

'*Candidatus Phytoplasma mali*', '*Candidatus Phytoplasma pyri*' and '*Candidatus Phytoplasma prunorum*', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively

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Apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY) are among the most economically important plant diseases that are caused by phytoplasmas. Phylogenetic analyses revealed that the 16S rDNA sequences of strains of each of these pathogens were identical or nearly identical. Differences between the three phytoplasmas ranged from 1.0 to 1.5% of nucleotide positions and were thus below the recommended threshold of 2.5% for assigning species rank to phytoplasmas under the provisional status '*Candidatus*'. However, supporting data for distinguishing the AP, PD and ESFY agents at the species level were obtained by examining other molecular markers, including the 16S–23S rDNA spacer region, protein-encoding genes and randomly cloned DNA fragments. The three phytoplasmas also differed in serological comparisons and showed clear differences in vector transmission and host-range specificity. From these results, it can be concluded that the AP, PD and ESFY phytoplasmas are coherent but discrete taxa that can be distinguished at the putative species level, for which the names '*Candidatus Phytoplasma mali*', '*Candidatus Phytoplasma pyri*' and '*Candidatus Phytoplasma prunorum*', respectively, are proposed. Strains AP15^R, PD1^R and ESFY-G1^R were selected as reference strains. Examination of available data on the peach yellow leaf roll (PYLR) phytoplasma, which clusters with the AP, PD and ESFY agents, confirmed previous results showing that it is related most closely to the PD pathogen. The two phytoplasmas share 99.6% 16S rDNA sequence similarity. Significant differences were only observed in the sequence of a gene that encodes an immunodominant membrane protein. Until more information on this phytoplasma is available, it is proposed that the PYLR phytoplasma should be regarded as a subtype of '*Candidatus Phytoplasma pyri*'.

INTRODUCTION

Phytoplasmas are plant-pathogenic, yet uncultured, mollicutes. In plants, they usually inhabit phloem sieve tubes and

are transmitted from plant to plant by phloem-feeding insects, primarily leafhoppers. They are associated with diseases in about 1000 plant species (Seemüller *et al.*, 2002). These diseases include three economically important disorders of temperate fruit trees: apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). AP is only known in Europe and was first described in Italy (Rui *et al.*, 1950). ESFY is mainly known in Europe, but has also been reported in Turkey (Jarausch *et al.*, 2000a) and was first described as a decline of Japanese plum (*Prunus salicina*) in Italy (Goidanich, 1933). Until recently, the disease was known by the names plum leptonecrosis, apricot chlorotic leaf roll and others, according to the crop affected and the symptoms induced (Lorenz *et al.*, 1994). PD was first reported in North America (McLarty, 1948; Woodbridge *et al.*, 1957) and seems to have been introduced

Abbreviations: AP, apple proliferation; BWB, buckthorn witches'-broom; ESFY, European stone fruit yellows; IMP, immunodominant membrane protein; PD, pear decline; PYLR, peach yellow leaf roll; SpaWB, spartium witches'-broom.

The GenBank/EMBL/DDBJ accession numbers for the P1/P7 amplimers (16S rRNA gene and 16S–23S rDNA spacer region) of phytoplasma strains AP15^R, AP1/93, PD1^R, ESFY-G1^R and ESFY-G2 are AJ542541, AJ542542, AJ542543, AJ542544 and AJ542545, respectively.

Restriction maps of rDNA obtained from P1/P7 PCR products from the apple proliferation, pear decline and European stone fruit yellows phytoplasmas are available as supplementary material in IJSEM Online.

from Europe (Shalla *et al.*, 1961; Seemüller, 1992). These data indicate that AP, PD and ESFY are of European origin and were present in Europe long before the first reports were published. In contrast, peach yellow leaf roll (PYLR), which is caused by a phytoplasma that is related closely to the AP, PD and ESFY agents, has only been reported in western North America (Seemüller, 1999).

Recent investigations, particularly sequence analysis of 16S rDNA, have revealed that phytoplasmas constitute a coherent, genus-level taxon. In the monophyletic phytoplasma clade, groups and subgroups have been delineated, many of which are being considered as putative species under the provisional status 'Candidatus' (Seemüller *et al.*, 2002) for incompletely described prokaryotes, according to Murray & Stackebrandt (1995). Several provisional species have been described to date and rules for future putative species delineation have been defined (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group, 2004). According to these recommendations, 'a phytoplasma can be described as a novel 'Candidatus Phytoplasma' species if its 16S rDNA sequence has <97.5 % similarity to that of any previously described 'Candidatus Phytoplasma' species'. In cases where phytoplasmas share $\geq 97.5\%$ 16S rDNA sequence similarity, description as different 'Candidatus' species is only recommended if there is an indication that these phytoplasmas clearly represent separate populations, as evidenced by significant differences based on molecular markers other than 16S rDNA, antibody specificity, host range and vector transmission specificity.

