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RESEARCH ARTICLE

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Epidemiological and molecular study on '*Candidatus* Phytoplasma prunorum' in Austria and Hungary

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Abstract

The epidemiology of '*Candidatus* Phytoplasma prunorum' was studied in Austria and Hungary from 2014 to 2018. Testing of root samples showed average infections rates of 61 and 40% of the Austrian *Prunus spinosa* and *Prunus domestica* spp. *insititia* samples, respectively. In Hungary, on average 21% of the *P. spinosa* and 13% of the feral *Prunus cerasifera* samples were infected. The pathogen was found in 18 out of 19 apricot orchards and PCR positive *Cacopsylla pruni* were observed at 11 out of 17 sampling locations in both countries. In cage experiments with *C. pruni* remigrants successful pathogen transmission to *Prunus armeniaca*, *P. domestica* and *P. spinosa* seedlings in budding and foliated developmental stages was recorded, an inoculation access period of 4 hr was sufficient for transmission. A field experiment with ungrafted apricot seedlings planted in 2012 and 2014 indicated a prominent role of the insect vectors for disease spread. In 2017, 40 and 28% of the trees planted in 2012 and 2014, respectively, were infected. Molecular characterisation based on the genes *aceF* and *imp* allowed the discrimination between 10 phytoplasma types in apricots. Around 70% of the phytoplasma types in apricots were also common in *P. spinosa*, in *P. domestica* spp. *insititia* and in remigrant *C. pruni* pointing to a possible pathogen exchange by insects between wild and cultivated *Prunus* spp. For disease control, vector management over the entire flight period of the remigrants seems necessary; when selecting active compounds, the short inoculation access period of not more than 4 hr should be considered.

KEYWORDS

aceF, *Cacopsylla pruni*, European stone fruit yellows, *imp*, transmission trial, wild *Prunus* species

1 | INTRODUCTION

'*Candidatus* Phytoplasma prunorum' ('*Ca*. Phytoplasma prunorum') is a widespread pathogen of *Prunus* spp. in Europe and the Mediterranean region (IPWG, 2018; Steffek, Follak, Sauvion, Labonne, & MacLeod, 2012). The phytoplasma induces European stone fruit yellows (ESFY; Lorenz, Dosba, Poggi Pollini, Llácer, & Seemülle, 1994), a disease associated with severe disorders in susceptible crops such as apricots (*Prunus armeniaca* L.), peaches (*Prunus persica* (L.) Batsch) and Japanese plums (*Prunus salicina* L.) (Marcone, Jarausch, & Jarausch, 2010).

Disease symptoms can vary greatly among plant species, rootstocks, climatic conditions, involved phytoplasma types or tree age, but generally infected plants share common symptoms in form of premature bud break, leaf yellowing and reddening in summer and eventually death of trees (Marcone et al., 2010). In apricots, the pathogen causes chlorotic, conically rolled leaves, sparse foliation, small, tasteless fruits as well as dieback and decline of trees (Lederer & Seemüller, 1992). Early bud break is typical for infected apricot trees in Mediterranean areas (Marcone et al., 2010; Morvan, 1977) but seems less common in Hungary (Süle, 1999). Based on sequence analysis of 16S rDNA, '*Ca*. Phytoplasma prunorum' is classified into the Apple proliferation group (16SrX), together with other important fruit tree phytoplasmas such as '*Ca*. Phytoplasma mali' and '*Ca*. Phytoplasma pyri' (Seemüller & Schneider, 2004).

The only known insect vector transmitting the pathogen is the plum psyllid *Cacopsylla pruni* (Scopoli, 1763) (Carraro, Osler, Loi, Ermacora, & Refatti, 1998). Molecular analyses suggest that *C. pruni* actually is a complex of two cryptic species that are not yet distinguished morphologically and have a similar biology (Peccoud, Labonne, & Sauvion, 2013; Peccoud, Pleydell, & Sauvion, 2018; Sauvion, Lachenaud, Genson, Rasplus, & Labonne, 2007). The plum psyllid is a univoltine species that completes its immature to adult life cycle on *Prunus* spp. In spring, the insects mate and lay eggs on wild and cultivated *Prunus* species. In late spring or at the beginning of summer, the new generation of *C. pruni* adults leaves the *Prunus* hosts and migrates to conifers in mountainous regions (emigrants). In the following year, at the end of winter or at the beginning of spring, the insects migrate back to *Prunus* spp. plants (remigrants), reproduce and die (Ossiannilsson, 1992; Thébaud, Yvon, Alary, Sauvion, & Labonne, 2009). These results on *C. pruni* life cycle were confirmed by observations in a permanent rearing of the species under experimental conditions (W. Jarausch & Jarausch, 2016). Recent investigations on the factors responsible for the regulation of migration revealed that migrating psyllids do feed on overwintering hosts but the nymphs are unable to develop on conifer diet (Gallinger & Gross, 2018).

C. pruni transmits '*Ca*. Phytoplasma prunorum' in a persistent propagative manner as the pathogen was shown to multiply in both immature and mature insects (Thébaud et al., 2009). Several studies suggest that in the field the remigrants are the crucial life stage for phytoplasma transmission (Carraro, Ferrini, Labonne, Ermacora, & Loi, 2004; Thébaud et al., 2009). For successful phytoplasma transmission, an acquisition access period of 2–4 days, minimum latent periods of 2–3 weeks and inoculation access periods (IAP) of 1–2 days were reported (Carraro, Loi, & Ermacora, 2001; Thébaud et al., 2009).

Basic strategies for ESFY management include the use of healthy propagating material and the uprooting of infected trees in orchards. In some regions, however, the success of these measures is compromised as wild *Prunus* species hosting both the vectoring insects and the phytoplasma might play a significant role as phytoplasma sources (Carraro, Ferrini, Ermacora, & Loi, 2002; Maier et al., 2013; Marcone et al., 2010). In a recent study in the Baix Llobregat area of Spain, feral *Prunus mahaleb* plants were suspected as the infection source for the nearby *P. salicina* orchards (Sabaté, Lavina, & Battle, 2016). Investigations in Germany estimated wild *Prunus spinosa* as a major source of infection in stone fruit orchards (W. Jarausch et al., 2019).

A multilocus sequence analysis approach based on the genes *aceF*, *pnp*, *secY* and *imp* allowing the analysis of genetic diversity of '*Ca*. Phytoplasma prunorum' has been developed by Danet et al. (2011). Among the four genetic loci used in this and a recent Slovenian (Dermastia, Dolanc, Mlinar, & Mehle, 2018) study, the genes *imp* (34 characterised haplotypes) encoding for an immunodominant surface protein and *aceF* (26 haplotypes) encoding for dihydrolipoamide acyltransferase, a protein involved in glycolysis, showed the highest variability and discriminative power.

In Austria, apricots are currently produced on an area of approximately 1,000 ha, of which more than 65% are located in the Federal Province of Lower Austria, approximately 16% in Styria and around 10% in Burgenland (Statistik Austria, 2018). In the past two decades, ESFY has become a major concern in all these areas, around the year 2000 infection rates of up to 40% were reported in apricot orchards (Laimer Da Câmara Machado et al., 2001; Richter, 1999, 2002). The highly susceptible "Klosterneuburger Marille" (variety group "Hungarian Best") is the most important apricot variety in Austria (30%), followed by "Orangered" (13%), Goldrich (10%), Bergeron (8%), "Pinkcot" and "Silvercot" (both 5%) (Statistik Austria, 2018). In intensive orchards, plants are predominantly grafted on plum rootstocks (mostly "TORINEL® avifel," "St. Julien A," "WAVIT® Prudom"), in extensive production plum seedlings "Brompton" and apricot seedlings are also used (Wurm, 2018). Often apricot orchards are small scale structured without any vector management. Many production areas are located in close vicinity to mountainous regions with high densities of conifers presumably serving as overwintering hosts for the vectors. From 2011 to 2013, PCR analyses in the Wachau region of Lower Austria based on leaf testing showed a disease incidence of 9.8 to 63.3% in apricot orchards, 20–40% in plum orchards and occasional infections of *P. spinosa* (Maier et al., 2013). High numbers of *C. pruni* (up to 25 individuals) were observed on blackthorns on a single branch with one sampling effort (beating tray method). Phytoplasma infection rates ranged between 11.5% for the remigrants and 3.4% for the emigrant insect generation (Maier et al., 2013).

