

'*Candidatus* Phytoplasma spartii', '*Candidatus* Phytoplasma rhamnii' and '*Candidatus* Phytoplasma allocasuarinae', respectively associated with spartium witches'-broom, buckthorn witches'-broom and allocasuarina yellows diseases

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Spartium witches'-broom (SpaWB), buckthorn witches'-broom (BWB) and allocasuarina yellows (AlloY) are witches'-broom and yellows diseases of *Spartium junceum* (Spanish broom), *Rhamnus catharticus* (buckthorn) and *Allocasuarina muelleriana* (Slaty she-oak), respectively. These diseases are associated with distinct phytoplasmas. The SpaWB, BWB and AlloY phytoplasmas share <97.5% 16S rDNA sequence similarity with each other and with other known phytoplasmas, including the closely related phytoplasmas of the apple proliferation group. Also, the SpaWB, BWB and AlloY phytoplasmas each have a different natural plant host. Based on their unique properties, it is proposed to designate the mentioned phytoplasmas as novel '*Candidatus*' species under the names '*Candidatus* Phytoplasma spartii', '*Candidatus* Phytoplasma rhamnii' and '*Candidatus* Phytoplasma allocasuarinae', respectively.

Plant-pathogenic phytoplasmas are non-culturable, wall-less prokaryotes of the class *Mollicutes* with a small genome size that ranges from 530 to 1350 kb (Marcone *et al.*, 1999). They are associated with diseases in several hundred plant species, including many woody shrubs or small trees (McCoy *et al.*, 1989; Seemüller *et al.*, 1998a). Sequence analysis of 16S rDNA and other conserved genes reveals that phytoplasmas comprise a discrete, monophyletic clade that is related more closely to acholeplasmas than to other mollicutes (Gundersen *et al.*, 1994; Seemüller *et al.*, 1994). According to phylogenetic data based on 16S rDNA sequences, approximately 20 major phylogenetic groups or subclades have been identified within the phytoplasma clade. This number is

generally in accordance with the phytoplasma strain clusters that have been established by RFLP analysis of PCR-amplified rDNA (Lee *et al.*, 1998, 2000; Seemüller *et al.*, 1998a). Each phytoplasma subclade is considered to represent at least one distinct species under the provisional taxonomic prefix *Candidatus* (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1997, 2000; Seemüller *et al.*, 2002).

Spartium witches'-broom (SpaWB), buckthorn witches'-broom (BWB) and allocasuarina yellows (AlloY) are lethal witches'-broom and yellows diseases of *Spartium junceum* (Spanish broom), *Rhamnus catharticus* (buckthorn) and *Allocasuarina muelleriana* (Slaty she-oak), respectively. SpaWB is known to occur in Italy and Spain (Marcone *et al.*, 1996; Torres *et al.*, 2002) and BWB disease is present in south-western Germany (Seemüller *et al.*, 1994; Mäurer & Seemüller, 1996), whereas AlloY disease occurs in Australia (Gibb *et al.*, 2003). The causal agents, the SpaWB, BWB and AlloY phytoplasmas, are distinct phytoplasmas that are phylogenetically related more closely to phytoplasmas of the apple proliferation (AP) group than to other phytoplasma subclades (Seemüller *et al.*, 1994, 1998a, 2002; Mäurer & Seemüller, 1996; Marcone *et al.*, 1996; Lee *et al.*, 1998; Gibb

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Abbreviations: AlloY, allocasuarina yellows; AP, apple proliferation; BWB, buckthorn witches'-broom; ESFY, European stone fruit yellows; PD, pear decline; PYLR, peach yellow leaf roll; SpaWB, spartium witches'-broom; SSWB, sarothamnus witches'-broom.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA and 16S–23S rDNA spacer region sequences of the AlloY phytoplasma and for the 16S–23S rDNA spacer region of the BWB phytoplasma are AY135523 and AJ583009, respectively.

