

# '*Candidatus Phytoplasma phoenicium*' sp. nov., a novel phytoplasma associated with an emerging lethal disease of almond trees in Lebanon and Iran

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Almonds (*Prunus amygdalus*) represent an important crop in most Mediterranean countries. A new and devastating disease of almond trees in Lebanon was recently reported, characterized by the development of severe witches'-brooms on which no flowers or fruits developed, and leading to tree death within a few years. A phytoplasma was detected in diseased trees by PCR amplification of rRNA operon sequences, and RFLP patterns of amplified DNA indicated that the phytoplasma belonged to the pigeon pea witches'-broom (PPWB) group. In the present work, the presence of a phytoplasma in symptomatic plants was confirmed by electron microscopy; this phytoplasma was graft-transmissible to almond, plum and peach seedlings. The phytoplasma was characterized by sequence analysis of rRNA genes and was shown to be different from the phytoplasmas previously described in the PPWB group. A 16S rDNA phylogenetic tree identified the almond tree phytoplasma as a member of a distinct subclade of the class *Mollicutes*. Oligonucleotides have been defined for specific detection of the new phytoplasma. The almond phytoplasma from Lebanon was shown to be identical to a phytoplasma that induces a disease called 'almond brooming' in Iran, but different from another PPWB-group phytoplasma that infects herbaceous annual plants in Lebanon. Based on its unique properties, the name '*Candidatus Phytoplasma phoenicium*' is proposed for the phytoplasma associated with almond witches'-broom in Lebanon and Iran.

During three surveys (in 2000, 2001 and 2002) to evaluate the sanitary status of fruit trees in Lebanon, samples were collected from almond trees showing small yellow leaves on proliferating shoots in various orchards of the Bekaa Valley (centre), Deir Amar (north) and Tamboureet (south) areas. All almond varieties were affected but some (e.g. Alwani

and Awja) were highly susceptible and developed severe witches'-brooms, leading to rapid tree death; others (e.g. Kachabi) were less severely affected, in that the witches'-brooms were limited to one part of the canopy and only a few trees were affected in orchards where all trees of other varieties were dying of the disease.

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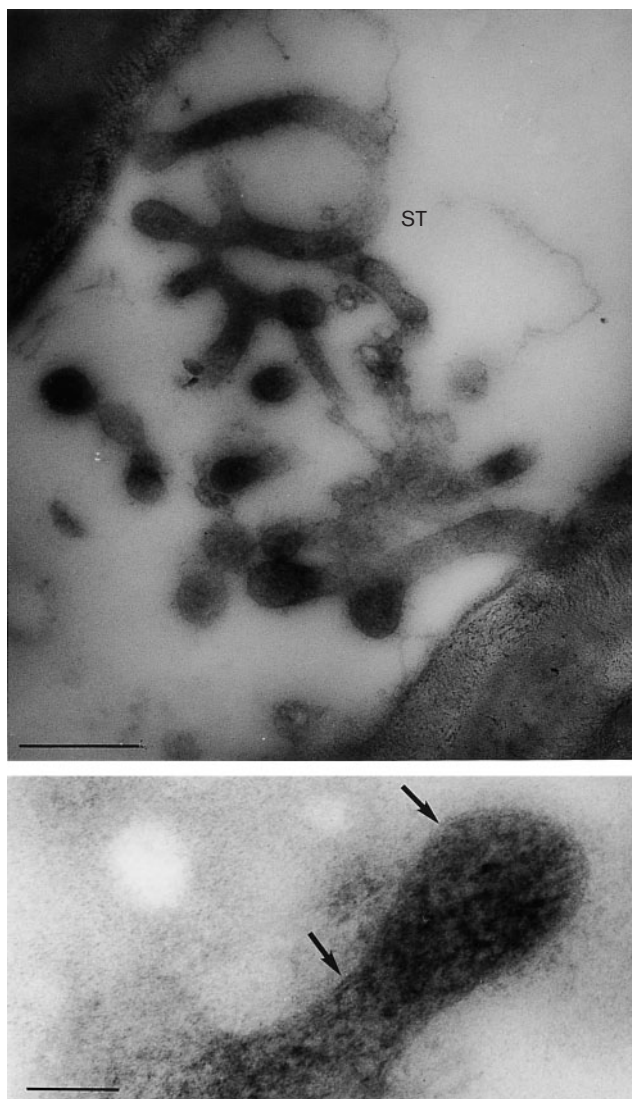
**Abbreviations:** ESFY, European stone fruit yellows; KAP, *Knautia arvensis* phyllody; PEY, *Picris echioides* yellows; PPWB, pigeon pea witches'-broom.

