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Composition, abundance and phytoplasma infection in the hawthorn psyllid fauna of northwestern Italy

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Abstract Hawthorn (*Crataegus monogyna*) is one of the natural hosts of *Cacopsylla melanoneura*, the acknowledged vector of '*Candidatus* Phytoplasma mali', the causal agent of Apple Proliferation disease, a serious and growing problem for apple production in Europe, particularly in northern Italy. Wild plants could be important sources of both insects and phytoplasmas, but their role in the epidemiology of phytoplasma diseases and their insect vectors has never been thoroughly examined. *Cacopsylla melanoneura*'s primary host is hawthorn, a plant closely related to apple which often grows wild near orchards. Other psyllid species feed on hawthorn, but no data are available on their possible role as phytoplasma vectors. We investigated the hawthorn's psyllid fauna in northwestern Italy using yellow sticky traps, beat trays, and molecular analyses from 2003–2005, to study the relationship between hawthorn, the phytoplasma and the insect vector. Population dynamics were monitored, and insects and hawthorn samples were analysed by polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and DNA sequencing for the presence of phytoplasmas. *Cacopsylla melanoneura* was the dominant psyllid species, followed by *C. peregrina*, *C. affinis* and *C. crataegi*. PCR and RFLP analyses revealed the presence of different fruit tree phytoplasmas in hawthorn plants, and in all four psyllid species.

Keywords *Cacopsylla affinis* · *Cacopsylla crataegi* · *Cacopsylla melanoneura* · *Cacopsylla peregrina* · Fruit tree phytoplasmas

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Introduction

Psyllids of the genus *Cacopsylla* are the main vectors of fruit tree phytoplasmas in temperate regions and recent studies have revealed vector-phytoplasma specificity. '*Candidatus* Phytoplasma pyri', the causal agent of pear decline (PD), is transmitted by *C. pyricola* in the UK (Davies et al. 1992) and by *C. pyri* in France (Lemoine 1991) and Italy (Carraro et al. 1998a). '*Ca.* Phytoplasma prunorum', the causal agent of European stone fruit yellows (ESFY), is

transmitted by *C. pruni* (Carraro et al. 1998b, 2001) while '*Ca.* Phytoplasma mali', the causal agent of apple proliferation (AP) is by *C. picta* (syn. *C. costalis*) (Frisinghelli et al. 2000; Jarausch et al. 2003) and by *C. melanoneura* (Tedeschi et al. 2002; Tedeschi and Alma 2004). These insects transmit phytoplasmas as both nymphs and adults (Carraro et al. 1998a; Tedeschi and Alma 2004). They complete one or more generations per year on wild or cultivated rosaceous plants (Pomaceae and Drupaceae) and often overwinter as adults on alternative host plants. Tedeschi et al. (2006) observed transovarial transmission of the European stone fruit yellows phytoplasma in *C. pruni*.

Recently, many research programmes have focused on the insect vectors of '*Ca.* Phytoplasma mali' because of the serious damage the disease causes in central Europe, particularly in Italy. Owing to the phytoplasma's rapid spread and ensuing economic losses, mandatory controls measures have been introduced into Italy by a Ministerial Decree. This action has resulted in increased research on the vectors.

The relationship between *C. melanoneura* and hawthorn (*Crataegus monogyna*) plants is particularly interesting. *Cacopsylla melanoneura* has always been reported as a primarily hawthorn-feeding species (Lal 1934; Ossiannilsson 1992) but has recently become an economically injurious pest of apple trees (Alma et al. 2000; Tomasi et al. 2000; Tedeschi et al. 2002). In northwestern Italy, *C. melanoneura* is considered the main vector of '*Ca.* Phytoplasma mali' (Tedeschi et al. 2003; Tedeschi and Alma 2004) and apple proliferation (AP) infection has been increasing steadily. Wild plants could be important as sources of both insects and phytoplasmas in the epidemiology of the phytoplasma diseases, yet this has never been thoroughly investigated.

The hawthorn is a deciduous shrub or small tree native to Europe and to the region from North Africa to the Himalaya. It is widespread in northwestern Italy up to 1,000 m a.s.l. in glades, hedgerows and woodland. The hawthorn psyllids have been studied in the past (Lal 1934; Missonnier 1956; Sutton 1983, 1984) but their ability to transmit phytoplasmas has recently given them new prominence.

