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Molecular diversity of '*Candidatus* Phytoplasma mali' and '*Ca*. P. prunorum' in orchards in Slovenia

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Abstract Phytoplasmas from the 16Sr-X apple proliferation (AP) group are quarantine species in Europe and causal agents of the most important diseases of fruit trees within the family *Rosaceae*, namely apple proliferation, European stone fruit yellows and pear decline. In this study, a detailed insight into the molecular diversity of isolates of two phytoplasmas from the AP group, i. e. '*Candidatus* Phytoplasma mali' and '*Ca*. P. prunorum' obtained from different orchards in Slovenia, was estimated by a multilocus sequence typing, based on analysis of the genomic regions of *aceF*, *pnp*, *secY* and *imp*. With seven and five genotypes defined for '*Ca*. P. mali' and *'Ca*. P. prunorum' isolates, respectively, *imp* was the most variable among the applied markers. On the other hand, *pnp* was the least variable with three genotypes defined for *'Ca*. P. mali' isolates and only one for *'Ca*. P. prunorum' isolates. The presented results complete the survey of the AP group phytoplasma diversity in Slovenia, which has started with the recent analysis of the '*Ca.* P. pyri'. The comparison of results with those from several European countries shows an important genetic diversity of the Slovenian genotypes with some previously unknown. The genotype distribution reflects the geographic position of Slovenia. Additional grafting experiments with apricot trees tolerant to '*Ca*. P. prunorum' demonstrated that the tolerance status is transmissible. Some possible mechanisms involved in the process are discussed.

Keywords Apple proliferation · '*Candidatus* Phytoplasma mali' · '*Candidatus* Phytoplasma prunorum' · Multilocus sequence typing · Phytoplasma diversity · Slovenia

Introduction

Phytoplasmas are obligate bacterial parasites of plant hosts and insect vectors, and they belong to the Mollicutes class (Lee et al. 2000). The apple proliferation (AP) group of phytoplasmas consists of '*Candidatus* Phytoplasma mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri', which are included within the 16Sr-X group of phytoplasmas based on the similarity of their 16S ribosomal DNA sequences (Seemuller and Schneider 2004). Those from the AP group share between 98.6 and 99.0% sequence identity of their 16S rRNA gene, which is above the 97.5% proposed as an arbitrary threshold to distinguish between phytoplasma species (IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma 2004). However, these phytoplasmas differ significantly on the basis of their biological and ecological properties, which can also be used for species delineation (IRPCM Phytoplasma/Spiroplasma Working Team–Phytoplasma Taxonomy Group 2004). They

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trigger different diseases and are transmitted by different psyllid vectors. Specifically, '*Ca*. P. mali' is a causing agent of AP and its vectors are *Cacopsylla picta* and *C. melanoneura* (Jarausch and Jarausch 2010; Križanac et al. 2017; Mehle et al. 2007). A vector of '*Ca*. P. prunorum', which causes European stone fruit yellows (ESFY) is *C. pruni* (Jarausch and Jarausch 2010; Marcone et al. 2014); and '*Ca*. P. pyri', a causing agent of pear decline, is transmitted in Europe mainly by *C. pyricola* and *C. pyri* (Jarausch and Jarausch 2010; Pavšič et al. 2014). These phytoplasmas thus cause major economic losses to the European apple and temperate stone fruit tree industry (Kirkpatrick et al. 2011). They are quarantine organisms in the European Union, and listed in the Annex I.A.II of the Council Directive 2000/29/EC/Annex I/A2.

The presence of symptoms of mycoplasma-like diseases has been documented in Slovenian orchards since the mid-1980's (Šarić and Cvjetković 1985). However, their causal agents were not confirmed in the laboratory until 2001 (Mehle et al. 2011). Since then, '*Ca*. P. mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri' have been constantly detected both in fruit production and in nurseries and mother plantations, where they are under official control (Mehle et al. 2011). To exclude the possibility of starting inoculum of phytoplasmas, the elimination of infected plants from nurseries and mother plantations is required by law.

Recently there have been some reports on the genetic diversity among the species within the AP group of phytoplasmas (Danet et al. 2011; Franova et al. 2013; Križanac et al. 2017; Pavšič et al. 2014). Multilocus sequence typing have revealed considerable variability among the strains and several yet unrecorded genotypes. Danet et al. (2011) reported on phytoplasmas of the AP group from several European and Mediterranean countries, including Azerbaijan. Molecular diversity of '*Ca*. P. pyri' was shown for Slovenia (Pavšič et al. 2014), and similarly for '*Ca*. P. mali' in Croatia and the Czech Republic (Franova et al. 2013; Križanac et al. 2017). This knowledge of the genetic diversity of different strains in the AP group has a practical value, as differences in virulence have been shown among isolates of '*Ca*. P. prunorum'(Kison and Seemuller 2001) and '*Ca*. P. mali' (Schneider et al. 2014; Erich Seemüller and Schneider 2007).