In Hungary, apricot is produced on about 5,400 ha (KSH, 2018). Commercial apricot production covers about 4,100 ha. The largest growing district (34% of the total apricot area) is located mainly in the northeastern hills, while another 35% are situated on the slopes of several counties in the western part (Transdanubia), where wild *Prunus* species, such as *P. spinosa* and *P. cerasifera* are common. Apricot is grown on sandy soils of the Great Plain (18%) where also wild *Prunus* species can be frequently found. Nowadays, the majority of varieties belong to the variety group of "Magyar kajszi" ("Hungarian Best"); however, the share of foreign varieties is continuously increasing in newly established plantations. Most often the trees are grafted on *P. cerasifera* seedlings (about 80%), for sandy soil preferably *P. armeniaca* is used (about 20%). In the last years, a new highly grafted interstem system approach gained importance. In this system, the apricot variety is grafted on *Prunus domestica* "Fehér besztercei" (interstem) grafted on *P. cerasifera* seedlings. A study on the occurrence of ESFY in 2009 revealed a widespread and frequent presence

of the disease in several areas of the country. Based on visual symptoms, disease incidences of up to 85% were reported for apricot orchards (Tarczali, Kövics, & Kiss, 2014). Recent surveys on the presence and the infection status of *C. pruni* at several locations in Hungary showed a wide distribution of the insect species and average rates of PCR-positive specimens of 15% (Mergenthaler, Kiss, & Viczián, 2017), 7% (Czibulyás, Koncz, & Pénzes, 2018) and 35% (Lepres, Mergenthaler, Viczián, & Tóth, 2018).

Extensive knowledge on the presence of the pathogen in orchards, on the relevance of vector-mediated pathogen spread as compared to transfer via propagation material, on the role of wild *Prunus* spp. as pathogen sources, on the exchange of phytoplasmas between the compartments of the epidemic cycle as well as on the exact times of the year at which the insects transmit the pathogen is a requirement for well-adapted management strategies. Knowledge on the length of the IAP is crucial for the selection of insecticides intended to reduce pathogen spread. In general, a control of pathogen vectors by insecticides aims to keep vector populations low and thus to minimise phytoplasma spread. However, in the case of pathogen vectors moving into a crop from outside, additionally a direct effect of the insecticide on pathogen transmission is essential. The treatment should ideally disrupt pathogen inoculation either by a rapid knockdown effect or by altering the phloem feeding activities to minimise transmissions (Paleskić et al., 2017).

As a basis for the development of suitable control strategies, our study focused on the following aspects of epidemiology and characteristics of vector transmission:

Infection rates of wild or feral (initially introduced as cultivated plants but now naturally spreading) *Prunus* species and apricot trees in orchards in Austria and Hungary were determined. Obtained phytoplasma types were characterised by aid of *aceF* and *imp* gene sequencing. Phytoplasma types in wild *Prunus* spp., *C. pruni* and cultivated apricots were analysed to describe the phytoplasma genotypes in the vector and the wild and cultivated host plant species. A further objective was to trace the vector-mediated pathogen spread under field conditions in order to assess the need of insect control measures. For this purpose, plantations consisting of ungrafted apricot seedlings were established and monitored for several years. The efficiency of phytoplasma transmission to different plant hosts at different developmental stages of trees and insect vectors and the duration of IAP were studied by aid of transmission experiments under semi field conditions.

2 | MATERIAL AND METHODS

2.1 | Sampling of plants and insects in Hungary and Austria

Sampling was restricted to apricot producing regions and apricot orchards in both countries over a wide area, as illustrated in Figure 1 and Supplemental Table S1. Samples of apricots (*P. armeniaca*), German plum (*P. domestica* ssp. *insititia* (L.) Bonnier & Layens), myrobalan plum (*P. cerasifera* Ehrh.) and blackthorn (*P. spinosa* L.) were collected in July–September 2015–2018. All sampled German plums, myrobalan plums and blackthorns were free of disease symptoms. For analysis of these plant species, roots located 10–30 cm below the surface were dug out by aid of a shovel. Each sample consisted of at least two root pieces (diameter 2–10 mm) collected from different sides of the tree (each tree sampled individually). At sites A2, A3, A5, and A6 in addition to root samples leaf samples of *P. spinosa* and *P. domestica* spp. *insititia* from identical plants were collected for comparison purposes (5–10 leaves from different branches).

In contrast to the wild and feral *Prunus* species at the time of sampling, the *P. armeniaca* trees showed disease symptoms such as rolling and/or yellowing of the leaves and sparse foliation. At sampling sites A10–A13 as well as at sites H1, H2 and H4–H11. sampling in apricot orchards was based on these symptoms. Suspicious and symptomless trees were included in the study (Tables 1 and 2, Table S1). In contrast to that, samples were randomly collected in the orchards at sites A2, A3 and A7–A9. The small orchard at site A2 was entirely inspected and included in the random sampling; in case of all other (bigger) orchards, a sector was randomly selected for inspection and within that sector random sampling was carried out (Table 1 and Table S1).

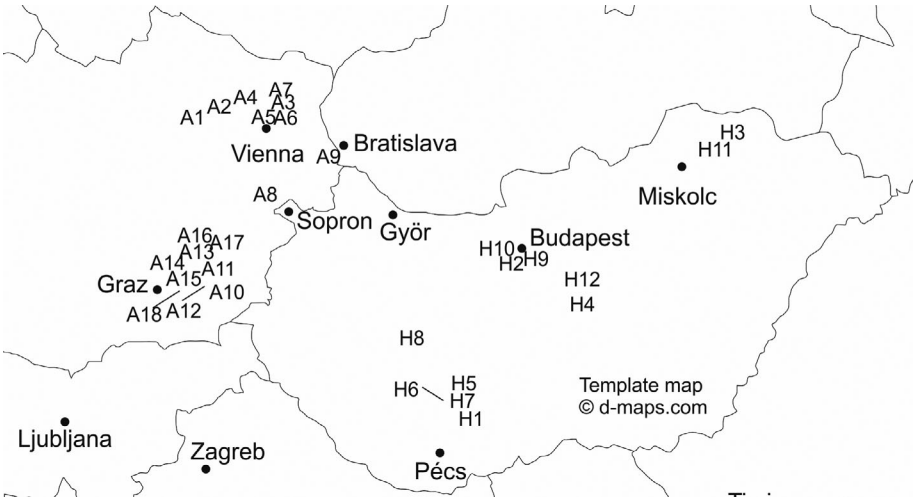

FIGURE 1 Map of sampling locations in Austria and Hungary; acronyms and description of locations according to Table S1

TABLE 1 Plant samples collected in Austria: numbers and description of samples and test results; r.s., random sampling

Location	*Prunus armeniaca* (orchards)			*Prunus spinosa*			*Prunus domestica* spp. *insititia* feral		
	N° tested plants	N° suspicious plants	PCR positive samples (leaves), N°/%	N° tested plants	PCR positive samples (roots) N°/%	PCR positive samples (leaves) N°/%	N° tested plants	PCR positive samples (roots) N°/%	PCR positive samples (leaves) N°/%
A2	12	r.s.	5/ 41.7	8	4/ 50	0	6	0/ 0	0/ 0
A3	50	r.s.	35/ 70	2	1/ 50	0	8	5/ 62.5	3/ 37.5
A5				3	2/ 66.7	0			
A6				10	7/ 70	0	6	5/ 50	1/ 16.6
A7	10	r.s.	5/ 50						
A8	10	r.s.	3/ 30						
A9	25	r.s.	9/ 36						
A10	2	2	2/ 100[a]						
A11	15	6	3/ 20[a]						
A12	18	6	4/ 22.2[a]						
A13	16	6	5/ 31.3[a]						
Total sumH1	158		71/ 44.9	23	14/ 60.9	0 / 0	20	8/ 40	4/ 20

[a]Sampling based on visual symptoms (presence or absence), no random sampling.

