (spectrum on right).

Morph- functional investigation of signal emission

Scanning electron microscopy (SEM) investigation shows the presence of two pairs of axillary cords, one pair on mesoscutellum and the other one on metascutellum, in males and females of both *C. picta* and *C. melanoneura*. The hypothesis is that the signal is produced by the rubbing of the "bow-shaped" part of forewing and hindwings with the axillary cords (Figure 5.7). By

visual observations, it appears clear that this corresponds to rapid wings movements occurred synchronously during signal emission.

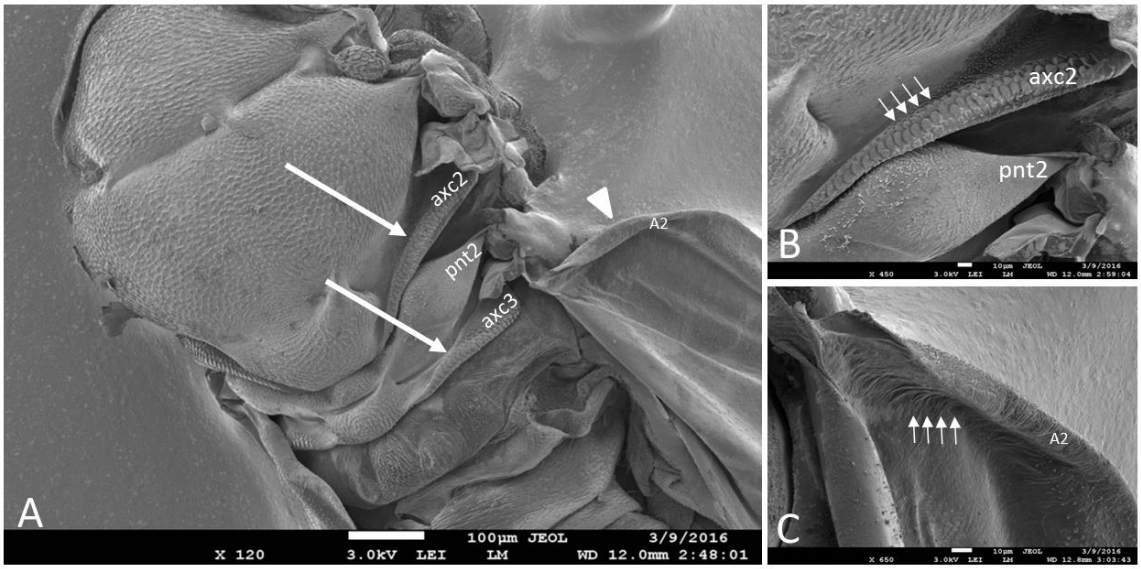


Figure 5.7. SEM investigation on *C. melanoneura* female. A. thorax and hindwing axillary cords (axc2, axc3), mesopostnotum (pnt2); B. detail of the axillary cord B; C. detail of hindwing (A2).

Discussion

This brief study explored sexual communication in *C. picta* and *C. melanoneura*, the two known psyllid vectors of apple proliferation (AP). The results obtained show for the first time the presence of substrate-borne vibrational signals produced during courtship behavior in these species. The signals seem to be species-specific, confirming what observed by Percy *et al.* (2006) in other psyllids. However, they are not a prerequisite for courtship, because mating happened even in absence of signal or duet, in both *C. picta* and *C. melanoneura*.

About morph-functional investigation, the imagines observed at SEM in both species are comparable to the structures described by Taylor (1985), Tishechkin (2006) and recently by Eben *et al.* (2014) in the pear psyllid *Cacopsylla pyri*. Mesoscutellum and metascutellum bear each a pair of axillary cords corresponding was detected in these structures. A correspondence between thorax morphology and the rapid wing movement was observed during signaling, suggesting a synchrony of the two parts.

These preliminary results provide new information about the sexual communication of *C. picta* and suggest a similar behavior in *C. melanoneura*. In conclusion, this work help to enrich the scientific background on biology of *C. picta* and *C. melanoneura*, and this is not only an ecological or evolutionary interest, but it is extremely important for the development of

ecologically sound control strategies for agricultural pest, such as apple proliferation.

References

Burckhardt D., Ouvrard D. (2012). A revised classification of the jumping plant-lice (Hemiptera: Psylloidea). Zootaxa, 3509(1): 1-34.

Cocroft R. B. (1999). Parent-offspring communication in response to predators in a subsocial treehopper (Hemiptera: Membracidae: Umbonia). Ethology, 105: 553-568.

Cocroft R. B., Rodríguez R. L. (2005). The behavioral ecology of insect vibrational communication. Bioscience, 55(4): 323-334.

Eben A., Mühlethaler R., Gross J., Hoch H. (2014). First evidence of acoustic communication in the pear psyllid *Cacopsylla pyri* L. (Hemiptera: Psyllidae). Journal of Pest Science, 88: 87-95.

Eriksson A., Anfora G., Lucchi A., Lanzo F., Virantdoberlet M., Mazzoni V. (2012). Exploitation of insect vibrational signals reveals a new method of pest management. PLoS ONE, 7: e32954.

Frisinghelli C., Delaiti L., Grando M. S., Forti D., Vindimian E. (2000). *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. Journal of Phytopathology, 148: 425-431.

Horton D. R., Landolt P. J. (2007). Attraction of male pear psylla, *Cacopsylla pyricola*, to female-infested pear shoots. Entomologia experimentalis et applicata, 123: 177-183.

Krysan J. L. (1990). Laboratory study of mating behavior as related to diapause in overwintering *Cacopsylla pyricola* (Homoptera: Psyllidae). Environmental Entomology, 19(3): 551-557.

Liao Y. C., Yang M. M. (2015). Acoustic communication of three closely related psyllid species: a case study in clarifying allied species using substrate-borne signals (Hemiptera: Psyllidae: Cacopsylla). Annals of the Entomological Society of America 108(5): 902-911.

Lubanga U. K., Drijfhout F. P., Farnier K., Steinbauer M. J. (2016). The long and the short of mate attraction in a psylloid: do semiochemicals mediate mating in *Aacanthocnema dobsoni* Froggatt?. Journal of chemical ecology, 42(2): 163-172.

Muther J., Vogt H. (2003). Sampling methods in orchard trials: a comparison between beating and inventory sampling. IOBC wprs bulletin, 26(5): 67-72.

Ossiannilsson F. (1992). The Psylloidea (Homoptera) of Fennoscandia and Denmark. Fauna Entomologica Scandinavica, vol 26. E. J. Brill, Leiden.

Percy D. M., Taylor G. S., Kennedy M. (2006). Psyllid communication: acoustic diversity, mate recognition and phylogenetic signal. Invertebrate Systematics, 20: 431-445.

Polajnar J., Eriksson A., Virant-Doberlet M., Mazzoni V. (2016). Mating disruption of a grapevine pest using mechanical vibration: from laboratory to the field. Journal of Pest Science, 89(4): 909-921.

