Cacopsylla melanoneura Has No Relevance as Vector of Apple Proliferation in Germany

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ABSTRACT

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Long-term field surveys on the distribution and natural infection rates of *Cacopsylla melanoneura* were carried out in commercial and abandoned apple-proliferation-infected orchards throughout Germany, northern Switzerland, and eastern France. Although the infection rates of some orchards reached up to 80%, only 0.09% of all *C. melanoneura* collected on apple were infected by the pathogen ‘*Candidatus Phytoplasma mali*’. Despite higher population densities, no infected individual was found on wild hawthorn. Individuals of *C. melanoneura* were not able to transmit phytoplasmas to healthy plants, and even the acquisition of ‘*Ca. P. mali*’ from infected plants was very inefficient. Quantitative

real-time polymerase chain reaction demonstrated that the very few infected individuals of *C. melanoneura* harbored phytoplasma concentrations 10,000 times lower than individuals of *C. picta*, the main vector species in Germany. Oviposition bioassays showed that hawthorn is the preferred reproduction host plant for *C. melanoneura* in Germany, not apple. Because hawthorn is not a suitable host plant for ‘*Ca. P. mali*’, it does not play a role in the spread of apple proliferation. In contrast to data reported from northwestern Italy, *C. melanoneura* developed on either apple or hawthorn has no relevance as a vector of apple proliferation in Germany. The existence of epidemiologically different populations is proposed.

Additional keywords: ‘*Candidatus Phytoplasma mali*’.

Phytoplasmas are bacteria lacking a cell wall that can live in two different hosts, plants and vector insects. They are responsible for several hundred plant diseases worldwide that have large economic impact (29). The phytoplasma species belonging to the apple proliferation (AP) group (AP, pear decline, and European stone fruit yellows) are the economically most important fruit tree phytoplasmas and are widespread in the temperate regions of Europe (48). ‘*Candidatus Phytoplasma mali*’ is associated with AP disease (49) which causes major economic yield losses by inducing ‘witches brooms’ and tasteless undersized fruit in apple (*Malus domestica* Borkh.) (24).

Different psyllid species belonging to the taxon *Cacopsylla* Ossiannilsson 1970 (Hemiptera: Psyllidae) are responsible for the transmission of European fruit tree phytoplasmas. *Cacopsylla pyricola* (Förster) and *C. pyri* (L.) have been reported to transmit pear decline, whereas *C. pruni* (Scopoli) is the vector of the European stone fruit yellows (9–12,14,26,31). Psyllid species are also considered the main vectors for AP even though the leafhopper *Fieberiella florii* (Stål) has also been reported as a vector (27,53). In recent years, it was published that the univoltine psyllid species *C. picta* (Förster) (synonym *C. costalis*) is the vector for ‘*Ca. P. mali*’ in northeastern Italy (19) and Germany (22). Additionally, the hawthorn psyllid *C. melanoneura* (Förster) was identified as another vector of this disease in northwestern Italy (52,54,55). Although the vector ability of *C. picta* is indisputable because successful transmission trials have been con-

ducted for populations from different regions (7,19,21,22), the role of the hawthorn psyllid *C. melanoneura* for the spread of AP in other apple-growing regions in Central Europe remains inconsistent. Cases of transmission of ‘*Ca. P. mali*’ by *C. melanoneura* were reported from the Aosta Valley (52,54) and from Trentino, Northern Italy, although yielding a transmission rate of only 0.36% in Trentino (37). Due to the high economic impact of AP in the important apple-growing regions of Northern Italy and due to the quarantine status of ‘*Ca. P. mali*’, a compulsory clearing of infected apple trees recently has been regulated by law in several Italian regions (17).

C. melanoneura is an oligophagous species which was recorded from apple and pear trees (13,43), although its main host is hawthorn (*Crataegus* spp.) (24,25,43). However, thus far, no precise data were published describing the risk which could arise from potentially infected hawthorn plants as well as from *C. melanoneura* populations on such infected plants with regard to the spread of ‘*Ca. P. mali*’ to apple orchards. Several authors described briefly the population dynamics of *C. melanoneura* on apple (37,47,48,54). Mayer and Gross (38) first analyzed the whole univoltine life cycle of this species after a thorough monitoring of two reproductive seasons. They described two obligatory migratory phases and the host plants mandatory during the life cycle of *C. melanoneura*. Here, according to Mayer and Gross (38), we introduced some new definitions in order to improve the clarity of the wording used by different authors. After their development from egg to adult on their reproduction host plants (plants on which the juvenile development occurs: apple and hawthorn), the newly hatched adults leave (first migratory phase) by switching primarily to transitional hosts plants (plants only used during migration from their reproduction host to their overwintering host or vice versa: lowland conifers) before reaching their overwinter-

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ing hosts (main host plant for feeding, but also used for overwintering: mountainous conifers). After overwintering, returning mature adults (second migratory phase) initially fly to lowland conifers (transitional host) before finally shifting to their reproduction hosts for oviposition. In this article, we will use the terms “reproduction hosts” and “overwintering hosts” for the two groups of host plant mandatory during the life cycle of *C. melanoneura*. Further, we introduce the terms “emigrant” for a young adult of *C. melanoneura* between hatching and maturation (this includes the first migratory phase, the emigration to overwintering host plants) and “remigrant” for a mature adult, including copulation, the second migratory phase (remigration to hawthorn or apple), and oviposition, until its death.