The AP, PD and ESFY agents are among the phytoplasmas that have been studied most intensively, using both molecular and biological methods. 16S rDNA sequence data indicated that each of them represents a relatively uniform organism (Lorenz *et al.*, 1995; Seemüller *et al.*, 1998b; Kison & Seemüller, 2001). The results also revealed that the three pathogens are closely related phylogenetically and form, together with the PYLR phytoplasma, a cluster designated the 'AP phytoplasma group' (Seemüller *et al.*, 1998b) or 16SrX group (Lee *et al.*, 2000) within the AP subclade, which is one of the major branches of the phytoplasma clade. The four phytoplasmas differ from each other in <2.5 % of 16S rDNA nucleotide sequence positions. Thus, the objective of this work was to examine whether the above-mentioned requirements for differentiation of closely related phytoplasmas are fulfilled. The data examined include sequence and/or RFLP analyses of the 16S–23S rDNA spacer region, protein-encoding genes and randomly cloned DNA fragments, serological comparisons and differences in insect vector and host-plant specificity. Further data were generated in this work. In particular, rDNA sequences of more strains were examined and previously sequenced strains were re-examined in order to obtain unambiguous sequences. Also, RFLP analyses of macrorestriction fragments and Western blot studies were performed to demonstrate inter- and intraspecific differences of the phytoplasmas examined.

METHODS

Most data on which these taxonomic descriptions are based were generated previously. Additional work was carried out as follows. Sequence and RFLP analyses of ribosomal fragments were performed by using rDNA that was amplified with the primer pair P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995) and extended from the 5' end of the 16S rRNA gene to the 5' region of the 23S rRNA gene, thus including the 16S–23S rDNA spacer region. RFLP analysis was carried out as described previously (Lorenz *et al.*, 1995; Kison & Seemüller, 2001). For sequencing of strains AP1/93, PD1, ESFY-G1^R and ESFY-G2 and resequencing of strains AT and AP15^R, P1/P7 amplicons were cloned by using the pGEM-T vector system (Promega). Sequencing of both strands was done by a commercial service (SEQLAB, Göttingen, Germany) by using M13 forward and reverse primers and six primers that were complementary to conserved 16S rDNA regions (Seemüller *et al.*, 1994). Sequencing data obtained with these primers usually provided threefold coverage of the sequences. To obtain unambiguous sequences, three or four clones were sequenced from each of strains AP15^R, AT, PD1^R and ESFY-G1^R, which represent the three taxa treated in this description. One clone was sequenced from each of strains AP1/93 and ESFY-G2. Sequence evaluation, including manual alignment, was done by using the software package HUSAR (Biocomputing Service Group, German Cancer Research Center, Heidelberg, Germany). Gaps and ambiguities were removed from the final dataset. Phylogenetic and molecular evolutionary analyses were conducted by using the neighbour-joining program of the genetic analysis software MEGA, version 2.1 (Kumar *et al.*, 2001). The data were resampled 500 times and bootstrap percentage values are given at the nodes of the tree. Phylogenetic distances were calculated by pairwise comparison. Numbering of nucleotide positions corresponds to that of the 16S rRNA gene of aster yellows phytoplasma strain OAY (Lim & Sears, 1989).

Full-length chromosomes of the AP, PD and ESFY phytoplasmas were isolated from diseased tobacco plants and RFLP analysis was performed as described previously (Lauer & Seemüller, 2000). ELISA and Western blot experiments were carried out by using standard procedures (Ausubel *et al.*, 1987). The strains examined in this work are listed in Table 1.

TAXONOMIC EVIDENCE

Phylogenetic relationships based on 16S rDNA sequences. Four AP phytoplasma strains (AP15^R, AT, AP1/93 and APS), three PD phytoplasma strains (PD1^R, PD2 and PD3), five ESFY phytoplasma strains (ESFY-G1^R, ESFY-G2, ESFY3, ESFY4 and ESFY5) and one strain from the PYLR agent (PYLR1) were used for 16S rDNA sequence analysis (Fig. 1). Of these phytoplasmas, which were collected in several European countries and California and, in the case of the ESFY agent, from several stone fruit species (Table 1), strains AP15^R, PD1^R and ESFY-G1^R were selected as reference strains. AP15^R is known to occur in several European countries and is probably the most common type of AP agent (Kison *et al.*, 1994; Jarausch *et al.*, 2000b).

The 16S rRNA gene of AP-group phytoplasmas consists of 1521 nt, of which 97.0–99.6 % was analysed. Alignment revealed that the sequences of strains of each phytoplasma type were identical or nearly identical, with similarity

Table 1. Designations and other details of the AP phytoplasma group strains examined