The GenBank accession numbers for the 16S rDNA and 16S–23S spacer sequences of '*Candidatus Phytoplasma phoenicium*' are AF515636 and AF515637 for the Lebanese and Iranian almond isolates, respectively. The accession number for the 16S rDNA and 16S–23S spacer sequence of the wild lettuce (*Lactuca serriola*) and Madagascar periwinkle (*Catharanthus roseus*) phytoplasma is AF515638.

Two supplementary figures showing PCR amplification profiles are available in IJSEM Online.

## Aetiology of the disease

We previously showed by PCR amplification of 16S rDNA that a phytoplasma was associated with diseased almond trees (Choueiri *et al.*, 2001). This is now confirmed by electron microscopy, as shown in Fig. 1. The phytoplasma cells were restricted to the sieve tubes and were found in leaf petioles and midveins of symptomatic, but not asymptomatic, almond trees. Cells had the typical phytoplasma pleomorphic ultrastructure, with predominantly filamentous and branched forms 0.1–0.2 µm in diameter (Fig. 1, top). Phytoplasma cells were surrounded by the triple-layered cytoplasmic membrane characteristic of the class *Mollicutes* (labelled on Fig. 1, bottom). The phytoplasma



**Fig. 1.** Transmission electron micrographs of longitudinal sections through the petiole of a symptomatic almond leaf showing (top) pleomorphic phytoplasma cells in a phloem sieve tube (ST); (bottom) a phytoplasma cell with its characteristic cytoplasmic membrane (arrows). Bars, 0.5 (top) and 0.1 (bottom)  $\mu\text{m}$ .

could be experimentally transmitted by graft-inoculation of infected almond shoots onto seedlings of almond (*Prunus amygdalus*), peach (*Prunus persicae* GF305) and plum

(*Prunus mariana* GF8-1). Symptoms developed after 1 month on the inoculated *Prunus* species, and consisted of axillary bud proliferation similar to that observed on naturally infected almond trees. As phytoplasmas cannot be cultured *in vitro*, Koch's postulates cannot be fulfilled. However, demonstration of the presence of phytoplasmas only in infected almond trees, by electron microscopy and PCR, is a strong indication that the phytoplasma is the causal agent of the disease. This was supported by testing for the presence of various fruit tree viruses, such as *Prunus necrotic ringspot ilarvirus* (PNRSV), *Prune dwarf ilarvirus* (PDV), *Apple mosaic ilarvirus* (ApMV), *Apple chlorotic leaf spot trichovirus* (ACLSV), *Plum pox potyvirus* (PPV), *Cherry leaf roll nepovirus* (CLRV), *Tomato ringspot nepovirus* (ToRSV) and *Strawberry latent ringspot nepovirus* (SLRV), which all gave negative results (E. Choueiri, unpublished data).

### PCR-RFLP analysis of phytoplasma DNA

For characterization of the phytoplasma, cetyltrimethylammonium bromide (CTAB)-extracted almond leaf midvein DNA (Maixner *et al.*, 1995) was used as template for PCR amplification of rRNA genes, using the phytoplasma universal primer pairs P1/P7 (Deng & Hiruki, 1991; Smart *et al.*, 1996) and fU5/rU3 (Seemüller *et al.*, 1994). PCR was performed for 35 cycles using the following conditions: denaturation for 45 s at 92 °C, annealing for 45 s at 58 °C (P1/P7) or 55 °C (fU5/rU3) and primer extension at 72 °C for 90 s (P1/P7) or 45 s (fU5/rU3). DNA extracted from European stone fruit yellows (ESFY) phytoplasma-infected plum midveins was used as a control. No amplification was observed in the absence of template DNA or when template DNA was extracted from asymptomatic almond midveins, whereas DNA fragments of the expected sizes (1.8 and 0.8 kbp for P1/P7 and fU5/rU3, respectively) were obtained with DNA from symptomatic almond midveins or ESFY phytoplasma-infected plum midveins. A supplementary figure showing PCR results with primers P1/P7 is available in IJSEM Online. In addition, to better understand the outbreak of the disease, PCR amplification was performed on DNA extracted from a symptomatic ornamental Madagascar periwinkle (*Catharanthus roseus*) plant at the El Abade experimental station, and from two stunted wild lettuce (*Lactuca serriola*) plants affected by rosetting that were growing as weeds in almond orchards (Choueiri *et al.*, 2002; Table 1). DNA from symptomatic, but not