In the present study, we evaluated the risk of *C. melanoneura* for apple-growing areas in Germany and neighboring countries. *C. melanoneura* spends most of the year on conifers, whereas reproduction and juvenile development takes place in early spring (28,43) on either hawthorn (*Crataegus* spp. L.) or apple (*M. domestica*). Thus, our study focused on two main objectives: (i) the investigation of hawthorn (*Crataegus monogyna* Jacq.) as reservoir for ‘*Ca. P. mali*’ and (ii) the evaluation of the German population of *Cacopsylla melanoneura* on transmission ability for ‘*Ca. P. mali*’.

Vector insects often transmit phytoplasmas in a persistent manner (8,44). Thus far, all evidence of phytoplasma dissemination within insects has been provided for leafhoppers only (30,32, 42,51). Following acquisition from the infected source plant, there is a latent period before the vector is able to transmit. During this time, the phytoplasmas multiply in midgut, haemocytes, and salivary glands of the insect (30,56). Transmission mechanisms of phytoplasmas by leafhoppers and psyllids are similar (36); therefore, we suspect that the multiplication mechanisms in these vectors are similar as well. To prove the real vector capacity of an insect species, transmission trials under controlled conditions are essential. Determination of the phytoplasma concentration in infected insects might be a supplementary tool to assess the vector ability of a species. Today, quantification of phytoplasma can be achieved by real-time polymerase chain reaction (PCR). Specific quantitative PCR assays for ‘*Ca. P. mali*’ (2,24) were used in the present study for measuring the phytoplasma titers in the two known insect vectors of ‘*Ca. P. mali*’, *C. melanoneura* and *C. picta*.

Recent studies demonstrate that *C. picta*, the main vector of ‘*Ca. P. mali*’ in Germany, uses olfactory cues (e.g., β -caryophyllene) emitted from plants for host recognition (39,40). Thus, we tested the role of chemical compounds emitted by the two reproduction host plants (apple and hawthorn) of *C. melanoneura* using oviposition bioassays.

MATERIALS AND METHODS

Collection of insect and plant material. From 2002 to 2007, regular insect collections were undertaken from February to September at least every 2 weeks. Insects were collected in cultivated and uncultivated apple orchards (*M. domestica*) in different apple-growing regions of Germany, eastern France (Alsace), and northern Switzerland. The number of collection sites per region and year are specified in Table 1. In total, 51 different sites were investigated, which corresponds to 32 commercial apple orchards managed according to the principles of integrated pest management (IPM), six commercial apple orchards managed according to the principles of organic production, and 13 untreated abandoned or scattered orchards. The infections by ‘*Ca. P. mali*’ were monitored by recording typical symptoms of AP disease such as witches’ brooms and enlarged stipules. The infection rates by ‘*Ca. P. mali*’ of sampling sites have been monitored in the years 2003 and 2005. These data were used to group the orchards into different infection classes.

Insects used in transmission trials were collected predominantly in one commercially managed (IPM) and two untreated orchards. The infection rate of AP in the commercial orchard was monitored regularly. Between 2002 and 2006, it increased from 41 to 57%. In the abandoned orchards, the unpruned trees hardly showed typical symptoms of infections by ‘*Ca. P. mali*’. However, PCR testing of random samples indicated that infection rates were between 40 and 80%.

Between 2002 and 2008, irregular collections of *C. melanoneura* were carried out on hawthorn (*Crataegus monogyna*) plants at specific locations next to infected apple orchards in southwest Germany (Table 2). The infection rates in the apple orchards were 10% in Rheinhessen, 20 to 80% in Vorderpfalz (abandoned untreated orchard), and 30% in Nordbaden. All insect collections were done using the beating tray method according to Muther and Vogt (41). After sampling, the collected insects were counted, stored in vials, and frozen at -20°C for later identification. Determination of the collected psyllids followed the key of Ossiannilsson (43).

Shoot and root samples of hawthorn bushes (*C. monogyna*) were collected at sites of psyllid collections (Table 3) in the period from September to February between 2001 and 2008. Their age ranged from young 4-year-old plants to old bushes for which no exact age could be estimated. For infection rates of neighboring apple orchards, see preceding paragraph. No data were available for Alsace.

Experimental infection of hawthorn with AP. Grafting experiments were carried out at Julius-Kühn Institute at Dossenheim, Germany, for testing the susceptibility of hawthorn to an

TABLE 1. Number of individuals of *Cacopsylla melanoneura* collected on *Malus domestica* in different apple-growing regions of Germany, eastern France (Alsace), and Northern Switzerland (Aargau, Solothurn) between 2002 and 2007^a

Region	No. of sites	2002	No. of sites	2003	No. of sites	2004	No. of sites	2005	No. of sites	2006	No. of sites	2007	Total
Schleswig-Holstein	nt	...	3	3	2	11	2	15	nt	...	nt	...	29
Rheinland	1	8	1	0	1	8	1	5	nt	...	nt	...	21
Rheinhessen	3	17	5	134	2	6	3	71	nt	...	nt	...	228
Vorderpfalz	7	155	14	594	8	253	6	169	2	32	2	15	1,218
Südpfalz	nt	...	nt	...	nt	...	4	60	4	2	4	13	75
Nordbaden	4	42	2	109	2	148	2	123	2	62	2	4	488
Mittelbaden	2	4	1	69	nt	...	2	832	nt	...	nt	...	905
Südbaden	nt	...	nt	...	nt	...	1	116	1	21	nt	...	137
Weinsberg	nt	...	1	2	1	1	nt	...	nt	...	nt	...	3
Bodensee	nt	...	4	189	5	91	1	58	3	10	nt	...	348
Elsass	nt	...	nt	...	nt	...	3	132	3	27	3	2	161
Aargau	nt	...	nt	...	nt	...	1	552	1	46	1	50	648
Solothurn	nt	...	nt	...	nt	...	1	151	1	29	1	0	180
PCR+/total tested	...	3/226	...	0/1,100	...	0/518	...	1/2,284	...	0/229	...	0/84	4/4,441

