Multilocus sequence analysis reveals the genetic diversity of European fruit tree phytoplasmas and supports the existence of inter-species recombination

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The genetic diversity of three temperate fruit tree phytoplasmas '*Candidatus* Phytoplasma prunorum', '*Ca. P. mali*' and '*Ca. P. pyri*' has been established by multilocus sequence analysis. Among the four genetic loci used, the genes *imp* and *aceF* distinguished 30 and 24 genotypes, respectively, and showed the highest variability. Percentage of substitution for *imp* ranged from 50 to 68 % according to species. Percentage of substitution varied between 9 and 12 % for *aceF*, whereas it was between 5 and 6 % for *pnp* and *secY*. In the case of '*Ca. P. prunorum*' the three most prevalent *aceF* genotypes were detected in both plants and insect vectors, confirming that the prevalent isolates are propagated by insects. The four isolates known to be hypo-virulent had the same *aceF* sequence, indicating a possible monophyletic origin. Haplotype network reconstructed by eBURST revealed that among the 34 haplotypes of '*Ca. P. prunorum*', the four hypo-virulent isolates also grouped together in the same clade. Genotyping of some Spanish and Azerbaijanese '*Ca. P. pyri*' isolates showed that they shared some alleles with '*Ca. P. prunorum*', supporting for the first time to our knowledge, the existence of inter-species recombination between these two species.

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Abbreviations: AP, apple proliferation; ESFY, European stone fruit yellows; MLST, multilocus sequence typing; PD, pear decline; SNP, single nucleotide polymorphism; SSH, suppression subtractive hybridization.

The GenBank/EMBL/DDBJ accession numbers for the *aceF*, *pnp*, *secY* and *imp* gene fragments isolated in this study are listed in Tables 3–6.

A supplementary table, showing the origin and haplotype of all phytoplasma isolates, is available with the online version of this paper.

INTRODUCTION

Phytoplasmas are plant-pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less micro-organisms phylogenetically related to low G+C-content, Gram-positive bacteria (Weisburg *et al.*, 1989). They are responsible for hundreds of crop diseases worldwide (Lee *et al.*, 2000) and are transmitted from plant to plant by sap-sucking hemipteran insects (Weintraub & Beanland, 2006). European temperate fruit trees are affected by diseases associated with phytoplasmas which cause important economic losses to the European fruit production. ‘*Candidatus* Phytoplasma prunorum’ is responsible for European stone fruit yellows (ESFY) in trees and shrubs of the genus *Prunus*, ‘*Candidatus* P. mali’ is the causative agent of apple proliferation (AP) in those of the genus *Malus* and ‘*Candidatus* P. pyri’ is responsible for pear decline (PD) in *Pyrus* (Seemüller & Schneider, 2004). AP affects European and Turkish apple orchards (Seemüller, 1990; Sertkaya *et al.*, 2008) and the highest economical impact occurs in Germany and northern Italy. PD has been reported in Europe, North America and the Middle East (Battle *et al.*, 1999; Blomquist & Kirkpatrick, 2002b; Choueiri *et al.*, 2007; Davies *et al.*, 1992; Lorenz *et al.*, 1995; Malinowski *et al.*, 1996; Sharbatkhari *et al.*, 2008) and also in Taiwan (Liu *et al.*, 2007). ESYF is present in the Euro-Mediterranean basin (Marcone *et al.*, 2010) and kills 5% of the apricot trees per year in southern France (Jarausch *et al.*, 2001) due to a severe form of apricot chlorotic leafroll. It also induces plum leptonecrosis on *Prunus salicina* (Japanese plum) and yellows on most of the peach accessions in southern Europe. These three phytoplasmas belong to the same phylogenetic cluster, the group 16SrX (Lee *et al.*, 2000), and form three closely related subgroups (Jarausch *et al.*, 1994; Lorenz *et al.*, 1994).

‘*Ca. P. mali*’, ‘*Ca. P. pyri*’ and ‘*Ca. P. prunorum*’ are transmitted from tree to tree by psyllids of the genus *Cacopsylla* (Carraro *et al.*, 1998; Frisinghelli *et al.*, 2000; Jarausch & Jarausch, 2009; Jensen *et al.*, 1964) and can be propagated by grafting or multiplication of infected material in nurseries. In addition, ‘*Ca. P. mali*’ can also be transmitted through rootbridges among neighbouring trees in an orchard (Ciccotti *et al.*, 2007). Difference in virulence has been shown between isolates of ‘*Ca. P. prunorum*’ (Kison & Seemüller, 2001) and of ‘*Ca. P. mali*’ (Seemüller & Schneider, 2007) giving the first evidence of the biodiversity in this clade of phytoplasmas. In the case of ‘*Ca. P. prunorum*’, hypo-virulent isolates of the same species have been reported to occur in France and attempts have been made to use them for cross protection (Cornaggia *et al.*, 1995; Morvan *et al.*, 1991). The biodiversity of ‘*Ca. P. mali*’ was recently investigated by single-strand conformation polymorphism and sequence analyses of the *hflB* gene (Schneider & Seemüller, 2009) as an outcome of the ‘*Ca. P. mali*’ genome sequencing (Kube *et al.*, 2008). However, little is known about the genetic diversity of ‘*Ca. P. prunorum*’ and ‘*Ca. P. pyri*’.

In the absence of typing tools, it is not possible to trace the route of propagation of phytoplasma strains. Typing tools are therefore needed to improve disease control. The most appropriate method for genotyping prokaryotes is multi-locus sequence typing (MLST) which is widely used in bacterial epidemiology and population genetics (Maiden *et al.*, 1998; Urwin & Maiden, 2003). However, it requires the identification of gene sequences. For this purpose, and because very few genes have been characterized in this group of phytoplasmas, genes of ‘*Ca. P. prunorum*’ were cloned using a subtractive method. To design a multilocus sequence analysis of phytoplasmas of the group 16SrX, four genes were targeted: two genes involved in carbon and nucleotide metabolism and two genes encoding the immunodominant surface protein Imp and SecY, a component of the protein secretion machinery. The results of this investigation are presented with special emphasis on the genetic diversity of ‘*Ca. P. prunorum*’.