Soroker V., Talebaev A., Harari A. R., Wesley S. D. (2004). The role of chemical cues in host and mate location in the pear psylla *Cacopsylla bidens* (Homoptera: Psyllidae). Journal of Insect Behavior, 17(5): 613-626.

Taylor K. L. (1985). A possible stridulatory organ in some Psylloidea (Homoptera). Journal of the Australian Entomological Society, 24: 77-80.

Tedeschi R., Alma A. (2004). Transmission of apple proliferation phytoplasma by Cacopsylla melanoneura (Homoptera: Psyllidae). Journal of Economic Entomology, 97(1): 8-13.

Tishechkin D. Y. (2005). Vibratory communication in Psylloidea (Hemiptera). In: Drosopoulos S, Claridge MF (eds) Insect sounds and communication. CRC Taylor and Francis, Boca Raton, pp 357-363.

Tishechkin D. Y. (2006). On the structure of stridulatory organs in jumping plant lice (Homoptera: Psyllinea). Russian Entomological Journal, 15(3): 335-340.

Virant-Doberlet M., Cokl A. (2004). Vibrational communication in insects. Neotropical Entomology, 33(2): 121-134.

Weintraub P. G., Beanland L. A. (2006). Insect vectors of phytoplasmas. Annual Review of Entomology 51: 91-111.

Wenninger E. J., Hall D. G. (2007). Daily timing of mating and age at reproductive maturity in *Diaphorina citri* (Hemiptera: Psyllidae). Florida entomologist, 90(4): 715-722.

Wenninger E. J., Stelinski L. L., Hall D. G. (2008). Behavioral evidence for a female-produced sex attractant in *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). Entomologia experimentalis et applicate, 128: 450-459.

Wenninger E. J., Stelinski L. L., Hall D. G. (2009). Roles of olfactory cues, visual cues, and mating status in orientation of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) to four different host plants. Environmental Entomology, 38(1): 225-234.

CHAPTER 6

Searching for potential new vectors of '*Candidatus* Phytoplasma mali' in apple orchards located in different landscapes

Abstract

Valsugana, a representative apple growing area of Trentino with a high apple proliferation (AP) incidence, was chosen as model region to investigate putative role of leafhoppers and planthoppers in the epidemiology of AP. This valley is enclosed by two mountain ranges and is characterized by a large habitat variability. Effect of landscapes on putative vectors spreading in apple agroecosystems were evaluated. For this reason, sampling sites were chosen in apple orchards with differential ecological features. In particular, they were divided into three categories, based on the landscape characteristics: prevalence of forest, grassland and other orchards. In addition, each orchard was divided in internal and external rows, where samplings were conducted on both plants and grasses. The specimens collected in the field were analyzed to detect the presence of '*Candidatus* Phytoplasma mali', the causal agent of AP. The results of this study indicate that landscapes influence the species richness and that grasses are visited by higher numbers of species and individuals in all landscapes considered. All insect collected were tested by real-time PCR and results indicate that three samples belonging to three different species tested positive to '*Ca.* P. mali'.

Key words: apple proliferation, leafhoppers, planthoppers, phytoplasma, landscape ecology

Introduction

Biodiversity in agricultural habitats is influenced by the surrounding landscapes (Perez-Bote and Romero, 2012). Landscape structure can explain much of the patterns of biodiversity in complex landscapes [i.e. those with >20% cover of semi- natural habitats, Batary *et al.* (2011)], whereas simpler landscape management practices could have important effects on biodiversity (Chamberlain *et al.*, 1999; Schmidt *et al.*, 2005). As a consequence, human activities that can seem ineffective in complex landscapes, may result pivotal in simpler ones (Batary *et al.*, 2011). Simplified systems, such as conventional apple orchards, host mainly generalist and common species, defined as 'those that are abundant and widespread' (Gaston, 2010). Despite the low contribution to community richness, common species are exceptionally influential in determining many macro-ecological patterns and in providing ecosystem services (Gaston,

2011). As an example, investigations on bird communities in Trentino vineyards showed that these animals provide fundamental services and economic benefits to humans, such as seed dispersal, pollination, and biocontrol (Assandri *et al.*, 2017, Sekercioglu *et al.*, 2004; Whelan *et al.*, 2015). The structural contrast between agricultural and semi-natural environments can thus result in species immigration and emigration between habitats (Schellhorn *et al.*, 2014). Studies on the ecology and distribution of insect populations in agroecosystems and in the surrounding landscapes, in association with epidemiological observations, may represent an alternative point of view in the research of potential vectors of insect-transmitted diseases.

Apple proliferation (AP) is a phytoplasma-caused disease and the etiological agent is '*Candidatus* Phytoplasma mali', a phloem-limited pathogen. Several studies conducted on the AP disease showed that two psyllids species *Cacopsylla picta* Föster (Homptera: Psyllidae) and *Cacopsylla melanoneura* Föster (Homoptera Psyllidae) are vector of this phytoplasma (Frisinghelli *et al.*, 2000; Tedeschi and Alma, 2004). Despite years of systematic control and the consequent reduction of population densities of the two species, AP is still an important threat for apple production in Trentino. It is known that '*Ca.* P. mali' occurs in a wide range of species of the genus *Malus* and has been detected occasionally in other plants too (Kartte and Seemüller, 1991). So far, apart from psyllids, the leafhopper *Fieberiella florii* Stål (Homoptera: Cicadellidae) have been demonstrated to be able to transmit AP (Tedeschi and Alma, 2006). With all these information, hypothesize the presence of other vectors involved in the AP spreading become almost automatic. Among candidates, some Homoptera groups, such as Auchenorrhyncha Cicadomorpha (leafhoppers) and Fulgoromorpha (planthoppers), display features, e.g. a selective feeding behaviour limited to the phloem system, that could make the transmission of '*Ca.* P. mali' possible. The first investigations about the transmission of '*Ca.* P. mali' by insect vectors was focused on spittlebug and leafhopper species. The species reported as vectors are *Philaenus spumarius* L. (Homoptera: Aphrophoridae) and *Artianus interstitialis* Germar (Homoptera: Cicadellidae), which were able to transmit apple proliferation phytoplasma from infected celery to apple seedlings and from infected to healthy celery (Marenaud *et al.*, 1978; Hegab and El-Zohairy, 1986; Nemeth, 1986). However, other experiments conducted with *P. spumarius* did not confirm the previous results (Refatti *et al.*, 1986).

Insects such as leafhoppers and planthoppers show frequent migrations (Della Giustina, 2002a, 2002b) that influence their population dynamics and spatial distributions. These migrations have to be taken into account for adequate Integrated Pest Management strategies (Matsumura and Suzuki, 2003; Orenstein *et al.*, 2003; Emmen *et al.*, 2004; Decante and van Helden, 2008). As a rule, only few insects are considered as key species of any crop. However, this minimalistic

approach fails to explain in detail the full range of relationships occurring in the entire crop system, where instead is the community, as a whole, to drive the final outcome in terms both of yield and socio-economic impact.