The widespread prevalence of hawthorn in wild areas and in the surrounding areas of apple orchards induced us to study its importance in the transmission of phytoplasmas to apples, by insect vectors. Furthermore, this work highlights the other psyllid species present on hawthorn and presents evidence for their possible role in phytoplasma transmission.

Materials and methods

Sites

Two sites (C1 and C2) were selected at altitudes between 480 and 580 m a.s.l., in the Aosta Valley (northwestern Italy). The region has an economically important tradition of apple production, and symptoms of '*Ca.* Phytoplasma mali' infection have often been recorded in the last 10 years. Sites were located near apple orchards, contained wild *Cr. monogyna,* and were surrounded by meadows, wild areas, and vineyards. Temperatures (mean, minimum and maximum) were recorded daily throughout the experimental period and mean weekly temperatures were calculated.

Insect sampling

In 2003, yellow sticky traps (150×80 mm, Rebell® giallo, Andermatt Biocontrol AG, Switzerland) were used to sample the psyllid fauna living on *Cr. monogyna*. Three traps were hung in the hawthorn canopy at each site from mid-February until the end of October, and changed weekly. To identify psyllids, samples were removed from sticky traps using a drop of Bio-Clear (Bio-Optica, Milan, Italy), dissected, and then classified by examining male and female terminalia (Hodkinson and White 1979). Surveys were repeated using the same methods in 2004 and 2005. Sampling using a beating tray was also carried out in the latter 2 years from mid-February to the end of October, to assess psyllid population densities and to collect material for molecular analyses. When not otherwise stated, beating tray collections occurred weekly. For population studies, hawthorn branches were beaten for 20 min at each site, but to collect insects for molecular analyses beating was often prolonged. When possible, 50 specimens per species were collected at each site on every sampling date. During beating tray sampling, plants were also carefully examined for eggs and nymphs. In late

summer, hawthorn samples were collected from all sites to test for phytoplasmas by molecular analyses.

Phytoplasma identification

Total DNA was extracted from batches of five adult psyllids following a protocol adapted from Marzachì et al. (1998). Plant DNA was isolated from phloem tissue from field-collected branch samples following the phytoplasma enrichment procedure described by Ahrens and Seemüller (1992). Approximately 1.0 g of fresh plant material was used for each sample. Insect and plant DNAs were amplified with the nested polymerase chain reaction (PCR) firstly with phytoplasma universal primer pair P1/P7 (Schneider et al. 1995) and then with the AP-group specific primer pair fO1/rO1 (Lorenz et al. 1995) after a 1:40 dilution. Reaction and cycling conditions were as described in the original papers. PCR amplification products were analysed by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualised on a U.V. transilluminator. Specific AP-group phytoplasma profiles were obtained by restriction fragment length polymorphism (RFLP) with the endonucleases *Ssp*I and *Rsa*I. Seven microliters of the amplicon were digested with 3 U of *Ssp*I for 4.5 h at 37°C and with 5 U of *Rsa*I at 37°C overnight, respectively.

Seventeen PCR-amplified DNA fragments fO1/rO1, two from each psyllid species and phytoplasma revealed by RFLP as well as from field-collected hawthorn samples were directly sequenced. PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. Purified products were then sequenced with the fO1 and rO1 primers using a DYEnamic ET Terminator Cycle Sequencing kit (Pharmacia) and an ABI 310 automated sequencer (Applied Biosystems). The resulting sequences were compared with the sequence database of the National Centre for Biotechnology Information using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical and phylogenetic analysis

The proportion of infected insects was estimated with the maximum likelihood estimator, $\hat{p}$, calculated with Swallow (1985): $\hat{p}=1-H^{1/k}$, where H is the observed fraction of uninfected insects and k is the number of insects per group, in this case five. The closest match to each sequence was found with BLASTN (Altschul et al. 1990). Chimera sequences were checked using the Chimera check programme at the Ribosomal Data Project II (RDPII, http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). Sequences were then aligned using ClustalX v.1.83 (Thompson et al. 1997). Alignments were checked manually and poorly aligned or divergent regions were eliminated using GBlocks v.0.91b (Castresana 2000) with a minimum block of five and allowed gap positions equal to half. Tree construction was done with Treecon v.1.3b (Van de Peer and Wachter 1994), using neighbour-joining methods and a bootstrap analysis with 1,000 replicates.

Accession numbers

Sequences were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under accession numbers from AM404163 to AM404176, AM746983, AM746984 and AM749199.