Designation*	Origin	Original host	Reference/collector
AP15†‡	Udine, Italy	<i>Malus domestica</i>	Carraro <i>et al.</i> (1988)
AT‡	Heidelberg, Germany	<i>Malus domestica</i>	Marwitz <i>et al.</i> (1974)
APS	Spain	<i>Malus domestica</i>	L. Avinent
AP2	Dossenheim, Germany	<i>Malus domestica</i>	E. Seemüller
AP1/93‡	Burgundy, France	<i>Malus domestica</i>	E. Seemüller
AP5/93	Hohenstaufen, Germany	<i>Malus prunifolia</i>	E. Seemüller
AP12/93	Dossenheim, Germany	<i>Malus domestica</i>	E. Seemüller
AP-Bx	Bordeaux, France	<i>Malus domestica</i>	F. Dosba
AP-Ro	Pitesti, Romania	<i>Malus domestica</i>	E. Seemüller
PD1†‡	Dossenheim, Germany	<i>Pyrus communis</i>	Marcone <i>et al.</i> (1999a)
PD2	Dossenheim, Germany	<i>Pyrus communis</i>	E. Seemüller
PD3	Basilicata, Italy	<i>Pyrus communis</i>	C. Marcone
PD5	Dossenheim, Germany	<i>Pyrus communis</i>	E. Seemüller
PD471	Dossenheim, Germany	<i>Pyrus communis</i>	E. Seemüller
PD477	Dossenheim, Germany	<i>Pyrus communis</i>	E. Seemüller
PD484	Dossenheim, Germany	<i>Pyrus communis</i>	E. Seemüller
PD489	Dossenheim, Germany	<i>Pyrus communis</i>	E. Seemüller
ESFY-G1†‡	Dossenheim, Germany	<i>Prunus persica</i>	Marcone <i>et al.</i> (1999a)
ESFY-G2‡	Dossenheim, Germany	<i>Prunus armeniaca</i>	Marcone <i>et al.</i> (1999a)
ESFY1‡	Udine, Italy	<i>Prunus salicina</i>	Loi <i>et al.</i> (1995)
ESFY2	Udine, Italy	<i>Prunus salicina</i>	R. Osler
ESFY3	Dossenheim, Germany	<i>Prunus persica</i>	E. Seemüller
ESFY4	Czech Republic	<i>Prunus armeniaca</i>	Z. Vorackova
ESFY5	Austria	<i>Prunus armeniaca</i>	M. Laimer
ESFY6	Dossenheim, Germany	<i>Prunus persica</i>	E. Seemüller
ESFY176	Kallstadt, Germany	<i>Prunus dulcis</i>	K.-H. Lorenz
ESFY163	Weinsberg, Germany	<i>Prunus salicina</i>	E. Seemüller
PYLR1	California, USA	<i>Prunus persica</i>	B. C. Kirkpatrick
PYLR2	California, USA	<i>Prunus persica</i>	B. C. Kirkpatrick

*For rDNA sequence GenBank accession numbers, see Fig. 1 and text.

†Reference strain.

‡Maintained in periwinkle.

values between 99.9 and 100 %. A difference of 0.2 % was determined between strains ESFY3 and ESFY5. In sequences of the AP phytoplasma strains, only one polymorphism occurred (at position 1442 where, in the AP15^R sequence, the C was replaced by a T residue). This substitution, the only polymorphism that was detected in the PD sequences, is shared by strain PD2. Three polymorphisms were observed in the ESFY strains, with the following substitutions: a G at position 30, present in all other AP-group members, that is replaced by a T residue in strain ESFY5; a G at position 645 that is replaced by an A in strains ESFY-G1^R and ESFY3; and an A at position 1251, present in other ESFY strains, that is replaced by a G in strain ESFY3. There was no indication of sequence heterogeneity between the two rRNA operons that are present in all phytoplasmas (Schneider & Seemüller, 1994a).

The AP, PD and ESFY phytoplasmas are closely related phylogenetically. In interspecific comparisons of the AP/PD, AP/ESFY and PD/ESFY agents, differences in 16S rDNA

sequences were 1.0–1.1, 1.3–1.5 and 1.2–1.3 %, respectively. Strain PYLR1 is related most closely to the PD strains, with which it shares 99.6 % sequence similarity, whereas similarity values between the AP and ESFY strains were 98.4–98.6 %. Sequence similarity values within taxa and divergence between taxa largely confirm the results of previous work (Seemüller *et al.*, 1994, 1998b).

Phylogenetic relatedness of the phytoplasmas examined is depicted in Fig. 1. Strains of each of the three pathogens cluster tightly together and form three distinct branches. The PYLR agent clusters with the PD strains. The closest relatives of the AP-group phytoplasmas are the spartium witches'-broom (SpaWB; Marccone *et al.*, 1996b) and buckthorn witches'-broom (BWB; Mäurer & Seemüller, 1996) agents, which compose, together with the fruit tree agents, the AP subclade. Their 16S rDNA sequence similarity with the fruit tree phytoplasmas is slightly less than 97.5 % and is thus below the defined 2.5 % threshold. Other selected phytoplasmas that represent other major

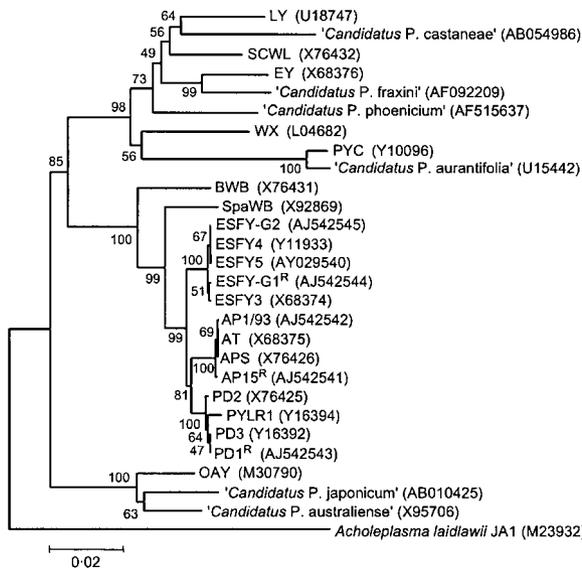


Fig. 1. Phylogenetic tree constructed by using the neighbour-joining method with 16S rDNA sequences from strains of the PD (PD1^R–PD3 and strain PYLR1 from PYLR phytoplasma), AP (AP15^R, APS, AT and AT1/93) and ESFY (ESFY-G1^R, ESFY-G2, ESFY3–ESFY5) agents and 14 reference phytoplasmas. *Acholeplasma laidlawii* was used as the outgroup.