^a Rate of polymerase chain reaction (PCR)-positive (PCR+) psyllids for ‘*Candidatus Phytoplasma mali*’ is shown on the last line; nt = not tested.

infection with ‘*Ca. P. mali*’. Ten 3-year-old potted hawthorn bushes were grafted each with two root scions of AP-infected apple plants (cv. Golden Delicious) in January 2007 and maintained in an insect-free temperate greenhouse chamber (day and night temperatures = 20 and 15°C, respectively). In the following months, the shoots of the hawthorn plants and the apple shoots which proliferated from the scions were examined for typical symptoms of AP disease (premature reddening of leaves, enlarged stipules, and witches’ brooms). Subsequently, the DNA of the phloem tissue was extracted to obtain material for PCR analysis to control whether ‘*Ca. P. mali*’ was transmitted to hawthorn 2, 5, and 11 months after grafting.

Transmission trials. All transmission trials were carried out at AIPanta-IPR at Neustadt/W., Germany, using psyllids captured in specific apple orchards mentioned above and on several hawthorn bushes adjacent to one of the abandoned apple orchards. Healthy and ‘*Ca. P. mali*’-infected micropropagated apple plants were used for the bioassays as described by Jarausch et al. (23,24). After inoculation by psyllids, all test plants were treated with insecticides (Confidor at 0.16 g/liter or Calypso at 0.2 ml/liter) and maintained in the greenhouse under insect-proof conditions. The numbers of test plants and psyllids are given in Tables 4 and 5. The test plants were regularly checked for symptom expression and analyzed by PCR for infection with ‘*Ca. P. mali*’ 6, 12, and 18 months after the inoculation process.

Transmission trials with field-collected *Cacopsylla melanoneura*. Natural infectivity was determined in remigrants (overwintered individuals) collected from both hawthorn plants and

infected apple trees at the beginning of the remigration period between end of February and middle of March. Additionally, emigrants (newly hatched adults) were collected in May from infected apple trees just after adult emergence. Groups of 5 to 20 specimens were caged on micropropagated healthy apple plants in glass vessels or cages for 2 to 4 weeks as described by Jarausch et al. (21). The vessels were closed with an anti-aphid mesh and, after the inoculation period, dead psyllids were recollected from cages and frozen at –20°C until DNA extraction.

Acquisition trials with reared *Cacopsylla melanoneura*. The acquisition and transmission capacity of remigrants and emigrants was examined under controlled conditions in the greenhouse. Field-collected remigrants of *Cacopsylla melanoneura* from apple and hawthorn were first exposed to infected apple plants for acquisition access periods (AAP) of 2 to 14 days. Dead specimens were promptly removed from the cages and frozen at –20°C until DNA extraction. Groups of 5 to 20 surviving individuals were subsequently transferred to single healthy micropropagated apple plants in glass vessels for transmission and collected after death for DNA extraction. The acquisition and transmission behavior of *C. melanoneura* emigrants was examined separately using newly hatched adults obtained from greenhouse rearing on caged plants of either healthy apple or hawthorn. Groups of 5 to 20 specimens were caged in glass vessels on micropropagated apple plants infected with ‘*Ca. P. mali*’ (23) for AAP of 2 to 14 days. After acquisition feeding, surviving individuals were collected from the infected plant and transferred to a healthy micropropagated apple test plant for transmission. After the inoculation period, dead

TABLE 2. Number of individuals of *Cacopsylla melanoneura* collected on hawthorn (*Crataegus monogyna*) at different sites in Germany and tested for phytoplasma infection by polymerase chain reaction (PCR)^a

Region	Site	Year ^b							Total
		2002	2003	2004	2005	2006	2007	2008	
Rheinhausen	Laurenziberg	...	...	...	...	...	37	...	37
Vorderpfalz	Neustadt/W.	1,660	139	857	1,827	920	473	...	5,876
Nordbaden	Dossenheim	...	...	...	...	...	...	240	240
PCR+/total tested	...	0/1,660	0/139	0/857	0/1,827	0/920	0/510	0/240	0/6,153

^a The rate of PCR-positive (PCR+) psyllids for ‘*Candidatus Phytoplasma mali*’ is shown on the last line.

^b Number of individuals of *C. melanoneura* tested.

TABLE 3. Screening of phloem samples from hawthorn (*Crataegus monogyna*) for infections with ‘*Candidatus Phytoplasma mali*’^a

Region	Site	Phloem from		PCR+ ^b	
		Root	Shoot	fU5/rU4	Nested PCR P1P7/fO1/rO1
Rheinhausen	Laurenziberg	0	1	0	nt
Vorderpfalz	Weisenheim	0	2	0	nt
	Meckenheim	0	7	0	nt
	Neustadt/W.	0	17	0	nt
Nordbaden	Ladenburg	7	12	nt	0
	Dossenheim	39	0	nt	0
Elsass (F)	Roppenheim	0	5	0	nt
Total	...	46	44	0	0

^a Root and shoot samples were collected around apple-proliferation-affected apple orchards in different apple-growing regions.

^b Polymerase chain reaction (PCR) positive; nt = not tested.