Results

Psyllid fauna

Ten species of psyllids were identified in traps during 2003 (Table 1). *Cacopsylla melanoneura* was the dominant species at both sites (70.24%), followed by *C. peregrina*, which was more abundant at site C2 than at site C1. Few specimens of *C. crataegi* (0.38%) were captured at either site, while other species were encountered sporadically. In-depth examinations of the apical part of the aedeagus, the shape of the paramere, and the forewing of 3,700 specimens of *C. melanoneura*, revealed that the *C. melanoneura* population in the Aosta Valley is mixed with *C. affinis*. The two species resemble each other so closely that females of the two species cannot be reliably distinguished. Considering the difficulty of discriminating between the two species, especially in females, the complex *C. melanoneura–C. affinis* has been denoted as *C. melanoneura* in the present work, unless otherwise specified. The proportion of the two species was assessed using only the males among the 3,700 specimens observed with *C. affinis* representing 21.6% of the overwintered population and 6.9% of

Table 1 Psyllid species caught on hawthorn plants by means of yellow sticky traps in 2003

Species	Sites			Proportion
	C 1	C 2	Tot	(%)
C. melanoneura–C. affinis complex	1,859	1,810	3,669	70.24
Cacopsylla peregrina	512	1,014	1,526	29.22
Cacopsylla crataegi	12	8	20	0.38
Cacopsylla pulchella	0	2	2	0.04
Cacopsylla pruni	2	0	2	0.04
Cacopsylla pyrisuga	1	0	1	0.02
Baeopelma foersteri	0	1	1	0.02
Psyllopsis fraxini	1	0	1	0.02
Trioza rhamni	0	1	1	0.02

Total number of adults per site.

newly-emerged adults. Abundances of the three main hawthorn-feeding psyllids were similar across both sites. Two peaks of abundance were observed for overwintered and newly-emerged *C. melanoneura* adults, one in the second half of March and another in the first half of May, respectively. *Cacopsylla peregrina* emerged at the end of April, reaching a first peak in mid-May. The population density then decreased rapidly and a second peak was observed in the second half of September. *Cacopsylla crataegi* specimens were collected only in March–May.

Psyllid population dynamics

In 2004 and 2005, the population sampling was focused on the two most abundant species, *C. melanoneura* and *C. peregrina*. As measured by beating, peak densities of overwintered *C. melanoneura* adults were reached between mid-March and the beginning of April, when the mean week temperature exceeded 10°C. Highest number of emerged new generation adults was recorded in mid-May, when the weekly mean temperature reached 20°C. By contrast, the first peak collected with sticky traps was generally delayed compared with beating trays (Figs. 1 and 2). Mating was observed from the second half of February until mid-March, while the presence of the eggs on the plants was recorded from the last week of March until the first week of April. Nymphs were found from the first week of April until mid-May.

Following winter, overwintered adults persisted at the two sites during an average of 13.5±0.29 (±SEM) weeks, compared with only 4.25±0.25 (±SEM) weeks for springtime adults. The data obtained from both sticky trap and beat tray samplings revealed that the overwintered population is more abundant on hawthorn plants than the newly-emerged population, as shown in Fig. 1.

Nymphs of *C. peregrina* were observed in the second half of April and adults emerged at the end of the month, with peak densities occurring between mid- and late-May. A particularly high density was recorded at site 2 at the end of May 2004 (1,061 specimens). After the end of July in 2004 and the end of June in 2005, when the mean weekly temperature exceeded 20°C, no specimens of *C. peregrina* were caught until late August. At the end of September, when the mean week temperature reached 15°C, the second peak of *C. peregrina* density was observed, while after the third week of October, when the mean weekly temperature fell below 10°C, no specimens were collected (Figs. 1 and 2). Males captured at the beginning of September were darker (brownish) than those collected in summer, whereas females did not darken for another 2 weeks. In the second half of September, mating specimens were observed.