subclades differ from the AP-group agents in >7% of 16S rDNA nucleotide positions.

16S rDNA signature sequences. The 16S rRNA gene sequences from the AP, PD and ESFY agents were aligned with those of phytoplasmas that represent most phylogenetic groups. Several oligonucleotides that are unique for the AP-group phytoplasmas were selected and used as query sequences in a BLAST 2.0 search (Altschul *et al.*, 1997). This analysis resulted in the determination of signature sequences that are characteristic for the AP-group phytoplasmas and are absent in other organisms or prokaryotes. Sequences identified as unique for the AP group were at positions 1393–1409, where 5'-AATACTCGAA-ACCAGTA-3' and 5'-AATACCCGAAACCAGTA-3' were specific for the AP and ESFY phytoplasmas, respectively, and 5'-AATACTCAAACCAGTA-3' was specific for the PD and PYLR agents. Another sequence that was only shared by PD and PYLR strains was 5'-TTAATAAGTC-TATGGTCT-3' (positions 570–587). PD and PYLR agents could be distinguished from each other by the sequence 5'-ATACGGCCCAAACATACGGA-3' at positions 329–351, which was specific for PYLR. A second ESFY-specific sequence was 5'-TGAAGTTTTGAGGCATCTCG-AA-3' at positions 159–180.

Sequence analysis of the 16S–23S rDNA spacer region. The 16S–23S rDNA spacer of AP-group phytoplasmas consists of about 210 bp. The gene that encodes tRNA^{Ile} was present and identical in all strains examined,

which comprised AP15^R, AT, AP1/93, APS (GenBank accession no. X76426), PD1^R, PD3, PD5 (accession no. U54989), ESFY-G1^R, ESFY-G2, ESFY3, ESFY4, ESFY6 (accession no. U54988), PYLR1 and PYLR2 (accession no. U54990) (see Table 1 for strain identities and Fig. 1 for other accession numbers). Flanking sequences within these phytoplasmas were also highly conserved; those of strains of each the AP and ESFY phytoplasmas were identical, whereas one polymorphism in the PD spacer (in the form of a deletion of an A residue in PD1^R and PD5, just downstream of the tRNA^{Ile} gene) was identified. Divergence between taxa was slightly higher in the 16S–23S rDNA spacer region than in the 16S rDNA sequence, being 1.9, 3.0 and 1.5% for the comparisons AP/PD, AP/ESFY and PD/ESFY, respectively. The sequence of PYLR strains PYLR1 and PYLR2 was identical to that of strain PD3. Relationships between the phytoplasmas examined here agree with results of previous comparisons that were made with fewer strains (Kirkpatrick *et al.*, 1994; Schneider *et al.*, 1995).

Sequence analyses of non-ribosomal DNA. Examination of putative translation products of immunodominant membrane protein (IMP) genes of the AP, PD, ESFY and PYLR phytoplasmas revealed a similar size for all. The IMPs consisted of a transmembrane region towards the N-terminus, with only a short N-terminal intracellular sequence (together, about 35 aa), and a large hydrophilic C-terminal domain (about 130 aa), probably held on the outside of the cell membrane. The extracellular hydrophilic domain, which may play a role in pathogen–host interactions, is not conserved. Similarities of nucleotide and deduced amino acid sequences were 47–76 and 31–57%, respectively. Sequence similarity values for the combined N-terminal and transmembrane domains were higher, ranging from 82 to 98% and 65 to 100%, respectively. Whilst the PD and PYLR phytoplasmas differed significantly in the nucleotide and amino acid sequences of their large hydrophilic domain (54 and 31% similarity, respectively), sequences of their combined N-terminal and transmembrane domains were nearly identical or identical. However, sequence similarity of the entire gene was highest between the PD and AP phytoplasmas (Berg *et al.*, 1999; Barbara *et al.*, 2001; Morton *et al.*, 2003). There is no sequence similarity in antigenic membrane proteins between AP-group and aster yellows-group phytoplasmas (Barbara *et al.*, 2002).

The DNA sequence of randomly cloned DNA fragment AT67 from strain AT of the AP phytoplasma (Schneider & Seemüller, 1994b), which contains three ORFs that encode a putative ATP-binding protein and two putative permease proteins, was compared with analogous, PCR-amplified sequences of Californian, German and Italian PD phytoplasma isolates and a Californian PYLR phytoplasma isolate. This analysis revealed that the Californian and German PD isolates and the Californian PYLR isolate had identical sequences. DNA sequence similarity of these

isolates to the Italian PD isolate was 98 % and to AP strain AT was 92 % (Guerra, 1997).