TABLE 4. Summary of transmission trials (2002–2007) with adults of *Cacopsylla melanoneura* collected on apple (*Malus domestica*)

Type of trial	PCR+/total ^a		Transmission (%)
	<i>C. melanoneura</i>	<i>Malus</i> test plants	
Transmission (natural infection)			
Overwintered remigrants	3/409 (0.7%)	0/72	0
Emigrants (young adults)	0/95	0/15	0
Acquisition feeding + transmission			
Overwintered remigrants	3/111 (2.7%)	0/4	0
Emigrants (young adults)	0/67	0/8	0

^a Polymerase chain reaction-positive (PCR+) *C. melanoneura*/total number tested. The three PCR+ individuals of field-collected *C. melanoneura* are identical to those mentioned in Table 1 for the year 2002.

psyllids were removed from the cages and frozen at -20°C until DNA extraction.

DNA extraction. *Insect material.* The extraction procedure followed mainly the protocol of Doyle and Doyle (16). Single insects collected from apple or batches of five insects of the same sex collected from hawthorn were homogenized with a conical pestle (PP 1.5 ml; Eppendorf, Hamburg, Germany) in 1.5-ml centrifugation tubes containing 50 to 250 µl of extraction buffer and in, some cases, a pinch of silicon carbide (Carborundum; Sigma-Aldrich, Munich, Germany) as grinding additive. The resulting DNA pellet was resuspended in 25 µl (single insects) or 50 µl (batches) of sterile water and stored at -20°C.

Plant material. Two different protocols were used for DNA extraction from plant tissues (0.3 g of leaf midribs, phloem from shoots or phloem from roots). DNA extraction of plant material generally followed the protocol described above by Doyle and Doyle (16). The second extraction method was employed because the reaction was inhibited by hawthorn compounds. In these cases, the silica capture method protocol for total nucleic acids extraction described by Rott and Jelkmann (46) was adopted. The resulting DNA was resuspended in 100 to 150 µl of sterile water and stored at -20°C. In both protocols (16,46), the extraction buffers contained 2-mercapto ethanol as antioxidation agent. In this study, this chemical was exchanged with sodium metabisulfide (2%).

PCR analysis. PCR amplification of phytoplasma 16S rDNA was routinely carried out using universal ribosomal primers fU5/P7 (34,47) on DNA samples from psyllids. Samples showing unclear results in direct PCR were further analyzed with nested PCR. For this, a 'Ca. P. mali'-specific assay described by Jarausch et al. (25) was used. Briefly, nonribosomal primers AP5/AP6 were employed for a first PCR and 1 µl of the PCR product was subjected to a second PCR with the specific primers AP3/AP4. The cycle conditions were as described by Jarausch et al. (25). Ribosomal DNA from insects collected from hawthorn in 2008 was amplified with nested PCR using the universal primers P1/P7 (15,47) and then the AP-group-specific primer pair fO1/rO1 on 1 µl of the first amplicon (34). DNA extracts of plant samples were analyzed in direct PCR with universal, ribosomal primers fU5/rU4 (1,34) or specific primers fAT/rAS (50) as described. DNA from some hawthorn samples (Dossenheim, Ladenburg, transmission trial after 11 months) was subjected to nested PCR using the primers P1/P7 and fO1/rO1 as mentioned above. Aliquots (5 to 8 µl) of each PCR product were electrophoresed on a 1% agarose gel containing ethidium bromide at 0.3 µg/ml in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The DNA was visualized and photographed while exposed to UV light (302 nm).

Quantitative real-time PCR. The phytoplasma concentrations in infected individuals of *C. melanoneura* and *C. picta* were determined by quantitative real-time PCR. For this, the assay based on SYBR Green technology published by Jarausch et al. (24) was used. This assay amplifies a specific gene fragment of 'Ca. P. mali' with the primers AP3/AP4. A 10-fold dilution series of 10⁸ to 10¹ of a plasmid carrying the target gene fragment of 'Ca. P. mali' was carried out in PCR-negative DNA extracts of

C. melanoneura. Naturally infected individuals of *C. picta* were used as a positive control. The quantitative PCR assay was carried out on a Chromo4 Real-Time PCR detector (MJ Research, Waltham, MA) as described. The phytoplasma concentration determined in the sample was referred to as the phytoplasma concentration in one single individual. The differences between phytoplasma titers of the two species of *Cacopsylla* were compared by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc tests (STATISTICA 5.5; StatSoft Inc., Tulsa, OK).

Oviposition bioassays. *Test insects.* Psyllids were collected from *Picea abies* L. (Weißer Stein, Dossenheim, Germany) on 28 February and 5 March before remigration to *Crataegus* spp. Even though the psyllids already showed frequent mating behavior, they were kept in a cage on potted *P. abies* plants in a tempered greenhouse chamber (10°C constant) until 9 to 17 March to increase their motivation for oviposition. Because only one *C. affinis* male (0.65%) was detected among all collected male psyllids, any risk of contamination with *C. affinis* females should be insignificant.

Experimental setup. Oviposition preferences of gravid *C. melanoneura* females were tested in dual choice bioassays. Individual females were set in transparent plastic boxes (Mepal-Rosti, 19 by 15 by 9 cm; Tischler Gastro, Heidelberg, Germany), each together with two twigs (length = 5 cm) of either apple and hawthorn, apple and plum, or hawthorn and plum, which were drawn into water-filled glass vials sealed with Nescofilm (Bando Chemical Inc. Ltd, Japan). The boxes were covered by a lid with a ventilation hole (≈9.5 cm, closed by Saran tissue, mesh width = 0.4 mm). Each experimental setting started with 15 females but, due to mortality, the number of valid repeats reduced. The oviposition set-up was placed in a climate chamber at 20°C and a light regimen of day and night = 12 h each. After 72 h, the number of eggs deposited on the twigs was counted by dissecting the material under a binocular.

