'Candidatus Phytoplasma pini', a novel taxon from *Pinus silvestris* and *Pinus halepensis*

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*Pinus silvestris* and *Pinus halepensis* trees grown in Germany and Spain, respectively, showing abnormal shoot branching, dwarfed needles and other symptoms were examined for the presence of plant-pathogenic mollicutes (phytoplasmas). While phytoplasmas could not be detected unambiguously with microscopical methods, PCR amplification using universal phytoplasma primers yielded positive results. Samples collected from symptomatic and non-symptomatic plant parts of both symptomatic *Pinus silvestris* and *Pinus halepensis* trees tested positive. Also, surrounding non-symptomatic trees proved to be phytoplasma-infected. Comparisons revealed that the 16S rRNA gene sequences of the phytoplasmas identified in *Pinus silvestris* and *Pinus halepensis* were nearly identical. However, the pine phytoplasma is only distantly related to other phytoplasmas. The closest relatives are members of the palm lethal yellowing and rice yellow dwarf groups and ‘Candidatus Phytoplasma castaneae’, which share between 94.5 and 96.6% 16S rRNA gene sequence similarity. From these data it can be concluded that the phytoplasmas identified in the two *Pinus* species represent a coherent but discrete taxon; it is proposed that this taxon be distinguished at putative species level under the name ‘Candidatus Phytoplasma pini’.

Phytoplasmas are wall-less, non-helical prokaryotes of the class *Mollicutes* (‘mycoplasmas’). These (as yet) uncultured plant pathogens reside mostly in plant phloem sieve tubes, are transmitted between plants by phloem-feeding insects and are associated with diseases in about a thousand plant species, almost exclusively angiosperms (Seemüller et al., 2002). There are, on the basis of electron microscopic studies, only a few unconfirmed reports of the detection of phytoplasmas in gymnosperms including conifer species of the families *Pinaceae*, *Taxodiaceae* and *Cupressaceae* (Gopo et al., 1989; Koyama, 1970; McCoy et al., 1989). The trees in which phytoplasma-like structures were detected showed yellowing symptoms and prolific branching of small shoots. Molecular data confirming the phytoplasma nature of the organisms observed and providing information on their phylogenetic relatedness to other phytoplasmas are absent in these reports. However, phytoplasmas related to the X-disease or 16SrIII group were identified in an unspecified cypress species by PCR-RFLP analysis (Paltrinieri et al., 1998).

In this article, the detection, characterization and taxonomic classification of a hitherto unknown phytoplasma from *Pinus silvestris* (Scots pine) and *Pinus halepensis* (Aleppo pine) in Germany and Spain, respectively, are reported. In Tübingen in southwestern Germany, a Scots pine was observed showing conspicuous shoot-proliferation symptoms in combination with dwarfed needles on one major branch. These aberrations gave the branch a dense, ball-like appearance. Other branches of the tree were non-symptomatic. In Tarragona in northeastern Spain, several Aleppo pines were observed showing abnormal shoot proliferation and short, yellowish and sometimes twisted needles. However, these aberrations did not result in the ball-like structures of affected branches described above for *Pinus silvestris*.

The GenBank/EMBL/DDBJ accession numbers for the P1/P7 amplicon (16S rRNA gene and 16S/23S rRNA spacer region) of phytoplasma strains Pin127S⁶, Pin190S and PinG are AJ632155, AJ632156 and AJ910849, respectively.
Recent investigations, in particular sequence analysis of 16S rRNA genes, have revealed that phytoplasmas constitute a coherent genus-level taxon (Lee et al., 1998; Seemüller et al., 2002). In the monophyletic phytoplasma clade, groups and subgroups have been delineated, many of them being considered as putative species under the provisional status 'Candidatus' for incompletely described prokaryotes described by 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 strain can be described as a novel ‘Ca. Phytoplasma’ species if its 16S rRNA gene sequence has <97.5 % similarity to that of any previously described ‘Ca. Phytoplasma’ species”. In this study, we analysed the rRNA gene sequences of three pine strains. The results showed that the phytoplasmas detected are virtually identical at the 16S rRNA level, but differ significantly from other phytoplasmas.

