Genetic diversity of European phytoplasmas of the 16SrV taxonomic group and proposal of 'Candidatus Phytoplasma rubi'

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In addition to the grapevine flavescence dorée phytoplasmas, other members of taxonomic group 16SrV phytoplasmas infect grapevines, alders and species of the genera *Clematis* and *Rubus* in Europe. In order to investigate which phytoplasmas constitute discrete, species-level taxa, several strains were analysed by comparing their 16S rRNA gene sequences and a set of five housekeeping genes. Whereas 16S rRNA gene sequence similarity values were >97.5%, the proposed threshold to distinguish two 'Candidatus Phytoplasma' taxa, phylogenetic analysis of the combined sequences of the *tuf*, *rplV-rpsC*, *rplF-rplR*, *map* and *uvrB-degV* genetic loci showed that two discrete phylogenetic clusters could be clearly distinguished. The first cluster grouped flavescence dorée (FD) phytoplasmas, alder yellows (AldY) phytoplasmas, *Clematis* (CL) phytoplasmas and the Palatinate grapevine yellows (PGY) phytoplasmas. The second cluster comprised *Rubus* stunt (RS) phytoplasmas. In addition to the specificity of the insect vector, the *Rubus* stunt phytoplasma contained specific sequences in the 16S rRNA gene. Hence, the *Rubus* stunt phytoplasma 16S rRNA gene was sufficiently differentiated to represent a novel putative taxon: 'Candidatus Phytoplasma rubi'.

Phytoplasmas are plant-pathogenic bacteria belonging to the class *Mollicutes*, a group of cell wall-less micro-organisms phylogenetically related to Gram-positive bacteria with a low DNA G+C content (Weisburg *et al.*, 1989). Phytoplasmas are still unavailable in culture. 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). Flavescence dorée (FD) of grapevine, a quarantine disease first reported in the 1950s in south-western France (Caudwell, 1957) is now present in southern Europe (Battlé *et al.*, 1997; Belli *et al.*, 1985; Boudon-Padieu, 2002; Duduk *et al.*, 2003). The causal agent of FD was shown to be a phytoplasma (Caudwell *et al.*, 1971) that is transmitted by

a grapevine leafhopper of north American origin, *Scaphoideus titanus* (Ball.) (Schvester *et al.*, 1961). The FD phytoplasma belongs to the 16SrV taxonomic group (Lee *et al.*, 1998). Members of this group share high 16S rRNA gene sequence similarity (Davis & Dally, 2001), but the group consists of phytoplasmas with an important variety of biological niches restricted to woody perennial hosts. 'Candidatus P. ulmi' in the 16SrV-A subgroup is responsible for yellows of elm species in North America and Europe (Lee *et al.*, 2004) and 'Candidatus P. ziziphi' in the 16SrV-B subgroup is the agent of jujube witches'-broom and cherry lethal yellows in Asia (Jung *et al.*, 2003; Lee *et al.*, 2004). In Europe, other phytoplasmas in the 16SrV group mainly infect grapevines (Maixner *et al.*, 1994), alder (Lederer & Seemüller, 1991; Mäurer *et al.*, 1993), blackberry (de Fluiter & van der Meer, 1953; Mäurer & Seemüller, 1995), species of the genus *Spartium* (Marccone *et al.*, 1996) and *Clematis vitalba* (Angelini *et al.*, 2004).

Most of the insect vectors that naturally disseminate group 16SrV phytoplasmas have been identified. The elm yellows phytoplasma is transmitted in north America by *Scaphoideus luteolus* (Van Duzee) (Baker, 1949) and in Europe by *Macropsis mendax* (Fieber) (Carraro *et al.*, 2004), whereas *Rubus* stunt phytoplasma is transmitted by *Macropsis fuscula*

Abbreviations: AldY, alder yellows; CL, *Clematis*; FD, flavescence dorée; PGY, Palatinate grapevine yellows; RS, *Rubus* stunt.

The GenBank/EMBL/DDBJ accession numbers for the *tuf*, *rplV*, *rplF*, *map* and *degV* gene sequences for the strains discussed in this paper are available in a supplementary table with the online version of this paper.

The origin and sequence accession numbers of phytoplasmas used in this study and the primers used for gene amplification and PCR conditions are available as supplementary tables with the online version of this paper.