RESULTS

Natural infection rates of *C. melanoneura*. For the monitoring of the field populations of psyllids, different apple orchards with a wide range of infestation by 'Ca. P. mali' have been selected. This spectrum included commercial orchards with integrated production or organic farming, as well as field sites of abandoned or scattered orchards without any treatment. The infection rates in orchards with integrated production varied from <1% to >50% (Table 6). The organically grown orchards showed lower infection rates of only 5%. In contrast, abandoned or scattered orchards were highly AP infected. The 7-year-long survey of the psyllid populations in these different orchards demonstrated that *C. melanoneura* was present in all investigated regions in Germany, eastern France, and northern Switzerland (Table 1). Despite the regular presence of *C. melanoneura*, its population densities and dynamics showed strong variations among the different areas and years of investigation. Coincidentally for all investigated areas, the highest number of individuals was collected in 2005 followed by a severe breakdown of the popu-

TABLE 5. Summary of transmission trials (2002–2007) with adults of *Cacopsylla melanoneura* collected on hawthorn (*Crataegus monogyna*)

Type of trial	PCR+/total ^a		Transmission (%)
	<i>C. melanoneura</i>	<i>Malus</i> test plants	
Transmission (natural infection)			
Overwintered remigrants	0/173	0/6	0
Emigrants (young adults)	nt	nt	nt
Acquisition feeding + transmission			
Overwintered remigrants	3/1,359 (0.2%)	0/10	0
Emigrants (young adults)	0/392	0/9	0

^a Polymerase chain reaction-positive (PCR+) *C. melanoneura*/total number tested; nt = not tested.

lation in the years 2006 and 2007. The examination by PCR of more than 3,400 individuals of *C. melanoneura* collected over a period of 6 years from different German sites, supplemented by 828 individuals from northern Switzerland and 161 individuals from eastern France collected and examined over 3 years, revealed only four natural infections by 'Ca. P. mali' (0.09%) (Table 1). In 2002, three infected individuals were found in the region Vorderpfalz and, in 2005, one individual captured in Alsace tested positive for 'Ca. P. mali'. As shown in Table 6, all infected psyllids were caught in highly AP-infected orchards. The examination of nearly 6,000 individuals of *C. melanoneura* collected in the region of Neustadt on hawthorn over a period of 7 years, and supplemented by collections in Rheinhessen (2007) and Dossenheim (2008), revealed no infections (Table 2).

Transmission trials with *C. melanoneura* collected on apple.

A small number of young emerged emigrants was analyzed just before starting their migration to the overwintering hosts in early summer. In all, 3 of 409 remigrants (0.7%) (Table 4) collected from apple were infected with 'Ca. P. mali', whereas none of the field-collected emigrants bore the agent. Likewise, no test plant became infected and, thus, no transmission occurred.

In order to elucidate the potential vector capacity of remigrants and emigrants of *C. melanoneura*, induced acquisition feeding and subsequent direct transmission were studied under controlled conditions. Only 3 of 111 remigrants and 0 of 67 young emigrants acquired the phytoplasma from infected plants under forced conditions (Table 4), corresponding to an acquisition rate of 2.7% for remigrants and no acquisition for emigrants, respectively. In no case did healthy test plants become infected during the incubation period.

Transmission trials with *C. melanoneura* collected on hawthorn. The natural infection rate and the retention of infectivity was investigated in overwintered adults of *C. melanoneura* ($n = 173$) collected from wild hawthorn bushes in the surroundings of apple orchards in early spring at the beginning of the remigration period. None of the overwintered adults was infected with the pathogen (Table 5) and, thus, no transmission appeared.

Further, the potential vector behavior was compared for individuals of *C. melanoneura* collected from apple with specimens derived from hawthorn in order to determine biological differences among these two populations. Comparable acquisition or transmission trials were carried out as described for *C. melanoneura* captured from apple (see above). The results of acquisition and transmission trials are summarized in Table 5. Only 3 of 1,359 remigrants as well as 0 of 392 emigrants, which were tested after both passages (acquisition and subsequent transmission feeding), acquired the agent from the inoculum plants. In no case did a transmission from an infected *Malus* to a healthy *Crataegus* test plant occur.

Determination of the concentration of 'Ca. P. mali' in infected individuals of *C. melanoneura* and *C. picta*. The mean

phytoplasma concentrations in infected individuals of *C. melanoneura* determined by quantitative real-time PCR are given in Figure 1 (white bars). The number of phytoplasmas in one individual of *C. melanoneura* collected from apple ($n = 1$) was <40,000 and could be increased in forced acquisition experiments on apple to an amount of $\approx 600,000$ (*C. melanoneura* collected from apple, $n = 3$) or 90,000 (*C. melanoneura* collected from hawthorn, $n = 3$) phytoplasmas per insect. These titers of phytoplasma concentrations in individuals of *C. melanoneura* are much lower than those determined in remigrated individuals of *C. picta* collected from apple (Fig. 1, black bar). The number of phytoplasmas ('Ca. P. mali') in individuals of *C. picta* ($n = 7$) reached an average of 325 million, corresponding approximately to a titer 10,000 times higher compared with the natural infection rates of *C. melanoneura*. The mean number of phytoplasmas found in *C. melanoneura* (total of all treatments = 311,414 phytoplasmas/insect) was significantly different from the number found in *C. picta* (one-way ANOVA: $df = 1$, $F = 36$, $P < 0.001$; followed by LSD post hoc test; $P < 0.001$).