Phytoplasma detection

Performing nested PCR with the specific primers fO1/rO1 allowed us to observe amplification products of the expected size (1,080 bp) and to detect phytoplasmas of the AP-group (16Sr-X) in all four of the focal psyllid species (Table 2), and in hawthorn plants. The proportion of infected insects ranged from 3.17% to 4.34% for *C. melanoneura* in the two study years,

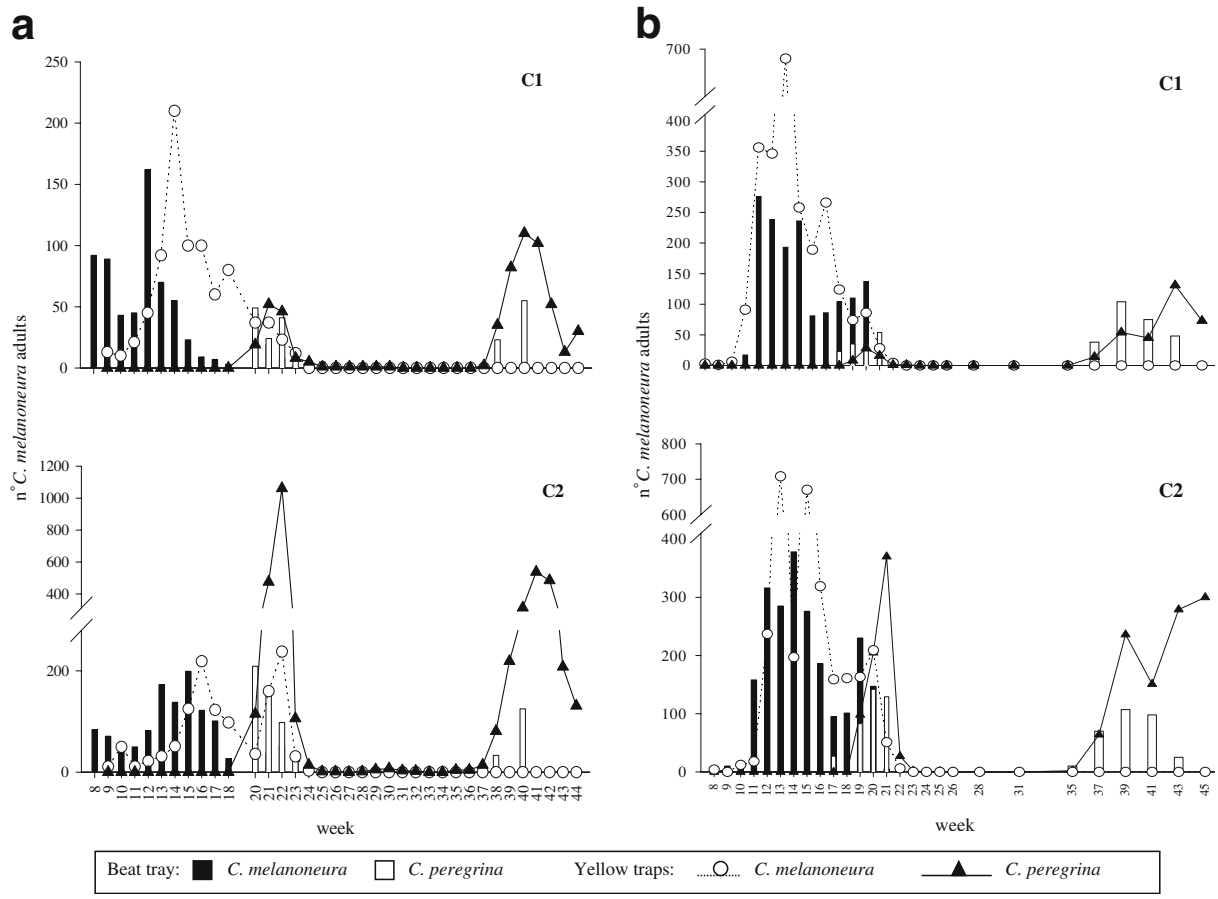

Fig. 1 Counts of *Cacopsylla melanoneura* and *Cacopsylla peregrina* adults caught by means of yellow sticky traps and beat trays in the two investigated sites in 2004 (**a**) and 2005 (**b**)

while lower percentages were recorded for *C. peregrina*. Estimates of 2.63% and 4.36% infection rate for *C. crataegi* were recorded in 2004 and 2005, respectively.

