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Detection of '*Candidatus* Phytoplasma mali' in different populations of *Cacopsylla melanoneura* in Italy

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Abstract

Cacospylla melanoneura Förster (Hemiptera Psyllidae) is one of the vectors of '*Candidatus* Phytoplasma mali', which is the causal agent of apple proliferation (AP) disease. In 2006 and 2007, overwintering adult psyllids were collected from different host plants (apple, hawthorn and conifers) in different localities to assess the natural infection of *C. melanoneura.* AP phytoplasma was detected in insects through the use of PCR amplification with specific primers (AP3/AP4). Eleven percent of the psyllids collected from apple in the Trentino region were infected with AP phytoplasma, as compared with 18.63% of the psyllids collected from apple in the Aosta Valley and none of the psyllids collected from apple in the Veneto region. The percentage of AP-positive overwintering adults was higher in the Aosta Valley than in the Trentino region. Furthermore, considering the level of AP presence in the monitored orchards, a positive correlation between the infection rates in the insects and the percentage of symptomatic plants was observed. Regarding psyllids collected from hawthorn, only few individuals tested positive in Trentino populations, while higher infection levels were found out in the Aosta Valley. Interesting percentages of positive psyllids resulted also in the insects collected from conifers (10.5%). Data obtained in this work demonstrate therefore that '*Ca*. P. mali' may overwinter in the body of *C. melanoneura* and that there are differences in the infection proportion among populations.

Key words: apple proliferation, phytoplasma, psyllids, *Cacopsylla melanoneura*, host plants.

Introduction

The agronomic importance of the Hemiptera genus *Cacopsylla* is linked to the role played by several species as vectors of phytoplasma-associated diseases. Apple proliferation (AP) disease is one of the most severe problems in Italian apple (*Malus domestica* Borkh.) orchards. In northeastern Italy, symptoms of AP have been observed since the 1950s (Rui, 1950), but the disease has only recently become widespread, particularly in Trentino (northeastern Italy), where it has struck the cultivars Golden Delicious, Florina and Renetta Canada (Vindimian and Delaiti, 1996). AP causes important economic losses, as infected trees produce small fruits with poor flavour.

The aetiological agent, '*Candidatus* Phytoplasma mali' (Seemüller and Schneider, 2004), is transmitted by two psyllids, with different efficiencies: *Cacopsylla picta* (Förster) is the main vector in north-eastern Italy (Carraro *et al.*, 2001b) and *C. melanoneura* (Förster) in north-western Italy (Tedeschi *et al*., 2002). Studies conducted in Germany confirm the role of *C. picta* in the epidemiology of AP disease, excluding the latter species (Jarausch B. *et al.*, 2004; Mayer *et al.*, 2009). Both species are present in Trentino, but with different distributions: *C. melanoneura* is ubiquist and shows the highest population densities in bottom valley environments (Adige Valley and Valsugana); the presence of *C. picta* is limited to hilly areas, such as Non and Sole Valley (Mattedi *et al.*, 2008). Transmission experiments with the two psyllids were therefore conducted also in this region over a six-years period (Mattedi *et al*., 2008). Owing to these trials, the role of *C. picta* was confirmed (transmission rate of 4.1%), while contradictory results emerged with *C. melanoneura*, with only one successful transmission out of 278 test plants (transmission rate of 0.36%) (Mattedi *et al.*, 2008).

Despite the incidence of '*Ca.* P. mali'-infected apple plants in all the apple growing areas of Trentino, the abundance of both species is dramatically decreasing in the last years. Interesting population levels are only present for *C. melanoneura* in Valsugana, while *C. picta* density has drastically dropped down in the whole region and therefore this study focuses on the role of the first species in AP epidemiology.

The biology of *C. melanoneura* in the apple orchards of Trentino has been studied in detail (Mattedi *et al.*, 2007; 2008). Overwintering adults migrate into apple orchards between the end of January and February. Oviposition begins between the end of February and the beginning of March and this activity lasts about 30-40 days. The first nymphs appear at the end of March and the new generation of adults emerges at the end of April. As the adults develop, they migrate to other shelter plants and disappear from the orchard before the end of June.