**Table 1.** Description of plant samples collected

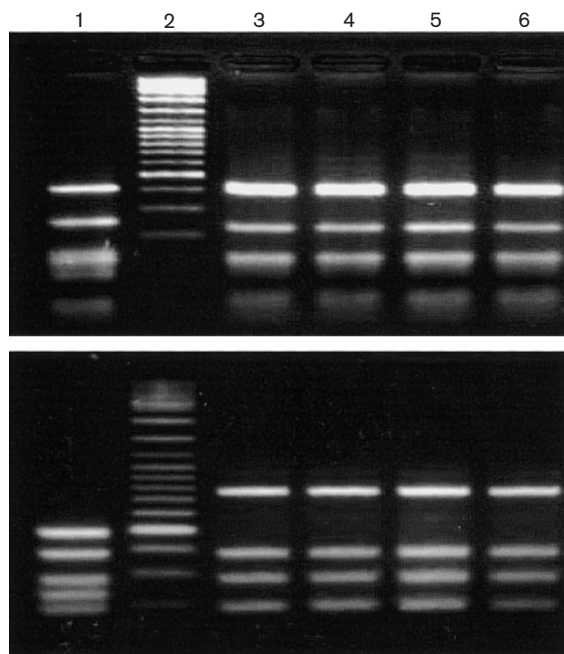
Common name	Scientific name	Symptoms	Location
Almond	<i>Prunus amygdalus</i>	Witches'-broom, yellowing, decline	Lebanon (Bekaa, Deir Amar, Tamboureet); Iran (Shiraz)
Wild lettuce	<i>Lactuca serriola</i>	Stunting, rosetting	Lebanon (Bekaa)
Madagascar periwinkle	<i>Catharanthus roseus</i>	Stunting, rosetting	Lebanon (Bekaa)
Peach	<i>Prunus persicae</i>	Yellowing, proliferation	Lebanon (Tamboureet)

asymptomatic, wild lettuce and periwinkle plants also led to the amplification of DNA fragments of the expected sizes with both primer pairs (results not shown).

The P1/P7-amplified products were digested with the restriction enzymes *Mse*I and *Alu*I (MBI Fermentas), following the manufacturer's instructions. Restriction profiles on 2.5% agarose gels are shown in Fig. 2, top (*Mse*I) and Fig. 2, bottom (*Alu*I) for almond and plum samples. Digestion with each enzyme led to patterns that were identical for the four symptomatic almond DNAs analysed (Fig. 2a and 2b, lanes 3–6), but were different from those obtained with ESFY phytoplasma DNA, in the case of *Alu*I digestion (Fig. 2b, lane 1). The profiles obtained with lettuce and periwinkle plants were identical to those of the almond phytoplasma (results not shown). These results indicated that the almond phytoplasma was different from ESFY, the major stone fruit-associated phytoplasma in Europe, and that RFLP analysis could not distinguish the almond phytoplasma from those in wild lettuce and periwinkle plants.

### Sequence and phylogenetic analysis

The complete sequence of P1/P7-amplified fragments was determined for two different Lebanese almond phytoplasma isolates, from the central Bekaa and northern Deir Amar regions. The sequence was also determined for an