RFLP analysis of ribosomal and non-ribosomal DNA.

rDNA, PCR-amplified with universal phytoplasma primer pairs fU5/rU3 or P1/P7 or with primers fO1/rO1, fPD/rO1 or P1/PYL Rint, which specifically amplify some or all phytoplasmas of the AP group, was predominantly used in RFLP analysis (Seemüller *et al.*, 1998a). Following *AluI* digestion, the AP, PD and ESFY phytoplasmas showed the same restriction pattern, which was also shared by the PYLR agent (Kison *et al.*, 1997). The AP, PD and ESFY phytoplasmas can be distinguished from each other by separate restriction of 16S rDNA with *RsaI* and *SspI*. Following *RsaI* digestion, the ESFY agent differs from the AP and PD phytoplasmas by one additional restriction site at position 420, whereas *SspI* digestion cleaves the AP phytoplasma at position 413, a site that is absent in the PD and ESFY agents. Differentiation of AP and PD from ESFY phytoplasmas is also possible with *BsaAI* and *SfcI*. The ESFY agent shows a *BsaAI* site at position 422 that is absent in the two other phytoplasmas, whilst both the PD and ESFY phytoplasmas have an *SfcI* site at position 624 that is absent in AP. In addition, the PD and ESFY agents share a unique *SspI* site in the 16S–23S rDNA spacer region that does not occur in the AP phytoplasma (Fig. 2). A supplementary figure showing restriction maps of rDNA from the AP, PD and ESFY agents is available in IJSEM Online. In all comparisons, the PYLR agent resembled the PD phytoplasma (Lorenz *et al.*, 1995; Marcone *et al.*, 1996a; Guerra, 1997; Kison *et al.*, 1997; Del Serrone *et al.*, 1998; Seemüller *et al.*, 1998a; Topchiiska *et al.*, 2000; Kison & Seemüller, 2001). Hundreds of AP-group phytoplasma isolates from various locations in European countries and California, including the ESFY agent taken from several *Prunus* species, have been examined to date by using these differentiation approaches. With the above-mentioned restriction enzymes and several others, no variation among the different AP-group phytoplasmas was observed.

DNA derived from randomly cloned fragment IH196 of strain AT, amplified with several primer pairs (Bonnet *et al.*, 1990), also proved to be useful for AP-group phytoplasma characterization and differentiation. Work by Jarausch *et al.* (1994, 2000a, 2000b) showed that this fragment contains three ORFs, including a putative nitroreductase gene. Restriction enzyme digestion with *RsaI* and *HincII* of a product obtained with primers AP9/AP10 enabled the differentiation of AP from PD and ESFY agents. All three phytoplasmas could be distinguished from each other by digestion of an AP3/AP10 amplicon with these enzymes. One hundred and seventy-five ESFY phytoplasma isolates, which were collected from many *Prunus* species and hybrids in four Mediterranean countries, responded identically in this work. However, by examining a 1.5 kbp fragment that was obtained from AP phytoplasma isolates with primers AP8/AP10, three RFLP types were identified by restriction

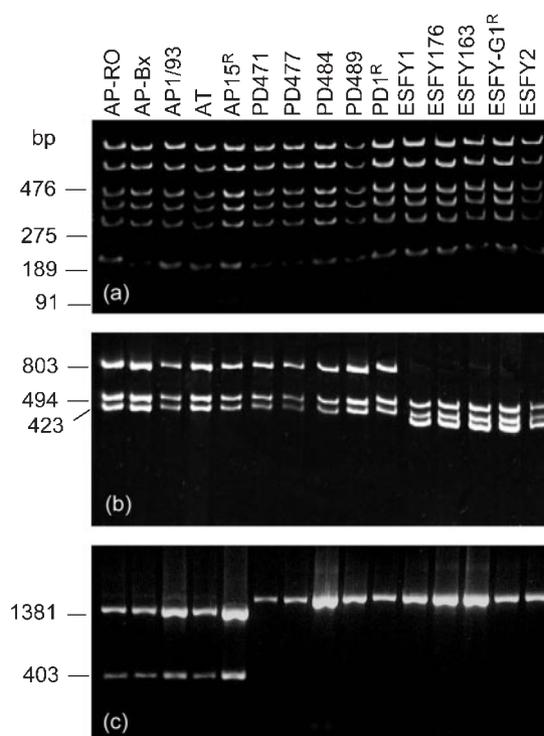


Fig. 2. RFLP analysis of rDNA, PCR-amplified with primer pair P1/P7, from strains of the AP (AP-RO–AP15^R), PD (PD471–PD1^R) and ESFY (ESFY1–ESFY2) phytoplasmas. PCR products were digested with *AluI* (a), *RsaI* (b) and *SspI* (c). See Table 1 for strain information.

digestion with *RcaI* and *HincII*. Nucleotide sequence analysis revealed that the three RFLP types are nearly identical and differ only by a point mutation in each of the two restriction sites that are responsible for the polymorphisms.