Aim of this research was to investigate the complex of Homoptera (Cicadomorpha and Fulgoromorpha) insects of apple agroecosystems in Valsugana. The effects of landscapes were evaluated on the presence of these communities inside the orchards. Moreover, their role as putative vector of '*Ca.* P. mali' was evaluated.

Material and Methods

Study area

Valsugana extends for approximately 970 km^2 in the South-Eastern Trentino. It is enclosed by two mountain ranges and has the peculiarity of containing within an enormous variability of habitats. The area of apple orchards is almost 800 ha and every year there is a mean fruit production about 450 q/ha. Moreover, AP represents a very big treat for apple production of the valley. All of these features make Valsugana a very interesting model region to study the relation among AP disease and the environmental factors that can influence it.

Sampling design

To study the Auchenorrhyncha communities inside the orchards and in the ecotone between the orchards and the surrounding ecosystems, sampling sites were divided into three categories. Twenty-seven apple orchards were selected in landscapes characterized by different dominance of crop and non-crop habitats. Nine orchards were selected in landscapes dominated by apple orchards, nine in landscapes dominated by forests and nine in landscapes dominated by grasslands (Figure 6.1). Selected orchards were confined in areas with >50% of quantified landscape composition within a 500 m radius. For each landscape, apple orchards were selected based on the same local management.

Figure 6.1. Localization of the 27 sampling points on the map of Valsugana.

During 2014, two samplings were carried out in each selected orchard, one in June and one in September. Samplings took place with favorable meteorological conditions (absence of wind and dry canopies and grasses). Inside each orchard, two habitats were chosen: plants and grass. Moreover, to evaluate the trend to move between the orchard and the ecotone, insects on plant and grass were collected in central and external rows, for a total of four samplings per orchard. The beating tray method (Muther and Vogt, 2003) was used to collect insects from plants. One hundred beatings were done on 50 branches (two beatings/branch) and both sides of the rows were covered in each sampling. The sweep netting method was adopted to collect insects from grasses, walking at a constant speed through vegetation and repeatedly sweeping the net from side to side for 30 times. Insects collected were transferred to labeled plastic bags.

Species determination

After collection, insects were stored at -20 °C and later, leafhoppers and planthoppers fauna were separated from all the other groups. Species recognition took place by using dichotomous keys and a stereomicroscope. So, to identify the Auchenorryncha species, the books "The Plant- and Leafhoppers of Germany: Identification Key to all Species" by Biedermann and Niedringhaus and "The Auchenorrhyncha of Central Europe. Volume 1: Fulgoromorpha, Cicadomorpha (excl. Cicadellidae.)" by Holzinger, Kammerlander and Nickel were the mainly used. When taxonomic keys required the identification by inspection or comparison of genital apparatus, terminalia tissues were cut off from the body and immerged in heated potassium hydroxide solution (KOH 10%). Few seconds in contact with KOH are enough to help clear teguments and soft tissues. After that, cleared genital samples were immerged in a drop of

glycerin on an excavated slide to make them easy to inspect during manipulation under stereomicroscope, avoiding tissues break.

Statistical analyses

Differences in the species composition and abundance between orchards in the three landscapes (forest-, grass- and orchard-dominated landscapes) and in the habitats (apple trees and grass; central rows and edges) were evaluated by chi-squared tests using the statistic software SPSS Base ver. 15.0.

Molecular analyses

After identification, the total DNA was extracted using the commercial kit NucleoSpin® Tissue (Macherey-Nagel) and samples were analyzed by real-time PCR following the method developed by Baric and Dalla Via (2004), as described in Chapter 1, to assess the presence of AP phytoplasma. When high number of individuals belonging to the same species in the same sampling occurred, specimens were grouped in pools prior to the analyses.

Results

So far, 1434 samples from 32 genera and 5 families of Auchenorrhyncha have been identified: Cicadellidae, Aphrophoridae and Membracidae in the infraorder Cicadomorpha and Delphacidae and Flatidae in the infraorder Fulgoromorpha. Regarding species richness, leafhoppers were more abundant than planthoppers with 32 and 10 species, respectively (Table 6.1)

Table 6.1. Summary of Auchenorrhyncha families sampled in apple orchards.

Auchenorrhyncha					
Fulgoromorpha			**Cicadomorpha**		
families	genera	species	families	genera	species
Delphacidae	9	9	Aphrophoridae	3	4
Flatidae	1	1	Membracidae	1	1
			Cicadellidae	18	27

The complete list of species and the abundance of individuals collected are shown in the Supplemetary material (Tables 1, 2 and 3).

Landscapes influence on species richness and abundance

Three different landscapes were considered in the monitoring, depending on the dominance of crop and non-crop habitats. In forest-dominated landscapes, the total number of collected species was 33 (29 leafhoppers and four planthoppers). The most common species were *Laodelphax striatella* Fallen (Delphacidae: Delphacinae), species belonging to the *Macrosteles sexnotatus* group (Cicadellidae: Deltocephalinae), *Muellerianella extrusa* Scott (Delphacidae: Delphacinae) and *Empoasca vitis* Göthe (Cicadellidae: Typhlocybinae), *Psammotettix confinis* Dahlbom (Cicadellaidae: Deltocephalinae), and *Zyginidia pullula* Boheman (Cicadellidae: Typhlocybinae) (Figure 6.2). In grass-dominated landscapes, 25 species (21 leafhoppers and four planthoppers) were sampled. The most common species were in the *Macrosteles sexnotatus* group (Cicadellidae: Deltocephalinae), followed by *Laodelphax striatella* Fallen (Delphacidae: Delphacinae), and *Psammotettix confinis* Dahlbom (Cicadellidae: Deltocephalinae) (Figure 6.3). Finally, in orchard-dominated landscapes, 27 species (19 leafhoppers and eight planthoppers) were collected. The most common species were *Laodelphax striatella* Fallen (Delphacidae: Delphacinae), *Macrosteles ossiannilssoni* Lindberg (Cicadellidae: Deltocephalinae), *Psammotettix confinis* Dahlbom (Cicadellidae: Deltocephalinae) and *Empoasca vitis* Göethe (Cicadellidae: Typhlocybinae) (Figure 6.4).

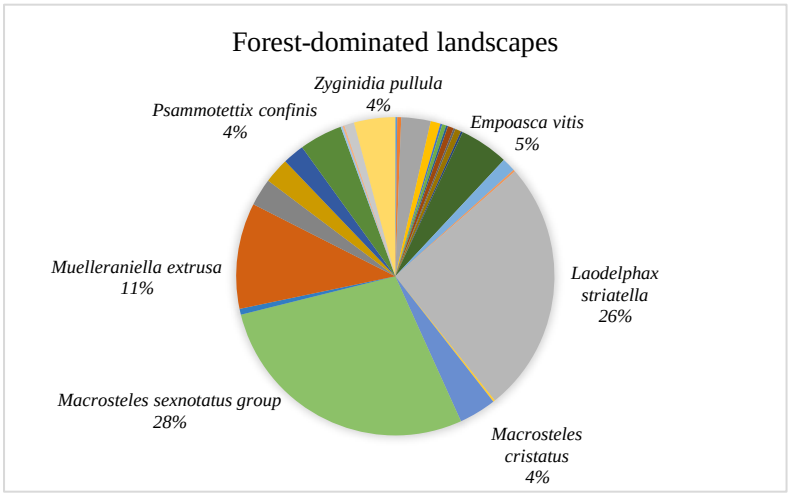


Figure 6.2. Species abundance in nine apple orchards surrounded by forest-dominated landscape in Valsugana.