To ascertain the infection frequency of *C. affinis*, 50 unambiguously identified male samples were individually screened for phytoplasma presence, and two of them were positive for phytoplasmas of the AP-group (4.00%). Two of eight hawthorn leaf samples in 2004 and 1 of 12 in 2005 tested positive for AP-group phytoplasma with fO1/rO1 nested PCR. RFLP analyses carried out with the endonucleases

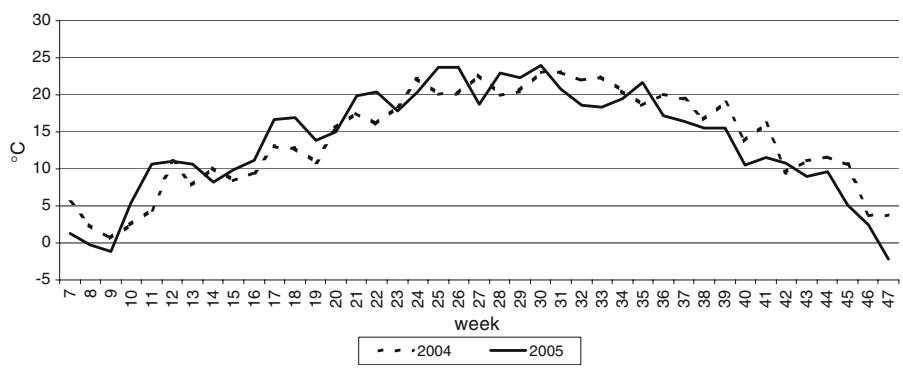

Fig. 2 Mean weekly temperature recorded throughout the experimental period in the years 2004 and 2005

Table 2 Number of hawthorn psyllid positive samples in nested PCR with the AP-group (16Sr-X) specific primers fO1/rO1 and estimated proportion ($\hat{p}$) of infected insects

Species	2004		2005	
	PCR positive	$\hat{p}$	PCR positive	$\hat{p}$
C. melanoneura–C. affinis complex	106/533	4.34%	59/396	3.17%
C. peregrina	25/246	2.12%	23/328	1.44%
C. affinis	–	–	2/50[a]	4.00%[b]
C. crataegi	1/8	2.63%	1/5	4.36%

[a] Individually tested

[b] Real proportion

*Ssp*I and *Rsa*I detected '*Ca.* Phytoplasma mali', '*Ca.* Phytoplasma pyri' and '*Ca.* Phytoplasma prunorum' in the psyllids, as well as the hawthorn plants (Fig. 3).

RFLP analyses carried out with *Ssp*I on most of the fO1/rO1 amplicons revealed that 70.80% and 69.50% of *C. melanoneura* samples in 2004 and 2005, respectively, carried '*Ca.* Phytoplasma mali' while the remaining samples were not digested by *Ssp*I. Similarly 53.85% and 21.74% of '*C. peregrina*' samples in 2004 and 2005, respectively, showed the presence of '*Ca.* Phytoplasma mali', while the rest of the samples were undigested. The RFLP performed with *Rsa*I on the samples undigested by *Ssp*I showed a restriction profile typical of '*Ca.* Phytoplasma prunorum' in three samples of *C. peregrina*, while all the other amplicons were ascribed, by elimination, to '*Ca.* Phytoplasma pyri' (Table 3). The amplicons derived from the two *C. affinis* males were undigested by *Ssp*I, while the RFLP profile obtained with *Rsa*I revealed the presence of '*Ca.* Phytoplasma prunorum' (Fig. 3). One of the hawthorn samples tested positive for '*Ca.* Phytoplasma mali' and two for '*Ca.* Phytoplasma pyri'.

Partial 16S rRNA gene sequences (800 bp) obtained from two samples per psyllid species, per phytoplasma type confirmed the RFLP analyses. In particular, the sequences of samples ascribed to '*Ca.* Phytoplasma mali' and '*Ca.* Phytoplasma prunorum' by means of RFLP analyses showed <1% difference with the sequences of the respective AP group members available in the GenBank databases. In contrast, all sequences of the psyllid and hawthorn samples ascribed to '*Ca.* Phytoplasma pyri' by RFLP analyses (from AM404169 to AM404176) showed an average of 98% identity with the '*Ca.* Phytoplasma pyri' sequences available in the GenBank databases and seem to represent a new subtype (Fig. 4).