TABLE 2 Plant samples collected in Hungary: numbers and description of samples and test results

	Prunus armeniaca (orchards)			*Prunus cerasifera* rootstock suckers (orchards)		*Prunus spinosa*		*Prunus cerasifera* feral	
Location	N° tested plants	N° suspicious plants	PCR positive samples (leaves), N°	N° tested plants	PCR positive samples (roots) N°/%	N° tested plants	PCR positive samples (roots) N°/%	N° tested plants	PCR positive samples (roots) N°/%
H1	6	4	2/ 33%[a]	1	0/ 0				
H2	6	4	5/ 83%[a]	4	2 / 50			10	1/ 10
H3						2	0/ 0	4	1/ 25
H4	11	7	3/ 27%[a]	5	0/ 0				
H5	3	3	3/ 100%[a]						
H6	6	3	3/ 50%[a]	1	1 /100	2	0/ 0		
H7	5	4	0/ 0%[a]	1	0	4	0/ 0	1	0/ 0
H8	3	2	2/ 66%[a]			11	1/ 9		
H9	2	0	1/ 50%[a]	1	1 / 100	4	1/ 25		
H10	2	1	2/ 100%[a]	2	0/ 0	3	0/ 0		
H11	5	3	2/ 40%[a]			12	6/ 50		
Total	49	31	23/ 47%[a]	15	4/ 26.6	38	8/ 21.1	15	2/ 13.3

[a]Sampling based on visual symptoms (presence or absence), no random sampling.

TABLE 3 *Cacopsylla pruni* samples (remigrant generation only) collected in Austria and Hungary

Acr.	Host plant from which insects were collected	N° tested individuals/ N° of PCR positive individuals (%)
A1	*P. spinosa*	107/ 10 (9.3)
A2	*P. spinosa*	66/ 5 (7.6)
A3	Plum rootstock suckers apricot orchard	60/ 2 (3.3)
A4	*P. spinosa*	223/ 13 (5.8)
A5	*P. spinosa*	64/ 7 (10.9)
A6	*P. spinosa*	44/ 3 (6.8)
A12	*P. spinosa*	29/ 1 (3.4)
A14	*P. spinosa*	60/ 3 (5)
A15	*P. spinosa*	32/ 3 (9.3)
A16	*P. spinosa*	51/ 0 (0)
A17	*P. spinosa*	13/ 0 (0)
A18	*P. spinosa*	11/ 0 (0)
Total Austria		760/ 47 (6.1)
H2	*P. armeniaca*, *P. cerasifera*, *P. domestica*	92/ 4 (4.3)
H3	*P. spinosa*, *P. domestica*	64/ 0 (0)
H4	*P. cerasifera*	1/ 0 (0)
H11	*P. spinosa*, *P. armeniaca*, *P. domestica*	39/ 0 (0)
H12	*P. cerasifera*	14/ 2 (14.2)
Total Hungary		210/ 6 (2.9)

All apricot samples consisted of 5–10 leaves collected from all sides of single trees. *C. pruni* were sampled from March to April in the years 2014–2017 on *P. spinosa*, *P. domestica*, and *P. cerasifera* by the beating tray method (Table 3).

2.2 | Transmission trials

All insects used for the transmission trials were field-collected from 2012 to 2014 and in 2016. Seed transmission of '*Ca.* P. prunorum' has never been reported, therefore *Prunus* spp. seedlings were used as host plants. One part of test plants (apricot, plum and blackthorn seedlings for experiments simulating field conditions after blooming and transmission by emigrant generation insects) was raised from seeds in our lab. Apricot seeds were harvested in an apricot orchard (variety "Klosterneuburger Marille"), *P. domestica* seeds were harvested from a plum orchard and *P. spinosa* seeds were collected in the hedgerows at site A4. The seeds were at first kept at 4°C in moist sand for 2–3 months and then cultivated in a growth chamber at 21–23°C for approximately 3 months until they had 8–10 fully expanded leaves. Because these seedlings were produced under controlled conditions, we were able to rule out any phytoplasma infections before the start of the experiments. The other part of test plants consisted of potted 1-year-old certified seedling rootstocks from a commercial nursery cultivated until BBCH stages 03–09 (end of bud swelling/development of green tips; BBCH scale according to Meier et al., 1994). The plants were produced from seeds in a certification system which allows to assume with a reasonable degree of probability that the plants were healthy at the beginning of the experiments. However, infections in the nursery cannot be entirely excluded. In the transmission experiments on the length of the IAP, control trees were included. At the end of the experiments, the surviving insects were collected and tested by PCR. Results are given as average of PCR positive insects per sampling site in Table 4.

2.3 | Experiments simulating field conditions in late winter and early spring

The objective of the first test series (2012–2014) was to study the ability of *C. pruni* to transmit the pathogen to trees at very early developmental stages at the end of winter or in early spring. Dormant, certified, 1-year-old apricot seedlings (without closer specification of cultivar) and "Brompton" plum seedlings (size approximately 50 cm) were obtained from a commercial nursery and cultivated in pots. The trials were carried out in March and at the beginning of April under outdoor conditions in a paved courtyard covered with a roof protecting trials from rain. Ten insects per experiment (remigrants collected by beating tray method on *P. spinosa* at site A4) were introduced into cylindrical cages (diameter 3 cm, length 10 cm) attached to the plants and allowed to feed for 7 days.

2.4 | Experiments simulating field conditions after bloom

The second group of tests carried out during 2012–2014 aimed to simulate vector activity after flowering at already warmer outdoor conditions on foliated trees. Foliated apricot, plum and blackthorn seedlings raised in our lab were entirely covered with cylindrical cages (diameter 9 cm, height 25 cm). Per cage, 10 remigrant insects (collected on *P. spinosa* at site A4 simultaneously with the insects for the experiments described above) were allowed to feed for 7 days. Before, during and for 2 months after the trials, the test plants were maintained in a growth chamber at 21–23°C.

2.5 | Transmission by emigrant generation insects

The third group of experiments carried out in June 2012–2014 targeted the question whether the emigrant generation of *C. pruni* is capable of transmitting the disease. The test plants and the experimental conditions were the same as for the second group of tests (experiments simulating field conditions after bloom). Emigrant generation insects were collected on suckers of the plum rootstocks in the apricot orchard at site A3 (40 experiments) and on *P. spinosa* at site A4 (46 experiments).