METHODS

Plant material and insects. Reference phytoplasma strains AT, AP15, GSFY2 and PD1 had been transmitted by dodder from fruit trees to periwinkle (*Catharanthus roseus* cv. Cooler) and since then have been maintained in periwinkle by successive graft inoculation (Carraro *et al.*, 1988; Marcone *et al.*, 1999; Marwitz *et al.*, 1974). Samples from infected *Prunus*, *Malus* or *Pyrus* species were collected from different regions of France, Spain, Austria, Germany, Croatia, Hungary, the UK, Bosnia and Herzegovina, Romania, Italy, Switzerland, Turkey, Lebanon and Azerbaijan (Table 1). *Cacopsylla pruni* psyllids were collected in Spring on *Prunus spinosa* (wild *Prunus*) in France and Spain, and on Japanese plum in Italy, by hitting branches above a lying net (Table 1). Plant DNA was tested by ECA1-ECA2 PCR for ‘*Ca. P. prunorum*’ (Jarausch *et al.*, 1998), by AP3-AP4 PCR for ‘*Ca. P. mali*’ (Jarausch *et al.*, 1994) and by universal phytoplasma 16SrDNA nested-PCR (Gundersen & Lee, 1996) followed by sequencing for ‘*Ca. P. pyri*’. Only positive samples were included in this study.

Double suppression subtractive hybridization (SSH) and genome walking. Double SSH was performed using the PCR-Select bacterial genome subtraction kit (BD Bioscience) except for the primers and adaptors which were modified as described previously (Cimerman *et al.*, 2006). Total DNA from healthy and GSFY2-infected periwinkle (2 µg) were digested overnight at 37 °C by *RsaI* (MBI Fermentas). *RsaI*-digested healthy plant DNA was used as the driver. Two populations of tester DNA were produced by ligating adaptors 1 and 2R to *RsaI*-digested DNA of infected plants. Aliquots of the ligation products were hybridized with an excess of driver according to the manufacturer’s instructions. These first hybridization mixtures were then hybridized together in the presence of an additional amount of driver. Hybrids carrying both adaptors 1 and 2R were amplified by nested PCR according to the manufacturer’s instructions using *Taq* polymerase (Promega). PCR amplification was performed in a 25 µl reaction volume with 0.4 µM primer P1. The templates were first heated for 2 min at 72 °C to fill extremities and then denatured for 25 s at 94 °C. PCR conditions were 30 cycles (10 s at 94 °C, 30 s at 62 °C, 1 min 30 s at 72 °C) with a single final extension of 7 min at 72 °C. Twenty identical cycles were used for nested amplification, with the sense and anti-sense primers NP1 and NP2R, and followed by a single final extension of 7 min at 72 °C. For reverse SSH, DNA from infected plants was used as the driver and DNA from healthy plants was used as the tester.

Table 1. Origin of the phytoplasma isolates tested

Country	Host	No. of isolates tested		
		' <i>Ca. P. prunorum</i> '	' <i>Ca. P. pyri</i> '	' <i>Ca. P. mali</i> '
Azerbaijan	<i>Prunus armeniaca</i>	2		
	<i>Prunus myrobalan</i>	1		
Croatia	<i>Prunus armeniaca</i>	8		
France	<i>Cacopsylla pruni</i>	26		
	<i>Prunus armeniaca</i>	10		
	<i>Prunus spinosa</i>	9		
	<i>Prunus salicina</i>	3		
	<i>Prunus persica</i>	2		
	<i>Prunus mariana</i>	5		
	GF8-1			
	<i>Prunus</i> sp.	10		
Germany	<i>Prunus armeniaca</i>	22		
Greece	<i>Prunus armeniaca</i>	1		
Italy	<i>Prunus armeniaca</i>	9		
	<i>Cacopsylla pruni</i>	4		
Spain	<i>Cacopsylla pruni</i>	2		
	<i>Prunus armeniaca</i>	9		
Turkey	<i>Prunus armeniaca</i>	3		
Azerbaijan	<i>Pyrus communis</i>		3	
Croatia	<i>Cacopsylla pyri</i>		2	
	<i>Cacopsylla pyrisuga</i>		1	
	<i>Pyrus communis</i>		8	
France	<i>Pyrus communis</i>		3	
Germany	<i>Pyrus communis</i>		8	
Italy	<i>Pyrus communis</i>		6	
Lebanon	<i>Pyrus communis</i>		4	
Spain	<i>Pyrus communis</i>		5	
Turkey	<i>Cacopsylla pyri</i>		1	
	<i>Pyrus communis</i>		2	
UK	<i>Pyrus communis</i>		3	
Austria	<i>Malus domestica</i>			1
Czech Republic	<i>M. domestica</i>			1
France	<i>M. domestica</i>			9
Germany	<i>M. domestica</i>			13
Italy	<i>M. domestica</i>			13
Romania	<i>M. domestica</i>			1
Switzerland	<i>M. domestica</i>			1

For double SSH, adaptors 1 and 2R were removed from the SSH product by *RsaI* digestion and replaced by the adaptors 3 and 4R before proceeding to the second round of subtraction. Then, *RsaI*-digested SSH product ligated to new adaptors NP3 and NP4 was used as tester and the *RsaI* reverse SSH product was used as the driver. PCR amplification was carried out with 0.4 µM primer P3. Nested amplification of the double SSH product was performed with primers NP3 and NP4R (Cimerman *et al.*, 2006). One microlitre of the double SSH product was ligated to pGEM-T Easy (Promega) and cloned in *Escherichia coli* DH10B according to standard procedures (Sambrook *et al.*, 1989).