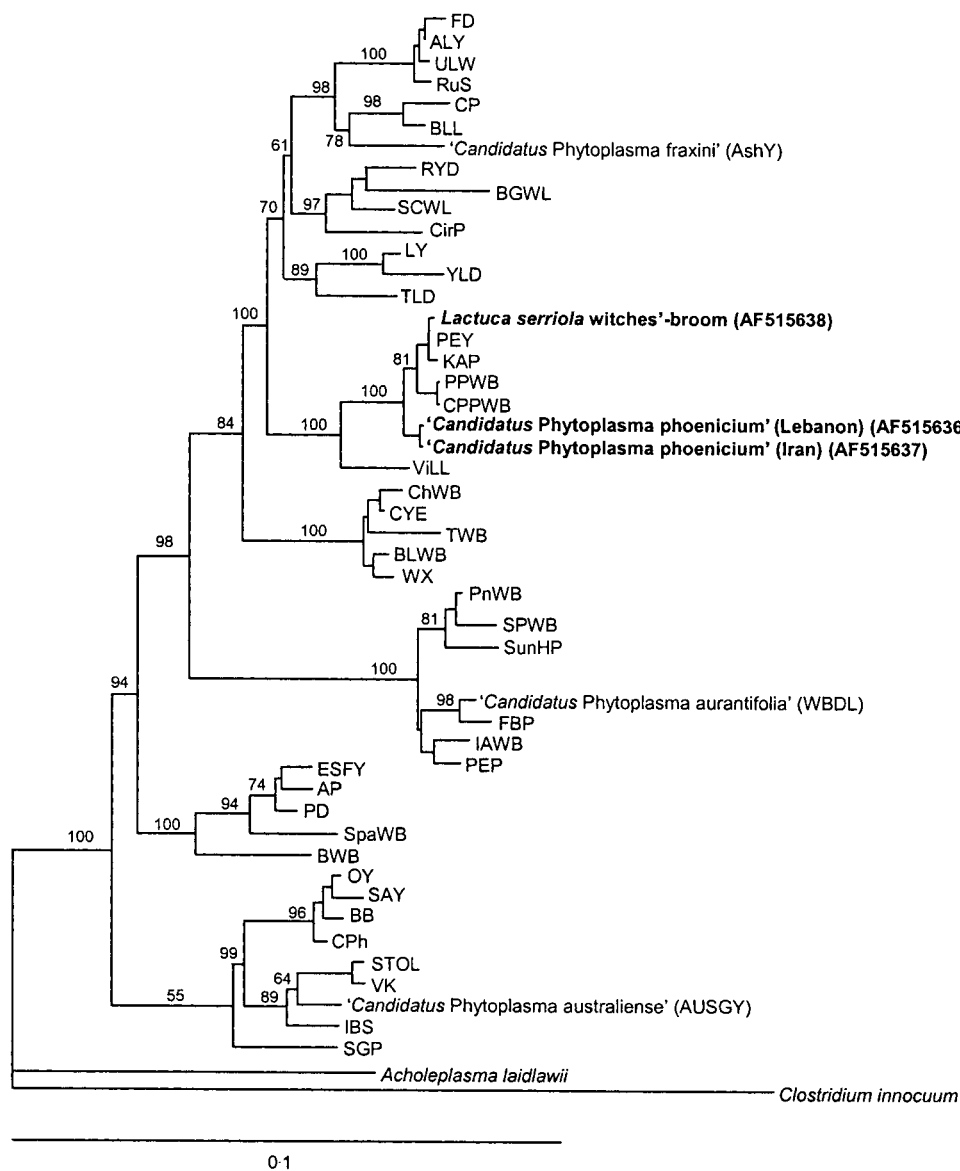
**Fig. 2.** Agarose gel electrophoresis of DNA amplified by PCR with primers P1/P7 and digested with (top) *Mse*I or (bottom) *Alu*I. DNA was extracted from ESFY-infected *Prunus mariana* (lane 1) or witches'-broom-affected almond (lanes 3–6). Lane 2, size standard (GeneRuler DNA Ladder mix, MBI Fermentas).

uncharacterized phytoplasma, detected in 1997 in an almond tree affected by almond brooming, in the Shiraz region of Iran (Bové *et al.*, 1999). The P1/P7 sequences of the phytoplasmas infecting wild lettuce and periwinkle plants were also determined. The nucleotide sequences were assembled with the CAP program (Huang, 1992) and compared with sequences of phytoplasmas and achleplasmas from GenBank using the BLASTN program (Altschul *et al.*, 1990). **The 16S rDNA sequences of the two almond phytoplasmas from Lebanon were identical and differed by only 4 nt (99.7% identity) from the sequence of the phytoplasma from Iran, showing that almond brooming in Iran and almond witches'-broom in Lebanon are caused by the same phytoplasma species.** This suggests that the disease might be present and spreading in additional areas of the Middle East. The closest phytoplasma relatives were members of the pigeon pea witches'-broom (PPWB) group (Schneider *et al.*, 1995), *Picris echioides* yellows (PEY) and *Knautia arvensis* phyllody (KAP), with which they share 99% identity, and the PPWB phytoplasma, with which they share 98.5% identity. The sequences of the wild lettuce and periwinkle phytoplasmas were identical and had a higher identity (99.8%) with the sequences of the PEY and KAP phytoplasmas than with those of the almond phytoplasmas (99.3% identity).