Discussion

The study of the hawthorn psyllid fauna of north-western Italy allowed us to ascertain the presence of two predominant species, the Holopalaearctic *C. melanoneura* and the Eurosiberian *C. peregrina*, both already known hawthorn feeders (Conci et al. 1992;

Table 3 Results of the RFLP analyses carried out with the endonucleases *Ssp*I and *Rsa*I on *Cacopsylla melanoneura–C. affinis* complex, *Cacopsylla peregrina*, *Cacopsylla crataegi* and *Crataegus monogyna* samples

Species	2004				2005			
	Sample tested	16SrX-A	16SrX-B	16SrX-C	Sample tested	16SrX-A	16SrX-B	16SrX-C
C. melanoneura–C. affinis complex	90	63	0	27	57	41	0	16
C. peregrina	13	7	1	5	22	5	2	15
C. affinis	–	–	–	–	2	0	2	0
C. crataegi	1	0	0	1	1	0	0	1
Cr. monogyna	2	1	0	1	1	0	0	1

16SrX-A = '*Ca.* Phytoplama mali', 16SrX-B = '*Ca.* Phytoplasma prunorum', 16SrX-C = '*Ca.* Phytoplasma pyri'

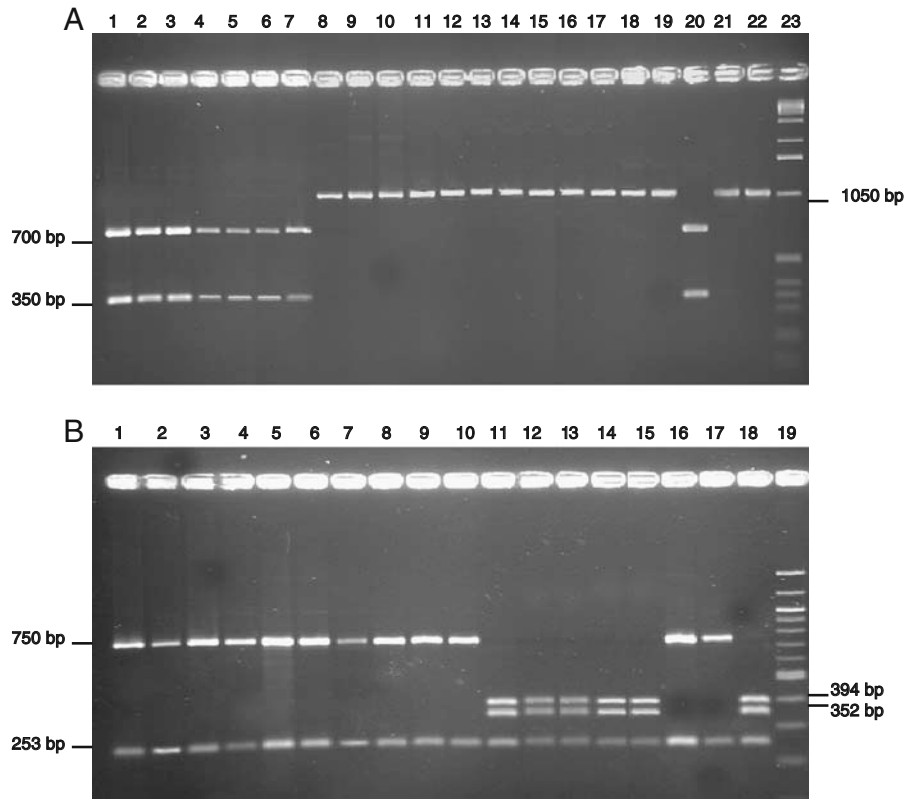

Fig. 3 RFLP analysis with *Ssp*I (**a**) and *Rsa*I (**b**) of PCR products from DNA extracted from: **a** *Cacopsylla melanoneura* (1–3, 8–10), *Cacopsylla peregrina* (4–6, 11–13), *Cacopsylla affinis* (14 and 15), *Cacopsylla crataegi* (16 and 17), *Crataegus monogyna* (7, 18 and 19), AP control (20), PD control (21), ESFY control (22), 1-kb DNA ladder (Invitrogen, Carlsbad, CA) (23). **b** *Cacopsylla melanoneura* (1–3), *Cacopsylla peregrina* (4–6, 11–13), *Cacopsylla affinis* (14 and 15), *Cacopsylla crataegi* (7 and 8), *Crataegus monogyna* (9 and 10), AP control (16), PD control (17), ESFY control (18), 100-bp ladder (Invitrogen) (19). (AP = Apple Proliferation, PD = Pear Decline, ESFY = European Stone Fruit Yellows)

Ossiannilsson 1992; Lauterer 1999). The third species found, *C. affinis*, is a Eurocaucasian species known from most European countries as oligophagous on *Crataegus* spp., predominantly *Cr. monogyna*, chiefly on free-growing shrubs and in woodland ecotones and shrubberies; it almost invariably occurs with the much more abundant *C. melanoneura* (Lauterer 1999). The difficulty of morphologically discriminating females of the two species constrained our description of population dynamics to the *C. melanoneura–C. affinis* complex, but some interesting information on the two species concerning the phytoplasma infections were recorded. On the contrary, the presence of *C. crataegi*, another hawthorn-specialist species, was negligible, compared with the other three psyllids.