The aim of the present study was the completion of the multilocus sequence typing for the AP group in Slovenia (Pavšič et al. 2014), based on analysis of the genomic regions of *aceF*, *pnp*, *secY* and *imp*.

Materials and methods

Samples and DNA extraction

The leaves of symptomatic apple trees (*Malus domestica*), and in the case of presumed latent infection, the roots of apple trees, were collected between 2012 and 2015 in eight locations in Slovenia (Supplementary Table S1). This formed part of the official monitoring by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection. Thirty-one of these samples were collected from different apple tree cultivars. In addition, five samples were obtained from the vector insect *Cacopsylla picta* and one from *Cacopsylla melanoneura*. These vectors were collected in two orchards: one in western Slovenia, and the other in the far north-east of Slovenia (Supplementary Table S1).

For '*Ca*. P. prunorum', samples of leaves and roots of apricot (*Prunus armeniaca*) and peach trees (*Prunus persica*) were collected between 2012 and 2014 (Supplementary Table S2). Four apricot trees that were included in the survey were of old Slovenian cultivars 'Catar' (1) and 'Debeli Flokar' (3) that tested positive for the presence of '*Ca*. P. prunorum' in 2012, although they showed no symptoms, and instead vigorous growth and good yield. These were the source for the graft inoculation of the rootstock Mirabolana 29 C. Following the graft inoculation, the trees were observed for 2 years for the appearance of the ESFY symptoms. In 2014, they were tested again for the presence of '*Ca.* P. prunorum'.

Initially, all of the samples included in the multilocus sequence typing were tested for the presence of '*Ca*. P. mali' (i.e., 37 samples) and '*Ca*. P. prunorum' (i.e., 20 samples). Total DNA was isolated from the samples using kits (QuickPick Plant DNA kits; Bio-Nobile, Finland) and a purification system (KingFisher mL; Thermo Scientific, USA) (Mehle et al. 2013b). The presence of phytoplasmas was determined using real-time PCR according to a protocol for specific detection of these three phytoplasmas from the AP group,

essentially as described previously (Mehle et al. 2013a; Nikolić et al. 2010). The negative extraction and amplification controls were always negative, and the Cq for positive samples were between 25 and 36.

PCR and nested PCR amplification

Amplification of the *aceF*, *pnp*, *secY* and *imp* genes was carried out by nested PCR, using the primers designed by Danet et al. (2011). All of the PCR assays were performed on a PCR cycler (PCR System 9700 Gene Amp) in 50 μL final reaction volumes that contained: 2 μL 10-fold diluted DNA sample; 1× High Fidelity buffer (Invitrogen); 2 mM $MgSO_4$ (Invitrogen); 200 μM dNTPs (Applied Biosystems); 0.03 U/μL Platinum Taq DNA Polymerase High Fidelity (Invitrogen); and 0.4 μM of each primer. The PCR conditions for the initial denaturation were 3 min at 94 °C, which was followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C, and extension for 1 min at 68 °C. The final extension was for 7 min at 68 °C. Nested PCR was carried out using 1 μL of the PCR amplification product. The conditions for the initial denaturation were 3 min at 94 °C, which was followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C, and extension for 1 min at 68 °C. The final extension was for 7 min at 68 °C. The nested PCR products were separated on 1% agarose gels stained with ethidium bromide, observed under UV light, and purified later (MiniElute PCR purification kits, Qiagen).

Sequencing and sequence analysis

The forward and reverse sequencing reactions for the nested PCR products were performed by Macrogen Europe using the Sanger method. The partial amplified sequences of *aceF*, *pnp*, *secY* and *imp* were compared with sequences of non-Slovenian '*Ca.* P. mali' and '*Ca.* P. prunorum' isolates from the GenBank database, using the BLAST algorithms (http://www.ncbi.nlm.nih.gov/blast). DNA sequence alignments of the Slovenian isolates were carried out by creating contiguities using the ContigExpress software (Vector NTI). Multiple alignments of the Slovenian and non-Slovenian isolates were carried out using the AlignX software (Vector NTI). Phylogenetic analyses were carried out with MEGA7 (Kumar et al. 2016) and haplotype network was constructed using the e-BURST programme (Feil et al. 2004).