TABLE 4 Transmission experiments. Insect numbers, description of insects, host plant species, numbers of tests and test results

Type of transmission experiment	N° insects per trial	Insect generation	Host plant for insect collection	Percentage of PCR positive insects (over all trials)	Feeding duration	Host plant in transmission experiment	N° / N° (%) of positive test plants
Simulation field conditions in late winter and early spring	10	Remigrants	*P. spinosa* (site A4)	14.3%	7 days	*P. armeniaca*	51/ 8 (15.7)
						P. domestica "Brompton"	13/ 1 (7.7)
Simulation field conditions after bloom	10	Remigrants	*P. spinosa* (site A4)	14.3%	7 days	*P. armeniaca*	57/ 3 (5.3)
						P. domestica	7/ 0 (0)
						P. spinosa	15/ 5 (33.3)
Transmission by emigrant generation insects	10	Emigrants	*P. domestica* rootstock suckers site A3	28.3%	7 days	*P. armeniaca*	40/ 0 (0)
			P. spinosa site A4	0%	7 days	*P. armeniaca*	46/ 0 (0)
Studies on length of IAP	20	Remigrants	*P. domestica* rootstock suckers site A3	3.3%	4 hr	*P. armeniaca*	9/ 0 (0)
					3 days	*P. armeniaca*	7/ 0 (0)
	20	Remigrants	*P. spinosa* site A4	5.8%	4 hr	*P. armeniaca*	22 /2 (9)
					3 days	*P. armeniaca*	5/ 1 (20)
	0	-	-	-	Control, no feeding	*P. armeniaca*	16/ 0 (0)

2.6 | Studies on length of IAP

The fourth series of tests carried out in April 2016 aimed to enlarge our insight into the length of the IAP in practically orientated conditions. In 2012–2014, the highest transmission rates were recorded in the tests simulating conditions in late winter or early spring. Therefore, the length of IAP was studied on certified 1-year-old apricot seedlings in BBCH stages 03–09 obtained from a nursery and maintained outdoors as described for the first test series. Per cage, 20 remigrant insects were allowed to feed for 4 hr and 3 days. Remigrant insects were collected on suckers of the plum rootstocks in the apricot orchard at site A3 (nine experiments feeding duration 4 hr, seven experiments feeding duration 3 days) and on *P. spinosa* at site A4 (22 experiments with feeding duration 4 hr; five experiments with feeding duration 3 days). Sixteen seedlings from the same batch of purchased seedlings served as control plants.

After the transmission trials, all plants were kept in pots in a foil tunnel under an insect proof net at outdoor temperatures. Annually in August–September, all plants were visually inspected and 2–3 root pieces (diameter 2–5 mm) per plant were excised taking particular care to maintain a functioning root system in order to keep the plants alive for further analyses.

2.7 | Monitoring of infection risk in the field

The objective of this experiment was to trace the vector-mediated pathogen spread and the development of ESFY under field conditions. The experiment was carried out in Krems (Lower Austria). At this location, an examination of the remigrant vector population in 2012 by direct PCR had demonstrated an infection rate of 8.6% (Maier et al., 2013). Dormant certified 1-year-old apricot seedling rootstocks (identical to plants described above for transmission experiments) were obtained from a commercial nursery and left ungrafted during the entire experiment. Plantings were carried out both in spring 2012 (March 28) and in spring 2014 (March 10) as illustrated in Figure 2. In the 2014 planting two times, five apricot seedlings were protected from *C. pruni* by an insect proof net. The nets entirely covered single plants and were installed every year from the beginning of March until July. Samples of each individual plant were taken annually in August or September. In 2013–2016 leaves (5–10 leaves randomly collected from all sides of the tree) and in 2017 roots (2–3 root pieces diameter 2–10 mm) were collected. Visual inspection of the trees based on yellowing and rolling of the leaves and sparse foliation was carried out in summer. Trees were classified as symptomatic or asymptomatic. Because of significant signs of drought stress, no visual inspection was carried out in 2017, but only in 2018. Dead trees were also recorded. In the area, early bud break seems less common than in other regions (Süle, 1999), thus an explicit assessment of early bud break was not included in the study. In any case, no observation in this regard was made while maintaining the test orchards.

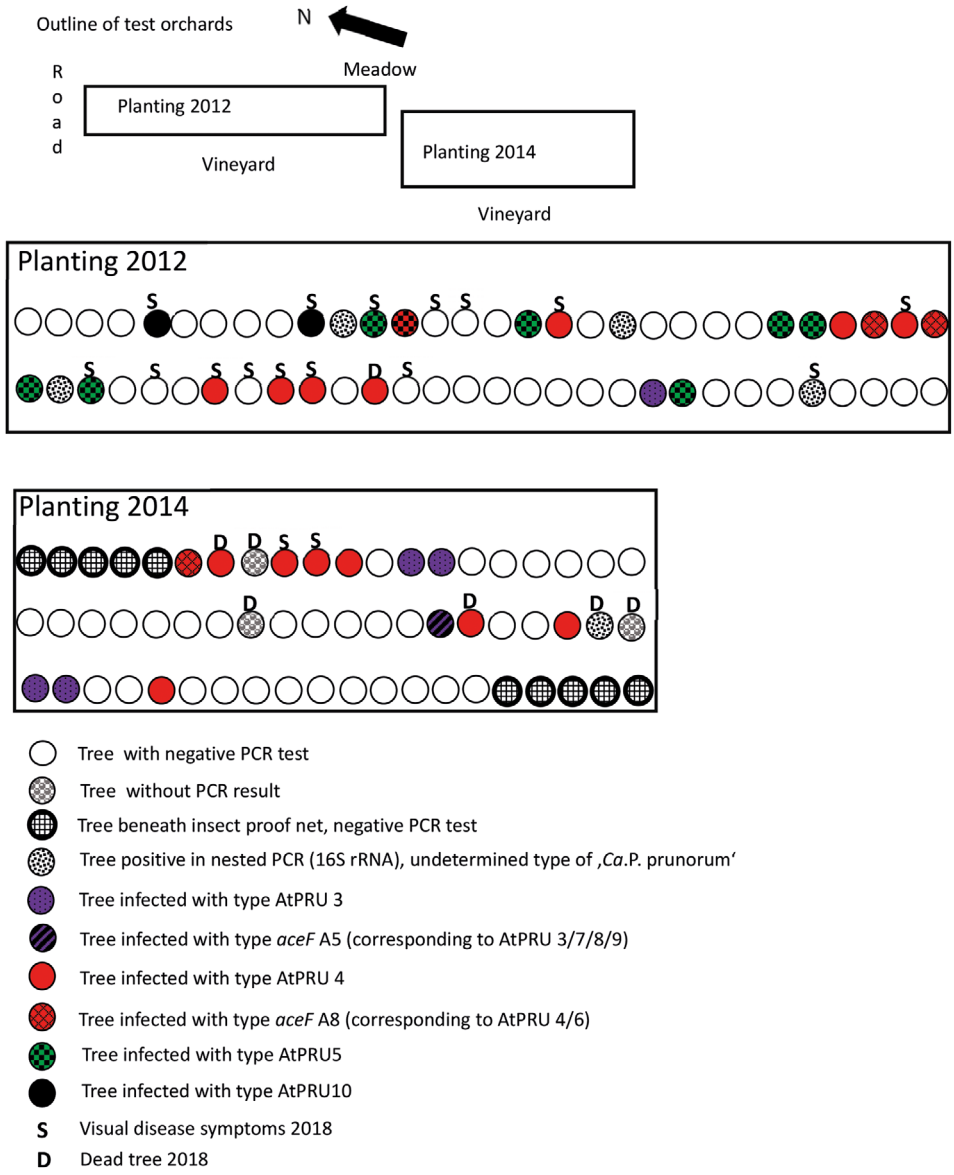

FIGURE 2 Map of the experimental orchard planted to monitor pathogen spread and ESFY development in the field: results of PCR analysis and molecular characterisation of phytoplasma types in 2017 as well as visual assessment of each tree in 2018 are indicated

2.8 | PCR analysis

DNA extraction from plant and insect samples was carried out by a CTAB procedure as described previously (Maixner, Ahrens, & Seemüller, 1995). Each plant or insect was analysed individually. In case of leaf samples, midrib and petiole tissue of five leaves per sample was combined. Roots were exhaustively washed by aid of a rough sponge. Each sample consisted of two cross sections per root piece.