During the same period, *C. melanoneura* can also be found on hawthorn (*Crataegus monogyna* Jacq. and *C. oxyacantha* L.) (Ossiannilsson, 1992), on which high population densities have been reported even in some locations of Trentino (Pedrazzoli *et al.*, 2005). Recently, experiments conducted in north-western Italy found that *C. melanoneura* individuals collected from hawthorn carried AP-group phytoplasmas (Tedeschi *et al.*, 2005; 2009).

Besides hawthorn and apple, *C. melanoneura* has also been found on other plants, particularly on conifers such as *Picea abies* (L.) H. Karst., *Pinus sylvestris* L., *P.*

mugo Turra, on which the new generation of adults is thought to overwinter (Conci *et al.*, 1992; Ossiannilsson, 1992; Lauterer, 1999). *P. abies* is the main forest species of Trentino region, covering more than one third of the total forest area (137203 ha out of 407500 ha) (INFC, 2005). To date, several studies have been conducted on the overwintering habits of this species in Trentino (Pedrazzoli *et al.*, 2005; Mattedi *et al.*, 2007). A regular presence has been found on Norway spruce and pine in only a few high-altitude locations (Pedrazzoli *et al.*, 2005).

The different roles assigned to *C. melanoneura* by the studies conducted to date suggest the existence of different populations, characterized by a different level of infectivity. To examine these differences thoroughly, molecular analyses were performed on overwintered *C. melanoneura* collected from different plant species (apple, hawthorn and conifers) in several areas of Italy. Hawthorn hedgerows are a common component of the rural landscape of Trentino region, so that often they are close to apple orchards. The knowledge of the infectivity of *C. melanoneura* collected from hawthorn plants could be informative about alternative inoculum sources.

In the present work, we report the results of these investigations, underlining the correlation between the infection level in the different plants and in the insects.

Materials and methods

Insect collection

The research was conducted in the Trentino and Veneto regions (northeastern Italy) and in the Aosta Valley (northwestern Italy) between 2005 and 2007, as reported in table 1. Overwintering adults of *C. melanoneura* were collected from apple trees and hawthorn bushes at the end of March and from conifers during December and January, using the beat tray method. The specimens were identified using Ossiannilsson's keys (1992).

Molecular analyses

Individual specimens were frozen, lyophilized, homogenized, and then stored at -80 °C until their DNA could be extracted. Total genomic DNA was extracted from the samples following the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1990).

To assess the presence of '*Ca*. P. mali', all of the collected insects were subjected to PCR amplification of a non-ribosomal DNA region of 162 bp with the specific primers AP3/AP4 (Jarausch *et al.*, 1994). The PCR assays were performed with 100-150 ng DNA, 375 nM of each primer and the 2x Go Taq® Green Master Mix (Promega, Madison, USA), in a final reaction volume of 20 µl. The PCR parameters were as follows: an initial denaturation of 2 min at 95 °C and 40 cycles with 30 sec at 95 °C, 30 sec at 57 °C, 30 sec at 72 °C and a final extension of 5 min at 72 °C. Amplifications were performed in a Gene Amp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The total DNA extracted from infected insects and a reaction blank (Milli-Q water) were included in the experiments as controls. PCR products were analyzed by electrophoresis on 1.5% w/v agarose gels stained with SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and visualized under UV light with the Gel Doc XR System molecular imager (BIO-RAD, Hercules, CA, USA; figure 1).

Data analysis

The correlation between the proportion of infected insects and the presence of symptomatic plants in apple orchards was analysed applying the median test for the correlation between two variables proposed by Blomqvist (1950). As there is not an official classification of the level of infected orchards, we did an arbitrary classification dividing apple orchards in four classes: low (with a percentage of symptomatic plants ranging from 0 to 10), middle (from 10% to 30% of symptomatic plants), high (from 30% to 50%) and very high (more than 50%). This analysis was not carried out for the other plant species, as no symptoms were detected on hawthorn and conifers.