Results and discussion

In this study, we genotyped the *aceF*, *secY*, *pnp* and *imp* genes from the Slovenian isolates of '*Ca.* P. mali' and '*Ca.* P. prunorum'. These genes encode dihydrolipoamide acetyltransferase, a component of the protein secretory machinery – preprotein translocase subunit SecY, polynucleotide phosphorylase, and immunodominant membrane protein, respectively. These have been established as suitable markers for genotyping of phytoplasma species within the AP group (Danet et al. 2011; Križanac et al. 2017; Pavšič et al. 2014). The sequences of these genes from the Slovenian isolates were compared with the sequences of the non-Slovenian ones deposited in the GenBank database. For easier comparisons, the Slovenian sequences were annotated according to those described in Danet et al. (2011). Phylogenetic analyses of partial gene sequences, including previously reported ones (Danet et al. 2011), confirmed the presence of two clusters corresponding to '*Ca.* P. mali' and '*Ca.* P. prunorum' (Fig. 1).

Diversity of the *aceF* genotype

For the *aceF* genomic region, considerably high variability was seen among the different isolates, with five and four different *aceF* genotypes for '*Ca.* P. mali' and '*Ca.* P. prunorum', respectively (Table 1, Fig. 1, Supplementary Figs. S1, S2). Similarity among the sequences of the different Slovenian genotypes of both species within the *aceF* genomic region ranged from 99.3–99.8%, and they differed by up to four single nucleotide polymorphisms (SNPs), in different parts of the approximately 600-bp-long PCR products of these phytoplasma isolates.

The most abundant *aceF* genotype of '*Ca.* P. mali' was A13, for 67.6% of the isolates. A13 is also the most widespread aceF genotype of '*Ca.* P. mali' in Europe, and it has been defined for isolates from Germany, France and Italy (Supplementary Fig. S1), (Danet et al. 2011). In the neighbouring country of Croatia, A13 has been detected at similar rates to those in Slovenia (i.e., 70.6%) (Križanac et al. 2017). The genotype defined for eight out of 37 isolates was A15, which has already been reported for France, as well as the countries that border Slovenia, as Austria, Italy (Danet et al. 2011) and Croatia, where it was defined for one sample (Križanac et al. 2017). Irrespective of the collecting site, the *aceF* genotypes A13 and A15 were also defined at the same