(Zetterstedt) (de Fluiter & van der Meer, 1953). Phytoplasmas associated with Palatinate grapevine yellows (PGY) and alder yellows (AldY) are both transmitted by the alder leafhopper *Oncopsis alni* (Schrank) (Maixner & Reinert, 1999; Maixner *et al.*, 2000).

PGY and AldY phytoplasmas were classified as members of the 16SrV group on the basis of their high 16S rRNA and *secY* gene sequence similarities to the corresponding genes of FD phytoplasmas (Angelini *et al.*, 2001, 2003). FD phytoplasmas have been classified into two distinct subgroups, 16SrV-C and 16SrV-D, on the basis of sequence differences in the 16S rRNA gene and the 16S–23S intergenic spacer (Davis & Dally, 2001; Martini *et al.*, 1999). However, genetic diversity studies using non-ribosomal genes have provided evidence for further FD strain clusters (Martini *et al.*, 2002). In order to trace the spread of FD strains and to identify possible passages between the vineyard and wild plant compartments, multilocus sequence typing of 16SrV phytoplasma strains has been developed. Phylogenetic analyses showed that FD, PGY and AldY phytoplasmas were members of the same phylogenetic subclade, which could have originated in Europe (Arnaud *et al.*, 2007). It has also been shown that the *Rubus* stunt (RS) phytoplasma is genetically distinct from the other members of the 16SrV group, confirming its classification as subgroup 16SrV-E (Davis & Dally, 2001). These recent data have consequences for the taxonomy of the FD, PGY, AldY and RS phytoplasmas. In this paper, the results of multilocus sequence analyses are reported that support the establishment of a novel taxon in the 16SrV phylogenetic group in agreement with the rules for the description of new taxa in the '*Candidatus* Phytoplasma' provisional taxon (Firrao *et al.*, 2004).

The phytoplasmas examined in this study are listed in Supplementary Table S1 (available in IJSEM online). Samples were collected in France, Italy and Germany. Grapevines collected in France in 2004 tested positive for FD phytoplasma using a non-ribosomal-specific PCR assay (Clair *et al.*, 2003). Alders, elms, *Rubus* and dog roses were sampled in France (Boudon-Padieu *et al.*, 2004; Jarausch *et al.*, 2001; Malembic-Maher *et al.*, 2007) and gave a positive result by PCR of the *map* gene (Arnaud *et al.*, 2007). Other elm and *Rubus* samples collected in north-eastern Italy had tested positive by 16S rRNA PCR and recognized RFLP methods. *Clematis* were sampled in Italy and tested positive by real-time PCR (Angelini *et al.*, 2007; Filippin *et al.*, 2009).

In order to investigate the phylogenetic relationships between phytoplasmas, the 16S rRNA gene sequence was amplified and sequenced using the primer pair P1/P7 (Schneider *et al.*, 1995) and the sequences were compared with each other. All sequences from grapevines, alder and *Clematis* were 99.9–100 % similar as only one nucleotide differentiated members of subgroups 16SrV-C and 16SrV-D. They also showed 99.6 % and 99.4 % sequence similarity with '*Ca. P. ulmi*' strain EY1 and *Rubus* stunt phytoplasma strain RuS, respectively. The 16S rRNA gene sequence similarity between *Rubus* stunt phytoplasma strain RuS and '*Ca. P. ulmi*' strain EY1 was 98.9 %. Phylogenetic analysis revealed four clusters (Fig. 1) corresponding to: (i) members of subgroups 16SrV-A, i.e. '*Ca. P. ulmi*'; (ii) *Rubus* stunt phytoplasmas, which are members of subgroup 16SrV-E; (iii) members of subgroups 16SrV-C (strain FD70) and (iv) 16SrV-D (strain FD92). Apart from the branch for subgroup 16SrV-E, the phylogenetic tree was not supported by high bootstrap values. This was due

Fig. 1. Evolutionary relationships of the 16S rRNA gene sequence genetic loci for 23 strains of group 16SrV phytoplasmas. Evolutionary history was inferred 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 <90 % are omitted. Trees are drawn to scale with branch lengths calculated using the average pathway method. Bar, 1 nucleotide change.

to the high degree of similarity between the 16S rRNA gene sequences. The description of a novel taxon usually requires the identification of a sequence specific to the 16S rRNA gene of the candidate taxon. In the case of the *Rubus* stunt phytoplasma, four specific oligonucleotides could be identified. For groups 16SrV-C and 16SrV-D, no specific oligonucleotide could be found in the 16S rRNA gene sequence, as all sequences that were different from those of the '*Ca. P. ulmi*' sequence were identical to those of the *Rubus* stunt phytoplasmas and vice versa.