One of the double SSH clones (SDR47) was extended by genome walking. Extension of the SDR47 sequence was performed using the

Biosciences Clontech BD GenomeWalker universal kit according to the manufacturer's instructions. Libraries for genome walking were composed of endonuclease-restricted DNA of periwinkles infected with '*Ca. P. prunorum*' isolate GSFY2.

Extraction of DNA from plants and insects, and PCR amplification. Nucleic acids were extracted from plants and insects as described by Maixner *et al.* (1995). The *aceF*, *pnp*, *secY* and *imp* genes were amplified by nested PCR using the primers described in Table 2. First amplifications were performed using an initial denaturation step at 95 °C for 3 min, followed by 20 cycles consisting of 94 °C for 30 s, 50 °C for 30 s and 66 °C for 45 s, and by a final extension step at 66 °C for 7 min. Nested amplification was carried out using 1 µl of the first amplification product with an initial denaturation step at 95 °C for 3 min followed by 35 cycles consisting of 94 °C for 30 s, 50 °C for 30 s and 66 °C for 45 s, and by a final extension step at 66 °C for 7 min. Nested PCR products were separated on a 1% agarose gel; DNA was stained with ethidium bromide and visualized under UV light.

Sequencing and sequence analysis. Sequencing reactions of the SSH plasmids and MLST PCR products were performed by Cogenics on ABI PRISM capillary sequencing instruments. Raw sequence chromatograms were assembled and edited using GAP4 (Bonfield *et al.*, 1995) or the PHRED-PHRAP-CONSED package (Ewing & Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998). All MLST PCR products were directly sequenced on both strands to achieve a 2 × coverage. Homologies between SSH clone sequences and known sequences were detected using the BLASTX algorithms against the non-redundant GenBank database at <http://www.ncbi.nlm.nih.gov/blast>. Multiple alignments were performed using CLUSTAL W (Thompson *et al.*, 1994) and the phylogenetic analyses were carried out with MEGA2 (Kumar *et al.*, 2001). Haplotype networks were constructed using the eBURST programme (Feil *et al.*, 2004).

RESULTS

Double SSH and PCR analysis of phytoplasmas

A library was constructed by cloning the *RsaI* double SSH product obtained by double-subtracting the total DNA of healthy periwinkle from the total DNA of a periwinkle infected with '*Ca. P. prunorum*' isolate GSFY2. Thirty plasmid inserts with sizes ranging from 536 to 971 bp were sequenced and identified by a BLASTX similarity search as being phytoplasma partial gene sequences. Most of the sequences were redundant in the library and finally corresponded to 11 unique sequences which are listed elsewhere (Danet *et al.*, 2008). The peptides predicted from SSH sequences shared 53–90% identity with proteins predicted from the '*Ca. P. asteris*' genome sequence (Oshima *et al.*, 2004). The sequence downstream of the *acoB* (insert SDR47) was determined by genome walking and used to identify the sequence of the neighbouring gene *aceF*. Among the '*Ca. P. prunorum*' genes identified, *aceF* and *pnp* were chosen as targets for the variability study because they presented the lowest conservation level when compared with the '*Ca. P. asteris*' homologous genes, which had 41 and 53% identity, respectively, at the predicted protein level. In addition to these two genes involved in sugar and nucleotide metabolism, the two genes *secY* and *imp* were selected to drive the genetic

Table 2. Oligonucleotides used for PCR and nested PCR

Gene	Length (bp)	Purpose	Primer	Sequence (5'–3')
<i>aceF</i>	797	PCR	AceFf1	TAAAATTCGCTGATGTTGGCG
			AceFr1	CATCTTCAATTCATTAATACTAG
		Nested-PCR	AceFf2	AGGTATTGAAGAAGGAAGCTG
			AceFr2	CAACCGCTTTCATAATAAAAG
<i>pnp</i>	549	PCR	Pnpf1	GAAGTTGGTATTACTGCTTTAC
			Pnpr1	GATAAATCTATTTGACCGCG
		Nested-PCR	Pnpf2	TACAATTAGATATTAAGTTAAAGG
			Pnpr2	ATTAATTTTAATACATTTTCGC
<i>secY</i>	664	PCR	SecYMalF1	TAGGACGTAGTATACAAATCCCNNTT
			SecYMalR1	ACAATAATTAATAATCCTGTNCC
		Nested-PCR	SecYMalF2	AAGAATGGCGTGAAACARGGNGA
			SecYMalR2	GCATCTGTTTAGATAAATGTTT
<i>imp</i> 'Ca. P. prunorum'	673	PCR	IMPF1	CAAATGATAAAGCTGATCAA
			IMPR1	CCTTTAAGACCACATCCTTTG
		Nested-PCR	IMPF2	CAGTGTATTAATTAACAATG
			IMPR2	CCATCATTACAATAAAACAT
<i>imp</i> 'Ca. P. pyri'	452	PCR	IMPF2bis	GTATTTAGTTACAATTATTTACTAG
			IMPR1bis	CAAGACCTTTAAGGCCACATC
		Nested-PCR	IMPF3pyr	AGGAGAAAAATAATGGAAGC
			IMPR4pyrA	TTGTGTTCTAAAAGTATCAC
<i>imp</i> 'Ca. P. mali'	670	PCR	IMPF1	CAAATGATAAAGCTGATCAA
			IMPR1bis	CAAGACCTTTAAGGCCACATC
		Nested-PCR	IMPF3	GTTTTATGTTATAATAAACAGTG
			IMPR3mal	CAAACATAGTTAAAATTAAGC

diversity study. *secY* is involved in protein secretion and its sequence was kindly provided by the 'Ca. P. mali' genome sequence consortium (Kube *et al.*, 2008). *imp* encodes the immunodominant surface protein and had been sequenced for the three species members of the 16SrX group (Morton *et al.*, 2003).