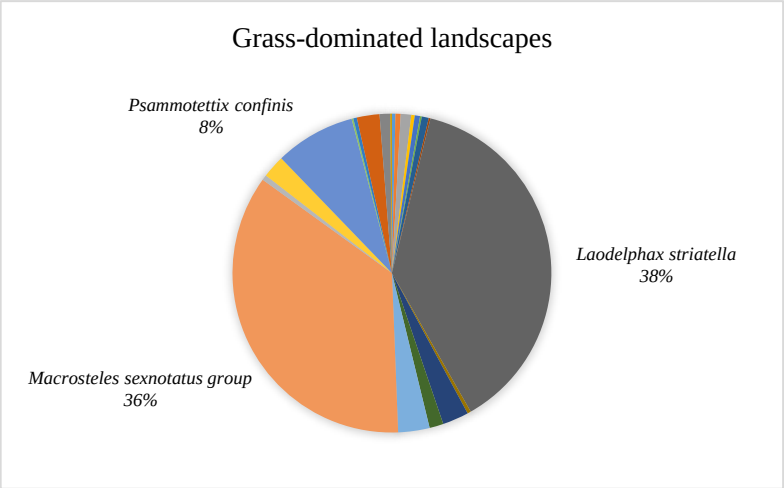


Figure 6.3. Species abundance in nine apple orchards surrounded by grass-dominated landscape in Valsugana.

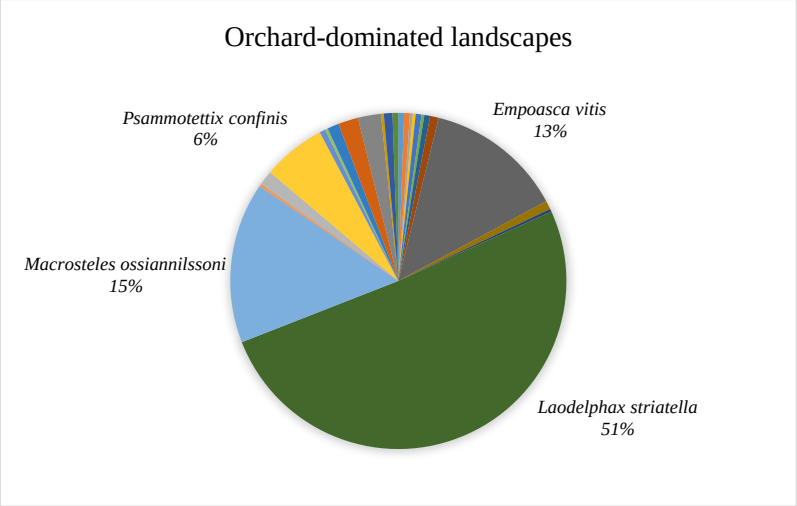


Figure 6.4. Species abundance in nine apple orchards surrounded by orchard-dominated landscape in Valsugana.

The numbers of species sampled in the three landscapes are similar ($\chi^2_{(2)}$= 0.69; p= 0.71). In contrast, regarding the total number of individuals found in whole valley, 509 were collected in forest-dominated areas, being Cicadellidae and Delphacidae the predominant families (56.8% and 36.5%, respectively); 562 individuals were collected in grass-dominated landscapes, where the predominant families were Cicadellidae and Delphacidae (56.6% and 38.8%, respectively); 363 individuals were sampled in orchard-dominated landscapes, with Delphacidae and Cicadellidae representing the most abundant families (76.1% and 20.9%, respectively). Significantly higher numbers of individuals were obtained in the first two landscapes compared to orchard-dominated ones ($\chi^2_{(2)}$= 44.44; p= 2,24E-10).

For each orchard monitored, different habitats were considered: grass and apple trees. Within habitats, insects were collected in central rows and along the edge, for a total of four samplings/orchard.

Regarding Auchenorrhyncha species richness, similar trends were observed in orchards belonging to the different categories (forest-, grass- or orchard-dominated landscapes), with higher numbers of species collected on grass compared to plants (Figure 6.5)

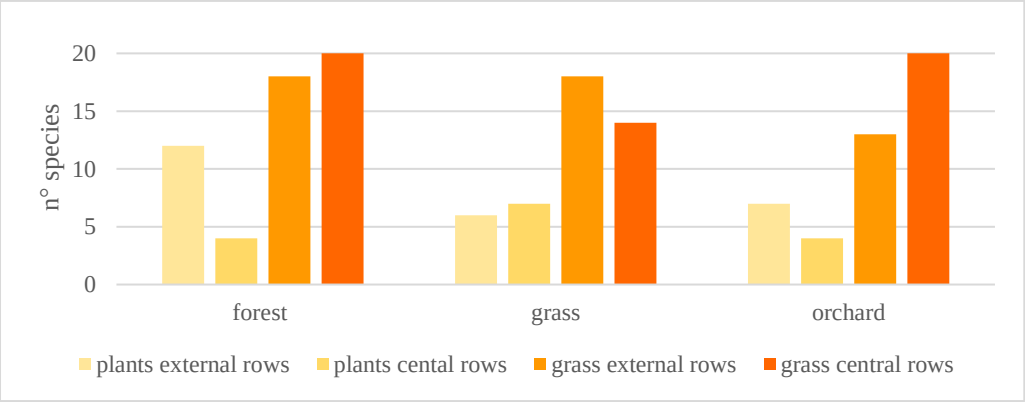


Figure 6.5. Species richness in the different habitats considered for each landscape. For the two habitats (apple plants and grass), two different positions (internal and external rows in the orchard) were sampled.

In forest-dominated landscapes, the most common species on plants was *E. vitis* in both central and external rows (n= 22); on grass, the most common species belong to the genus *Macrosteles* (n= 158), followed by the planthopper *L. striatella* (n= 125). Interestingly, *M. extrusa*, which was present on grass in external rows (n= 54), was never collected in internal rows.

In grass-dominated landscapes, only few individuals were collected on apple trees. The most common species was *Metcalfa pruinosa* Say (Flatidae: Flatinae) (n= 6) and only three individuals belonging to the genus *Macrosteles* were collected in the central rows. Regarding sampling on grass, the leafhoppers belonging to *Macrosteles* genus and the planthoppers belonging to the species *L. striatella* were the most common, both in central and external rows (n= 236 and n= 213, respectively).