Phloem and surrounding bark tissue from branches and roots of Pinus silvestris were fixed in a glutaraldehyde/formaldehyde mixture (Karnovsky, 1965). Specimens were sectioned with a freezing microtome and the sections stained with 4’,6-diamidino-2-phenylindole (Seemüller, 1976). In parallel, ultra-thin sections were prepared using standard methods and examined by electron microscopy. Although all cell types of the phloem were examined in many specimens, unequivocal identification of phytoplasmas was not possible with either method. This may be explained by very low phytoplasma titres and/or poor preservation of the organisms in the tissue examined. It is well established that phytoplasma numbers in infected plants may be so low that microscopic detection is difficult or impossible (Berges et al., 2000; Caudwell & Kuszala, 1992; Kartte & Seemüller, 1991).

For PCR detection, samples were collected several times over a 2-year period from the symptomatic branch from non-symptomatic branches and roots of the diseased tree and from aerial parts and roots of three non-symptomatic neighbouring trees. All trees were approximately 80 years old. Phloem tissue was prepared as aseptically as possible and DNA extracted as described previously (Ahrens & Seemüller, 1992, 1994). From Pinus halepensis, samples from symptomatic branches were collected from one tree several times over a 3-year period. In addition, samples from symptomatic and non-symptomatic branches of three neighbouring trees and one tree grown at another location were taken. Also, samples from aerial parts of a non-symptomatic tree were collected. All Pinus halepensis trees were approximately 25 years old. DNA extraction was performed using an initial enrichment step (Ahrens & Seemüller, 1992) followed by a procedure employing the NucleoSpin Plant kit (Macherey-Nagel). PCRs in Germany and Spain were carried out as described by Kison et al. (1997) and Torres et al. (2003), respectively, using phytoplasma-specific ribosomal primer pair fU5/rU3 (Lorenz et al., 1995) or P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995). Primers fU5/rU3 mediate amplification of an 880 bp fragment of the 16S rRNA gene and the pair P1/P7 primes amplification of a 1800 bp product extending from the 5’ end of the 16S rRNA gene to the 5’ region of the 23S rRNA gene, thus including the 16S/23S rRNA spacer region. A nested PCR for detecting phytoplasmas in Pinus halepensis was performed by re-amplifying the P1/P7 product with the internal primers R16F2/R2 (Lee et al., 1995). PCR products of the expected sizes were obtained from nearly all samples taken from roots and from symptomatic and non-symptomatic aerial parts of symptomatic trees. In addition, most root and stem samples collected from the non-symptomatic Scots pine and Aleppo pine trees were PCR-positive (Fig. 1 and data not shown).

P1/P7 products from two phytoplasma strains from Pinus halepensis (Pin127S<sup>R</sup> and Pin190S) and one strain from Pinus silvestris (PinG) were used for rRNA gene sequence analysis. Sequence evaluation, including manual alignment, was done using the software package HUSAR (Biocomputing Service Group, German Cancer Research Centre, Heidelberg). Gaps and ambiguities were removed from the final data set. Phylogenetic and molecular evolutionary analyses were conducted 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 the bootstrap percentage values are given at the nodes of the tree. Phylogenetic distances were calculated by pairwise comparison.

Analysis of the aligned DNA revealed that the 16S rRNA genes of the pine phytoplasma strains consist of 1531 nucleotide residues, more than 99 % of which were analysed. The sequences of the three strains are nearly identical, having similarity values between 99.7 and 99.9 %. Strain Pin190S differed by one polymorphism at position 153 from the selected reference strain Pin127S<sup>R</sup> and the German strain PinG. In addition, three substitutions were

![Fig. 1. PCR amplification of an 880 bp fragment from the 16S rRNA gene of the pine phytoplasma isolated from Pinus silvestris using primer pair fU5/rU3. M, 100 bp ladder (MBI); 1, healthy periwinkle; 2, apple proliferation, as positive control; 3, symptomatic shoot; 4, non-symptomatic shoot of symptomatic tree; 5, root from symptomatic tree; 6, shoot from non-symptomatic tree; 7, root from non-symptomatic tree.](https://example.com/fig.png)
observed in strain PinG at positions 30, 264 and 362 in comparison with the Spanish strains.