The molecular analyses performed on insect and hawthorn samples gave new information on the relationships between phytoplasmas, hawthorn, and psyllids. First of all, all four psyllid species were able to harbour phytoplasmas of the AP-group, although the transmission ability has been proven only with *C. melanoneura* and '*Ca.* Phytoplasma mali' (Tedeschi and Alma 2004). The estimated proportion of positive *C. melanoneura* specimens was similar to that recorded from specimens collected on apple trees (3.17–4.34% vs 2.9–3.6%) (Tedeschi et al. 2003), while lower frequencies were found for *C. peregrina*, suggesting a different ability for acquiring the AP-group phytoplasmas. Another interesting dataset was obtained by RFLP analyses and demonstrated the presence in the insects of not only '*Ca.* Phytoplasma mali' but also of the other phytoplasmas belonging to the same taxonomic group. Almost 30% of the *C. melanoneura* samples subjected to RFLP analysis with *Ssp*I and *Rsa*I tested positive for '*Ca.* Phytoplasma pyri' while in *C. peregrina* the percentage of '*Ca.* Phytoplasma pyri'-positive samples was even higher (68% in 2005). Some *C. peregrina* were also positive for '*Ca.* Phytoplasma prunorum'. These results are supported also by the detection of '*Ca.* Phytoplasma mali' and '*Ca.* Phytoplasma pyri' in *Cr. monogyna* plants that can now be considered as a new source of inoculum for these diseases.

The co-presence in the investigated area of apple and hawthorn plants suggests a possible shift of *C. melanoneura* between the two hosts and this is the possible