In orchard-dominated landscapes, sampling on internal and external rows of plants showed *E. vitis* (n= 43) as predominant species, despite the low number of insects collected. On grass, the higher number of individuals collected in both external and internal rows belonged to *L. striatella* (n= 176) and to species of *Macrosteles* genus (n= 56).

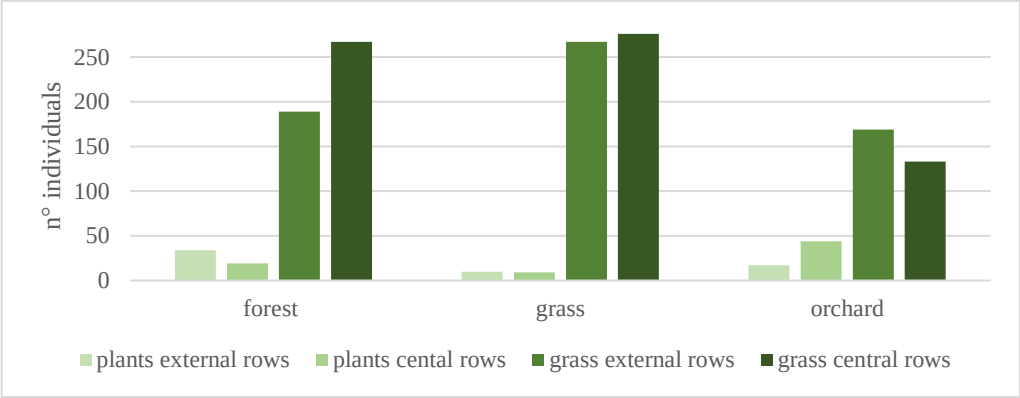


Figure 6.6. Abundance in the different habitats considered for each landscape. For the two habitats (apple plants and grass), two different positions (internal and external rows in the orchard) were sampled.

The comparison between samplings from apple plants and grasses indicates higher numbers of species and individuals when sampling on grasses in all of the considered landscapes (for the number of species, $\chi^2_{(1)}$= 10.29; p= 0.001; for the number of individuals, $\chi^2_{(1)}$= 951.34; p= 6.78E-209). On the other hand, we did not find significant differences in either quantitative or qualitative diversity between internal and external rows of orchards (for the number of individuals, $\chi^2_{(1)}$= 2.68; p= 0.10; for the number of species, $\chi^2_{(1)}$= 0.40; p= 0.53).

First occurrence of a non-native species

Hishimonus Ishihara is mostly an oriental genus of leafhoppers with a remarkable distributional extension into the eastern Palaearctic region (Dai *et al.*, 2013). During this study, a specimen of *Hishimonus hamatus* Kuoh (Homoptera: Cicadellidae) was collected for the first time in Trentino. The first recorded in Europe occurred in western Slovenia in 2012, where adults and nymphs were found on several ornamental trees. Impacts of *H. hamatus* on the european plant health situation are still known and unpredictable, but its phytoplasma transmission potential is highlighted (Seljak, 2013).

Molecular analyses to assess potential new vector

Real-time PCR analyses were conducted on 1305 individuals. So far, two leafhoppers of the family Cicadellidae were found infected by '*Ca*. P. mali': one individual of *Empoasca vitis* and one of *Orientus ishidae* Matsumura (Cicadellidae: Deltocephalinae). Moreover, two specimens belonging to the species *Stictocephala bisonia* Kopp and Yonke (Membracidae: Membracinae)

were found slightly positive to AP phytoplasma. Some biological characteristics of these species are summarized below.

Empoasca vitis

Empoasca vitis Göthe is a widespread polyphagous insect, known in Europe as vine pest. The adults overwinter outside vineyards on evergreen trees and shrubs (Decante and van Helden, 2006). They immigrate during early spring into vineyards, where they remain during two to four sexual generations (depending on regions), before returning to winter host-plants (Cerutti *et al.*, 1991; Bosco *et al.*, 1996; van Helden, 2000). *E. vitis* feeds by puncturing phloem vessels of the leaves. This induces an obstruction of the vessels, a reddening and necrosis of leaves, thus reduced photosynthesis, resulting in delayed maturity (Candolfi *et al.*, 1993) or a reduced sugar content of the harvest. Whereas the gender *Empoasca* is known as vector of phytoplasma (Acosta *et al.*, 2017; Galetto *et al.*, 2011; Pastore *et al.*, 2004; Batlle *et al.* 2000), no data are available about this species so far.

Orientus hishidae

Orientus ishidae Matsumura is an Asian species recently associated with 16SrV phytoplasmas, related to grapevine "*flavescence dorée*". It is monovoltine and overwinters in the egg stage (probably laid close to buds). This species is polyphagous, feeding mainly on broadleaf trees. Little is known about the ecology and dispersal abilities of the mosaic leafhopper (Lessio *et al.*, 2016). After the first report in Slovenia, where many specimens were found to be positive to phytoplasmas (Mehle *et al.*, 2011) there was a second report from Italy (Gaffuri *et al.*, 2011) and a third is from Switzerland (Trivellone *et al.*, 2015).

Stictocephala bisonia

Stictocephala bisonia Kopp et Yonke is a Membracidae species native to North America and quite common also in northern Italy. This univoltine pest overwinters as eggs, which are laid in crescent-shaped slits (6-12 eggs per slit) in bark of trees at the end of summer. Nymphs drop to ground where they feed on alfalfa and grasses. Adults, which develop after five nymphal instars, move to grapevine and fruit trees. Damages to cultivated species are due to the piercing trophic activity on young shoots and leaves.

Discussion

So far, biodiversity of apple agroecosystem has received scarce attention from ecologists. It could be due to its strong habitat simplification, especially in those areas characterized by an extensive monoculture. To compensate for these poor knowledges, in a historical period in which environmental conservation is always more mandatory, faunistic investigations result essential. This research had the objective to deepen the knowledges on apple proliferation spread, in particular focusing on the research of potential new vectors among leafhoppers and planthoppers communities in apple agroecosystem.

An outbreak of AP disease was reported to affect Valsugana in the last years, despite the measures applied to control the population levels of the two already known vectors of the disease (Dallago, 2016). Several studies conducted on the epidemiology of AP in Trentino showed that the psyllids *Cacopsylla picta* and *Cacopsylla melanoneura* transmit the disease with a low efficiency (Mattedi *et al.*, 2008; Pedrazzoli, 2009). Moreover, the leafhopper *Fieberiella florii* was demonstrated to be able to transmit the disease (Krczal *et al.*, 1989; Tedeschi and Alma, 2006), but its presence in Trentino apple orchards seems to be only occasional (Mattedi *et al.*, 2008). Therefore, the monitoring activity was associated to molecular analyses to assess the ability of other Auchenorrhyncha species to acquire the phytoplasma.

Even if species in these taxonomic groups are known as agricultural pests, few information is available on their ecological role in the agroecosystems and on the exchange between orchards and the surrounding landscapes. Hence, a purpose of the research conducted on the biodiversity

of leafhoppers and planthoppers in apple orchards of Valsugana was the compilation of a checklist of the species visiting apple orchards.