The presence of phytoplasmas was detected by direct PCR, nested PCR and/or qualitative real real-time PCR. For the direct PCR procedure, we used the primer pair f01/r01 (Lorenz, Schneider, Ahrens, & Seemüller, 1995). All samples yielding the expected PCR fragment were characterised by restriction fragment length polymorphism (RFLP) using *Rsa*I to differentiate '*Ca.* Phytoplasma prunorum' from other 16SrX phytoplasmas and to exclude unspecific PCR products (Seemüller & Schneider, 2004). For nested PCR, primers P1/P7 (Deng & Hiruki, 1991; Smart et al., 1996) and R16(X) F1/ R16(X)R1 (Lee, Bertaccini, Vibio, & Gundersen, 1995) were used. R16(X) fragments were further characterised by RFLP as described above. The assignation of *C. pruni* to genetic groups A or B was performed with PCR of the ITS2 region using primer sets ITS set 1 and ITS set 3 as described (Peccoud et al., 2013). For qualitative real-time PCR, we followed the procedure of Christensen, Nicolaisen, Hansen, & Schulz (2004) using SensiFAST Probe No.Rox Kit (Bioline, London, UK) according to the manufacturer's instructions and an MIC thermocycler (Biomolecular Systems, Upper Coomera, Australia). One microliter of template preparation was included in 20 µl reaction volumes. Samples yielding Ct levels ≤30 were considered as phytoplasma-infected.

The three PCR methods have different characteristics. The nested PCR protocol presumably is the most sensitive, but in general nested protocols suffer from the risk of contamination and thus false positive results. The direct PCR protocol is less sensitive, and the qPCR protocol is not specific for '*Ca.* Phytoplasma prunorum' but a universal phytoplasma test. We have tested different types of samples with all

protocols as listed in Table S2a and tested for significant differences as described in Table S2b. Overall, data presented for *C. pruni* samples were obtained by direct PCR (Austrian samples) or qPCR (Hungarian samples). The phytoplasma species in positive qPCR samples was confirmed by the nested PCR protocol. All data for plant samples were derived from nested PCR.

2.9 | Characterisation of phytoplasma strains

Infected plant and insect samples from Austria were further characterised by aid of the genes *aceF* and *imp*. The analysis comprised plant and insect samples from the locations shown in Figure 1 and Table S1 and in addition *P. armeniaca* samples identified as infected in previous investigations in Lower Austria (Maier et al., 2013), namely from Rohrendorf (43 samples), Krustetten (four samples), Krems (two samples) and Rossatz (three samples). All in all, 211 samples were analysed. Fragments of the genes *aceF* and *imp* were amplified in nested PCR procedures using the primers AceFf1/AceFr1, AceFf2/AceFr2 and IMPF1/IMPR1, IMPF2/IMPR2 (Danet et al., 2011) and sequenced (Sanger method performed by GATC Biotech, now Eurofins Genomics, Ebersberg, Germany) with the primers AceFr2 and IMPR2 after purification by the Qiaquick® PCR purification kit (Mo Bio, Qiagen).

3 | RESULTS

3.1 | Suitability of PCR methods

As illustrated in Table S2a, in case of *P. armeniaca* (leaves and roots) and *P. cerasifera* root samples, all three PCR methods led to largely comparable results. In contrast, in case of *P. spinosa* and *P. domestica* nested PCR gave higher numbers of positive PCR reactions than the other two methods. Statistical analysis confirms a significant higher number of positive nested PCR results compared to single PCR in *P. spinosa* and *P. domestica* ssp. *insititia* (Table S2c,d).

3.2 | Analysis of plants and insects in Hungary and Austria

PCR analyses of apricot and *P. cerasifera* rootstocks samples from both countries demonstrated the presence of the pathogen in all orchards except site H7 (Tables 1 and 2). A random sampling effort in the apricot orchards at sites A2, A3 and A7–A9 revealed infection rates between 30 and 70%.

In both countries, the phytoplasma was found in roots of wild or feral *Prunus* spp. In Austria, the infection rates of *P. spinosa* reached up to 70% and in Hungary up to 50%. In Austria, the infection rates of *P. domestica* spp. *insititia* were as high as 62.5% (Table 1), in Hungary up to 25% of the feral *P. cerasifera* samples also showed a positive PCR reaction (Table 2).

In case of *P. spinosa* and *P. domestica* spp. *insititia*, comparative analyses of root and leaf material taken from identical plants at identical time points were carried out. In *P. spinosa*, the phytoplasma was detected in roots only, in case of *P. domestica* spp. *insititia*, roots samples resulted in higher rates of PCR positive plants (Table 1). Statistical analysis confirmed the significant effect of the plant material on the PCR result for both plant species (Table S2d,e).

Phytoplasma infections of *C. pruni* were detected at 9 out of 12 locations in Austria and 2 out of 5 locations in Hungary. Infection rates amounted up to 10.9% in Austria (average of 12 sites 6.1%) and 14.2% in Hungary (average over five sites 2.9%) (Table 3). All phytoplasma positive *C. pruni* and at least five individuals from each origin (except H4 with only one collected individual) were tested by PCR with ITS set 1 and ITS set 3 primers. All individuals belonged to genetic group B of the *C. pruni* complex.

3.3 | Transmission trials

3.3.1 | Experiments simulating field conditions in late winter and early spring

In transmission trials with 1-year-old seedlings obtained from a commercial nursery at BBCH stages 03–09 and 10 *C. pruni* remigrants per cage feeding for 7 days, the pathogen was transmitted to 15.7% of the *P. armeniaca* and to 7.7% of the *P. domestica* plants (Table 4). PCR testing of the insects showed an infection rate of 14.3% for the remigrants collected at site A4 on *P. spinosa*. The median value for the number of insects surviving the test duration of 7 days was six.

3.3.2 | Experiments simulating field conditions after bloom

The experiments with foliated test plants raised from seeds in our lab and 10 remigrant *C. pruni* per plant feeding for 7 days led to 5.3% of PCR-positive *P. armeniaca* seedlings and 33.3% of PCR-positive *P. spinosa* seedlings, plum seedlings were not infected (Table 4). Insects in this study were from the same collections as for simulating field conditions in late winter and early spring, thus infection rates of insects were identical to the ones stated above (14.3%). A median value of six insects per experiments survived the test duration of 7 days.

3.3.3 | Transmission by emigrant generation insects

No phytoplasma transmission by new generation psyllids was observed (Table 4). A 28.3% of the insects originating from plum rootstock suckers in the apricot orchard at site A3 were PCR positive, but 0% of the individuals captured at site A4 on *P. spinosa*. A median value of six individuals per cage survived the test period of 7 days.