In order to develop a polyvalent PCR test for the entire 16SrX group, three nested PCR tests were designed from *aceF*, *pnp* and *secY* genes with primer pairs selected in regions encoding conserved domains of the proteins. To check the effectiveness of PCR primers, they were challenged against periwinkles infected with Italian and German 'Ca. P. prunorum' isolates and German 'Ca. P. mali' and 'Ca. P. pyri' isolates. Each primer pair allowed the direct amplification of *aceF*, *pnp* and *secY* DNA fragments for all phytoplasma tested provided that annealing and elongation temperatures were 50 and 66 °C, respectively (data not shown). For *imp*, due to its important variability among 16SrX phytoplasmas (Morton *et al.*, 2003), species-specific primer pairs had to be designed.

aceF, *pnp*, *secY* and *imp* nested PCR assays were applied to a collection of 197 phytoplasma isolates originating from France, Germany, Italy, Spain, Croatia, Switzerland, the UK, Hungary, Turkey, Greece, Azerbaijan and Lebanon. All samples had previously tested positive for 16SrX phytoplasmas by 16S rDNA PCR diagnosis. Most of the samples (157 of 197) reacted positively for the four nested PCR tests

including all the 32 infected *Cacopsylla pruni* tested ('Ca. P. prunorum' insect vector).

Sequences of the PCR products were determined and compared by multiple alignments. The presence of species-specific sequence motifs and important genetic distances were in agreement with the existence of three distinct phytoplasma species. Sequence alignments indicated that four deletions (total 15 bp) and one 3 bp insertion took place in the 'Ca. P. pyri' *aceF* sequence by comparison with 'Ca. P. prunorum', whereas five deletions totalling 48 bp distinguished the 'Ca. P. mali' *aceF* sequence. The proportion of substitutions in *aceF* ranged from 9.6 % between 'Ca. P. pyri' and 'Ca. P. mali' to 12 % between 'Ca. P. pyri' and 'Ca. P. prunorum'. No insertion or deletion occurred in *pnp* sequences and substitutions affected 5–6 % of nucleotide positions depending on the 'Ca. Phytoplasma' species compared. In the *secY* gene, sequences diverged about 5 % between species and one 3 bp deletion was observed for 'Ca. P. pyri'.

imp was the most variable gene with 34.3 sequence identity between 'Ca. P. prunorum' and 'Ca. P. pyri', and 32.1 % between 'Ca. P. prunorum' and 'Ca. P. mali', while 'Ca. P. mali' and 'Ca. P. pyri' *imp* sequences were 50.8 % identical. Two 3 bp deletions took place in *imp* of 'Ca. P. prunorum'; one 9 bp deletion and two 3 bp insertions characterize *imp* of 'Ca. P. mali'; whereas a 3 bp insertion was specific to the 'Ca. P. pyri' *imp* gene.

Sequencing *aceF*, *pnp*, *secY* and *imp* identifies geographically related genotypes

The sequence of the *aceF* marker was determined for 197 isolates of the three *Candidatus* species. Maximum-parsimony analysis of *aceF* sequences confirmed the existence of three phylogenetic clusters corresponding to '*Ca. P. prunorum*', '*Ca. P. mali*' and '*Ca. P. pyri*' (Fig. 1, Table 3). For '*Ca. P. prunorum*', 11 genotypes were discriminated, most of them differing by one or two single nucleotide polymorphisms (SNPs). Only genotype A9 [an apricot isolate and a *Prunus cerasifera* (myrobalan plum) isolate from Azerbaijan] presented 10–12 SNPs compared with the other '*Ca. P. prunorum*' genotypes. The genotype A3 represented 57 % of infection cases. This genotype was found in isolates from France, Germany, Spain and Italy (Table 3). Two other genotypes, A8 and A6, accounted for 19 and 14 % of the isolates, respectively. These genotypes were detected in isolates from France, Croatia, Germany, Italy, Turkey, Spain, Greece and Azerbaijan (Table 3). Seven *aceF* genotypes were only found in a single country, for example, genotypes A7, A9 and A17 were only detected in Croatia, Azerbaijan and Turkey, respectively. Interestingly, the four hypo-virulent isolates of '*Ca. P. prunorum*' tested (namely PVC-LA8 and PVC-LA9, B7

and E22) clustered together within the same A6 genotype. This monophyletic clustering of hypo-virulent isolates indicates their possible single origin. Five '*Ca. P. prunorum*' genotypes A3, A5, A6, A8 and A21, out of 11 genotypes, could be detected in *C. pruni* insect vectors (Table 3). For '*Ca. P. mali*', among six genotypes differing in between one and three SNPs, the genotype A13 was predominant (65 %) and was detected in isolates from France, Italy and Germany (Table 3). For '*Ca. P. pyri*', the seven genotypes showed a genetic divergence higher than that of '*Ca. P. prunorum*' genotypes, in the range of 1 to 24 SNPs. Genotype A10 represented 72 % of the isolates and was detected in isolates from eight of the nine countries surveyed (Table 3). The six other genotypes were each found in a single country. Again, countries in the eastern part of the Mediterranean basin, such as Turkey or Lebanon, had specific '*Ca. P. pyri*' genotypes.

Sequences of the *pnp* and *secY* markers were determined for 172 and 157 isolates of the three *Candidatus* species, respectively. Maximum-parsimony analyses were conducted with the sets of *pnp* and *secY* sequences (Fig. 2). Phylogenetic trees obtained for both genes were consistent with the existence of the three described taxonomic species; all isolates of a given species were clustered on the same branch supported by high bootstrap values. The diversity of *pnp* and *secY* was lower than that of *aceF*: 15 genotypes for *pnp* and 12 genotypes for *secY* could be discriminated (Tables 4 and 5). For '*Ca. P. prunorum*', two and three genotypes could be distinguished with *pnp* and *secY*, respectively, a much lower diversity when compared with the 11 *aceF* genotypes. The two *pnp* genotypes were also detected in *C. pruni* psyllids whereas only two of three *secY* genotypes were detected in *C. pruni* (Tables 4 and 5). For '*Ca. P. mali*', five and four genotypes were distinguished with *pnp* and *secY* respectively, whereas for '*Ca. P. pyri*', eight and five genotypes were differentiated with the same markers. The most prevalent *pnp* genotype was P1 in '*Ca. P. prunorum*' isolates originating from plant and insect. For *secY*, '*Ca. Phytoplasma*' species-specific prevalences were seen: S1 and S2 in '*Ca. P. prunorum*' isolates, S7 in '*Ca. P. pyri*' isolates and S12 in '*Ca. P. mali*' isolates.