The results obtained show the existence of important relationships between the presence of Auchenorrhyncha populations and surrounding natural habitats. In particular, even though the species richness seems to be similar in the different landscapes considered, orchards in forest- and grass- dominated landscapes show higher numbers of individuals, with lower numbers of species and individuals visiting apple trees compared to grass. These findings suggest that inter-rows in apple orchards could act as a biodiversity supply for Auchenorrhyncha species and deserves further studies also aimed at a more responsible weed management.

Therefore, it will be interesting to better examine the ecological contribution of insects such as Auchenorrhyncha in simplified agroecosystems, to deepen the knowledge on biodiversity with the aim to preserve crop and surrounding natural habitat.

The main aim of this research was to search for potential new vectors of '*Ca.* P. mali'. The results obtained by this ecological approach show that at least two species can be found infected. Nevertheless, these species showed low infection rates and populations collected on apple tree were very low. Therefore, these data are the first step in the research of new vectors and further investigations focused on their acquisition ability under controlled conditions are required.

References

Acosta K. I., Zamora L., Piñol B., Quiñones M. L., Ramos P. L., Luis M., Leyva-López N. L., Arocha Y. (2017). *Empoasca papayae* Oman, 1937 (Hemiptera: Cicadellidae) the simultaneous vector of phytoplasmas and rickettsia associated with "Bunchy Top Symptom" in Cuba. Anales de Biología, 39: 35-42.

Assandri G., Bogliani G., Pedrini P., Brambilla M. (2017). Assessing common birds' ecological requirements to address nature conservation in permanent crops: Lessons from Italian vineyards. Journal of Environmental Management, 191: 145-154.

Baric S., Berger J., Cainelli C., Kerschbamer C., Letschka T., Dalla Via J. (2011). Seasonal colonisation of apple trees by '*Candidatus* Phytoplasma mali' revealed by a new quantitative TaqMan real-time PCR approach. European. Journal of Plant Pathology, 129: 455-467.

Baric S., Dalla Via J. (2004). A new approach to apple proliferation detection: a highly sensitive real-time PCR assay. Journal of Microbiological Methods, 57: 135-145.

Batary P., Baldi A., Kleijn D., Tscharntke T. (2011). Landscape-moderated biodiversity effects of agri-environmental management: a meta-analysis. Proceedings of the Royal Society of London Biological, 278: 1894-1902.

Battle A., Angeles Martinez M., Laviña A. (2000). Occurrence, distribution and epidemiology of Grapevine yellows in Spain. European Journal of Plant Pathology, 106: 811-816.

Biedermann R., Niedringhaus R. (2009). The plant-and leafhoppers of Germany: identification key to all species. WABV Fründ, 410 pp.

Bosco D., Alma A., Bonelli S., Arzone A. (1996). Phenology and within-vineyard distribution of *Empoasca vitis* Goethe adults (Cicadellidae Typlocybinae). Redia, 79: 1-9.

Candolfi M. P., Jermini M., Carrera E., Candolfi-Vasconcelos M. C. (1993). Grapevine leaf gas exchange, plant growth, yield, fruit quality and carbohydrate reserves influenced by the grape leafhopper *Empoasca vitis*. Entomologia Experimentalis et Applicata, 69: 289-296.

Cerutti F., Baumgartner J., Delucchi V. (1991). The dynamic of grape leafhopper *Empoasca vitis* Goethe populations in southern Switzerland and the implications for habitat management. Biocontrol Science and Technology, 1: 177-194.

Chamberlain D. E., Wilson J. D., Fuller R. J. (1999). A comparison of bird populations on organic and conventional farm systems in southern Britain. Biological Conservation, 88: 307-320.

Dai W., Fletcher M., Zhang Y. (2013). First records of the genus *Hishimonus* Ishihara from Thailand (Hemiptera: Cicadellidae: Deltocephalinae: Opsiini) including description of three new species. Zootaxa, 3670(3): 301-316.

Dallago G. (2016). Scopazzi del melo: siamo sulla strada giusta. L'Informatore Agrario, 41: 36-39.

Decante and van Helden 2008). Spatial and temporal distribution of *Empoasca vitis* within a vineyard. Agricultural and Forest Entomology, 10: 111-118.

Decante D., Van Helden M. (2006). Population ecology of *Empoasca vitis* (Goethe) and *Scaphoideus titanus* (Ball) in Bordeaux vineyards: influence of migration and landscape. Crop Protection, 25: 696-704.

Della Giustina W. (2002a). Les cicadelles nuisibles à l'agriculture 1^{e} partie. Insectes, 126: 3-6.

Della Giustina W. (2002b). Les cicadelles nuisibles à l'agriculture 2^{e} partie. Insectes, 127: 25-28.

Emmen D. A., Fleischer S. J., Hower A. (2004). Temporal and spatial dynamics of *Empoasca fabae* (Harris) (Homoptera: Cicadellidae) in Alfalfa. Environmental Entomology, 33: 890-899.

Frisinghelli C., Delaiti L., Grando M. S., Forti D., Vindimian M. E. (2000). *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. Journal of Phytopathology, 148: 425-431.

Gaffuri F., Sacchi S., Cavagna B. (2011). First detection of the mosaic leafhopper, *Orientus ishidae*, in northern Italian vineyards infected by the flavescence dorée phytoplasma. New Disease Reports, 24: 22.

Galetto L., Marzachì C., Demichelis S., Bosco D. (2011). Host plant determines the phytoplasma transmission competence of *Empoasca decipiens* (Hemiptera: Cicadellidae). Journal of Economic Entomology, 104(2): 360-6.

Gaston K. J. (2010). Valuing common species. Science 327: 154-155.

Gaston K. J. (2011). Common ecology. Bioscience 61: 354-362.

Hegab A. M., El-Zohairy M. M. (1986). Retransmission of mycoplasma-like bodies associated with apple proliferation disease between herbaceous plants and apple seedlings. Acta Horticulturae, 193: 343.

Holzinger W. E., Kammerlander I., Nickel H. (2003). The Auchenorrhyncha of Central Europe. Die Zikaden Mitteleuropas, Volume 1 Fulgoromorpha, Cicadomorpha (excl. Cicadellidae). Brill, 674 pp.

Kartte S., Seemüller E. (1991). Susceptibility of grafted *Malus* taxa and hybrids to apple proliferation disease. Journal of Phytopathology, 131: 137-148.

Krczal G., Krczal H., Kunze L. (1989). *Fieberiella florii* (Stål), a vector of apple proliferation agent. Acta Horticulturae, 235: 99-104.

Lessio F., Picciau L., Gonella E., Tota F., Mandrioli M., Alma A. (2016). The mosaic leafhopper *Orientus ishidae*: host plants, spatial distribution, infectivity, and transmission of 16SrV phytoplasmas to vines. Bulletin of Insectology, 69: 277-289.

Marenaud C., Mazy K., Lansac M. (1978). La prolifération du pommier: une maladie curieuse et dangereuse. PHM Revue Horticole, 188: 41-51.