Table 1. Samples of *C. melanoneura* populations collected from different host plants in different locations. The total number of psyllids analysed, the proportion of AP-infected samples and infection level in the sampled orchards are given.

Host plant	Locality	Location	Insects		Infection level in the sampled orchards
			infected/tot.	percentage	
Apple	Borgo (Trentino)	46°3'N 11°29'E	11/96	11.46%	very high
"	Oltrecastello (Trentino)	46°4'N 11°9'E	27/113	23.87%	high
"	S. Michele (Trentino)	46°11'N 11°8'E	5/96	5.14%	middle
"	Vervò (Trentino)	46°18'N 11°7'E	3/90	3.33%	middle
"	Vigalzano (Trentino)	46°4'N 11°14'E	10/96	10.42%	high
"	Aosta (Aosta Valley)	45°44'N 7°18'E	11/59	18.63%	very high
"	Schio (Veneto)	45°42'N 11°24'E	0/12	0%	low
"	Verona (Veneto)	45°39'N 10°53'E	0/15	0%	low
Hawthorn	Cles (Trentino)	46°21'N 11°2'E	2/96	2.08%	-
"	Mezzolombardo (Trentino)	46°11'N 11°6'E	2/63	3.17%	-
"	Rumo (Trentino)	46°26'N 11°1'E	0/44	0%	-
"	Chambave (Aosta Valley)	45°44'N 7°33'E	8/53	15.10%	-
Conifers	Sopramonte (Trentino)	46°4'N 11°4'E	4/49	8.16%	-
"	Vason (Trentino)	46°2'N 11°3'E	5/38	13.15%	-

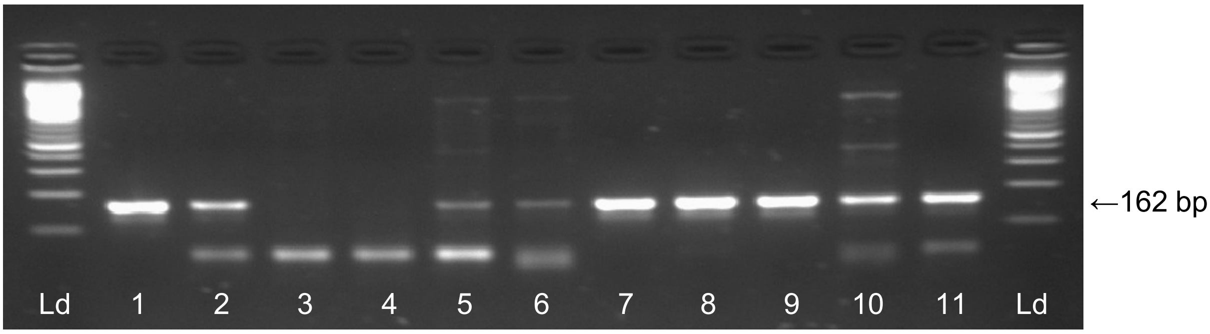

Figure 1. Agarose gel electrophoresis of PCR amplifications with primer pair AP3/AP4. Ld = molecular size marker (100 bp DNA Ladder, New England Biolabs, Inc., Beverly, MA, USA); 1-2 = positive controls (infected psyllids); 3 = negative control (water); 4-5-6 = psyllids collected from hawthorn; 7-8-9 = psyllids collected from apple plants; 10-11 = psyllids collected from Norway spruce. A 162 bp fragment is the expected amplification product, while the lower bands in some of the lanes are primer dimers, which are present especially in negative or weakly positive samples.

Results

The results of PCR amplifications performed on the different *C. melanoneura* populations are reported in table 1, where the percentages of infected individuals and the mean infection levels of the apple trees are listed for each population. A total number of 920 insects belonging to 14 different localities (10 located in Trentino, two in Aosta Valley and two in Veneto) were analysed.