Determination of natural infection of hawthorn by 'Ca. P. mali'. The examination of roots and shoots of 90 hawthorn bushes sampled in different regions showed neither visual symptoms nor positive results in single PCRs, regardless of which general phytoplasma primers were used (P1/P7 or fU5/rU4). Moreover, the samples from Nordbaden which were additionally analyzed by nested PCR using primers specific for fruit tree phytoplasmas (fO1/rO1) revealed no infections as well (Table 3).

Experimental inoculation of hawthorn with 'Ca. P. mali'. In all, 9 of 10 hawthorn plants developed connections to both grafted apple scions. Eleven scions on eight test plants had sprouted 2 months after grafting. Some of the grafted apple twigs developed typical symptoms of AP disease. Nevertheless, this was not observed with hawthorn twigs. Furthermore, leaves of apple scions of six plants were colored red. Additionally, on two plants, the leaves of the scions had enlarged stipules and, on one plant, a witches' broom was formed. PCR analysis of phloem material from newly developed shoots of the apple scions with 'Ca. P. mali'-specific primers fAT/rAS revealed infections on six plants. The observed symptoms on the shoots of the scions corresponded mainly with the results of the PCR analysis. PCR analyses of the hawthorn rootstocks were carried out with the same primers 2 and 5 months after grafting of the infected apple scions. 'Ca. P. mali' was never detected in any of the 10 test plants. In order to ensure that negative results of the PCR analysis were not generated by a very low titer of 'Ca. P. mali', the more sensitive nested PCR (3) was used for the analysis 11 months after grafting (primers P1/P7 followed by fO1/rO1), but no infection of hawthorn could be determined.

Natural population densities of *C. melanoneura* on apple or hawthorn. The comparison of the number of *C. melanoneura* remigrants collected from one specific hawthorn site in south-

TABLE 6. Relationship between infection status of the orchard and number of phytoplasma-infected *Cacopsylla melanoneura*

Type of orchard	No. of orchards	Years of sampling	Classes of infection	No. tested ^a	No. PCR+ ^b	Infection (%)
Organic farming	2	2004–2005	<1	20	0	0
Organic farming	4	2002–2005	1–5	112	0	0
Integrated production	3	2003–2007	<1	44	0	0
Integrated production	8	2002–2006	1–5	616	0	0
Integrated production	7	2002–2007	5–10	1208	0	0
Integrated production	7	2002–2007	10–20	1049	0	0
Integrated production	4	2002–2007	20–30	313	0	0
Integrated production	2	2005–2007	30–50	218	1	0.5
Integrated production	1	2002–2007	>50	313	2	0.6
Abandoned (untreated)	7	2002–2006	25–50	369	0	0
Abandoned (untreated)	6	2002–2005	>50	179	1	0.6
Total	51	...	...	4,441	4	0.09

^a Number of *C. melanoneura* individuals tested.

^b Polymerase chain reaction-positive (PCR+).

western Germany (Vorderpfalz) at the beginning of the remigration period demonstrated that the population density on hawthorn was much higher than on apple (Table 7). The data assembled in Table 7 picture the situation of the population density of overwintered *C. melanoneura* which were collected in the vicinity of Neustadt in a commercial apple orchard and an adjacent hawthorn site during the peak of population of remigrants (March). Influence of pesticide treatment could largely be excluded because sampling took place before the season's first treatments.

To permit a reliable comparison, numbers of captured individuals are presented as individuals per branch. This value takes into account the heterogeneous numbers of branches sampled on apple (100 per date) and hawthorn (25 per date) as well as the nonidentical frequencies for psyllid sampling on the two host plants. The calculated number of individuals per branch clearly

shows that, during the peak of remigration (middle March), the population density on hawthorn was ≈ 50 to 150 times higher than on apple. This result underlines that hawthorn is the main host plant for *C. melanoneura* and that, under natural conditions, it is preferentially frequented by remigrants (28,43).

Oviposition bioassays. The numbers of eggs laid by gravid females of *C. melanoneura* caught on *P. abies* just before remigrating to their reproduction hosts were determined in oviposition bioassays and are presented in Figure 2. The *C. melanoneura* females significantly preferred leaves of hawthorn to those of apple for oviposition (Fig. 2A). Furthermore, they also preferred leaves of hawthorn and apple (confirmed reproduction host plants) to those of the nonhost plant plum (Fig. 2B and C). The number of eggs laid on apple compared with hawthorn was significantly reduced.

Fig. 1. Mean concentrations and standard deviations of ‘*Candidatus Phytoplasma mali*’ numbers in the body of *Cacopsylla melanoneura* remigrants collected from *Malus* or *Crataegus* spp. and of naturally infected *C. picta* remigrants from *Malus* spp. as determined by quantitative real-time polymerase chain reaction. *C. melanoneura* was either naturally infected or experimentally infected by feeding on infected *Malus* spp. The limit for successful transmission as published by Jarausch et al. (20) is indicated by a dotted line. The mean number of phytoplasmas found in *C. melanoneura* (total of all treatments [white bars]: 311,414 phytoplasmas/insect) differs from the number found in the hemolymph of *C. picta* (statistically significant; one-way analysis of variance: $df = 1$, $F = 36$, $P < 0.001$, followed by least significant difference post hoc test, $P < 0.001$).