The sequence of the *imp* marker was determined for 151 isolates of the three *Candidatus* species. Phylogenetic analysis using maximum parsimony showed 30 different genotypes (Fig. 3a). Clustering was again in agreement with the description of three *Candidatus* species; all isolates of a given species were clustered on the same branch supported by bootstrap values of 100. Seven, nine and 14 genotypes could be distinguished for '*Ca. P. mali*', '*Ca. P. pyri*' and '*Ca. P. prunorum*', respectively. The number of mutations between genotypes of the same species was much higher in '*Ca. P. pyri*' and '*Ca. P. mali*' than in '*Ca. P. prunorum*'. For '*Ca. P. prunorum*', the most prevalent genotype, genotype I1, was detected in 56 plant and insect samples coming from six countries (Table 6). Six genotypes were detected in two to four countries and eight genotypes were found in only one country. This was the case for genotypes

Fig. 1. Evolutionary relationships of *aceF* for isolates of the group 16SrX. The evolutionary history was inferred by using the maximum-parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap values below 90 are omitted. The trees are drawn to scale; branch lengths were calculated using the average pathway method and represent the number of nucleotide changes over the whole sequence. Major genotypes are indicated (%). HYPV, Hypo-virulent.

Table 3. Origin of phytoplasma isolates with the *aceF* genotype

Reference isolate	Geographical origin*	EMBL accession no.	Pathogen	<i>aceF</i> genotype	No. of plants	No. of insects
WJ3703/30	Germany	FN598166	' <i>Ca. P. prunorum</i> '	A1	1	0
Psalor	France	FN598167	' <i>Ca. P. prunorum</i> '	A2	1	0
GSFY2	Germany, France, Italy, Spain	FN598168	' <i>Ca. P. prunorum</i> '	A3	47	24
ESFYSpa01	Spain	FN598169	' <i>Ca. P. prunorum</i> '	A4	3	0
40-04Cr	Croatia, Italy	FN598170	' <i>Ca. P. prunorum</i> '	A5	1	1
B7	France, Croatia, Germany, Italy, Turkey	FN598171	' <i>Ca. P. prunorum</i> '	A6	15	3
61-04Cr	Croatia	FN598172	' <i>Ca. P. prunorum</i> '	A7	1	0
ESFY	Italy, Azerbaijan, Croatia, France, Germany, Greece, Spain	FN598173	' <i>Ca. P. prunorum</i> '	A8	21	3
Azer10	Azerbaijan	FN598174	' <i>Ca. P. prunorum</i> '	A9	2	0
Ya15	Turkey	FN598175	' <i>Ca. P. prunorum</i> '	A17	2	0
E60-11B	Italy	FN598176	' <i>Ca. P. prunorum</i> '	A21	0	1
PD	Germany, Azerbaijan, Croatia, France, Germany, UK, Italy, Turkey	FN598177	' <i>Ca. P. pyri</i> '	A10	24	2
PD121Lib	Lebanon	FN598178	' <i>Ca. P. pyri</i> '	A11	4	0
PD2Luca	Italy	FN598179	' <i>Ca. P. pyri</i> '	A12	1	0
PP/ZG/1	Croatia	FN598180	' <i>Ca. P. pyri</i> '	A18	2	0
PI/ZG/1	Croatia	FN598181	' <i>Ca. P. pyri</i> '	A19	0	1
PTK7	Turkey	FN598182	' <i>Ca. P. pyri</i> '	A20	0	1
TR/1	Germany	FN598183	' <i>Ca. P. pyri</i> '	A24	1	0
AP-AT	Germany, France, Italy	FN598184	' <i>Ca. P. mali</i> '	A13	22	0
AP13	Romania	FN598185	' <i>Ca. P. mali</i> '	A14	1	0
AP032-10	France, Austria, Italy	FN598186	' <i>Ca. P. mali</i> '	A15	7	0
AP1Luca	Italy	FN598187	' <i>Ca. P. mali</i> '	A16	2	0
TN/1	Italy	FN598188	' <i>Ca. P. mali</i> '	A22	1	0
NW/2	Germany	FN598189	' <i>Ca. P. mali</i> '	A23	1	0

*The *aceF* gene was isolated from samples isolated from the country/countries listed.

I5 and I26, respectively, which were detected in Azerbaijan and Turkey. Five genotypes were specifically detected in France. A gradient in the geographical distribution appeared between western and central Europe (Fig. 3b). Two of the Spanish genotypes (red and pale blue in Fig. 3) were only found in isolates from France and Germany, whereas the prevalent I1 and I2 genotypes (dark blue in Fig. 3) were absent in isolates from Spain. Genotypes of isolates from the eastern part of the Mediterranean basin, such as those detected in isolates from Turkey and Azerbaijan (brown and orange in Fig. 3) were not detected in central or western Europe.