Regarding populations collected from apple in Trentino, data showed differences in the percentage of '*Ca.* P. mali'-infected psyllids, which ranged from 3% (Vervò) to 24% (Oltrecastello) (table 1). Few infected psyllids were found among populations collected from hawthorn and none of the examined hawthorn hedgerows ever showed disease symptoms or tested positive for the presence of the pathogen (unpublished data). A relatively large number of positive insects were collected in the Aosta Valley, from both apple (18.63%) and hawthorn (15.10%). The percentage of infected psyllids of Aosta Valley was higher than that of Trentino region for both hawthorn and apple, with the only exception of the apple population from Oltrecastello. PCR amplification revealed no infected insects among the psyllids collected in two different areas in the Veneto region. Eight to 13% of the psyllids collected from conifers in northeastern Italy were tested positive for '*Ca.* P. mali' (table 1).

A positive correlation was found with the Blomqvist median test (1950) between the proportion of infected psyllids and the infection level of the apple orchards where captures took place (P = 0.0143; figure 2).

Discussion

The present work provides evidence that the overwintering populations of *C. melanoneura* in northern Italy can carry '*Ca.* P. mali', since the PCR analyses revealed the presence of infected psyllids. Tedeschi *et al.* (2003, 2006) already suggested the possibility that the phytoplasma overwinters in the bodies of this insect vector, but nobody has ever tested this hypothesis. In this research we detected for the first time '*Ca.* P. mali' in individuals collected from conifers during the winter. As these host plants have never been found infected by '*Ca.* P. mali' (unpublished data), it is reliable that these individuals had acquired the phytoplasma before overwintering. These results are consistent with the behaviour of other psyllid vectors of phytoplasma, such as *C. pyricola* and *C. pyri* which may retain infectivity during the winter season (Davies *et al.*, 1998; Carraro *et al.*, 2001a; Thébaud *et al.*, 2009). Therefore, some overwintering *C. melanoneura* adults are already infected, even in high percentages, with the phytoplasma when they come back to the apple orchards. The high mean percentage of infected insects collected on conifers could depend on the restricted number of samples analysed. A wider sampling activity on conifers is needed to confirm these data and to obtain more consistent values.

Moreover, psyllids can acquire '*Ca.* P. mali' after a period of feeding on infected trees. Psyllids were collected in the Trentino region at the peak of overwintering adults, when the population densities are the highest,

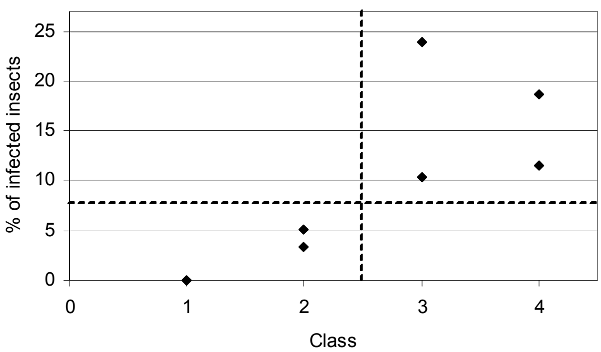

Figure 2. Scatter diagram of the observed infection percentage in the insect populations and the corresponding infection level in the orchards (1 = low infection, 2 = middle infection, 3 = high infection, 4 = very high infection). The broken lines indicate the sample medians of the values.