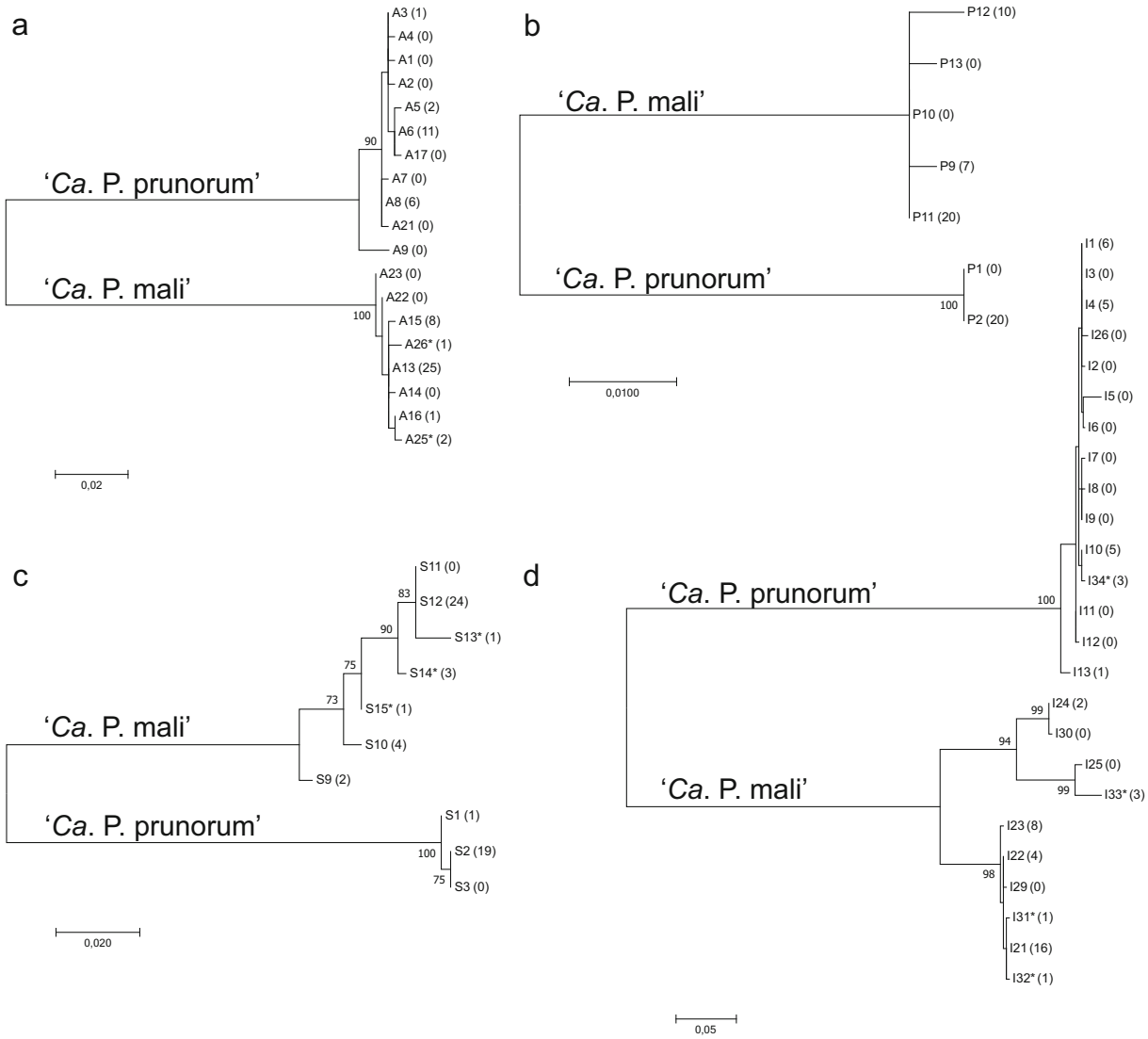

Fig. 1 Genetic variability of '*Ca.* P. prunorum and '*Ca.* P. mali' *aceF* (**a**), *pnp* (**b**), *secY* (**c**) and *imp* (**d**) genotypes. The evolutionary history was inferred by using the Maximum Likelihood method (1000 bootstrap replicates) with a best-fit model: Tamura 3-parameter model (aceF and pnp) or Tamura 3-parameter model and discrete Gamma distribution (imp and secY). There were a total of 567 (aceF), 392 (pnp), 478 (secY) and 410 (imp) positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. Bootstrap values below 70 are omitted. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Number of Slovenian isolates are shown in the bracket, after the name of the genotype. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)

frequencies in samples from the vectors. In addition to one occurrence of A16, which is known for Italy (Danet et al. 2011) and Croatia (Križanac et al. 2017), two previously unknown *aceF* genotypes were also defined that differed from A16 by one and three SNPs (Table 1, Fig. 1, Supplementary Fig. S1). The variability of the *aceF* genotypes for '*Ca.* P. mali' was comparable to that in Italy and in Croatia (Danet et al. 2011; Križanac et al. 2017), with three out of five identical genotypes, although it was higher than in the other countries from a previous study (Danet et al. 2011) (Supplementary Fig. S1).

The prevailing *aceF* genotype of '*Ca.* P. prunorum' was A6 (55% share), which is fairly common in France, Germany, Turkey and Croatia (Danet et al. 2011) and the major genotype in Friuli Venezia Giulia (Northern) near the border with Slovenia (Osler et al. 2016). Of note, all of apricot trees in this study infected with '*Ca.*

Table 1 '*Ca.* P. mali' and '*Ca.* P. prunorum' isolates with the *aceF* genotype

NCBI accession no.	Pathogen (no. of isolates)	Identical (100%) to isolates from other countries (accession no.)	*aceF* genotype (Danet et al. 2011) and this study*
MG972413	'*Ca.* P. mali' (25)	FN598184	A13
MG972414	'*Ca.* P. mali' (8)	FN598186	A15
MG972415	'*Ca.* P. mali' (1)	FN598187	A16
MG972416	'*Ca.* P. mali' (2)		A25*
MG972417	'*Ca.* P. mali' (1)		A26*
MG972418	'*Ca.* P. prunorum' (1)	FN598168	A3
MG972419	'*Ca.* P. prunorum' (2)	FN598170	A5
MG972420	'*Ca.* P. prunorum' (11)	FN598171	A6
MG972421	'*Ca.* P. prunorum' (6)	FN598173	A8

P. prunorum' *aceF* genotype A6 were symptomless. In addition, in the peach tree that was infected with the '*Ca.* P. prunorum' isolate of *aceF* genotype A6, the plant symptoms were mild and atypical (Supplementary Table S2). However, the haplotypes that included *aceF* genotype A6 had the same *secY* and *pnp* genotypes, plus three different *imp* genotypes (see below).