Matsumura M., Suzuki Y. (2003). Direct and feeding-induced interactions between two rice planthoppers, *Sogatella frucifera* and *Nilaparvata lugens*: effects on dispersal capability and performance. Ecological Entomology, 28: 174-182.

Mattedi L., Forno F., Cainelli C., Grando M. S., Jarausch W. (2008). Research on *Candidatus* Phytoplasma mali transmission by insect vectors in Trentino. Acta Horticulturae, 781: 369-374.

Mehle N., Rupar M., Seljak G., Ravnikar M., Dermastia M. (2011). Molecular diversity of 'flavescence dorée' phytoplasma strains in Slovenia. Bulletin of Insectology, 64: 29-30.

Muther J., Vogt H. (2003). Sampling methods in orchard trials: a comparison between beating and inventory sampling. IOBC Wprs Bulletin, 26(5): 67-72.

Nemeth M. (1986). Virus, mycoplasma, and rickettsia diseases of fruit trees. Martinus Nijhoff, Dordrecht (The Netherlands).841 pp.

Orenstein S., Zahavi T., Nestel D., Sharon R., Barkalifa M., Weintraub, P. G. (2003) Spatial dispersion patterns of potential leafhopper and planthopper (Homoptera) vectors of phytoplasma in wine vineyards . Annals of Applied Biology, 142: 341-348.

Pastore M., Raffone E., Santonastaso M., Priore R., Paltrinieri S., Bertaccini A, Simeone A. M. (2004). Phytoplasma detection in *Empoasca decedens* and *Empoasca* spp. and their possible role as vectors of European Stone Fruit Yellows (16 SRX-B) phytoplasma. Acta Horticulturae, 657: 507-511.

Pedrazzoli F. (2009). Investigations on the psyllid (Hemiptera: Psyllidae) vectors of '*Candidatus* Phytoplasma mali' in Trentino. 192 pp. Ph.D. Thesis, University of Padova, Italy.

Perez-Bote J. L., Romero A. (2012). Epigeic soil arthropod abundance under different agricultural land uses. Spanish Journal of Agricultural Research, 10(1): 55-61.

Refatti E., Osler R., Loi N., Roggero P. (1986). Reasearch on transmission of apple proliferation. Acta Horticulturae, 193: 345-350.

Schellhorn N. A., Bianchi F. J. J. A., Hsu C. L. (2014). Movement of entomophagous arthropods in agricultural landscapes: links to pest suppression. Annual Review of Entomology, 59: 559-581.

Schmidt M. H., Roschewitz I., Thies C., Tscharntke T. (2005). Differential effects of landscape and management on diversity and density of ground-dwelling farmland spiders. Journal of Applied Ecology, 42: 281-287.

Sekercioglu C. H., Daily G. C., Ehrlich P. R. (2004). Ecosystem consequences of bird declines. Proceedings of the National Academy of Sciences, 101: 18042-18047.

Seljak G. (2013). *Hishimonus hamatus* Kuoh (Hemiptera: Cicadellidae): a new alien leafhopper in Europe. Acta Entomologica Slovenica, 21(2): 123-130.

Tedeschi R., Alma A. (2004). Transmission of apple proliferation phytoplasma by *Cacopsylla melanoneura* (Homoptera: Psyllidae). Journal of Economic Entomology, 97(1): 8-13.

Tedeschi R., Alma A. (2006). *Fieberiella florii* (Homoptera: Auchenorrhyncha) as a vector of '*Candidatus* phytoplasma mali'. Plant Disease, 90(3): 284-290.

Trivellone V., Filippin L., Jermini M., Angelini E. (2015). Molecular characterization of phytoplasma strains in leafhoppers inhabiting the vineyard agroecosystem in Southern Switzerland. Phytopathogenic Mollicutes, 5: 45-46.

van Helden, M. (2000). La cicadelle verte (*Empoasca vitis* Goethe). In: Les ravageurs de la vigne. pp. 121-129. J. Stockel ed. Editions Féret, Bordeaux, France.

Whelan C. J., Sekercioglu Ç. H., Wenny D. G. (2015). Why birds matter: from economic ornithology to ecosystem services. Journal of Ornithology, 156: 227-238.

Supplementary Table 1. Species abundance and richness in forest-dominated landscapes.

habitat		species richness	abundance
plants	external rows	*Aphrophora alni*	2
		Arboridia ribaudi	1
		Empoasca vitis	12
		Laodelphax striatella	3
		Macrosteles sexnotatus group	1
		Metcalfa pruinosa	3
		Orientus ishidae	6
		Philaenus spumarius	2
		Strictocephala bisonia	3
		Zyginidia pullula	1
	central rows	*Arboridia emeta*	1
		Empoasca vitis	10
		Laodelphax striatella	1
		Orientus ishidae	7
grass	external rows	*Aphrodes* sp.	8
		Aphrophora alni	3
		Arboridia sp.	1
		Cicadella viridis	2
		Empoasca vitis	2
		Eupteryx vittata	1
		Forcipata citrinella	1
		Jassargus sp.	1
		Laodelphax striatella	49
		Lepyronia coleoptrata	1
		Macrosteles cristatus	4
		Macrosteles sexnotatus group	91
		Muelleraniella extrusa	54
		Philaenus spumarius	10
		Psammotettix alienus	1
		Psammotettix confinis	13
		Psammotettix sp.	5
		Strictocephala bisonia	2
		Zyginidia pullula	11
		Zyginidia sp.	1
	central rows	*Anaceratagallia ribauti*	1
		Anaceratagallia venosa	2
		Aphrodes sp.	7
		Arthaldeus striifrons	1
		Cicadella viridis	1
		Deltocephalus maculiceps	1
		Dicranotropis hamata	3
		Empoasca vitis	1
		Forcipata citrinella	6
		Laodelphax striatella	76
		Macrosteles cristatus	15
		Macrosteles sexnotatus group	48
		Orientus ishidae	1
		Philaenus spumarius	1
		Psammotettix alienus	4
		Psammotettix confinis	8
		Psammotettix poecilius	1
		Psammotettix sp.	2
		Recilia coronifer	1
		Zyginidia pullula	8

Supplementary Table 2. Species abundance and richness in grass-dominated landscapes.