DISCUSSION

The first objective of our work was to study the possibility of whether hawthorn (*Crataegus monogyna*), the main reproduction host of *Cacopsylla melanoneura*, could serve as an additional reservoir for 'Ca. P. mali', promoting the spread of the phytoplasma within the vector population. This would be of epidemiological relevance and could be detrimental to apple growers. Thus, we analyzed the natural infection rates of wild hawthorn plants by 'Ca. P. mali'. No natural infection of hawthorn could be revealed and it was not possible to infect hawthorn plants experimentally; therefore, we concluded that wild *Crataegus monogyna* plants do not represent a suitable reservoir for 'Ca. P. mali'.

The results of the behavioral bioassays revealed that remigrants of *Cacopsylla melanoneura* which returned from their overwintering hosts preferred hawthorn plants to apple trees for oviposition. This implies that they will reproduce preferentially on hawthorn and will settle on apple plants only occasionally. As a result, there is a reduced probability for transmission of 'Ca. P. mali' between *C. melanoneura*'s two host plants, apple and hawthorn. Our investigations of *C. melanoneura* populations in southwestern Germany support these findings by showing that densities on hawthorn were more than 10 times higher than on apple in abandoned orchards (38) and 50 to 100 times higher than on apple in conventionally managed orchards. Only when females had no possibility to oviposit on hawthorn did they accept apple (Fig. 2). However, the number of deposited eggs decreased to only half the number laid on hawthorn. In conclusion, *C. melanoneura* strongly prefers hawthorn as its main host plant for reproduction. In case no hawthorn is available for oviposition or after accidental settling on apple, females are capable of learning host-specific cues and changing their preference to apple, another suitable host plant for reproduction (38). These results explain the small but existing numbers of *C. melanoneura* reproducing on apple in

Germany. Consequently, because *C. melanoneura* primarily reproduces on hawthorn (38), the major proportion of the population will never get into contact with 'Ca. P. mali' and, therefore, will neither acquire nor transmit it.

The second objective of the present study was to investigate the vector ability of *C. melanoneura* in Germany and neighboring regions and, thus, to assess the risk for the spread of 'Ca. P. mali' by this psyllid species. Although *C. picta* was first described as a vector of 'Ca. P. mali' in Trentino (19), Tedeschi et al. (54) identified *C. melanoneura* as the main vector of the agent in northwestern Italy. During a 3-year survey, they determined the population dynamics of *C. melanoneura* in apple orchards by regular collections between January and July. Using the beating tray method, they always found more remigrants than emigrants, showing the highest population densities of the overwintered remigrants in mid-March (54,55). Similar population dynamics were reported for Trentino (37). These data correspond to the population dynamics of *C. melanoneura* in Germany (38). In that study, a population peak of the remigrants was detected in mid-March but only low numbers of emigrants from mid-May to the beginning of July. In the present study, $\approx 4,500$ individuals were collected in more or less affected apple orchards in different German and neighboring European regions. The overwintered remigrants constituted the main portion of these collections while the number of springtime individuals (emigrants) was negligible, confirming the observations of Tedeschi et al. (54,55). Among the 4,441 specimens tested by PCR with specific primers, we found only four remigrants and no emigrant naturally infected with 'Ca. P. mali'. This represents a natural infection rate of only 0.09% in Germany compared with mean infection rates of 3.6 and 2.8% of remigrants reported from northern Italy (55). During our long-term study in Germany, naturally infected emigrants were never found, whereas Tedeschi and coworkers calculated a natural infection rate of $\approx 0.8\%$ (55). Because the infection with the

TABLE 7. Population densities of overwintered remigrants of *Cacopsylla melanoneura* collected on apple (*Malus* spp.) and hawthorn (*Crataegus* spp.) at untreated sites in the surroundings of Neustadt during the peak of population in the middle of March^a

Year	<i>Malus</i> spp.			<i>Crataegus</i> spp.		
	No. of branches	Total individuals	Individuals/branch	No. of branches	Total individuals	Individuals/branch
2002	400	50	0.13	75	1,623	21.64
2003	200	57	0.29	50	774	15.48
2004	200	14	0.07	125	1,558	12.46
2005	100	20	0.2	25	310	12.40
2006	200	3	0.02	75	170	2.27
2007	100	1	0.01	25	180	7.20

^a There were 100 branches per sample on apple and 25 branches per sample on hawthorn.

Fig. 2. Mean number and standard deviation of eggs deposited by *Cacopsylla melanoneura* on different plant species in dual-choice tests. The gravid females were collected from spruce just before remigrating to reproduction host plants. Cm = *Crataegus monogyna*; Md = *Malus domestica*; Pd = *Prunus domestica*. The differences are statistically significant (** and * indicate $P < 0.01$ and 0.05 , respectively, Wilcoxon matched-pairs test).

pathogen was always higher in the more abundant remigrants, they suggested that the overwintered generation plays a crucial role in vectoring 'Ca. P. mali'.

All phytoplasma-infected *C. melanoneura* were caught in highly AP-infected orchards. Despite the high probability of phytoplasma acquisition, only four PCR-positive individuals could be identified. These field-collected data reflect the results of the acquisition-transmission experiments, which demonstrate that phytoplasma acquisition by *C. melanoneura* in Germany is very inefficient.