The phylogenetic relatedness of the three pine strains to each other and to selected reference phytoplasmas (Table 1) is depicted in Fig. 2. The three pine phytoplasma strains form a distinct branch in the phytoplasma phylogenetic tree and are only distantly related to other phytoplasmas. The closest relatives, sharing between 94-5 and 93-6% 16S rRNA gene sequence identity with the pine strains, are almost exclusively phytoplasmas associated with diseases of monocotyledonous plants. These pathogens belong to the coconut lethal yellowing or 16SrIV group and the rice yellow dwarf or 16SrXI group (Lee et al., 1998). The diseases caused by the first group include, among others, various types of coconut diseases such as coconut lethal yellowing, Yucatan lethal decline, Tanzanian coconut lethal disease and diseases of palm-like trees such as those affecting Carludovica palmata and Pandanus utilis (Harrison et al., 2002; Jung et al., 2002). The rice yellow dwarf group members are associated with diseases of gramineae, such as sugarcane white leaf, Bermuda grass white leaf, brachiaria grass white leaf and rice yellow dwarf (Marcone et al., 2004). ‘Candidatus Phytoplasma castaneae’ infecting the broadleaf tree Castanea crenata is similarly related to the pine strains. In previous work, this phytoplasma appeared to cluster in a larger group together with the palm pathogens (Jung et al., 2002). However, when the pine strains are included in the analysis, ‘Ca. Phytoplasma castaneae’ forms a group with the pine phytoplasma in which each of them constitutes a distinct branch (Fig. 2). Phytoplasmas of other phylogenetic groups show lower levels of 16S rRNA gene sequence similarity to the pine phytoplasma, ranging from 93-3 to 86-9%.

The 16S rRNA gene sequences from the pine strains were aligned with phytoplasmas representing most phylogenetic groups. Oligonucleotides occurring only in the pine phytoplasma were selected and used as query sequences in a BLAST 2.0 search (Altschul et al., 1997). This analysis resulted in the following signature sequences, which are unique to this phytoplasma and absent in other organisms: 5'-G-GAAAATCTTGCGGGATTTAGT-3' (positions 67–88) and 5'-TCTCAGTGCTTAACGCTGTTCT-3' (positions 603–624).

Like the 16S rRNA gene sequences, the sequences of the 16S–23S rRNA spacer region of the three pine strains proved to be very homogeneous. Only one polymorphism was observed, located just downstream of the tRNA\text{Ile} gene, whereby in strain Pin190S an A present in the other strains was substituted by a G. In the spacer, the differences with

Table 1. Phytoplasma strains and/or putative phytoplasma species used in this study, and associated diseases or plant hosts