et al., 2003). In this paper, it is proposed to consider the SpaWB, BWB and AlloY phytoplasmas as novel 'Candidatus Phytoplasma' species. Most of the data on which this proposal is based were published previously. Additional data were generated in this work. In particular, the rDNA of the AlloY phytoplasma, for which a partial 16S rDNA sequence (GenBank accession no. AY135523) had been determined previously by Gibb *et al.* (2003), was resequenced in order to obtain the full-length 16S rDNA and 16S–23S rDNA spacer region sequences. For this purpose, rDNA of the AlloY pathogen was amplified by PCR using the universal phytoplasma primer pairs P1/rU3 (Deng & Hiruki, 1991; Lorenz *et al.*, 1995) and fU5/m23SR (Lorenz *et al.*, 1995; Padovan *et al.*, 1995). PCR products were purified by using a QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions. Sequencing of both strands was performed by using an ABI Prism Big Dye Cycle Sequencing Ready Reaction kit (Applied Biosystems) with the following primers: P1, fP3, fU5, 16R723f and m23SR (Deng & Hiruki, 1991; Lorenz *et al.*, 1995; Padovan *et al.*, 1995). Sequences were then assembled and edited by using MacVector and Assembly Lign (Eastman Kodak) and a consensus sequence was generated. The full-length AlloY sequence has been deposited in GenBank (accession no. AY135523). Phylogenetic distances were calculated by pairwise comparisons using the GCG 8.1.0 (Genetics Computer Group, Madison, WI, USA) program GAP (gap weight, 5.0; length weight, 0.3) and multiple alignments were performed by using PILEUP, obtained through the Australian National Genomic Information Centre, Sydney, NSW, Australia. The rDNA sequences of the following phytoplasmas were retrieved from GenBank and used in this study: SpaWB, accession no. X92869; BWB, X76431; AP, AJ542541; pear decline (PD), AJ542543; European stone fruit yellows (ESFY), AJ542544; peach yellow leaf roll (PYLR), Y16394 and U54990; aster yellows, M30790; stolbur, X76427; X-disease, L04682; 'Candidatus Phytoplasma aurantifolia', U15442; Bermuda grass white leaf, AJ550984; elm yellows, X68376; 'Candidatus Phytoplasma fraxini', AF092209; clover proliferation, L33761; 'Candidatus Phytoplasma phoenicium', AF515636; coconut lethal yellowing, U18747; loofah witches'-broom, L33764; and sugarcane white leaf, X76432. Also, the 16S–23S rDNA spacer region sequence of the BWB phytoplasma, which had been determined earlier by Mäurer & Seemüller (1996), was deposited in GenBank (accession no. AJ583009) and included in the study. Numbering of nucleotide positions corresponds to that of the 16S rDNA molecule of aster yellows phytoplasma strain OAY (Lim & Sears, 1989).

Nucleotide sequence analysis of the PCR-amplified rDNA revealed that the SpaWB phytoplasma is related most closely to the AP, PD, ESFY and PYLR phytoplasmas of the AP group. The SpaWB phytoplasma shows 97.1–97.2% 16S rDNA sequence similarity to the mentioned fruit tree phytoplasmas. Greater differences exist in the sequences of the 16S–23S rDNA spacer region. In this fragment of about 210 bp, the SpaWB phytoplasma differs from the AP and PD phytoplasmas in 12% of positions and from the ESFY and

PYLR phytoplasmas in 13 and 18% of nucleotide positions, respectively. These findings largely confirm the results of previous work (Marccone *et al.*, 1996; Seemüller *et al.*, 1998a). Like all members of the AP group, the SpaWB agent has a unique *PvuII* site following position 518 of the 16S rDNA, which is lacking in phytoplasmas of other groups (Fig. 1). It differs from the four fruit tree phytoplasmas by the presence of two additional *AluI* sites following positions 234 and 627. The SpaWB phytoplasma can be further distinguished from the AP–fruit tree phytoplasmas by differences in other putative restriction sites that are suitable for characterizing members of the AP group (Lorenz *et al.*, 1995; Kison *et al.*, 1997). The SpaWB agent lacks the *SspI* site following position 419, which is present in the AP phytoplasma, but not in the PD, ESFY or PYLR phytoplasmas. It also shows an *SfcI* site following position 630, whereas the ESFY, PD and PYLR phytoplasmas have an additional *SfcI* site following position 1000 and the AP phytoplasma shows an *SfcI* site only at the latter position (Fig. 1).