The magnitude of natural infection only indicates that an insect may naturally acquire a pathogen to a certain extent but does not demonstrate effective transmission behavior of a species (57,58). For receiving clear evidence on the transmission ability of a species, transmission trials have to be conducted (36). Therefore, we examined the ability of *C. melanoneura* for vectoring 'Ca. P. mali' within variable experimental designs using ≈ 100 test plants and ≈ 500 adults collected from apple. The experimental designs corresponded to those used to demonstrate the transmission capacity of *C. picta* (19–21). However, there was no successful transmission of 'Ca. P. mali' to healthy test plants. This result may be due to the very low percentage (0.7%) of naturally infected remigrants used in the transmission trials. This low portion could be raised only slightly by induced acquisition feeding to 2.7%, which is lower than the natural infection of remigrants measured by Tedeschi et al. (55) in northwestern Italy. Experiments with emigrants resulted in neither any natural infection nor any induced acquisition. Unlike our results, Tedeschi et al. (52) obtained positive transmissions with naturally infected remigrants as well as with experimentally infected nymphal instars and emigrants.

Interplant spread of phytoplasma is naturally accomplished by insect vectors. In order to convert an infected vector individual to an effective vector, phytoplasmas must permeate two physical barriers within the insect: the basal lamina and surrounding cells of the gut and salivary glands. Vector morphology and physiology, as well as the phytoplasmas' molecular tool kit, may likewise influence the efficiency of a potential vector (6,18,51,58). Furthermore, the ability for multiplication of phytoplasmas in their vector insects may also influence their capability as a vector. Quantitative evidence of phytoplasma multiplication in the vector insects has been provided using enzyme-linked immunosorbent assay (5,30), competitive PCR (33), dot hybridization using DNA probes (42), and real-time PCR (4). According to these articles, an increase in phytoplasma titer in insects transferred to healthy plants after feeding on infected plants is direct evidence of phytoplasma multiplication in different organs of insects. Thus, we assume that a high phytoplasma titer in PCR-positive psyllids could be an assessment of its vector capacity. We used a quantitative real-time PCR assay based on SYBR green developed for the quantification of 'Ca. P. mali' in insects and plants (24). Jarausch et al. (20) showed for *C. picta*, the indisputable vector of 'Ca. P. mali' in Germany (20,21), that the phytoplasma load in the insect body seems to be crucial for a successful transmission and proposed the determination of a minimum threshold of phytoplasma for an efficient transmission. To evaluate the concentration of 'Ca. P. mali' in individuals of *C. melanoneura*, which gave positive signals in standard PCR, we applied the same approach and used several individuals of naturally infected *C. picta* as reference. The phytoplasma titer in overwintered *C. melanoneura* always ranged below 10^6 , even after an induced acquisition on infected apple plants (Fig. 1). The mean titer found for all examined *C. melanoneura* was 311,414 copies of phytoplasma DNA, displaying only one-third of the minimum threshold found for an effective transmission by *C. picta*. The populations of *C. melanoneura* in Germany and neighboring countries were capable of acquiring 'Ca. P. mali' by feeding on infected apple trees in certain circumstances but, for some reasons, the phytoplasma titer

did not increase to a threshold permitting transmission. Thus, we conclude that, in Germany, *C. melanoneura* has to be considered to be a nonvector for the agent of the AP disease.

Thus far, no comparable quantification data are available from the effective vector population of *C. melanoneura* from the Aosta Valley in northern Italy. Pedrazzoli et al. (45) quantified the titer of 'Ca. P. mali' in overwintered remigrants of *C. picta*, the main vector in Trentino, and of *C. melanoneura* after variable acquisition periods. Interestingly, they found that the two species did not differ in the mean phytoplasma titer during the acquisition period. However, the phytoplasma titer increased in *C. picta* but not in *C. melanoneura* during the latent period. They hypothesized that the low titer measured directly after acquisition feeding represents the amount of the pathogen uptake from the phloem sap. After having reached the specific organs where the main multiplication could take place, the pathogen titer would have risen. This hypothesis supports our observation that a certain minimum phytoplasma load in the insect body is necessary for an efficient transmission and that the rating of the phytoplasma titer is important to estimate whether an insect species is an efficient vector or not.

The data obtained in the present study are not in agreement with the results reported from a specific region in northwestern Italy. This raises the question about the existence of different populations of *C. melanoneura* with different capacities to acquire and transmit 'Ca. P. mali'. Intermediary data have been obtained in another important apple-growing region in northeastern Italy. In Trentino, the population of *C. melanoneura* on apple was able to transmit 'Ca. P. mali' but to a very low efficiency of 0.36% (37). Furthermore, the Trentino population of *C. melanoneura* was able to acquire the phytoplasma from infected test plants (45). Thus, genetic analyses are needed in order to confirm the postulated existence of different populations. For this, microsatellite markers for *C. melanoneura* have been developed (35) and respective studies are under way.

In conclusion, our data clearly show that neither remigrants (overwintered adults) nor specimens of the new generation (emigrants) had the capacity to acquire 'Ca. P. mali' from apple in an efficient way. Furthermore, the phytoplasmas were not able to multiply to a titer required for successful transmission in *C. melanoneura*, probably because they cannot reach the salivary glands and multiply there. Consequently, for Germany and neighboring regions, *C. melanoneura* has to be considered to be a non-vector for the agent of AP disease. Future work should stress phytoplasma infection of different organs of *C. melanoneura* of German origin with Italian 'Ca. P. mali' strains and vice versa as well as of *C. picta* for elucidating the reasons for the incapability of the German *C. melanoneura* in vectoring 'Ca. P. mali'.

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