3.3.4 | Studies on length of IAP

To evaluate the necessary IAP, 20 *C. pruni* remigrants were kept on 1-year-old apricots seedlings at BBCH stages 03–09 for 4 hr and 3 days, respectively. Inoculation access for 4 hr resulted in successful phytoplasma transmission to 2 out of 31 test plants (6.5%), inoculation access for 3 days led to 1 infected test plant out of 12 (8.3%). No infections of

control plants were found (Table 4). Positive transmissions were only observed with insects captured on *P. spinosa* (site A4, rate of PCR positive individuals 5.8%), whereas all experiments including insects collected on *P. domestica* rootstock suckers (site A3, rate of PCR positive individuals 3.3%) remained uninfected. A median number of 17 individuals were still alive after the 4 hr transmission experiments, after the 3 day experiments the median value of living individuals per cage was eight.

3.4 | Monitoring of pathogen spread and ESFY development in the field

The objective of the experiment was to monitor pathogen spread and appearance of ESFY symptomatic trees under field conditions. Testing of leaf samples collected from 2013 to 2016 resulted in no detection of phytoplasmas in any of the trees (data not shown). In contrast, the analysis of roots in 2017 demonstrated that 24 out of 60 trees (40%) planted in 2012 and 14 out of 50 trees (28%) planted in 2014 and grown in open air were infected by the phytoplasma (Figure 2). As opposed to that, no phytoplasma was detected in any of the trees protected by an insect proof net. In 2018, 25.4% of the trees (15 out of 59) planted in 2012 showed disease symptoms, one had died. Of the trees planted in 2014 and maintained in open air, 4.5% (two out of 44) were symptomatic and 13.6% (six) had died. A 54.1% (13 out of 24) of the PCR-positive trees planted in 2012 were without symptoms, and out of the trees tested as healthy by PCR, 4 of 36 (11.1%) had been scored as symptomatic. The trees planted in 2014 showed an even higher rate of latent infection, 81% of the PCR positive trees (nine out of 11) appeared healthy. All symptomatic trees planted in 2014 also gave a positive PCR result (Figure 2).

3.5 | Phytoplasma strains

A total of 211 phytoplasma infected samples was characterised by aid of the genes *aceF* and *imp*. Sequence analysis led to the discrimination of five different *aceF* and five different *imp* variants (Figure 3) resulting in 10 different phytoplasma types ATPRU 1–10 (Figure S1 and Table 5). All of them were present in apricot trees, and ATPRU 1–6 were found in remigrant *C. pruni*. For some *P. domestica* spp. *insititia* and *P. spinosa* samples as well as for samples from transmission trials in the field and in the lab, successful amplification of the *imp* gene was not obtained, despite positive 16S rRNA and *aceF* gene amplifications. In such cases, based on a*ceF* gene only, an unambiguous ascription of the phytoplasma to a type was only partially possible. Nevertheless the analysis allowed to identify at least five different types (ATPRU1-ATPRU5) in *P. domestica* spp. *insititia* and four (ATPRU2-ATPRU5) *P. spinosa* plants. ATPRU2-ATPRU5 accounted for almost 70% of the infections of *P. armeniaca* trees in orchards and for 87% of the phytoplasma types in *C. pruni* remigrants. Less than 5% of the phytoplasmas in commercial apricot orchards were not detected in any other compartment of the epidemic cycle. ATPRU1 accounts for 20% of the phytoplasma samples in *P. armeniaca*, but has been detected only once with *Prunus* spp., namely in *P. domestica* spp. *insititia* (Figure S1 and Table 5). At least

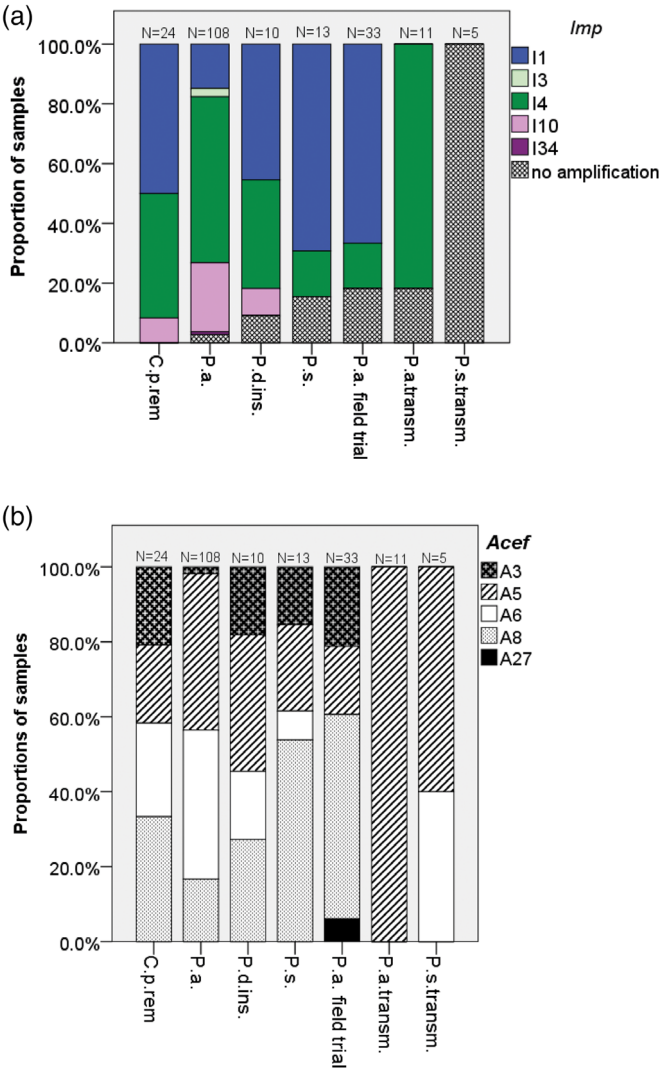

FIGURE 3 Occurrence of *imp* (a) and *aceF* (b) haplotypes of '*Ca.* Phytoplasma prunorum' detected in remigrant *Cacopsylla pruni* (C.p. rem.), *P. armeniaca* (P.a.), *P. domestica* spp. *insititia* (P. dom. ins.), *P. spinosa* (P.s.), *P. armeniaca* from the field trial to monitor infection risk (P.a. field trial) and transmission experiments with *P. armeniaca* (P.a. transm.) and *P. spinosa* (P.s. transm.) as host plants are illustrated. The colour code for the imp genotypes in (a) is according to Danet et al. (2011) with modifications to show the rarer types I3 and I34

four types (ATPRU 2–4, ATPRU 10) were transmitted in the field experiment aiming to monitor pathogen spread and at least two types were found in the host plants included in laboratory transmission experiments (Figure 3 and Figure S1). I1 was the most prevalent *imp* haplotype in *P. domestica* spp. *insititia* (>40%), *P. spinosa* (67%) as well as in *C. pruni* remigrants (50%), while 15% of the ESFY positive *P. armeniaca* contained this genotype. I4 was the most common type in *P. armeniaca* (55%) and it was also present in *P. domestica* spp. *insititia* (30%), *P. spinosa* (17%) and *C. pruni* (42%). I10 was regularly found in *P. armeniaca* (23%) but only occasionally in *C. pruni* (8%), in a single case in *P. domestica* spp. *insititia* and not at all in *P. spinosa* (Figure 3a). A similar pattern was observed with *aceF*, where the most

TABLE 5 Classification of phytoplasma genotypes based on *aceF* and *imp* sequences of '*Ca.* Phytoplasma prunorum'