Primers that were derived from the non-ribosomal DNA fragment AT67 (see above) of strain AT were used to amplify the analogous sequence of the PD phytoplasma. The primers designed from the sequence obtained were PD-specific and were used to examine 11 Californian PD isolates and a German reference strain. All showed the same RFLP profile following digestion with *AluI* and *RsaI* (Guerra, 1997). In another experiment, AP and PD phytoplasmas were distinguished by digestion of an 835 bp *tuf* gene fragment with both *HinfI* and *HpaII* (Berg, 1998). The PYLR agent also showed the same profiles as the PD phytoplasma in the present study.

Southern blot hybridizations. Randomly cloned chromosomal DNA fragments from strain AT were employed in Southern blot analysis to characterize and differentiate AP-group phytoplasmas. *HindIII* fragments IH184 and IH196 (Bonnet *et al.*, 1990) and *EcoRI* fragments AT17, AT27, AT67 and AT72 (Schneider & Seemüller, 1994b) were used as probes (see above for inserts IH196 and AT67). With both sets of fragments, which were employed mostly as

cocktail probes, distinctly different RFLP patterns were obtained with *Hind*III- and *Eco*RI-digested DNA from plants that were infected with the AP or ESFY agents (Ahrens *et al.*, 1993; Lorenz *et al.*, 1994; Kison *et al.*, 1997). The *Eco*RI probes also enabled clear differentiation of the PYLR agent from the AP and ESFY phytoplasmas. In contrast, following hybridization with probes IH184 and IH196, *Hind*III-digested PYLR phytoplasma DNA showed the same RFLP profile as strain AP15^R, which was different from that of strain AT (Kison *et al.*, 1997). Hybridization experiments using *Hind*III- or *Eco*RI-digested DNA from PD-diseased pear trees with both sets of probes gave inconclusive results; this is probably due to too low phytoplasma DNA concentrations in extracts from PD-diseased trees. The probes used were specific for AP-group phytoplasmas and did not hybridize or hybridized only weakly to DNA of phytoplasmas from other phylogenetic groups. When Southern blots of *Eco*RI-digested DNA were probed with IMP gene 318B from strain AT, hybridization signals were also obtained from the PD and ESFY phytoplasmas. However, the fragment size was different for each phytoplasma (Berg *et al.*, 1999).

Southern blot analysis also revealed sequence heterogeneity between isolates of the AP phytoplasma. After hybridization of *Hind*III-digested DNA from 34 isolates of the AP agent with *Hind*III fragments IH184 and IH196, five different RFLP patterns were observed. Two of them were represented by strains AT and AP15^R (Bonnet *et al.*, 1990; Kison *et al.*, 1994). These two genotypes correspond to PCR-RFLP subtypes AT-1 and AP of Jarausch *et al.* (2000b). Sequence heterogeneity within the AP agent was also observed when *Hind*III-digested DNA from three AP phytoplasma isolates was hybridized with a 5 kbp probe from an aster yellows phytoplasma, which comprised part of an rDNA operon and a 2.8 kbp upstream region. In this case, each strain showed a different RFLP pattern (Schneider & Seemüller, 1994a). However, no polymorphisms were observed following hybridization of *Eco*RI-digested AP DNA with *Eco*RI fragments AT17, AT27 and AT67 (Kison *et al.*, 1997). By hybridization of *Hind*III- or *Eco*RI-digested DNA from over 50 ESFY phytoplasma isolates with cocktail probes IH184 and IH196 or AT17, AT27 and AT67, polymorphisms were only observed in two samples following hybridization with the *Hind*III fragments as probes. No heterogeneity was observed in the PYLR phytoplasma following hybridization with both sets of probes (Ahrens *et al.*, 1993; Lorenz *et al.*, 1994; Kison *et al.*, 1997).

Genome sizes, chromosome restriction maps and G+C content. PFGE was used to estimate genome sizes of the AP, PD and ESFY phytoplasmas by resolving full-length chromosomes and/or chromosomal macrorestriction fragments that were generated by single or double digestions with rare-cutting enzymes (Marcone *et al.*, 1999b; Lauer & Seemüller, 2000). PFGE analysis showed that the chromosome sizes of the three fruit-tree pathogens are in the lower range for phytoplasmas. Estimated sizes of the

chromosomes of strains AT and AP15^R of the AP agent were 645 and 690 kbp, respectively, whereas the chromosome length of ESFY phytoplasma strains ESFY1, ESFY-G1^R and ESFY-G2 was uniformly 630 kbp and that of strain PD1^R was 660 kbp. Physical maps were constructed from the chromosomes of strains AT and ESFY-G1^R (Lauer & Seemüller, 2000; Marcone & Seemüller, 2001). Although there were differences in the restriction enzymes that were suitable for map construction and in the frequency and location of restriction sites, the overall arrangement of the chromosomes was similar, as evidenced by gene positions on the maps. Interspecific variations were detected by digestion of full-length chromosomes from strains AT, AP15^R, PD1^R and ESFY-G2 with *Bs*SIII. Also, polymorphisms were observed between strains AT and AP15^R (Fig. 3). The G+C content of the chromosome of strain AT was estimated to be 23.7 mol% (Kollar & Seemüller, 1989).