Identification of phytoplasma haplotypes reveals no geographical association

For all four genes and all three phytoplasma candidate species, a total of 73 different haplotypes were detected (Supplementary Table S1, available with the online version of this paper). In the case of '*Ca. P. prunorum*', for which 118 isolates were fully genotyped, the haplotype network combining the four genetic loci *aceF*, *pnp*, *imp* and *secY* was built (Fig. 4). Thirty-four haplotypes were organized into a continuous network with A3-P1-I1-S1 and A3-P1-I9-S1

(Fig. 4, in grey) as founders representing 28 % of the isolates. No relationship was found between the haplotype and the geographical origin of the plant (apricot, peach, Japanese plum) or psyllid. Interestingly, the four hypo-virulent isolates clustered on the same part of the haplotype network (Fig. 4, in black), again suggesting their common origin. Fifteen of the haplotypes were detected in psyllids and could possibly be epidemic if these insects were to be vectors. It was also noticeable that the major haplotype A3-P1-I1-S1 was only detected once in psyllids, whereas the less abundant haplotypes A3-P1-I1-S2 and A3-P1-I9-S2 were found twelve and seven times in the insect vector, respectively.

Evidence for '*Ca. P. pyri*' and '*Ca. P. prunorum*' inter-species recombination

The most striking result was obtained with three '*Ca. P. pyri*' isolates collected in Spain and Azerbaijan. Multilocus sequence analysis revealed that they possess both '*Ca. P. pyri*' and '*Ca. P. prunorum*' genetic markers (Fig. 5). For example, isolate Spa3 had *aceF* and *imp* genes of '*Ca. P. prunorum*' while its *pnp* and *secY* genes corresponded to a '*Ca. P. pyri*' genotype. Isolate Spa2 also had two genes corresponding to '*Ca. P. prunorum*' genotypes and two

Fig. 2. Evolutionary relationships of *pnp* (a) and *secY* (b) for isolates of the group 16SrX. The evolutionary history was inferred by using the maximum-parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap values below 90 are omitted. The trees are drawn to scale; branch lengths were calculated using the average pathway method and represent the number of nucleotide changes over the whole sequence.

genes clustering within the '*Ca. P. pyri*' branch of the phylogenetic trees, whereas AZ-POI45 had only a '*Ca. P. prunorum*' *pnp* gene and three other gene markers corresponding to '*Ca. P. pyri*'. Altogether, these results indicated that these isolates of '*Ca. P. pyri*' are inter-species recombinants. Surprisingly, for the Spa2 isolate, two different *imp* sequences could be detected, one corresponding to '*Ca. P. prunorum*' and one clustering with '*Ca. P. pyri*' *imp* sequences. This could not be associated with a

mixed infection as only the 16S rDNA sequence of '*Ca. P. pyri*' could be detected.

DISCUSSION

An MLST method has been developed and applied for typing temperate fruit tree phytoplasmas of the phylogenetic group 16SrX. The most variable markers were *imp* and

Table 4. Origin of phytoplasma isolates with the *pnp* genotype

Reference isolate	Geographical origin*	EMBL accession no.	Pathogen	<i>pnp</i> genotype	No. of plants	No. of insects
WJ3703-30	Germany, Azerbaijan, Croatia, France, Italy, Spain	FN598190	' <i>Ca. P. prunorum</i> '	P1	75	30
ESFY	Italy, France, Germany, Azerbaijan	FN598191	' <i>Ca. P. prunorum</i> '	P2	14	2
PD	Germany, France, Lebanon, Spain	FN598192	' <i>Ca. P. pyri</i> '	P3	5	0
PD121Lib	Lebanon, Azerbaijan, Croatia, UK, Spain	FN598193	' <i>Ca. P. pyri</i> '	P4	9	1
PD84	Italy, Lebanon	FN598194	' <i>Ca. P. pyri</i> '	P5	7	0
PD356-1	France, UK	FN598195	' <i>Ca. P. pyri</i> '	P6	2	0
PD36AZ	Azerbaijan	FN598196	' <i>Ca. P. pyri</i> '	P7	1	0
PD2Luca	Italy, Spain	FN598197	' <i>Ca. P. pyri</i> '	P8	2	0
PP/ZG/1	Croatia	FN598198	' <i>Ca. P. pyri</i> '	P14	2	0
PI/ZG/1	Croatia	FN598199	' <i>Ca. P. pyri</i> '	P15	1	1
AP-AT	Germany, France	FN598200	' <i>Ca. P. mali</i> '	P9	2	0
AP15	Italy, France	FN598201	' <i>Ca. P. mali</i> '	P10	3	0
AP28	Switzerland, France, Italy, Romania	FN598202	' <i>Ca. P. mali</i> '	P11	6	0
AP032-10	France, Austria, Italy	FN598203	' <i>Ca. P. mali</i> '	P12	4	0
AP1Luca	Italy	FN598204	' <i>Ca. P. mali</i> '	P13	2	0

*The *pnp* gene was isolated from samples isolated from the country/countries listed.

Table 5. Origin of phytoplasma isolates with the *secY* genotype

Reference isolate	Geographical origin*	EMBL accession no.	Pathogen	<i>secY</i> genotype	No. of plants	No. of insects
GSFY	Germany, France, Italy, Spain	FN598205	' <i>Ca. P. prunorum</i> '	S1	52	3
ESFY	Italy, Azerbaijan, Croatia, France, Spain	FN598206	' <i>Ca. P. prunorum</i> '	S2	29	29
14MY	France, Croatia, Germany, Spain	FN598207	' <i>Ca. P. prunorum</i> '	S3	6	0
PDSpa2	Spain	FN598208	' <i>Ca. P. pyri</i> '	S4	1	0
PD36Az	Azerbaijan	FN598209	' <i>Ca. P. pyri</i> '	S5	1	0
PD356-1	France, UK	FN598210	' <i>Ca. P. pyri</i> '	S6	2	0
PD	Germany, France, UK, Italy, Lebanon, Spain	FN598211	' <i>Ca. P. pyri</i> '	S7	10	0
PD2Luca	Italy, Spain	FN598212	' <i>Ca. P. pyri</i> '	S8	2	0
AP-AT	Germany	FN598213	' <i>Ca. P. mali</i> '	S9	3	0
AP4Luca	Italy, Austria	FN598214	' <i>Ca. P. mali</i> '	S10	2	0
AP032-10	France	FN598215	' <i>Ca. P. mali</i> '	S11	1	0
AP15	Italy, France, Romania	FN598216	' <i>Ca. P. mali</i> '	S12	10	0