In order to determine whether these phytoplasmas showed significant molecular diversity at the genome level, the following five genetic loci distributed at different locations on the FD chromosome were chosen (Malembic-Maher *et al.*, 2008): the *tuf* gene, encoding the translation elongation factor EFTu; the *rplV-rpsC* locus, encoding ribosomal proteins L22 and S3; the *rplF-rplR* locus, encoding ribosomal proteins L6 and L18; the *map* gene, encoding the methionine aminopeptidase; and the *uvrB-degV* gene encoding excinuclease B and DegV protein. Each genetic locus was amplified in all samples (see Supplementary Table S1) and sequenced according to the conditions described in Supplementary Table S2. For each sample, the sequences of the five genetic loci were concatenated to form a 4229 bp long sequence, which was then subjected to comparative and phylogenetic analyses. Sequences from FD, AldY, PGY and *Clematis* phytoplasma (CL) displayed 98.8–100 % sequence similarity with each other, but showed only 96.9–97.3 % similarity

with the corresponding sequence of strains of '*Ca. P. ulmi*' and 97.9–98.2 % similarity with strains of *Rubus* stunt phytoplasma, including strains from dog rose. It was possible to define 24 *Rubus* stunt phytoplasma-specific oligonucleotides, as well as eight oligonucleotides specific to FD, AldY, CL and PGY phytoplasmas (Table 1). Phylogenetic analysis of the concatenated gene sequences clearly distinguished three separate clusters supported by a bootstrap value of 100 % (Fig. 2). The first cluster corresponded to all FD strains, AldY, PGY, *Spartium* and *Clematis* phytoplasmas and had a clear monophyletic origin. The second cluster grouped all *Rubus* stunt and dog rose strains and was genetically very homogeneous, with all strains sharing at least 99.9 % gene sequence similarity. The third cluster corresponded to strains of '*Ca. P. ulmi*'. In conclusion, the data indicated genomic diversification between the strains of FD, AldY, *Spartium*, PGY and *Clematis* phytoplasma on one side and the strains of *Rubus* stunt and dog rose phytoplasma on the other side. Both groups were also clearly diversified in comparison with '*Ca. P. ulmi*'. Strain HD1 of hemp dogbane yellows was clearly different and clustered on a separate branch.

According to the convention proposed by Murray & Schleifer (1994) for prokaryotes that can be only incompletely described, phytoplasmas have been described as candidate taxa within the genus-level provisional taxon '*Candidatus Phytoplasma*' (Firrao *et al.*, 2004). The primary rule for the description of '*Candidatus Phytoplasma*' taxa is that they must have a 16S rRNA gene sequence similarity of

Table 1. Oligonucleotides specific to '*Ca. P. rubi*' and groups 16SrV-C and 16SrV-D

Gene	' <i>Ca. P. rubi</i> '	Groups 16SrV-C and 16SrV-D
<i>tuf</i>	TATTGAAGG <u>C</u> GTTATTAACG,* AACTGGCTTGCAAATGTTTC, GATAAAGAAGGTGCTTTGGCT, GTTATATGTAAACCAGGATCTGT, GCACTTGCTTTCGTGATAATTAT, TCTTAAATAGTAAATCCAGGAG	TAGACAAAC <u>C</u> TTTTTTTAAT
<i>rpsC-rplV</i>	TTATTAATAAGCGCTGTTGCAA, TATTTGTCGA <u>C</u> GAAGGTTTGCG, TAGTTTAATCAATAAAATAGAA	
<i>rplF-rplR</i>	AAAGGAATTC <u>T</u> TTTAGCTGGT, GCAGGAAAAAGTTCTAAAAAAT	
<i>secY-map</i>	AACATAAAGGTTATTTGTAGAT, CATGGTATTGGAAAAAATTACA	AATAATTAAGGTTGTTTAT, AATGAAAGAGGC <u>C</u> GGRAA
<i>uvrB-degV</i>	AACCAACTATTATTATAAAAAA, TTAATGAAAAAATTTCTAAAAA, CTTTTATACATTATTATTATTAT, AAAATCATAATATCGATTAAATTAG, GAAACTATAACATCTAAAGAATTT, CCTTCTATAGGGGAATTAA, AAAAGCAATTTAAAGACAAAA, AAATTATTCCTTACAATACTAATA	ATAAAAAATGAAGATATTGAA, TTTAAAAAACTACAATCTTT, TAATTATACAAATCAGAAAAA

*Specific nucleotide is underlined.