Antibody specificity. Two mAbs that were derived from antigens of strain AP15^R proved to be highly specific for the AP agent. In ELISAs, they detected both the homologous strain and strain AT, but did not react with antigen preparations from the PD or ESFY phytoplasmas, nor with extracts from plants infected with phytoplasmas from other phylogenetic groups (Loi *et al.*, 2002). In our Western blot experiments, in which five different strains of the AP agent were included, these mAbs reacted with only three strains (AP15^R, AP1/93 and AP12/93) and poorly with IMP 318B from strain AT (see above). A

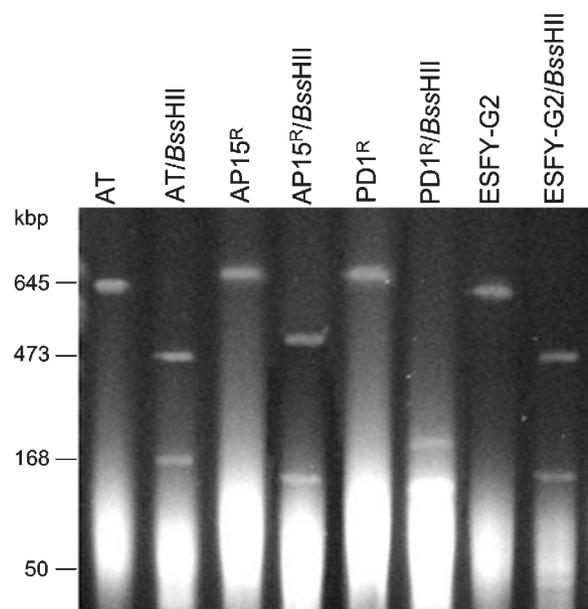


Fig. 3. PFGE of full-length chromosomes from strains AP15^R and AT of the AP phytoplasma, strain PD1^R of the PD phytoplasma and strain ESFY-G2 of the ESFY phytoplasma, before and after digestion with *Bs*SIII.

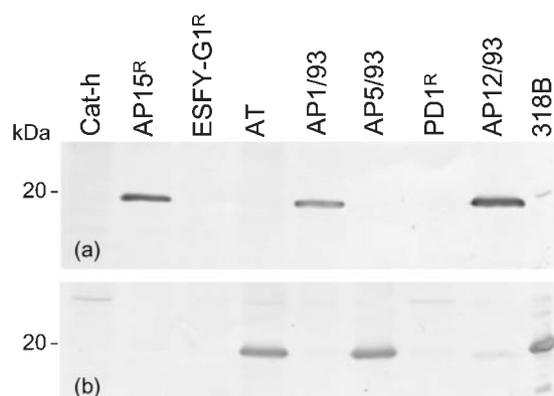


Fig. 4. Western blot analysis of five AP phytoplasma strains using a mAb derived from strain AP15^R (a) and a polyclonal antiserum raised to IMP B318 of strain AT (b). Cat-h, Healthy periwinkle; 318B, IMP 318B. ESFY-G1^R and PD1^R, ESFY and PD phytoplasma strains. See Table 1 for phytoplasma strain information.

polyclonal antiserum that was raised to IMP 318B of strain AT (Berg *et al.*, 1999) gave different results when the same samples were analysed in Western blot experiments. This antiserum reacted with the recombinant protein and antigens of strains AT and AP5/93, but not with antigens of strains AP15^R, AP1/93 or AP12/93 (Fig. 4). In another experiment, polyclonal antibodies purified from antisera that were raised to antigens from the AP, PD and ESFY phytoplasmas recognized, in Western blot assays, only the homologous antigens and did not cross-react (Davies & Adams, 2001).

Host-plant and vector transmission specificity. By using PCR, DNA hybridization and serological techniques, the AP, PD and ESFY phytoplasmas were almost exclusively detected in the genera *Malus* (apple), *Pyrus* (pear) and *Prunus* (stone fruits), respectively. Within these genera, several species are known to be natural or experimental hosts of the respective pathogen and develop disease following infection (Kartte & Seemüller, 1991; Seemüller, 1992; Jarausch *et al.*, 2001; Kison & Seemüller, 2001; Carraro *et al.*, 2002). Using universal phytoplasma primers or AP group-specific primers, phytoplasmas showing rDNA RFLP profiles that were specific for the three fruit-tree agents were occasionally identified in plants other than the typical host. On the basis of these patterns, additional AP phytoplasma hosts include stone fruits and both European and Asian pear (*Pyrus communis* and *Pyrus pyrifolia*) (Lee *et al.*, 1995; Del Serrone *et al.*, 1998; E. Seemüller, unpublished results). AP-group phytoplasmas were also detected in hazel (*Corylus avellana*) (Marcone *et al.*, 1996c), ash (*Fraxinus excelsior*), dog rose (*Rosa canina*), hackberry (*Celtis australis*) (Jarausch *et al.*, 2001), hawthorn (*Crataegus monogyna*), oak (*Quercus robur* and *Quercus rubra*), hornbeam (*Carpinus betulus*) and bindweed (*Convolvulus arvensis*) (Schneider *et al.*,

1997; Berges, 1999; Seemüller, 2002). However, the identity of the phytoplasmas detected in these plants remains to be confirmed by pathological and vector transmission studies and by examining additional molecular markers.