*The *secY* gene was isolated from samples isolated from the country/countries listed.

aceF but should not be solely used, because different clusterings were obtained with the markers *pnp* and *secY*. The efficiency of this typing system was illustrated by the discrimination of 34 different haplotypes within the '*Ca. P. prunorum*' species. MLST is particularly useful for epidemiological studies especially to compare the genotypes present in the wild and cultivated compartments, for example in the case of '*Ca. P. prunorum*' in *Prunus spinosa* versus apricot or Japanese plum orchards. MLST of the phytoplasmas detected in the psyllid vector will then show whether '*Ca. P. prunorum*' is transmitted from wild *Prunus* to orchards. Fine genotyping can also be useful to trace the route of propagation by nurseries. The dominant haplotype A3-P1-I1-S1 that was very rarely detected in psyllid vectors could have spread by the propagation of infected plant material. Phylogenetic studies can also help to explore the origin of related phytoplasmas living in different hosts as has been illustrated with the common origin of alder and grapevine phytoplasmas in the 16SrV phylogenetic group (Arnaud *et al.*, 2007). MLST may contribute to functional studies of the biological traits of phytoplasmas. For example, the hypo-virulence trait in '*Ca. P. prunorum*' (Kison & Seemüller, 2001) has been well characterized in the four French isolates used in this study and all of them had the same *aceF* genotype, A6, but different *pnp*, *secY* and *imp* genotypes. This shows that the A6 genotype is not carried by a clonal strain but by a diversified cluster of isolates. It is not yet known to what extent the A6 genotype is linked to the hypo-virulent trait. Are the other '*Ca. P. prunorum*' isolates with genotype A6 also hypo-virulent? Biological characterization of more isolates is needed to establish a firm correlation between the A6 genotype and the hypo-virulent trait. Two of the '*Ca. P. prunorum*' isolates tested in the present study infect sensitive apricot varieties that remained symptomless for many years. However, these isolates are genotype A8, showing at least that the hypo-virulent trait does not have a monophyletic origin. The *aceF* gene may be directly

responsible for hypo-virulence since the AceF protein is involved in glycolysis. Alternatively, a gene genetically linked to *aceF* may determine hypo-virulence. In the A6 genotype, a punctual mutation replaces a threonine with an asparagine at position 189 in the E2 component of the pyruvate dehydrogenase. The effect of this mutation is unknown. The possibility that hypo-virulent isolates are impaired in carbon metabolism can be envisaged. Glycolysis is a pathway essential for phytoplasmas which have a reduced physiology as shown by the analysis of their small genome content (Bai *et al.*, 2006; Oshima *et al.*, 2004; Tran-Nguyen *et al.*, 2008). This pathway is only partially present in '*Ca. P. mali*' strain AT, as deduced from the sequence of its linear chromosome (Kube *et al.*, 2008) even though the functional activity of the remaining half of the glycolysis pathway is still unknown.

Due to the low level of similarity between *imp* sequences of the different *Candidatus* species, it could be stressed that they do not correspond to orthologous genes. Despite the low identity at the nucleotide level, the three *imp* genes are certainly orthologues and not paralogues, because identity at the protein level ranges from 43 to 49% between the three phytoplasma species. In addition, only one copy of *imp* exists in the sequenced genome of '*Ca. P. mali*'. It has recently been demonstrated that *imp* genes are highly variable and submitted to strong diversifying selection (Kakizawa *et al.*, 2009). Nevertheless we cannot exclude the possibility of hidden paralogy, in which orthologues would have been duplicated and would have been lost further. It does not, however, affect the results of the genotyping and its analysis.

For the first time to our knowledge, a recombination between phytoplasmas has been shown. We showed that '*Ca. P. pyri*' and '*Ca. P. prunorum*' have undergone recombination. When the results were first obtained, we envisaged the possibility of a PCR cross-over contamination despite the numerous measures taken in our laboratory to

Fig. 3. Genetic variability of *imp* (a) and geographical distribution of 'Ca. P. prunorum' *imp* genotypes (b). (a) Evolutionary relationships of *imp* for isolates of the group 16SrX. The evolutionary history was inferred by using the maximum-parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap values below 90 are omitted. The trees are drawn to scale; branch lengths were calculated using the average pathway method and represent the number of nucleotide changes over the whole sequence. (b) Distribution of 'Ca. P. prunorum' *imp* genotypes in the northern Euro-Mediterranean basin. Numbers below the circles indicate the total number of isolates per country. The colour legend is given in (a).

prevent such artefacts. So, the experiment was repeated but the same results were obtained and still the negative PCR controls remained negative. So our results were reproducible and we finally excluded the possibility of a PCR contamination when looking in detail at the result of the Azerbaijani isolate POI45. This isolate is recombinant for the *pnp* gene and its *pnp* sequence is unique, with

several mutations distinguishing this isolate from the other known *pnp* genotypes (P1 and P2). If this result was due to PCR contamination, the sequence should have been that of P1 or P2. For isolate Spa2, in which we detected two different copies of *imp*, we could exclude mixed infection between 'Ca. P. pyri' and 'Ca. P. prunorum' after 16SrDNA sequencing, but we could not