Genotype	*aceF* accession	*aceF* geno-type[a]	*aceF* identical accession[b]	*imp* accession	*imp* geno-type[a]	*imp* identical accession[b]	Strain	Hosts and collection regions[c,d]
ATPRU1	MK101108	A6	FN598171 (B7)	MK101113	I10	FN600716 (13MY)	GD_135_pruni	C.p., P.a.; BGLD, NÖ, STMK
ATPRU2	MK101108	A6	FN598171 (B7)	MK101114	I4	FN600710 (40-04cr)	GD_16_prunus	C.p., P.a., P.d., P.s.; BGLD, NÖ, STMK
ATPRU3	MK101109	A5	FN598170 (40-04cr)	MK101114	I4	FN600710 (40-04cr)	GD_23_prunus	C.p., P.a., P.d., P.s., P.a.ft., P.a.tr.; BGLD, NÖ, STMK
ATPRU4	MK101110	A8	FN598173 (ESFY)	MK101115	I1	FN600707 (ESFY)	STMK_487_pruni	C.p., P.a., P.d., P.s., P.a.ft.; BGLD, NÖ, STMK
ATPRU5	MK101111	A3	FN598168 (GSFY2)	MK101115	I1	FN600707 (ESFY)	GD_46_prunus	C.p., P.a., P.d., P.s., P.a.ft.; NÖ, STMK
ATPRU6	MK101110	A8	FN598173 (ESFY)	MK101114	I4	FN600710 (40-04cr)	GD_48_prunus	C.p., P.a.; NÖ
ATPRU 7	MK101109	A5	FN598170 (40-04cr)	MK101113	I10	FN600716 (13MY)	RO_39_prunus	P.a.; STMK
ATPRU8	MK101109	A5	FN598170 (40-04cr)	MK101116	I3	FN600709 (WJ3889-42)	RO_37_prunus	P.a.; STMK
ATPRU9	MK101109	A5	FN598170 (40-04cr)	MK101117	I34	MG972433 (D598/14)	GD_1182_prunus	P.a.; NÖ
ATPRU10	MK101112	A27[e]	-	MK101115	I1	FN600707 (ESFY)	KL_2162_prunus	P.a. ft; NÖ

[a]*aceF* and *imp* genotypes based on previously published nomenclature (Danet et al., 2011; Dermastia et al., 2018).
[b]Acession numbers and corresponding strains with 100% sequence identity.
[c]Representative strains/samples, hosts and collection regions are indicated. Remigrant *C. pruni*: C.p., *P. armeniaca*: P.a., *P. domestica* ssp. *insititia*: P.d., *P. spinosa*: P.s., *P. armeniaca* from field trials: P.a.ft. and transmission experiments with *P. armeniaca*: P.a.tr.
[d]Regions: Styria: STMK; Lower Austria: NÖ; Burgenland: BGLD.
[e]No corresponding entry in the NCBI database.

common haplotype in *P. armeniaca*, A6, occurred at 40%. This haplotype was also identified in *P. domestica* spp. *insititia* (20%), *P. spinosa* (7.,7%) and in *C. pruni* (25%) (Figure 3b). An unambiguous detection of different strains from single individuals (multiple infections) was not recorded.

4 | DISCUSSION

The data obtained in the present study provide an insight into the current dissemination and the epidemiology of '*Ca.* Phytoplasma prunorum' in Hungary and in Austria. Strikingly high proportions of wild and feral *Prunus* species at several locations in both countries were found to be infected, although none of the tested plants showed any disease symptom. A fundamental role of wild *Prunus* spp. for disease epidemiology was already emphasised previously (Carraro et al., 2002; W. Jarausch et al., 2019; Maier et al., 2013). The data obtained in the current study support the assumption that beside infested nursery plants also natural contaminations play a role in ESFY occurrence in apricot orchards in several respects. First, at some locations the root testing carried out in this study showed pathogen presence in up to 70% of the *P. spinosa* and up to 62.5% of the feral *P. domestica* plants. Infection rates comparable to our findings were also reported from Belarus, where root analysis led to the detection of phytoplasmas in two out of three *P. spinosa* samples (Valasevich & Schneider, 2016). Having regard also to the fact that myrobalans, blackthorns and plums are among the most preferred hosts for the vector (B. Jarausch, Fuchs, et al., 2007; B. Jarausch, Mühlenz, et al., 2007; Labonne & Lichou, 2004; Maier et al., 2013; Mergenthaler et al., 2017), the role of wild or feral *Prunus* spp. as sources for infectious psyllids seems crucial. Second, as illustrated in Figure S3 and Figure 3 and discussed in detail below, the molecular characterisation of the phytoplasma types in this study showed a high degree of correspondence of pathogen types in wild and cultivated *Prunus* spp., in *C. pruni* as well as in the test plants in the transmission experiments. Although only a descriptive observation, this indicates a possible phytoplasma exchange between wild (blackthorn, German plum) and cultivated *Prunus* spp. in a joint epidemic cycle. Third, in our transmission experiments, the highest rate (33%) of infected plants was observed for *P. spinosa*. Apart from the conclusion that blackthorn is a suitable host both for the insect species and the pathogen, this observation indicates that pathogen dissemination, maybe also to so far pathogen-free regions, could also happen independently from cultivated *Prunus* spp.

Corresponding to the infection rates of their preferred host plants, a significant share of the remigrant *C. pruni* at the majority of sampling sites in both countries was also phytoplasma positive. The average of all sites in Austria was 6.1%, in Hungary 2.9%, at specific sites up to 11% in Austria and up to 14% in Hungary. In previous investigations in Italy, remigrant insects in areas with infected orchards were in a similar range of up to 9% infection (Carraro et al., 2004), whereas, for example, in Germany recently lower rates have been recorded (1–2%; W. Jarausch et al., 2019). *C. pruni* consists of a complex of cryptic

species, of which in Europe East of France, Western Switzerland and Italy, only genotype B has been reported (Peccoud et al., 2013). In confirmation of this result, all analysed *C. pruni* collected in Austria and Hungary were genotype B of the *C. pruni* complex.

In accordance with the high infection rates in vector insects and in wild host plants, the pathogen was identified in the majority of analysed apricot orchards in both countries, as reported previously (Steffek et al., 2012). Random sampling in several Austrian orchards proved infection rates ranging between at least 30 and 70%, a dimension capable of seriously compromising apricot cultivation. The infection rates observed in this study coincide with previous reports on high infection rates both in Austria and in Hungary (Maier et al., 2013; Tarczali et al., 2014).

In our transmission experiments, *C. pruni* remigrants transmitted the pathogen to *P. armeniaca*, *P. domestica* and *P. spinosa* plants. In case of *P. armeniaca*, the highest infection rate (15.7%) was observed for the trees in BBCH stages 03–09 kept under outdoor conditions. Applied to practical field conditions, this indicates that there is a high infection risk from the start of insect remigration in early spring even when trees are still dormant or growth has hardly started.

The trials limiting the stay of the remigrant insects on trees in BBCH stages 03–09 to 4 hr only led to successful transmission of the pathogen in 2 out of 26 experiments. It can therefore be concluded that the IAP can be much shorter than the previously reported 1–2 days (Carraro et al., 2001). In none of our experiments, the insects started to feed right after their transfer into the cages. The insects seemed to be under stress and walked around in the cages for 1–2 hr before they settled. Therefore, IAP under field conditions could be even shorter than the experimental 4 hr.

In our trials, no phytoplasma transmission by field captured springtime generation insects was recorded, a finding coinciding with previous reports indicating no or at the best a very low transmission rate by adults before overwintering (Carraro et al., 2004; B. Jarausch, Mühlenz, et al., 2007; Poggi Pollini et al., 2010; Thébaud et al., 2009). Interestingly, in this experiment, 28.3% of the emigrant insects captured at site A3 on plum rootstocks suckers of apricot trees, but 0% of the insects collected at site A4 on *P. spinosa* gave a positive PCR result.