Whilst other phytoplasmas are transmitted by either leafhoppers (Cicadellidae) or planthoppers (Xixiidae), the three fruit-tree phytoplasmas are vectored by psyllids (Psylloidea). AP is transmitted by both *Cacopsylla picta* (synonym, *Psylla costalis*) (Frisinghelli *et al.*, 2000) and *Cacopsylla melanoneura* (Tedeschi *et al.*, 2002), ESFY by *Cacopsylla pruni* (Carraro *et al.*, 1998b) and PD by both *Cacopsylla pyricola* (Jensen *et al.*, 1964) and *Cacopsylla pyri* (Carraro *et al.*, 1998a). *C. pyricola* is also a vector of PYLR (Blomquist & Kirkpatrick, 2002).

DISCUSSION

This taxonomic study showed that the AP, PD and ESFY phytoplasmas are unique, coherent entities. At the 16S rDNA and 16S–23S rDNA spacer levels, strains of each of these phytoplasmas were identical or nearly identical. Also, by RFLP analysis of randomly cloned fragments IH196 and AT67 (which contained several ORFs), none or very little variation was observed. The same was true for Southern blot hybridization of DNA from ESFY-diseased plants, with various randomly cloned fragments of the AP agent as probes. Whilst the PD and ESFY phytoplasmas showed little variation in the comparisons made, intraspecific heterogeneity was more pronounced in the AP agent, as revealed by Southern blot analysis with certain random probes, RFLP analysis of macrorestriction fragments and serological tests. Intraspecific heterogeneity and differences in variation within taxa are also known from other phytoplasmas and cultivable mollicutes (Christiansen, 1992; Lee *et al.*, 1992; Schneider & Seemüller, 1994b; Griffiths *et al.*, 1999).

Phylogenetic analyses revealed that the AP, PD and ESFY phytoplasmas are closely related, differing by only 16–19 nucleotide positions in their 16S rDNA, which corresponds to 98.6–99.1% sequence similarity. An even closer relationship occurs in other mollicute species. For example, *Mycoplasma cottewii* and *Mycoplasma yeatsii* share 99.7% sequence similarity (Heldtander *et al.*, 1998) and *Mycoplasma gallisepticum* and *Mycoplasma imitans* differ by only 2 nt in their 16S rDNA sequences. However, the latter two taxa have other characteristics that show that they can be regarded as different species (Bradbury *et al.*, 1993). Similarly, the AP, PD and ESFY phytoplasmas differed clearly in all comparisons made, in addition to 16S rDNA analyses. From this, it can be concluded that the requirements for defining phylogenetically closely related phytoplasmas as putative species are fulfilled and that the AP, PD and ESFY phytoplasmas represent discrete, coherent taxa.

In contrast to the AP, PD and ESFY agents, there is not sufficient evidence to justify a formal taxonomic classification of the PYLR phytoplasma at present. This pathogen is

phylogenetically closely related to, but not identical to, the PD agent. In most molecular analyses in which ribosomal and non-ribosomal DNA sequences were included, this pathogen was indistinguishable from the PD agent. Only recent comparisons of structurally similar IMP genes showed distinct differences between the two phytoplasmas (Morton *et al.*, 2003). This supports geographical and pathological evidence that indicates that PD and PYLR are caused by different organisms. Based on these results, it appears to be most appropriate to regard the PYLR agent as a subtype of the PD phytoplasma.

Taxonomic descriptions

To facilitate reference to unique phytoplasma lineages and to have names by which distinct phytoplasmas can be known, we propose to designate the AP, PD and ESFY phytoplasmas as novel '*Candidatus*' species, according to the convention proposed by Murray & Schleifer (1994) for prokaryotes that can be only incompletely described.

We propose that the AP, PD and ESFY phytoplasmas be designated as novel, distinct '*Candidatus*' species: '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', respectively, with the following descriptions.

'*Candidatus* Phytoplasma mali' (mali, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AJ542541); oligonucleotide sequence complementary to unique region of the 16S rRNA gene is 5'-AATACTCGAAACCAGTA-3'; P (*Malus*, phloem); MJ].

'*Candidatus* Phytoplasma pyri' (pyri, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AJ542543); oligonucleotide sequences complementary to regions of the 16S rRNA gene, which are only shared by the PYLR agent, are 5'-AATACTCAAACCAGTA-3' and 5'-ATACGGCCAAA-CTCATA CGGA-3'; P (*Pyrus*, phloem); MJ]. We propose to regard the PYLR agent as a subtype of '*Candidatus* Phytoplasma pyri'.

'*Candidatus* Phytoplasma prunorum' (prunorum, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AJ542544); oligonucleotide sequences complementary to unique regions of the 16S rRNA gene 5'-AATACCCGAAACCAGTA-3' and 5'-TGAAGTTTTGAGGCATCTCGAA-3'; P (*Prunus*, phloem); MJ].

Reference strains AP15^R, PD1^R and ESFY-G1^R and other strains mentioned herein that are maintained in *Catharanthus roseus* are available from the authors.

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