Table 6. Origin of phytoplasma isolates with the *imp* genotype

Reference isolate	Geographical origin	EMBL accession no.	Pathogen	<i>imp</i> genotype	No. of plants	No. of insects
ESFY	Italy, Azerbaijan, Croatia, France, Germany, Greece	FN600707	' <i>Ca. P. prunorum</i> '	I1	41	15
Pru33	France	FN600708	' <i>Ca. P. prunorum</i> '	I2	1	0
WJ3889-42	Germany	FN600709	' <i>Ca. P. prunorum</i> '	I3	1	0
40-04Cr	Croatia, France, Italy, Hungary	FN600710	' <i>Ca. P. prunorum</i> '	I4	8	0
Azer10	Azerbaijan	FN600711	' <i>Ca. P. prunorum</i> '	I5	2	0
ESFY014-1	France	FN600712	' <i>Ca. P. prunorum</i> '	I6	1	0
05MY	France	FN600713	' <i>Ca. P. prunorum</i> '	I7	1	0
G32	France	FN600714	' <i>Ca. P. prunorum</i> '	I8	1	0
B7	France, Germany, Spain	FN600715	' <i>Ca. P. prunorum</i> '	I9	20	11
13MY	France, Croatia, Italy	FN600716	' <i>Ca. P. prunorum</i> '	I10	3	3
ESFYSpa01	France, Spain	FN600717	' <i>Ca. P. prunorum</i> '	I11	3	2
01MY	France	FN600718	' <i>Ca. P. prunorum</i> '	I12	1	0
T13	France, Croatia, Germany, Spain	FN600719	' <i>Ca. P. prunorum</i> '	I13	6	1
ETK-Ya15	Turkey	FN600720	' <i>Ca. P. prunorum</i> '	I26	5	0
PD280-4	France, UK	FN600721	' <i>Ca. P. pyri</i> '	I14	3	0
PD20	UK	FN600722	' <i>Ca. P. pyri</i> '	I15	1	0
PD36AZ	Azerbaijan, Croatia	FN600723	' <i>Ca. P. pyri</i> '	I16	2	0
PD121Lib	Lebanon	FN600724	' <i>Ca. P. pyri</i> '	I17	3	0
PD33Lib	Lebanon, Azerbaijan	FN600725	' <i>Ca. P. pyri</i> '	I18	1	0
PD	Germany, Croatia, Italy	FN600726	' <i>Ca. P. pyri</i> '	I19	4	0
PDSpa2	Spain, France	FN600727	' <i>Ca. P. pyri</i> '	I20	1	0
KT/1	Croatia	FN600728	' <i>Ca. P. pyri</i> '	I27	2	1
PI/ZG/1	Croatia	FN600729	' <i>Ca. P. pyri</i> '	I28	0	1
AP032-10	France, Austria, Germany, Italy	FN600730	' <i>Ca. P. mali</i> '	I21	12	0
AP13	Romania	FN600731	' <i>Ca. P. mali</i> '	I22	2	0
AP15	Italy, Germany	FN600732	' <i>Ca. P. mali</i> '	I23	4	0
AP-AT	Germany, France	FN600733	' <i>Ca. P. mali</i> '	I24	2	0
AP1Luca	Italy	FN600734	' <i>Ca. P. mali</i> '	I25	2	0
TN2	Italy	FN600735	' <i>Ca. P. mali</i> '	I29	1	0
HD/1	Germany	FN600736	' <i>Ca. P. mali</i> '	I30	1	0

*The *imp* gene was isolated from samples isolated from the country/countries listed.

reject the possibility of a mixed infection between two '*Ca. P. pyri*' isolates, one isolate being recombinant, the other not. Another possibility could be that an isolate carries two *imp* genes, one copy having diverged in its function. But the question remains about the mechanism of such recombinations. Until the publication of the '*Ca. P. mali*' genome, the ability of phytoplasmas to perform recombination was

questionable because they lack the genetic information to perform homologous recombination (Bai *et al.*, 2006; Oshima *et al.*, 2004). Nevertheless, it was discovered that '*Ca. P. mali*' has an extended set of genes for homologous recombination, including the essential gene *recA* (Kube *et al.*, 2008). In addition, all members of the 16SrX group have a linear chromosome, a property that in combination with the

Fig. 4. Haplotype network of '*Ca. P. prunorum*' isolates. Grey indicates founder haplotypes and black indicates hypo-virulent isolates.

Fig. 5. Evolutionary relationships of *aceF* (a), *pnp* (b), *secY* (c) and *imp* (d) for recombinant 'Ca. P. pyri' and isolates of the group 16SrX. The evolutionary history was inferred by using the maximum-parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap values below 90 are omitted. The trees are drawn to scale; branch lengths were calculated using the average pathway method and represent the number of nucleotide changes over the whole sequence. Arrows indicate 'Ca. P. pyri' recombinants.

presence of multiple repeated genes such as *hflB* (Schneider & Seemüller, 2009), a gene known to be present as multiple copies in all known phytoplasma genomes (Kube *et al.*, 2008; Oshima *et al.*, 2004), can facilitate the exchange of a full chromosome arm through a single cross-over. Such a simple

recombination event would lead to the exchange of many gene markers at the same time. However, to allow recombination between two species, they have to share a common host. 'Ca. P. pyri' can be transmitted and multiplies in peach where it induces the peach yellow leaf

roll (Blomquist & Kirkpatrick, 2002a; Purcell *et al.*, 1981). Peach is a common host for 'Ca. P. prunorum' in the north of the Euro-Mediterranean area; it is therefore probable that peach was the host in which the inter-species recombination occurred. Recombination might also have occurred in a common insect vector, such as *C. pyri*, which is more polyphagous in autumn and might acquire both phytoplasmas from their respective host plant. Because none of the three 'Ca. P. pyri' recombinants had the same MLST genotype, it is likely that the recombination event is quite frequent. Indeed, it represented three cases from two distinct geographical areas over 19 different 'Ca. P. pyri' isolates tested. After their appearance, recombinants had been naturally transmitted back to pear by psyllids, in which they were finally detected. One could certainly consider that if recombination occurs between species it could also occur within species. We cannot confirm such a phenomenon because, despite incongruence between the phylogenetic trees of the different markers, the bootstrap validity levels were not high enough to consider it evident.

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