The transmission rates observed in the current study are lower than those in previous reports. In experiments in the Friuli Venezia Giulia region of Italy, groups of 3–20 remigrant insects transmitted the pathogen to 20–100% of micropropagated *P. salicina* plants, groups of 50 individuals infected 80% of *P. spinosa* and *P. cerasifera* seedlings and 100% of apricot seedlings cultivated in the greenhouse (Carraro et al., 2002, 2004). In Germany, field captured remigrants transmitted the pathogen to 23% of micropropagated *P. marianna* plants (B. Jarausch, Mühlenz, et al., 2007). The lower transmission rates in our experiments (15.7% for 1-year-old apricot seedlings in BBCH stages 03–09, 7.7% for plum seedlings in BBCH stages 03–09 and 5.3 and 33.3% for foliated *P. armeniaca* and *P. spinosa* seedlings, respectively) could be because of differences in the vector capacity of the insects in different parts of Europe, to varying numbers of insects in the transmission experiments, to different ages and stages of the recipient test plants in the experiments or to the cultivation under outdoor conditions.

The marked role of vector transmission for disease spread was confirmed by the experiments aiming to monitor the infection risk and the appearance of ESFY symptoms under field conditions. A 40% of the seedlings planted in 2012 and 28% of the seedlings planted in 2014 were PCR positive after 5 and 3 years, respectively. Around a quarter of the trees planted in 2012 had developed disease symptoms after 6 years. Four different phytoplasma types were transmitted. Extrapolated over the lifetime of an apricot orchard, these results show clearly that in regions with high infection pressure substantially all trees become infected within their desired life cycle. For this field study, we considered it as necessary to establish a plantation of ungrafted 1-year-old apricot seedlings. Generally, fruit trees are generated using vegetatively propagated planting material (scions and in most cases also rootstocks) as in commercial orchards uniformity of planting material and predictability of genetic traits are essential. For observations on phytoplasma spread via insect vectors, however, such orchards are less appropriate. The danger of disseminating the phytoplasma by vegetative propagating material can be relevant (Marcone et al., 2010; Riedle-Bauer et al., 2012) and, as shown in previous studies, PCR does not always give sufficiently clear-cut results to exclude low infections of young trees.

A deeper characterisation of phytoplasmas in plants and insects in Austria revealed the presence of 10 different types based on *aceF* and *imp* genotypes (Table 5), of which five, namely the types ATPRU1-ATPRU5, were frequent (Figure 3 and Figure S1). These frequent types consist of the *aceF* genotypes (according to Danet et al., 2011) A3, A5, A6 and A8 and the *imp* genotypes I1, I4 and I10 (Table 5). These more common genotypes have been also documented in several other Central and South European countries (Danet et al., 2011; Dermastia et al., 2018) and have been more frequently found in a recent investigation in Slovenian orchards (Dermastia et al., 2018). In contrast to that, the rarer *aceF* and *imp* genotypes (A27, I3, I34) have been previously reported at most from few individuals in single countries or not at all (A27). ATPRU2-ATPRU5 were the four types in wild or feral *P. spinosa* and *P. domestica* spp. *insititia* and in the field and laboratory assays monitoring vector transmission. Also in remigrant *C. pruni*, the types ATPRU2-5 were prevalent. It is therefore possible that these types spread via an epidemic cycle in which *C. pruni* transmits the pathogen from wild *Prunus* spp. to cultivated *P. armeniaca*, and likewise between wild *Prunus* spp. ATPRU1 with *imp* genotype I10 was frequent and widespread (at 8 of 12 tested locations all over Eastern Austria) in *P. armeniaca* and was sometimes found in remigrant *C. pruni*. However, we have detected the corresponding *imp* type (I10) only once in wild *Prunus* spp. (in *P. domestica* spp. *insititia*) and never in the transmission trials. The corresponding *aceF* type of ATPRU1, B7, has also only rarely been found in the wild *Prunus* spp. Although in our experiments we have not been able to successfully amplify the *imp* gene of all samples, it appears that vector transmission of ATPRU1 does less likely occur. Therefore, the question arises whether some phytoplasma types such as those containing I10 are prevalently disseminated by propagating material. The frequent occurrence of ATPRU1 could indicate that in addition to vector transmission in practice, propagating material is also a relevant source of

the phytoplasma in the Austrian and Hungarian *P. armeniaca* orchards. Previous experiments have shown that grafting of visually healthy but latently infected plant material leads to significantly infected progeny and that infected progeny may remain free of symptoms for several years (Riedle-Bauer et al., 2012).

Simultaneous analysis of blackthorn and German plum leaf and root samples led to higher rates of positive PCR results, the role of the plant material was statistically significant. It seems that root samples are particularly appropriate for the detection of weak phytoplasma infections, for example, in wild *Prunus* hosts, even though the sampling procedure is more time consuming.

The results of the current study allow a number of practical conclusions with regard to control strategies. In Austria and, to a lesser extent, also in Hungary, wild *Prunus* species were found infected to a large extent. Phytoplasma types observed in wild *Prunus* spp. were also prevalent in apricot orchards. Being at the same time, a preferred host for the insect vectors wild *Prunus* species likely accounts for considerably infected *C. pruni* populations threatening the health status of cultivated *Prunus* trees. Our field experiments with apricot seedlings clearly show the speed of pathogen dissemination and underline the need for efficient vector control. According to the transmission experiments, there is a high risk of phytoplasma transmission from the first day of remigration onwards as in our experiments the most efficient transmission was recorded on trees in BBCH stage 03–09 kept outside. In consequence, vector management by insecticides or repellents must start at this time point and last until the end of the remigrant flight period. In contrast to that, control of the emigrant generation does not appear necessary.

The duration of the IAP is a crucial point for successful management of the pathogen spread by insecticides. The principal goal of insecticide application is to keep vector populations inside the orchard as low as possible and in consequence to minimise the pathogen spread. However, in case of high vector numbers and continuous immigration from overwintering sites, a direct effect of the insecticide on pathogen transmission is essential as well. Only insecticides with a "knock down" or instant effect on feeding behaviour and acting faster than the minimum IAP seem suitable to provide at least some protection against inoculation of healthy trees (Paleskić et al., 2017). Based on our results, compounds disrupting the insect feeding behaviour in less than 4 hr are necessary to keep infection rates low. Last but not least, our study indicated at least some spread via propagation material; thus, continued efforts are needed to warrant the health status of the planting material.

All in all, control of ESFY is impeded by several factors. First, large-scale eradication of wild *Prunus* species with the aim to minimise vector populations seems hardly feasible and from an ecological point of view which is not desirable. Second, infectious vectors migrate into orchards for many weeks at the end of winter and at the beginning of spring at cool temperatures and during early developmental stages of trees. Successful vector control therefore requires active ingredients that are effective at low temperatures at early developmental stages of the trees and have a long lasting effect allowing insect control over several weeks with a small number of treatments. Third, it can be assumed that only the minority of the available insecticides disrupts insect feeding behaviour in less than 4 hr at cool temperatures. This applies especially for the effect of aged spraying residues (Paleskić et al., 2017).

Strategies based on clearing measures in orchards together with the use of certified propagating material and insecticides directed against the remigrants will likely reduce pathogen spread, but they will not entirely stop new infections in orchards. Future research should focus on long-standing field experiments proving the benefit of insecticides and repellents against pathogen spread (Marcone et al., 2010; Paleskić et al., 2017), on the effects of antagonistic microorganisms (Trivedi, Trivedi, Grinyer, Anderson, & Singh, 2016) and on the selection and/or the breeding of suitable rootstocks (Marcone et al., 2010).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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