<table>
<thead>
<tr>
<th>Putative species and/or strain</th>
<th>Associated disease or plant host</th>
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<tr>
<td>PinG</td>
<td>Pinus silvestris (Germany)</td>
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<tr>
<td>Pin127S\textsuperscript{R}</td>
<td>Pinus halepensis (Spain)</td>
</tr>
<tr>
<td>Pin190S</td>
<td>Pinus halepensis (Spain)</td>
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<tr>
<td>‘Ca. Phytoplasma asteris’</td>
<td>Aster yellows</td>
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<tr>
<td>‘Ca. Phytoplasma castaneae’</td>
<td>Chestnut witches’ broom</td>
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<tr>
<td>‘Ca. Phytoplasma cynodontis’</td>
<td>Bermuda grass white leaf</td>
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<tr>
<td>‘Ca. Phytoplasma oryzae’</td>
<td>Rice yellow dwarf</td>
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<tr>
<td>‘Ca. Phytoplasma phoenicium’</td>
<td>Almond witches’ broom</td>
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<tr>
<td>CPLY</td>
<td>Carludovica palmata leaf yellowing</td>
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<tr>
<td>CTD</td>
<td>China tree decline</td>
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<tr>
<td>LD</td>
<td>Coconut lethal disease</td>
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<td>LFWB</td>
<td>Loofah witches’ broom</td>
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<tr>
<td>LY</td>
<td>Coconut lethal yellowing</td>
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<td>PDF</td>
<td>Florida Pandanus decline</td>
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<tr>
<td>SCWL</td>
<td>Sugar-cane white leaf</td>
</tr>
<tr>
<td>ViLL</td>
<td>Vigna little leaf</td>
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Fig. 2. Phylogenetic tree constructed using the neighbour-joining method with 16S rRNA gene sequences from three strains of the pine phytoplasma (Pin127S\textsuperscript{R}, Pin190S, PinG) and 13 neighbouring reference phytoplasmas. Numbers on branches are confidence values obtained from 500 bootstrap replicates. ‘Candidatus Phytoplasma asteris’ was used as the outgroup. See Table 1 for phytoplasma strain abbreviations.
respect to other phytoplasmas were more pronounced than those in the 16S rRNA gene. Comparisons of the sequences of the pine strains with those of the Ca. luthovica agent, ‘Candidatus Phytoplasma cynodontis’, ‘Candidatus Phytoplasma fraxinii’, ‘Candidatus Phytoplasma auranti-folia’ and ‘Candidatus Phytoplasma phoenicium’ revealed similarity values between 72-1 and 57-6%.

16S rRNA gene sequences of the pine phytoplasmas were examined for the presence of putative restriction sites for Alu, Rsal, Msel, Hhal and Hinfi endonucleases, which are widely used in RFLP analysis of phytoplasma rRNA genes. The Spanish strains Pin1278R and Pin190S showed an identical pattern, whereas the German strain PinG differed by the lack of an Msel restriction site at position 30. Phytoplasmas of the coconut lethal yellowing group and the rice yellow dwarf group showed restriction map patterns that were, overall, similar to those of the pine strains. However, all of them differ, in terms of several restriction sites, from the pine phytoplasmas, and can be readily distinguished by these means.

The data presented show that the three strains examined, obtained from two different Pinus species and collected in two different countries, are nearly identical at the 16S rRNA gene level. They differ from other phytoplasmas in at least 5-5% of the nucleotide positions. From this it can be concluded that the requirements for defining putative phyto-plasma species are fulfilled and that the pine strains form a coherent and distinct taxon under the provisional status ‘Candidatus’ according to the convention proposed by Murray & Schleifer (1994) for prokaryotes that can be described only incompletely. In our work, independently carried out in Spain and Germany, the occurrence of phytoplasmas in conifers was clearly demonstrated. Hitherto, the suitability of gymnosperms as hosts has been questioned because of the small pore sizes in the sieve cells, which might hinder phytoplasma spread. Pore sizes between 1 and 14 μm were determined for the sieve elements of dicotyledons (Esau & Cheadle, 1959). Phytoplasmas, which are usually between 200 and 800 nm in diameter, are supposed to pass without difficulty through such pores (McCoy, 1979). However, the sieve pores of gymnosperms are considerably smaller (Esau, 1969). In Metasequoia, pore sizes between 50 and 85 nm were observed (Kollmann & Schumacher, 1963). Thus, it is possible that in gymnosperms phytoplasma spread and tissue colonization differ from those in dicotyledon plants. As the pine phytoplasma was also detected in non-symptomatic plants, its pathological role remains similarly unclear.

**Description of ‘Candidatus Phytoplasma pini’**

‘Candidatus Phytoplasma pini’ (L. gen. n. *pini* of a pine tree, of a tree of the genus *Pinus*).

Reference strain is Pin1278R.

‘Candidatus Phytoplasma pini’ [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank/EMBL/DDBJ accession no. AJ632155); oligonucleotide sequences complementary to unique regions of the 16S rRNA gene are 5’-GGAAA-TCTTTCGAGATTATTAGT-3’ and 5’-TCTCAGTGCTT- AACGGCTTCTT-3’; P (Pinus); M]. Schneider et al., this study.

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**References**


