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## Detection of ‘*Candidatus Phytoplasma pini*’ in *Pinus sylvestris* trees in Poland

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### Abstract

Abnormal shoot branching was observed in *Pinus sylvestris* trees in Poland. These abnormalities resulted in the formation of dense, ball-like structures with dwarfed needles. The presence of phytoplasma in the needles of branched and surrounding symptomless shoots was demonstrated using nested-polymerase chain reaction (PCR) with universal primer pairs that amplified phytoplasma 16S rDNA, as well as using restriction fragment length polymorphic analysis of PCR products. Comparison of nucleotide sequences of DNA samples from three *P. sylvestris* trees with ball-like structures revealed that their fragments of 16S rDNA were identical. The nucleotide sequence showed more than 99% similarity with the corresponding fragments of sequence of ‘*Candidatus phytoplasma pini*’.

### Introduction

Diseases of forest trees of uncertain aetiology are distributed worldwide. However, little is known about the nature of the virus-like symptoms of coniferous plants in Europe (Nienhaus and Castello, 1989). During the last decade the economic importance of some plant diseases associated with phytoplasma infection has increased considerably in many countries. Using molecular techniques for detection and identification, several phytoplasmas were found to be associated with diseases in about a thousand plant species, almost exclusively angiosperms (Lee et al., 2000). The three reports on the electron microscopic detection of phytoplasmas in conifer plants of the families Pinaceae, Taxodiaceae and Cupressaceae (Koyama, 1970; Gopo et al., 1989; McCoy et al., 1989) need to be confirmed. The conifer species in which phytoplasma-like structures were observed showed leaf yellowing, shoot proliferation and stunting. More recently, Paltrinieri et al. (1998) reported that in Italy *Cypress* species were naturally infected with a phytoplasma related to the X-disease phytoplasma by polymerase chain reaction restriction fragment length polymorphic (PCR-RFLP)

analysis. More recently, on the basis of PCR amplification of 16S rDNA and sequence analysis, Schneider et al. (2005) indicated that *Pinus sylvestris* (Scots pine) and *P. halepensis* (Aleppo pine) trees with shoot proliferation symptoms or ball-like structures were associated with phytoplasma infection. Comparisons revealed that the 16S rRNA gene sequences of the phytoplasmas from two *Pinus* species were nearly identical and they represent a new taxon designated ‘*Candidatus Phytoplasma pini*’. This phytoplasma was found in symptomatic branches as well as in symptomless parts of pines and apparently healthy *Pinus* trees in Germany and Spain.

*Pinus sylvestris* is one of the oldest living plants on earth. Of all needle-type evergreens, pines seem to show the greatest diversity of habit, distribution and ornamental characteristics. In Poland, some shoot proliferation and stunting causing dense, ball-like structures on some branches were observed in *P. sylvestris* trees. These aberrations, named also witches' brooms, are potentially attractive and they are used for propagation to get new selections of dwarf types of pine (Vrgoc, 2002). We report here the occurrence of ball-like structures in pine trees in Poland and their association with phytoplasma infection using molecular methods.

### Materials and Methods

#### Symptom observation and plant material

The observations were carried out in 2000–2004 on *P. sylvestris* trees grown in pine forests in Poland. Several hundred hectares of forests were surveyed visually and 198 trees with abnormally branched shoots were observed in different parts of the country. Eight pine trees forming ball-like structure on a branch were selected for further experimentation. All the selected trees were approximately 60–100 years old. The shoots obtained from the dense structures of selected trees were propagated by grafting in March–April 2000 using 3-year-old seedlings of *P. sylvestris* as a rootstock. The

plants were maintained in an insect-proof greenhouse of the Agriculture University in Poznań.

For PCR detection, samples of needles from ball-like structures and the surrounding symptomless branches were collected from eight *P. sylvestris* plants in March 2000. Sample of needles from eight selected grafted plants with dwarfed and abnormally branched shoots were collected in April 2004. The samples of collected needles were maintained at  $-70^{\circ}\text{C}$ . Samples of leaves of *Catharanthus roseus* inoculated by grafting with the reference strains of aster yellows phytoplasma (AY1, 16SrI-B, kindly supplied by Dr I.-M. Lee, Beltsville, USA) and the reference strain of apple proliferation phytoplasma (AP, 16SrX-A, kindly supplied by Dr A. Bertaccini, Bologna, Italy), were also included in this study.