While *aceF* genotype A3 is the most widespread in Germany, France, Italy and Spain, it was detected in only one apricot tree sample in Slovenia. The *aceF* genotype A8 was detected in two peach tree samples and four apricot tree samples. This genotype was defined for all of the countries included in the previous study, from Spain in the West of Europe to Azerbaijan in the East (Danet et al. 2011). In addition, two apricot trees were infected with '*Ca.* P. prunorum' genotype A5, which was previously detected in one plant and one insect, from Croatia and Italy, respectively.

The distribution of the *aceF* genotypes of '*Ca*. P. prunorum' in Slovenia corresponds to their reported European geographic distribution (Supplementary Fig. S2). The identity with the distribution of *aceF* genotypes in Italy might reflect the main Slovenian regions for the growing of apricot and peach trees, which are near the border with Italy.

Diversity of the *pnp* genotype

In agreement with the previous study by Danet et al. (2011), *pnp* was the least variable marker here. The approximate size of the PCR products of the *pnp* genomic regions was 400 bp for both of these phytoplasmas. While three different *pnp* genotypes were defined for '*Ca*. P. mali' isolates (Table 2, Fig. 1, Supplementary Table S1), all of the '*Ca.* P. prunorum' isolates showed only one *pnp* genotype (Table 2, Fig. 1, Supplementary Table S2). All of the *pnp* genotypes from the Slovenian '*Ca*. P. mali' and '*Ca.* P. prunorum' isolates have been shown in isolates from several European countries (Danet et al. 2011). However, the prevailing genotype of '*Ca.* P. mali' isolates was P11, which was detected in 20 samples from apple trees (Table 2, Fig. 1). For all of the '*Ca.* P. prunorum' isolates, only the *pnp* genotype P2 was defined. Although this genotype is not uncommon, it is not the most frequent *pnp* genotype in Europe (Danet et al. 2011).

Diversity of the *imp* genotype

Among the markers applied, *imp* was the most variable, with seven and five genotypes defined for '*Ca*. P. mali' and '*Ca.* P. prunorum', respectively. The size of the PCR products was approximately 510 bp for both of these phytoplasmas, with up to 73 SNPs in different parts of the sequences. Similarity within the *imp* genomic region among the different Slovenian genotypes of '*Ca*. P. mali' was between 85.7 and 99.8%, and of '*Ca*. P. prunorum' between 97.9 and 99.8% (Fig. 1).

As in France, Austria, Germany and Italy (Danet et al. 2011), the most prevalent *imp* genotype of '*Ca.* P. mali' was I21, which was detected in 12 apple tree samples and also prevailed in isolates from *C. picta* (i. e. in four out of six samples) (Table 3, Supplementary Table S1). The *imp* genotype I23 previously known from Italy and Germany (Danet et al. 2011) was found in seven isolates from apple trees and in one from *C. picta.* In four apple tree isolates the *imp* genotype I22, which has been previously detected only in

Table 2 '*Ca*. P. mali' and '*Ca*. P. prunorum' isolates with the *pnp* genotype

NCBI accession no.	Pathogen (no. of isolates)	Identical (100%) to isolates from other countries (accession no.)	*pnp* genotype (Danet et al. 2011)
MG972442	'*Ca*. P. mali' (7)	FN598200	P9
MG972443	'*Ca*. P. mali' (20)	FN598202	P11
MG972444	'*Ca*. P. mali' (10)	FN598203	P12
MG972445	'*Ca*. P. prunorum' (20)	FN598191	P2

Romania (Danet et al. 2011), was revealed (Table 3). In addition, *imp* genotype I24, which has been detected in two apple tree plants from Germany and France (Danet et al. 2011), was also present in two apple tree samples from Slovenia (Table 3). On the other hand, in a few samples, three new and previously unreported *imp* genotypes were defined (Table 2), one of which was only in *C. melanoneura* (i.e., I31; accession no. MG972426).

Comparison of the Slovenian *imp* genotypes in '*Ca*. P. prunorum' isolates with those from other European countries (Danet et al. 2011) confirmed their gradient in the geographic distribution (Supplementary Fig. S3). I1 was the prevailing *imp* genotype in all of these analysed isolates, but its share was lower than in Germany, France, Italy and Croatia (Fig. 1, Supplementary Fig. S3). Genotypes I6, I7, I8, I11 and I12 have been defined for France, Spain, and Germany, but were not defined here for Slovenia. In addition, I13 was detected, which is also known for Croatia, Spain, France and Germany, but not for Italy (Fig. 1, Supplementary Fig. S3). A previously unknown *imp* genotype was also defined that differed by one SNP from I10 and represented 15% of all of the detected genotypes (Fig. 1, Supplementary Fig. S3).