Multiple alignments of near-full-length 16S rDNA sequences from 48 phytoplasmas, one *Acholeplasma* and one *Clostridium* species were examined using CLUSTALW (Thompson *et al.*, 1994). Fig. 3 presents the phylogenetic tree constructed by parsimony analysis with the PHYLIP software package, version 3.5c (Felsenstein, 1989). Bootstrapping was performed 500 times for estimation of clade stability. **The tree shows, as expected, that the Lebanese and Iranian almond phytoplasmas cluster together and that they belong to the PPWB cluster,** but that they represent a new lineage in the PPWB group defined by Schneider *et al.* (1995) or in the 16S rIX-A group defined by Lee *et al.* (1998), and a new subclade in the 'Candidatus Phytoplasma' phylogenetic tree (Lee *et al.*, 1998; Seemüller *et al.*, 1998). In contrast, the wild lettuce and periwinkle phytoplasmas cluster with all other phytoplasmas of the PPWB group.

These results also indicate that the almond phytoplasmas are different from phytoplasmas infecting other *Prunus* species in Europe or the United States, such as the ESFY (90.4% identity) and Western X (94.3% identity) phytoplasmas. They are also different from phytoplasmas infecting other fruit trees, such as the apple proliferation (AP) and pear decline (PD) phytoplasmas (with 90.35 and 90.84% identity, respectively; mean, 90.7%).

Rules for the description of new phytoplasma species have been established by the International Research Program on Comparative Mycoplasma (IRPCM, 2000). A new species may be described when a 16S rDNA sequence (>1200 bp) has <97.5% identity with any previously described 'Candidatus Phytoplasma' species. As yet, none of the phytoplasmas of the PPWB group has been described



**Fig. 3.** Phylogenetic tree constructed using 16S rDNA sequences from 48 phytoplasmas, *Acholeplasma laidlawii* (M23932) and *Clostridium innocuum* (M23732, used as the outgroup). Phytoplasma strain abbreviations and sequence accession numbers (in parentheses): ALY, alder yellows (Y16387); AP, apple proliferation (X68375); AshY, ash yellows (X68339); BB, tomato big bud/Arkansas (L33760); BGWL, Bermuda grass white leaf (Y16388); BLL, brinjal little leaf (X83431); BLWB, black locust witches'-broom (AF244363); BWB, buckthorn witches'-broom (X76431); 'Candidatus Phytoplasma aurantifolia' (U15442); 'Candidatus Phytoplasma australiense' (L76865); ChWB, Chayote witches'-broom (AF147706); CirP, *Cirsium* phyllody (X83438); CP, clover proliferation (L33761); CPh, clover phyllody/Canada (L33762); CPPWB, Caribbean pigeon pea witches'-broom (U18763); CYE, clover yellow edge (AF173558); ESFY, European stone fruit yellows/Germany (X68374); FBP, faba bean phyllody (X83432); FD, flavescence dorée (X76560); IAWB, Italian alfalfa witches'-broom (Y16390); IBS, Italian bindweed stolbur (Y16391); KAP, *Knaulia arvensis* phyllody (Y18052); LY, coconut lethal yellowing (U18747); OY, onion yellows (D12569); PD, pear decline/Germany (X76425); PEP, *Picris echioides* phyllody (Y16393); PEY, *Picris echioides* yellows (Y16389); PnWB, peanut witches'-broom (L33765); PPWB, pigeon pea witches'-broom (AF248957); RuS, *Rubus* stunt (Y16395); RYD, rice yellow dwarf (D12581); SAY, western severe aster yellows (M86340); SCWL, sugar cane white leaf (X76432); SGP, strawberry green petal (U96614); SpaWB (formerly SPAR), *Spartium* witches'-broom (X92869); SPWB, sweet potato witches'-broom (L33770); STOL, stolbur of pepper (X76427); SunHP, sunn hemp witches'-broom (X76433); TLD, Tanzanian lethal decline of coconut (X80117); TWB, tsuwabuki witches'-broom (D12580); ULW, elm yellows/France (X68376); ViLL, Vigna little leaf (Y15866); VK, Vergilbungskrankheit (grapevine yellows) (X76428); WX, Western X-disease (L04682); YLD, Yucatan lethal decline of coconut (U18753). Confidence values (bootstrap percentage) are indicated on the branches. Bar, 1 nucleotide substitution per 10 nucleotides.