The BWB phytoplasma shows about 96% 16S rDNA similarity to the fruit tree phytoplasmas of the AP group (Seemüller *et al.*, 1994, 1998a; Mäurer & Seemüller, 1996) and 95% to the SpaWB agent. There are greater differences in the sequences of the 16S–23S rDNA spacer region, where the BWB agent differs from the AP, PD, ESFY and PYLR phytoplasmas in 14–17% of nucleotide positions and from the SpaWB phytoplasma in 16% of positions. The BWB phytoplasma lacks the *SspI* and *SfcI* restriction sites in the 16S rDNA. Like the ESFY phytoplasma, the BWB agent shows an additional *BsaAI* site following position 428. The 16S–23S rDNA spacer region of the BWB phytoplasma shows a *PvuII* site that is not present in the AP–fruit tree agents or related phytoplasmas, as well as an additional *AluI* site (Fig. 1).

The AlloY phytoplasma is related most closely to the BWB agent, sharing 96% 16S rDNA sequence similarity, whereas 16S rDNA sequence similarity with each of the SpaWB, AP, PD, ESFY and PYLR phytoplasmas is 94%. These sequence similarity values are lower than those reported previously by Gibb *et al.* (2003), who examined only a 1152 bp 16S rDNA fragment. At the 16S–23S rDNA spacer region level, the AlloY agent differs from AP–fruit tree phytoplasmas in 11–15% of nucleotide positions and from the SpaWB and BWB phytoplasmas in 17 and 18% of nucleotide positions, respectively. The AlloY phytoplasma lacks the *SspI* site in the 16S rDNA, as well as the *AluI* site following position 1000, which is present in the AP–fruit tree agents SpaWB and BWB (Fig. 1). However, it has the unique *PvuII* site following position 518. Also, it shows the same *SfcI* restriction sites as the ESFY, PD and PYLR phytoplasmas and has an additional *HpaII* restriction site in the 16S–23S rDNA spacer region. Phytoplasmas from other phylogenetic groups usually differ from the SpaWB, BWB and AlloY agents in >8% of 16S rDNA positions.

In previous work, RFLP analysis of a PCR-amplified 1800 bp rDNA fragment that included the 16S rDNA and