Fig. 2. Evolutionary relationships of the concatenated *tuf*, *rpsC-rplV*, *rplF-rplR*, *map* and *degV* genetic loci for 35 strains of the group 16SrV. Details of tree construction are as given for Fig. 1. Bar, 10 nucleotide changes.

<97.5% with an already described taxon. However, as various taxonomic groups within the phytoplasma display higher 16S rRNA gene sequence similarities, it has been established that, in such cases, the taxon should represent an ecologically separated population and therefore should have different plant-host range and a different insect vector and should show evidence of molecular diversity. Such criteria have been used to describe '*Ca. P. ulmi*', '*Ca. P. mali*', '*Ca. P. pyri*' and '*Ca. P. prunorum*' (Lee *et al.*, 2004; Seemüller & Schneider, 2004).

Rubus stunt phytoplasma has a specific ecology when compared with the other members of the 16SrV taxonomic group, as it is transmitted mostly to species of the genus *Rubus*, but also to the dog rose *Rosa canina*, in the Euro-Mediterranean basin (Davies, 2000; Jarausch *et al.*, 2001; Mäurer & Seemüller, 1995) by the leafhopper *M. fuscus* (de Fluiter & van der Meer, 1953). Due to its distinct biological niche and its genomic differentiation, it is proposed that the *Rubus* stunt phytoplasma represents a novel, distinct candidate taxon: '*Candidatus Phytoplasma rubi*'.

Members of subgroups 16SrV-C and 16SrV-D form a homogeneous genomic cluster and have a more diverse ecology. The FD, PGY, AldY and *Clematis* phytoplasmas are transmitted by *Scaphoideus titanus*, *Oncopsis alni* (AldY and PGY) and *Dictyophara europaea* (Filippin *et al.*, 2009; Maixner & Reinert, 1999; Maixner *et al.*, 2000; Mori *et al.*,

2002; Schvester *et al.*, 1961), respectively, and have natural plant host ranges that are restricted to *Vitis vinifera*, *Alnus glutinosa*, and *Clematis vitalba*, respectively. However, AldY phytoplasma can also be transmitted from alder to grapevine by its natural vector *O. alni* and CL phytoplasma can be transmitted from *Clematis* to grapevine by *D. euroapaea* (Filippin *et al.*, 2009; Maixner *et al.*, 2000). According to the sequence of the five genetic loci studied, *Spartium witches* broom phytoplasma can be included in this cluster. However, despite the monophyletic origin of the members of groups 16SrV-C and 16SrV-D, no common specific oligonucleotide could be found in their 16S rRNA gene sequences, and hence this phytoplasma cannot be described as a candidate taxon.

Description of '*Candidatus Phytoplasma rubi*'

'*Candidatus Phytoplasma rubi*' [ru'bi. L. n. *rubus* a blackberry, and also the generic name of blackberry (*Rubus*); L. gen. n. *rubi* of blackberry, of *Rubus*].

[(Mollicutes) NC; NA; O; wall-less; NAS (GenBank accession no. AY197648), oligonucleotide sequence complementary to unique region of the 16SrRNA gene is 5'-AGTCAAGATAGTTTCTATAAC-3'; P (*Rubus* sp., *Rosa canina*, phloem); M]. Reference strain RuS, maintained in *Catharanthus roseus*, is available from the authors. The genomic diversification of '*Ca. P. rubi*' is indicated by the

numerous specific oligonucleotides found in the five genetic loci studied (Table 1).

Acknowledgements

This work was supported by the Conseil Interprofessionnel des Vins de Bordeaux (CIVB) and the Regional council of Aquitaine with research grant number 19336. We thank Assunta Bertaccini, Cristina Marzachi, Michael Maixner and Carmine Marcone for providing some phytoplasma strains.

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