as 'Candidatus Phytoplasma' species, and all of the organisms in this group have <97.5% identity with phytoplasmas in other subclades (Lee *et al.*, 1998; Seemüller *et al.*, 1998). The phytoplasma infecting the wild lettuce and periwinkle plants and the other phytoplasmas of the PPWB group have >97.5% identity with the almond phytoplasma and at this stage cannot be described as separate *Candidatus* species, even though they have been found in different host plants (a condition required for species distinction of closely related phytoplasmas). Indeed, two additional properties must also be fulfilled: the phytoplasmas should be transmitted by different insect vectors and should have significant molecular or serological diversity. This has not yet been documented, as the insect vectors of phytoplasmas in the PPWB group are unknown, and genes other than ribosomal operon genes have not been isolated.

### Specific detection of the almond phytoplasma by PCR

In order to distinguish the almond phytoplasma from others in the PPWB group, a specific PCR test was developed. A forward primer, AlmF1 (5'-CCTTTTTC-GGAAGGTATG-3') within the 16S rRNA gene (position 170) and a reverse primer, AlmR1 (5'-GATAACACGCTT-AAGACG-3') within the 16S–23S spacer region (position 1682), were defined after comparison with published phytoplasma rDNA sequences (and more precisely those in the PPWB cluster, including the wild lettuce/periwinkle phytoplasma sequences determined during this work). Primer AlmR1, in combination with primers AlmF1 or fU5, was evaluated for PCR amplification (cycling protocol: 35 cycles of 92 °C for 45 s, 53 °C for 45 s and 72 °C for 60 s) using DNA from asymptomatic and symptomatic almond samples from Lebanon and Iran, PEY-infected periwinkle and infected wild lettuce. The results of PCR amplification with primers fU5/AlmR1 indicated that a DNA fragment of the expected size (1.4 kbp) was amplified using DNA from all symptomatic almond trees tested. No amplification occurred with DNA from asymptomatic almond trees, PEY phytoplasma or wild lettuce phytoplasma-infected plants (figure available as supplementary data in IJSEM Online). PCR with primers AlmR1/AlmF1 or fU5 was also applied to the experimentally graft-inoculated almond, peach and plum seedlings, as well as to a peach tree with proliferation symptoms from an orchard close to Tamboureet in southern Lebanon. PCR using all samples led to the amplification of bands of the expected sizes (results not shown), confirming the successful transmission of the phytoplasma to other *Prunus* species, both experimentally and in nature. However, during our surveys, we found only one positive peach tree among many affected almond trees, and no affected plum trees, which may indicate that the insect vector is preferentially attracted by almond trees.

The specific PCR will be particularly useful for identification of both the insect vector(s) and reservoir plant(s) of the almond phytoplasma, as we know that vectors and

reservoirs of other phytoplasmas in the PPWB group are also present in Lebanon. Indeed, we were recently able to identify a leafhopper carrying the wild lettuce/periwinkle phytoplasma but, at the time of writing, none of the insects collected gave positive tests for the almond phytoplasma (J.-L. Danet, P. Salar, E. Choueiri, F. Jreijiri, S. El Zammar, J. M. Bové & M. Garnier, unpublished data).

Based on its distinctive properties and the development of a specific detection test, we propose that the phytoplasma of almond witches'-broom in Lebanon should be the reference strain for the new phytoplasma subclade in the PPWB cluster, and according to the rules for the description of putative taxa of uncultured bacteria as defined by Murray & Schleifer (1994), should be named 'Candidatus Phytoplasma phoenicium' [(Mollicutes) NC; G+; F; NAS (GenBank AF515636), oligonucleotide sequence complementary to unique region of 16S rDNA 5'-CCTTTTTCGGAAGGTA-TG-3', S (*Prunus amygdalus*, phloem); M;].