Diversity of the *secY* genotype

The general length of the PCR products of the *secY* genomic region was 500-bp for both phytoplasmas, with up to eight SNPs in different parts of these sequences (Table 3). The isolates of '*Ca*. P. mali' shared 98.4 to 99.4% sequence identity for the *secY* genomic region. On the other hand, similarity within the *secY* genomic region between two detected genotypes of '*Ca*. P. prunorum' was 99.8% (Fig. 1).

S*ecY* was a relatively variable marker for the '*Ca*. P. mali' isolates, for which six different *secY* genotypes were defined. The most common was S12, which was detected in 64.9% of isolates (Table 4, Supplementary Table S1). The *secY* genotype S12 is also the most

Table 3 '*Ca*. P. mali' and '*Ca*. P. prunorum' isolates with the *imp* genotype

NCBI accession no.	Pathogen (no. of isolates)	Identical (100%) to isolates from other countries (accession no.)	*imp* genotype (Danet et al. 2011) and this study*
MG972422	'*Ca*. P. mali' (16)	FN600730	I21
MG972423	'*Ca*. P. mali' (4)	FN600731	I22
MG972424	'*Ca*. P. mali' (8)	FN600732	I23
MG972425	'*Ca*. P. mali' (2)	FN600733	I24
MG972426	'*Ca*. P. mali' (1)		I31*
MG972427	'*Ca*. P. mali' (1)		I32*
MG972428	'*Ca*. P. mali' (3)		I33*
MG972429	'*Ca*. P. prunorum' (6)	FN600707	I1
MG972430	'*Ca*. P. prunorum' (5)	FN600710	I4
MG972431	'*Ca*. P. prunorum' (5)	FN600716	I10
MG972432	'*Ca*. P. prunorum' (1)	FN600719	I13
MG972433	'*Ca*. P. prunorum' (3)		I34*

Table 4 'Ca. P. mali' and 'Ca. P. prunorum' isolates with the *secY* genotype

NCBI accession no.	Pathogen (no. of isolates)	Identical (100%) to isolates from other countries (accession no.)	*secY* genotype (Danet et al. 2011) and this study*
MG972434	'Ca. P. mali' (24)	FN598216	S12
MG972435	'Ca. P. mali' (2)	FN598213	S9
MG972436	'Ca. P. mali' (4)	FN598214	S10
MG972437	'Ca. P. mali' (1)		S13*
MG972438	'Ca. P. mali' (3)		S14*
MG972439	'Ca. P. mali' (1)		S15*
MG972440	'Ca. P. prunorum' (1)	FN598205	S1
MG972441	'Ca. P. prunorum' (19)	FN598206	S2

widespread in Italy, France and Romania (Danet et al. 2011). S12 shared 100% sequence identity with the *secY* genomic region from the reference strain AP15 (accession no. HM237294), which has been detected in '*Ca*. P. mali' populations in orchards in northwestern Italy (Casati et al. 2011). Two new, previously unreported, *secY* genotypes were defined that differed from S12 in four and three SNPs: S13 and S14, respectively. An additional new *secY* genotype S15 was distinguished by four SNPs from S10, which was previously reported for Italy and Austria (Danet et al. 2011) (Table 4, Fig. 1). None of the unreported *secY* genotypes were associated with the '*Ca*. P. mali' isolates from the insects. Genotype S9, which was defined for two isolates (Table 4, Fig. 1), and also appears in Germany (Danet et al. 2011).

In comparison with the '*Ca*. P. mali' isolates, only two *secY* genotypes were defined for the isolates of '*Ca*. P. prunorum'(Table 4, Fig. 1). Both of these are frequent in plants and insect vectors in Germany, France, Italy, Spain, Azerbaijan and Croatia (Danet et al. 2011).