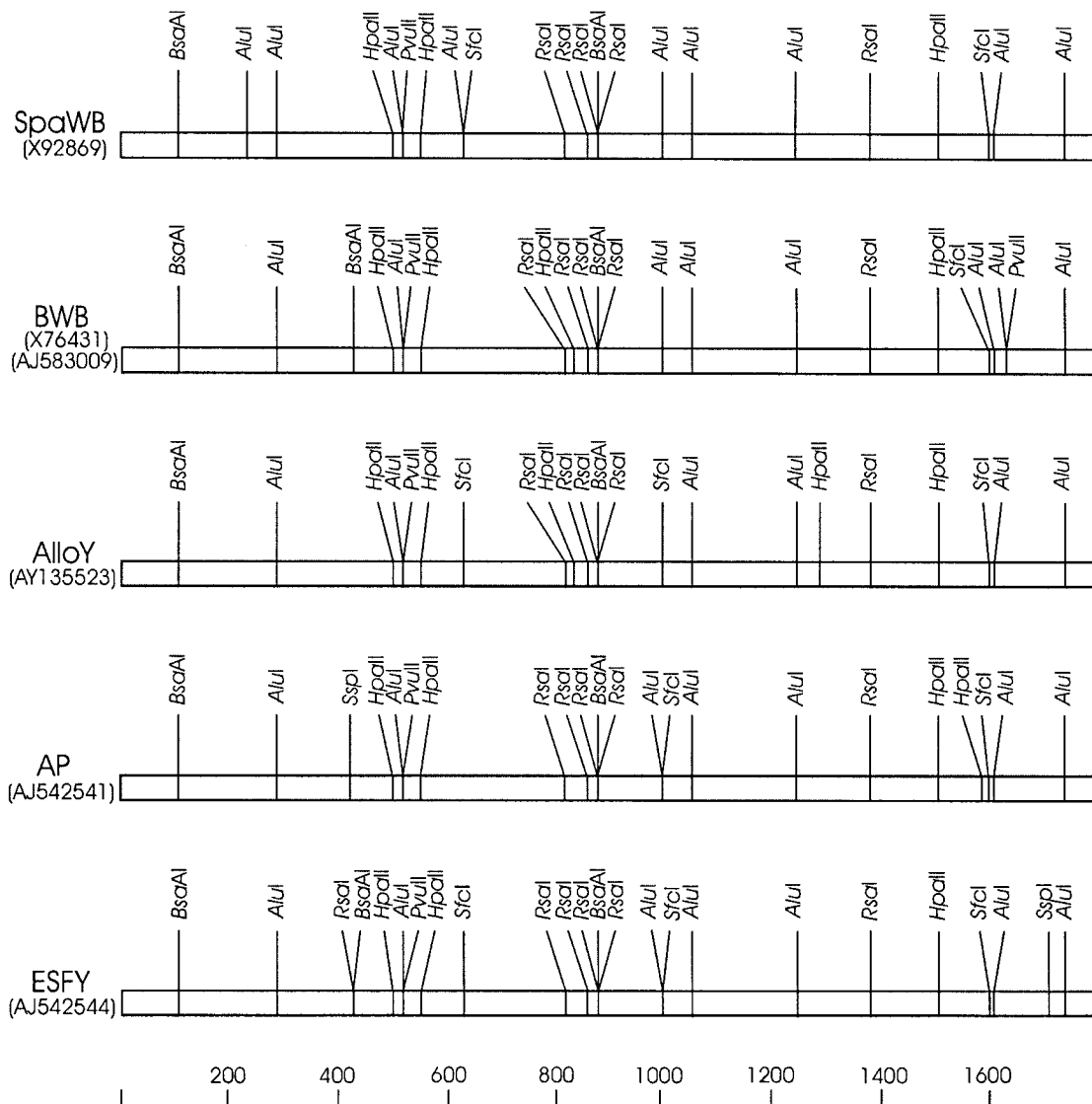


Fig. 1. Restriction map of 16S rDNA and 16S–23S rDNA spacer region sequences of spartium witches'-broom (SpaWB), buckthorn witches'-broom (BWB), allocasuarina yellows (AlloY), apple proliferation (AP) and European stone fruit yellows (ESFY) phytoplasmas. Locations of restriction sites were determined by sequence analysis using the GCG 8.1.0 (Genetics Computer Group, Madison, WI, USA) program package. The locations of *AluI*, *RsaI*, *SspI* and *BsaAI* restriction sites of SpaWB, AP and ESFY phytoplasmas, *AluI* and *RsaI* sites of the BWB agent, and *HpaII* and *SfcI* sites in the 16S rDNA of AlloY phytoplasma were also estimated from previously published RFLP analysis data.

the 16S–23S rDNA spacer region revealed that the SpaWB phytoplasma had the same *RsaI*, *Sau3AI*, *HhaI* and *BsaAI* restriction profiles as the AP phytoplasma. However, the SpaWB phytoplasma differed from the AP agent when the same rDNA fragments were digested with the restriction endonucleases *AluI* and *SspI* (Marcone *et al.*, 1996, 1997). The fragment sizes obtained by enzymic RFLP analysis of PCR-amplified rDNA are in agreement with the deduced fragment sizes based on the putative restriction site analysis that was carried out in this work (Fig. 1). Also, RFLP analysis of the PCR-amplified 16S rDNA and the 16S–23S rDNA spacer region sequences by using the endonucleases

AluI, *RsaI*, *Sau3AI*, *MseI*, *TaqI*, *SspI* and *BsaAI* revealed that the SpaWB agent is indistinguishable from the phytoplasma associated with the sarothamnus witches'-broom (SSWB) disease of *Sarothamnus scoparius*, which is known to occur in Italy (Marcone *et al.*, 1996, 1997; Seemüller *et al.*, 1998a). Distinction of the SpaWB and SSWB phytoplasmas is possible by digestion of the amplified sequences with *HhaI*. In this case, the profile of the SpaWB phytoplasma is different, as it lacks a cleavage site (Marcone *et al.*, 1997). Following separate digestion of the P1/P7 amplicon (Schneider *et al.*, 1995) with *AluI* and *RsaI*, the BWB phytoplasma showed restriction patterns that were identical

to those of the AP phytoplasma with each of these enzymes (Mäurer & Seemüller, 1996). These findings are consistent with data obtained by putative restriction site analysis. RFLP analysis of a PCR-amplified 880 bp 16S rDNA fragment by using *MseI* revealed that the AlloY phytoplasma is indistinguishable from the AP and PD phytoplasmas (Gibb *et al.*, 2003). However, when the same rDNA fragments were digested with *HpaII*, the AlloY phytoplasma proved to be different from both the AP and PD phytoplasmas. Following digestion with *SfiI*, the AlloY phytoplasma showed the same restriction patterns as the PD phytoplasma, but a different pattern from those of the AP agent.