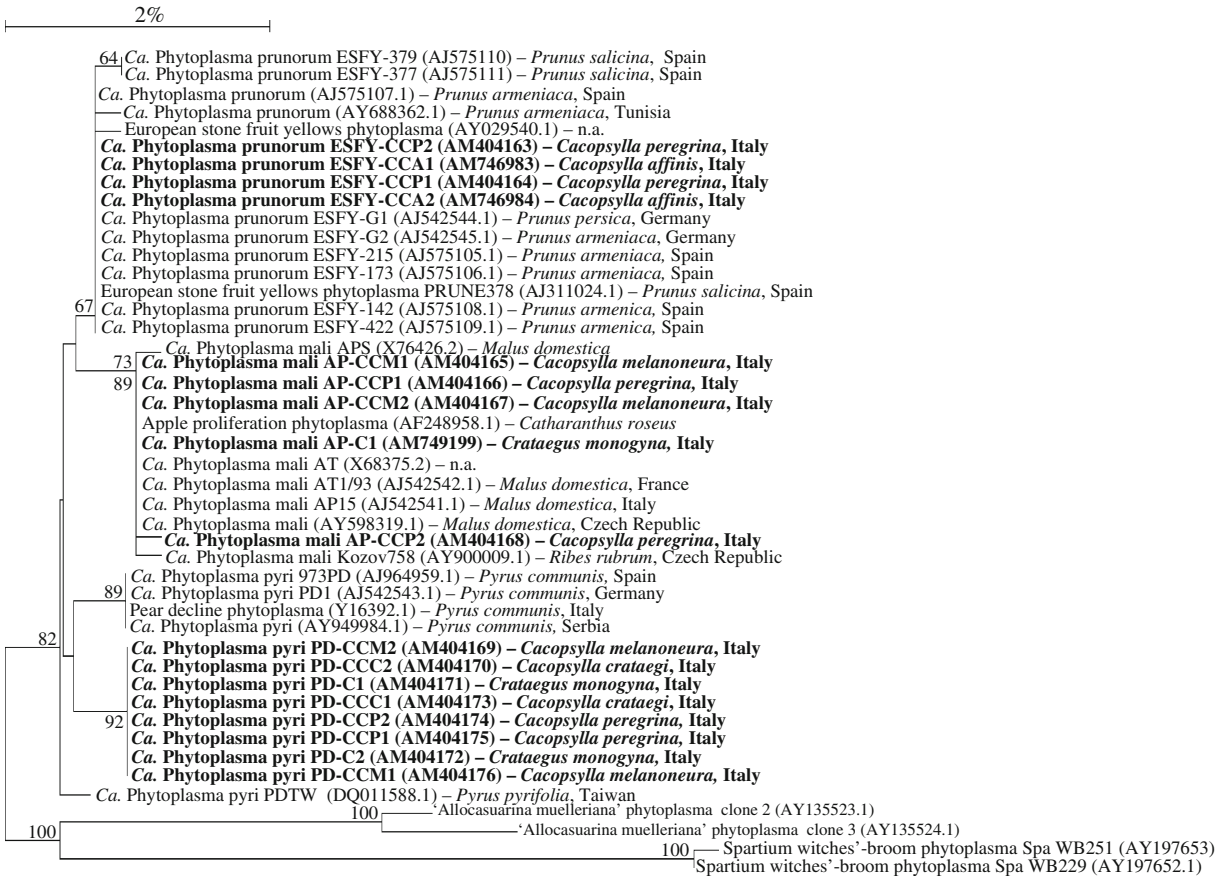

Fig. 4 Phylogenetic relationship of members of the apple proliferation phytoplasma group determined by neighbour-joining algorithm of partial 16s rRNA gene sequences. Bootstrap values were determined from 1,000 replicates. Bar represents 2% substitutions/site rate

explanation of the infection of *Cr. monogyna* by '*Ca.* Phytoplasma mali'. On the contrary, the absence of pear and stone fruit orchards in the region make old abandoned fruit trees and those in family gardens the only possible original infection source of '*Ca.* Phytoplasma pyri' and '*Ca.* Phytoplasma prunorum'.

Cacopsylla melanoneura collected on *Cr. monogyna* was biologically similar to that collected on apple trees and colonises the two hosts in the same periods (Tedeschi et al. 2002). Also on *Cr. monogyna* the overwintered adults have an important role because of their prolonged residence and their greater abundance than newly-emerged adults. In fact, the new generation is larger than the overwintering one, which loses many individuals to estivation and hibernation; however, it appears smaller because these individuals rapidly move to alternative hosts after emerging (Tedeschi et al. 2002).

Cacopsylla peregrina, that overwinters as eggs, emerged in our region at the end of April, almost simultaneously with the new generation of *C. melanoneura*, but the two species are easily distinguishable because of the colouring. After the population peaked at the beginning of June, densities rapidly decreased. This is due to the migration to alternative host plants during the summer (Lauterer 1999).

The temporal differences in seasonal colour changes confirmed the observations of Sutton (1983) and were correlated with the development of gonads in both sexes. Sutton asserted that males become sexually mature and migrate to the inner hawthorn crowns about 3 weeks earlier than the females, before mating and laying eggs; on the contrary the dark colouration of mature individuals mimics the bark of the basal regions of branches, their preferred resting spot. Immature adults are green and live on hawthorn leaves.

At present no information is available on the seasonal abundance of phytoplasmas in hawthorn plants, but it is likely that the phytoplasma concentration is higher at the end of the summer as happens in other rosaceous plants (Shaper and Seemüller 1982; Jarausch et al. 1999). This should be taken into account should the transmission activity of *C. peregrina* be proven, considering the second peak occurring in the second half of September.

The present work opens new perspectives in the study of the epidemiology of fruit tree phytoplasmas. First, transmission trials are now required to prove the ability of the four psyllid species to transmit to healthy plants. Moreover the ability of *C. peregrina*, *C. affinis* and *C. crataegi* to feed on plants different from *Cr. monogyna*, even occasionally, should also be investigated, to define the possible risk of phytoplasmas spreading. Moreover, since AP group phytoplasmas were detected in hawthorn samples, in-depth studies should be carried out on *Cr. monogyna* to better understand its role as a source of both the vectors and the phytoplasmas. Finally, the analysis of the partial 16S rRNA gene sequences confirmed the homology of the phytoplasmas detected in the psyllids with the AP and ESFY agents, while unusual results were obtained with samples ascribed to '*Ca.* Phytoplasma pyri'. The lower homology observed in this species, and the analysis of its phylogenetic relationship to other phytoplasmas, suggests the presence of a new phytoplasma subtype related to *Cr. monogyna* and its psyllid fauna or a divergent '*Ca.* Phytoplasma pyri' strain of the region. Further investigations are required to confirm these assumptions.

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