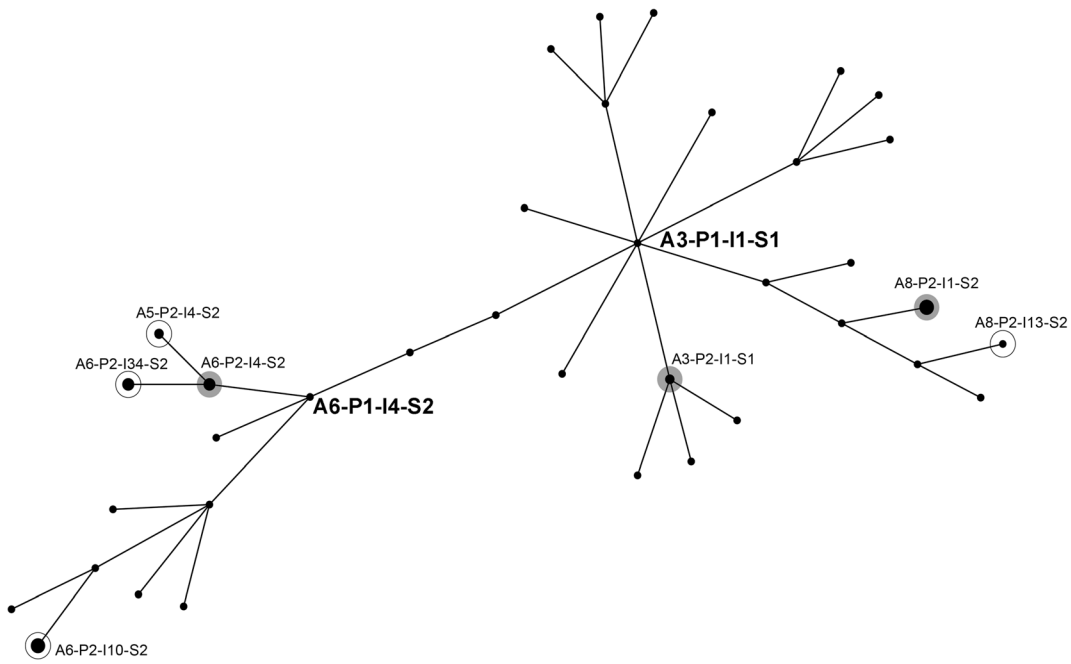

Fig. 2 Haplotype network of '*Ca*. P. prunorum' isolates constructed using the eBURSTv3 programme (http://eburst.mlst.net) (Feil et al. 2004). Isolates described by Danet et al. 2011 were used as a reference dataset. Only haplotypes found in this study are highlighted. Haplotypes indicated by white circles are previously unreported ones. The size of black dots is related to the number of samples. The founder haplotypes are indicated in bold case

High diversity of phytoplasma haplotypes

Due to the high numbers of genotypes associated with '*Ca*. P. mali' in Slovenia, the number of haplotypes unique for Slovenia was accordingly high (Supplementary Table S1). For 37 isolates of '*Ca*. P. mali' genotyped for four genes, 17 different haplotypes were detected. Two of them, A15-P12-I31-S10 and A13-P12-I21-S12 were unique for psyllid vectors *C. melanoneura* and *C. picta*, respectively (Supplementary Table S1). The haplotypes A13-P11-I21-S12 and A15-P12-I21-S10 detected in apple trees from orchard in Selo were also found in two specimens of *C. picta* at the same location, indicating the role of this insect as a vector (Supplementary Table S1). The same haplotype A13-P11-I23-S12 was found in apple tree samples both from Selo and from the very near location in Fokovci (Supplementary Table S1).

In the case of '*Ca*. P. prunorum' seven different haplotypes were identified and four of them (i. e. A3-P2-I1-S1, A6-P2-I34-S2, A6-P2-I10-S2, A8-P2-I13-S2) have not been described before (Fig. 2, Supplementary Table S2). They were organized into a network (Fig. 2), which also includes haplotypes from other countries (Danet et al. 2011). The network revealed A3-P1-I1-S1, previously found in Germany, France and Italy (Danet et al. 2011), as a founder haplotype. Haplotypes A8-P2-I1-S2 and A8-P2-I13-S2 associated with symptomatic phenotypes (Table 5, Supplemenatry Table S2) clustered separately from those related to asymptomatic phenotypes (Fig. 2). A6-P1-I4-S2 (Danet et al. 2011) is a founder haplotype for all haplotypes associated with asymptomatic phenotypes (Table 5, Fig. 2; Supplementary Table S2). The novel haplotype A5-P2-I4-S2 (Supplementary Table S2), originated in A6-P2-I4-S2 (Fig. 2). Both haplotypes were associated with symptomless apricot trees (Table 5).