so that they had spent a few weeks of feeding on highly infected apple trees. A period of four days is sufficient for *C. melanoneura* to acquire AP phytoplasma (Pedrazzoli *et al.*, 2007). At the end of March, when captures took place, '*Ca.* P. mali' is present in the aerial part of apple trees with an uneven distribution which in some cases reaches high concentration levels (Pedrazzoli *et al.*, 2008a). The percentages of infected psyllids are variable among Trentino populations collected from apple; the mean proportion of '*Ca.* P. mali'-positive insects (10.84%) in overwintering psyllids collected in 2006 on apple was comparable to the rates already reported for overwintering *C. melanoneura* adults in this region (Pedrazzoli *et al.*, 2008b). The highest infection levels for Trentino populations are comparable to that of Aosta Valley population. In both cases, psyllids were collected in orchards with high percentages of symptomatic apple trees. On the other hand, lower infection rates were detected in populations collected in low symptomatic plantations; moreover, in the Veneto region there was no evidence of infected psyllids, but there were also few AP-symptomatic apple trees. Goodwin *et al.* (1999) found out that the presence of infected *Macrosteles quadrilineatus* (Forbes) individuals is a better predictor of aster yellows infection in lettuce fields than the total number of leafhoppers and the quantity of phytoplasmas in the vector insects. Therefore, the positive correlation between the infection rates in plants and psyllids, which was confirmed by the statistical analysis performed in this work, could be a useful tool in the disease management. For instance, within the phytosanitary measures adopted to reduce the inoculum source, such as uprooting diseased plants, the number of infected psyllids should be taken into account as a predictor of latent infections in an orchard together with the presence of symptoms in the plants.

Regarding the infection level among the psyllids collected from hawthorn, the percentages obtained for the Trentino region (mean value 1.75%) are lower than that for the Aosta Valley (15.1%). The proportions of infected psyllids reported in this study for populations from Aosta Valley are similar in individuals collected from both hosts (apple and hawthorn). This result is consistent with preliminary ecological observations carried out in north-western Italy, indicating that the populations of *C. melanoneura* collected from apple can survive and reproduce also on hawthorn, while *C. melanoneura* collected from hawthorn seems to be more selective (R. Tedeschi, personal communication). A certain exchange in the populations could therefore take place, resulting in a more homogeneous infection level in the insects. In Trentino region, apple orchards are often close to stands so that *C. melanoneura* populations are sympatric. However, they seem more linked to their original host, showing a significantly reduced fitness when moved onto the alternative host plant (Malagnini *et al.*, unpublished data). High populations of *C. melanoneura* were recorded on hawthorn, while no psyllids were observed in the close apple orchard (unpublished data). This could also lead to different percentages of infected individuals for the two plant species in this region, even though the two plant species are close to each other.

The infection rates observed in this survey for the populations collected from apple and hawthorn in Aosta Valley are higher than those previously reported for the same region (Tedeschi *et al.* 2003; 2009; Tedeschi and Alma, 2007). These different data could reflect the use of different primers (AP3/AP4 vs. P1/P7, followed by nested PCR with fO1/rO1 primers) in the molecular analyses and/or the different number of psyllids analyzed (Tedeschi *et al.*, 2003; Tedeschi and Alma, 2007), the size of the sampled populations or the period of collection and the infection levels of the plants.
The results obtained in this work suggest the presence of a high variability in the infection level within the different populations of *C. melanoneura* in northern Italy and even within the Trentino region. These differences could depend on the affinity between the phytoplasma and the insects, due to the presence of various '*Ca.* P. mali' strains (AT-2 is predominant in Trentino, while AT-1 is more widespread in Aosta Valley), or to the characteristics of the psyllid populations (Cainelli, 2007; Malagnini *et al.*, 2008). Furthermore, the differences in the natural infection rates could explain the differences observed in previous transmission trials carried out in Aosta Valley and Trentino regions (Tedeschi and Alma, 2004; Mattedi *et al.*, 2005; 2007; 2008). This hypothesis is also supported by data obtained in Germany where *C. melanoneura* never transmitted '*Ca.* P. mali' (Jarausch W. *et al.*, 2004, Mayer *et al.*, 2009); in this region overwintering adults of *C. melanoneura* collected in apple orchards show a very low infection rate (Jarausch *et al.*, 2008; Mayer *et al.*, 2009). Further studies of transmission efficiency should take into account these differences among populations of *C. melanoneura*.

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