	habitat	species richness	abundance
plants	external rows	*Aphrophora alni*	1
		Laodelphax striatella	1
		Metcalfa pruinosa	4
		Orientus ishidae	1
		Philaenus spumarius	2
		Strictocephala bisonia	1
	central rows	*Empoasca vitis*	1
		Forcipata citrinella	1
		Macrosteles sexnotatus group	3
		Metcalfa pruinosa	2
		Zyginidia pullula	2
grass	external rows	*Anaceratagallia venosa*	1
		Aphrophora alni	1
		Euscelis incisus	1
		Forcipata citrinella	1
		Laodelphax striatella	94
		Lepyronia coleoptrata	2
		Macrosteles cristatus	13
		Macrosteles sexnotatus group	112
		Macrosteles sp.	2
		Megadelphax sordidula	3
		Philaenus spumarius	8
		Psammotettix confinis	28
		Psammotettix sp.	4
		Zyginidia pullula	6
	central rows	*Anaceratagallia venosa*	1
		Aphrophora alni	1
		Cicadula quadrinotata	6
		Empoasca vitis	1
		Eupteryx atropunctata	3
		Forcipata citrinella	2
		Javasella obscurella	1
		Laodelphax striatella	119
		Macrosteles laevis	5
		Macrosteles ossiannilssoni	18
		Macrosteles sexnotatus group	86
		Philaenus spumarius	3
		Psammotettix cephalotes	1
		Psammotettix confinis	6
		Psammotettix sp.	7
		Recilia coronifer	1
		Strictocephala bisonia	1
		Zyginidia pullula	5

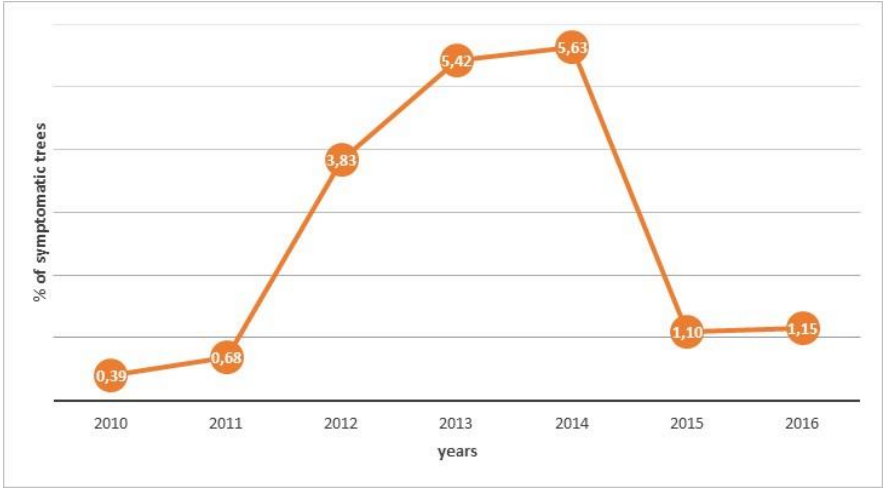
Supplementary Table 3. Species abundance and richness in orchard-dominated landscapes.

habitat		species richness	abundance
plants	external rows	*Aphrophora salicina*	1
		Balclutha punctata	1
		Empoasca vitis	7
		Laodelphax striatella	2
		Metcalfa pruinosa	2
		Orientus ishidae	2
		Strictocephala bisonia	2
	central rows	*Aphrophora alni*	1
		Empoasca vitis	36
		Laodelphax striatella	6
		Metcalfa pruinosa	1
grass	external rows	*Anaceratagallia venosa*	1
		Aphrophora alni	1
		Arthaldeus striifrons	1
		Balclutha punctata	1
		Cicadella lasiocarpae	1
		Cicadella viridis	2
		Dicranotropis hamata	2
		Jassargus sp.	1
		Laodelphax striatella	56
		Macrosteles ossiannilssoni	9
		Macrosteles sexnotatus group	21
		Muelleraniella extrusa	1
		Psammotettix alienus	2
		Psammotettix confinis	9
		Psammotettix sp.	7
		Ribautodelphax albostriata	2
		Stenocranus fuscovittatus	1
		Strictocephala bisonia	1
		Toya propinqua	7
		Zyginidia pullula	7
	central rows	*Anaceratagallia venosa*	1
		Arboridia ribaudi	1
		Conomelus anceps	1
		Dicranotropis hamata	1
		Empoasca vitis	5
		Forcipata citrinella	3
		Laodelphax striatella	120
		Macrosteles ossiannilssoni	18
		Macrosteles sexnotatus group	8
		Psammotettix confinis	6
		Psammotettix sp.	3
		Strictocephala bisonia	1
		Zyginidia pullula	1

CONCLUSION

A multidisciplinary approach was adopted in this research to delve into epidemiological and biological features of apple proliferation, a disease involving a triangular relationship between plant, pathogen and vector. As no direct control measures are available to fight phytoplasmas, this project focused on the knowledge of spread mechanisms, vectors' (and putative vectors) biology, and genetic variability of phytoplasma. These issues represent an essential starting point to develop innovative and sustainable control strategies.

Monitoring the incidence of symptomatic apple trees and the psyllid populations is necessary in epidemic and in endemic phases to assess the effectiveness of the phytosanitary measures, which must be initially aimed at reducing the inoculum source and vector populations. The graph below shows the results of the survey of AP-symptomatic apple plants in Valsugana and confirms that a combined strategy, in which researchers and growers were directly involved, is efficient in controlling the spread of the disease.



Percentage of symptomatic apple trees observed in Valsugana in the years 2010-2016.

In a wider context, as the disease showed in Trentino an up-and-down trend in the last 15 years, long-term investigations are required to study in detail the transmission dynamics. Moreover, insect vectors like psyllids, characterized by a univoltine biological cycle involving different (and only partially known) host plants, and small organisms with high evolution rates, like phytoplasmas, make any attempt of finding general conclusions even more challenging. Therefore, epidemiological monitoring and investigations on this three-way system (pathogen-plant-vector) should continue also if an endemic equilibrium has been reached, in order to explore biological factors that cannot be deeply studied in the emergency phase.

The results of the transmission trials with psyllids suggest that, even though none of the two species showed a high transmission efficiency, both have the potential to play a role in the disease spread in Valsugana, especially at high population levels and in presence of high inoculum sources. However, a potential role of other Homoptera, such as Auchenorrhyncha, in AP transmission cannot be excluded, as a few individuals tested positive for the phytoplasma. In addition, different phytoplasma genotypes were identified in the psyllid populations and plants analyzed and, hence, further research is necessary to disentangle this evolutionary relationship. Looking at the future, the next step should point to unravel the correlation between phytoplasma strain, vectoring ability, and virulence.

All control strategies applied so far in Trentino focused on vector populations' management by mean of multiple chemical treatments. The biological insights achieved in this study open new intriguing possibilities for the development of novel and more sustainable control measures. The characterization of some behavioral features, such as the use of the vibrational communication during courtship behavior of psyllids, suggests the possibility of new mating disruption strategies for vector management.

In addition, the investigations on leafhoppers' and planthoppers' biodiversity confirm the importance of investing in environmentally-friendly technological advances. Even simplified and exploited environments, like conventional apple orchards, can be visited by several species. The higher species richness observed in forest-dominated landscapes compared to crop-dominated ones suggests an exchange of individuals between orchards and natural surrounding environments, highlighting to ecological value of apple agroecosystems.

So, the research conducted during these three years, ranging from epidemiology to genetics, passing through insect biology and agroecosystem biodiversity, provided a scientific background that represents a useful tool to create a collaboration between research and agriculture in the frame of the development of sustainable strategies for the control of apple proliferation.

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