The A6 genotype of '*Ca*. P. prunorum' is associated with symptomless trait

In 2014, nine of the 12 trees grafted in 2012 with '*Ca*. P. prunorum' infected apricot scions from the symptomless old Slovenian cultivars 'Catar' and 'Debeli Flokar' tested positive for this phytoplasma. However, only some of the infected trees expressed the ESFY symptom of early bud burst (Ulubas Serce et al. 2007). The multilocus sequence typing study of the samples from

Table 5 The '*Ca*. P. prunorum' genotypes and symptom appearance in the source trees and the trees grafted with '*Ca*. P. prunorum' infected scions. Genotype designations are according to Danet et al. 2011; *this study; +independent samples from the same tree

Symptomless graft (sample ID)	Cultivar	Material for phytoplasma detection	Original haplotypes				After graft inoculation (sample ID)	Material for phytoplasma detection	Derived haplotypes				Symptoms
D586/12	Catar	Leaves and roots	A6	P2	I10	S2	D629/14-1	Roots	A6	P2	I34*	S2	Symptomless
							D629/14-2	Roots	A6	P2	I10	S2	Symptomless
D583/12+	Debeli Flokar	Leaves	A5	P2	I4	S2	D629/14-4	Roots	A6	P2	I10	S2	Symptomless
D584/12+	Debeli Flokar	Roots	A5	P2	I4	S2	D630/14-1	Roots	A8	P2	I1	S2	Early budding
							D630/14-2	Roots	A8	P2	I1	S2	Early budding
D582/12	Debeli Flokar	Leaves and roots	A6	P2	I10	S2	D630/14-3	Roots	A6	P2	I34*	S2	Symptomless
D581/12	Debeli Flokar	Roots	A3	P2	I1	S1	D637/14-2	Roots	A6	P2	I4	S2	Symptomless
							D637/14-3	Roots	A8	P2	I1	S2	Early budding
							D637/14-4	Roots	A8	P2	I13	S2	Early budding

the sources and the grafted trees revealed diversity of the haplotypes obtained (Table 5, Supplementary Table S2). Although the occurrence of random mutations or new infection transmitted with the infected vectors cannot be excluded, revealing of several haplotypes likely indicates that in the source apricot trees several different isolates co-occurred simultaneously. It has been previously shown for apple trees infected with '*Ca*. P. mali' that such multiple infections are common (Seemüller et al. 1984, 2011). Moreover, increased or, more often, reduced virulence of the infected phytoplasma strains was observed due to antagonistic interactions between mild and severe strains.

While only the P2 *pnp* genotype was defined for all samples, the genotypes of *aceF*, *secY* and *imp* varied. The haplotype of one graft from 'Catar' remained the same as in the source, but for the other graft, one SNP was detected in the *imp* genotype. All of the '*Ca*. P. prunorum' isolates from 'Catar' were characterized with the A6 *aceF* and S2 *secY* genotypes, and all of these trees were symptomless. It is of note that in four French isolates (Castelain et al. 1997; Cornaggia et al. 1995) with the same *aceF* A6 and S2 *secY* genotypes (Danet et al. 2011), the hypovirulence trait in '*Ca*. P. prunorum' (Kison and Seemuller 2001) has been characterized.

The source graft haplotypes from 'Debeli Flokar' were more diverse, with three different *aceF* genotypes (i.e., A3, A5, A6), and two *secY* and three *imp* genotypes (Table 5). After the grafting, all of the original haplotypes changed. Nevertheless, same as in cv. 'Catar', only when a derived haplotype included the A6 *aceF* and S2 *secY* genotypes did the early budding not develop, regardless of the alterations in *imp* genotype. However, it has been suggested that the hypovirulent trait is not monophyletic for its origin, as it is also expressed in *aceF* genotype A8 (Danet et al. 2011). In our case, all of the plants infected with an isolate of '*Ca*. P. prunorum' with *aceF* genotype A8 showed the symptom of early budding. On the other hand, among the Slovenian isolates of '*Ca*. P. prunorum', *aceF* genotypes A3 and A5 were also associated with symptomless apricot trees, which might indicate that the A6 genotype by itself is not a prerequisite for hypovirulence. It is also possible that mild and aggressive strains with different haplotypes co-exist in the same tree. In this case, the mild strain might supress more aggressive strains in cross-protecting way as has been shown for *Catharanthus roseus* and *Nicotiana occidentalis* infected with different strains of '*Ca*. P. mali' (Schneider et al. 2014). However, Osler et al. (2016) have showed that the genotype *aceF* occurs at similar frequency in the symptomatic and asymptomatic apricot trees infected with '*Ca*. P. prunorum' and based on the results hypothesized an induced phenotypic resistance. The results of our study neither confirm nor reject these suggestions. Nevertheless, taking into account that most of the Slovenian samples were from the orchards on the southwestern part of Slovenia, on the border with Italy, and that some were taken from the same trees in different years (see below), more sampling and further analyses are needed to provide a clearer picture of this situation.

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Compliance with ethical standards The authors bear all the ethical responsibilities of this manuscript. They declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest and that it does not include any animal and/or human trials.

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