Specific detection of SpaWB and AlloY phytoplasmas is possible by using the reverse primers rSP and rAllo1 (Marccone *et al.*, 1996; Gibb *et al.*, 2003). The rSP primer was designed from the 16S–23S rDNA spacer sequence of the SpaWB phytoplasma (Marccone *et al.*, 1996). This oligonucleotide (5'-GCTAATTAGAAATATCAACTA-3'), which is complementary to a downstream region of the conserved tRNA^{le} gene, amplified the target DNA from SpaWB and SSWB phytoplasmas when paired with the universal forward primers fU5 or P1. No amplification products were obtained from either the AP phytoplasma or more distantly related phytoplasmas (Marccone *et al.*, 1996, 1997). Primer rAllo1 (5'-GAGTCCCCAACTAAATGAT-3'), derived from the 16S rDNA sequence of the AlloY agent, amplified the target DNA from AlloY and AP phytoplasmas when used in combination with the AP group-specific primer fo1 (Lorenz *et al.*, 1995). However, DNA from the PD agent or from more distantly related phytoplasmas was not amplified (Gibb *et al.*, 2003). This confirmed other reports (Lorenz *et al.*, 1995; Seemüller *et al.*, 1998b) that ribosomal primers designed for specific detection of a given pathogen or group of pathogens cross-amplified target DNA from other related phytoplasmas. PCR primers for specific detection of the BWB phytoplasma have not been reported. Thus far, host range testing for the SpaWB, BWB and AlloY phytoplasmas by PCR amplification using universal and specific phytoplasma primers indicates that these phytoplasmas have only been detected in diseased Spanish broom, buckthorn and Slaty she-oak plants, respectively (Mäurer & Seemüller, 1996; Marccone *et al.*, 1996; Gibb *et al.*, 2003).

Conclusion and taxonomic description

The great importance given to 16S rDNA sequences in mycoplasma phylogeny, taxonomy and species identification (Razin *et al.*, 1998) has led the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (1997) to recommend the inclusion of 16S rDNA sequences in any description of a novel mollicute species. For uncultured phytoplasmas, a novel putative species may be described when its 16S rDNA sequence (>1200 bp) has $\leq 97.5\%$ similarity to any previously described 'Candidatus Phytoplasma' species. The SpaWB, BWB and AlloY phytoplasmas share $< 97.5\%$ 16S rDNA sequence similarity with each other and with other known phytoplasmas, including the AP group phytoplasmas. Also,

the SpaWB, BWB and AlloY phytoplasmas each have a different natural plant host that seems to be unique. The difference in geographical distribution of the AlloY phytoplasma from the closely related AP group phytoplasmas supports its placement in a distinct taxonomic unit. Based on their unique properties, we propose to designate the SpaWB, BWB and AlloY phytoplasmas as novel 'Candidatus' species, according to guidelines proposed by Murray & Schleifer (1994) for uncultivated prokaryotes whose uniqueness is defined only by very limited characteristics.

The SpaWB phytoplasma is proposed as a distinct, novel species with the following description: 'Candidatus Phytoplasma spartii' (*spartii*, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. X92869; oligonucleotide sequence complementary to a unique region of the 16S rDNA is 5'-TT-ATCCGCGTTAC-3'; P (*Spartium junceum*, phloem); M]. Reference strain is SpaWB. DNA samples from this strain are available from C. M.

The BWB phytoplasma is proposed as a distinct, novel species with the following description: 'Candidatus Phytoplasma rhamni' (*rhamni*, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession nos X76431 and AJ583009; oligonucleotide sequence complementary to a unique region of the 16S rDNA is 5'-CGAAGTATTCGATAC-3'; P (*Rhamnus catharticus*, phloem); M]. Reference strain is BWB. DNA samples from this strain are available from B. S.

The AlloY phytoplasma is proposed as a distinct, novel species with the following description: 'Candidatus Phytoplasma allocasuarinae' (*allocasuarinae*, epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AY135523; oligonucleotide sequence complementary to a unique region of the 16S rDNA are 5'-TTTATTCGAGAGGGCG-3'; P (*Allocasuarina muelleriana*, phloem); M]. Reference strain is AlloY. DNA samples from this strain are available from K. S. G.

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