### Note added upon submission of manuscript

Before submitting this manuscript for publication, a paper concerning the almond witches'-broom phytoplasma was published (Abou-Jawdah *et al.*, 2002). The phylogenetic relationship of the phytoplasma is in full agreement with the characterization described in the present manuscript; only the phytoplasma of almond trees in Lebanon was studied. The authors also indicated that a phytoplasma belonging to the same phylogenetic group was present in symptomatic peach and nectarine trees. This is in agreement with our PCR and graft-transmission results.

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### References

- Abou-Jawdah, Y., Karakashian, A., Sobh, H., Martini, M. & Lee, I. M. (2002). An epidemic of almond witches'-broom in Lebanon: classification and phylogenetic relationships of the associated phytoplasma. *Plant Dis* **86**, 477–484.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Bové, J. M., Danet, J. L., Bananej, K., Hassanzadeh, N., Taghizadeh, M., Salehi, M. & Garnier, M. (1999). Witches' broom disease of lime (WBDL) in Iran. In *Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV)*, pp. 207–212. Edited by J. V. da Graça, R. F. Lee & R. K. Yokomi. Riverside, CA: IOCV.
- Choueiri, E., Jreijiri, F., Issa, S., Verdin, E., Bové, J. M. & Garnier, M. (2001). First report of a phytoplasma disease of almond (*Prunus amygdalus*) in Lebanon. *Plant Dis* **85**, 802.
- Choueiri, E., Jreijiri, F., El Zammar, S., Verdin, E., Salar, P., Danet, J. L., Bové, J. M. & Garnier, M. (2002). First report of grapevine

“Bois Noir” disease and a new phytoplasma infecting solanaceous plants in Lebanon. *Plant Dis* **86**, 697.

**Deng, S. & Hiruki, C. (1991)**. Amplification of 16S rRNA genes from ultratable and nonculturable *Mollicutes*. *J Microbiol Methods* **14**, 53–61.

**Felsenstein, J. (1989)**. PHYLIP – Phylogeny Inference Package (version 3.2). *Cladistics* **5**, 164–166.

**Huang, X. (1992)**. A contig assembly program based on sensitive detection of fragment overlaps. *Genomics* **14**, 18–25.

**IRPCM. (2000)**. Report of the working team for phytoplasmas, spiroplasmas, mesoplasmas and entomoplasmas. In *Report of Consultations of the International Research Programme of Comparative Mycoplasmaology (IRPCM) of the International Organization for Mycoplasmaology (IOM)*. Fukuoka, Japan: IOM.

**Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E. & Bartoszyk, I. M. (1998)**. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int J Syst Bacteriol* **48**, 1153–1169.

**Maixner, M., Ahrens, U. & Seemüller, E. (1995)**. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *Eur J Plant Pathol* **101**, 241–250.

**Murray, R. G. E. & Schleifer, K. H. (1994)**. Taxonomic notes: a proposal for recording the properties of putative taxa of procaryotes. *Int J Syst Bacteriol* **44**, 174–176.

**Schneider, B., Seemüller, E., Smart, C. D. & Kirkpatrick, B. C. (1995)**. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In *Molecular and Diagnostic Procedures in Mycoplasmaology*, vol. 1, pp. 369–380. Edited by S. Razin & J. G. Tully. New York: Academic Press.

**Seemüller, E., Schneider, B., Mäurer, R. & 8 other authors (1994)**. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. *Int J Syst Bacteriol* **44**, 440–446.

**Seemüller, E., Marccone, C., Lauer, U., Ragozzino, A. & Göschl, M. (1998)**. Current status of molecular classification of the phytoplasmas. *J Plant Pathol* **80**, 3–26.

**Smart, C. D., Schneider, B., Blomquist, C. L., Guerra, L. J., Harrison, N. A., Ahrens, U., Lorenz, K. H., Seemüller, E. & Kirkpatrick, B. C. (1996)**. Phytoplasma-specific PCR primers based on sequences of the 16S–23S rRNA spacer region. *Appl Environ Microbiol* **62**, 2988–2993.

**Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994)**. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.