#### DNA extraction and PCR amplification

DNA was extracted from approximately 1.5 g of frozen needles using the procedure described by Stange et al. (1998). Total nucleic acids were used as templates for direct PCR with universal primers P1/P7 (Deng and Hiruki, 1991; Kirkpatrick et al., 1994). Products from the first PCR were diluted 25 times and then used in nested reactions as templates for amplification with universal primers: R16F2n/R16R2 (Davis and Lee, 1993; Lee et al., 1993) and fAT/rA (Ahrens and Seemüller, 1992) and group-specific primer pairs: R16(I)F1/R16(I)R1, R16(III)F2/R16(III)R1, R16(V)F1/R16(V)R1 (Lee et al., 1994) and fAT/rAS (Smart et al., 1996). All the PCR assays were run under parameters described previously (Kamińska et al., 2003). PCR products (5  $\mu\text{l}$ ) were analysed by 1% agarose gel electrophoresis in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.3) buffer followed by staining with ethidium bromide (0.5  $\mu\text{g/ml}$ ) and visualized with UV transilluminator (Syngen Biotech, Wrocław, Poland).

#### RFLP analysis of PCR products

The RFLP analysis of nested-PCR products was carried out with endonucleases *AluI*, *HhaI*, *MseI* or *RsaI* (GibcoBRL, Life Technologies, Warsaw, Poland) according to the manufacturer's instruction. The digested DNA was resolved by electrophoresis through 6% polyacrylamide gel, stained with ethidium bromide and observed under UV light. The lengths of DNA fragments were estimated by comparison of the position of DNA bands with those of molecular weight marker – Gene Ruler 100 bp DNA Ladder Plus (Fermentas, Vilnius Lithuania).

#### Sequencing and computer analysis

The PCR amplified products (obtained with primers R16F2n/R16R2) from three samples of *P. sylvestris* (OB1, NR1 and CL2) were used for phytoplasma 16S rRNA gene sequence analysis. Sequencing was performed in the Maria Skłodowska Memorial Cancer Center and Institute of Oncology, Warsaw, using ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Warsaw, Poland). A consensus sequence was compiled from

overlapping fragments, using the SEQMAN v.1.5 program LASERGENE 5.0 software package (DNASTAR Inc., Madison, WI, USA). The sequence was first checked for the similarity to known sequences, using the BLAST service available at <http://www.ncbi.nlm.nih.gov/BLAST/> (Altschul et al., 1997). Further analysis and calculations of percentages of sequence identity was performed with the MEGALING program (DNASTAR software package).

## Results

### Symptoms

During inspection of pine forests, very conspicuous symptoms on *P. sylvestris* trees were observed. They included abnormal shoot proliferation in combination with their retarded growth (Fig. 1) on one major branch. These aberrations resulted in forming dense ball-like structures with dwarfed needles. The symptoms were observed on 198 pine trees in different regions of Poland.

### Graft transmission experiment

Eighty *P. sylvestris* seedlings were grafted in 2000 with scions from ball-like structures. The disease symptoms of stunted growth, abnormal shoot proliferation and



Fig. 1 Branched and dwarfed shoots of *Pinus sylvestris* with shortened and chlorotic needles (at left) and shoots of pine tree without disease symptoms (at right)

leaf reduction were observed on 30–70% of the grafted plants.

#### Phytoplasma detection and identification

Specific products were obtained in direct PCR with the universal primer pair P1/P7 (expected length approximately 1.8 kb) only for the control samples of the reference strain AY1. No visible product was amplified by the direct PCR from samples obtained from pines and healthy periwinkle plants. However, in nested-PCR with fA/rA or R16F2n/R16R2 primers pairs a specific DNA bands were obtained for DNA samples isolated from five of 16 symptomatic pines. Using fA/rA primer pair, specific product (0.56 kb) was obtained for DNA samples collected from abnormally branched and dense shoots of five of eight plants and for samples collected from non-symptomatic parts of two of eight tested pines (Fig. 2) and for samples isolated from two of eight grafted plants. Using R16F2n/R16R2 primer pair a product of 1.24 kb was obtained only in DNA samples collected from needles of ball-like structures of three tested pines.

The restriction profiles of phytoplasmas isolated from three tested pines were identical (Fig. 3). The profiles were similar to those of 'Candidatus Phytoplasma pini' (computer-predicted from sequences of isolates Pin 127S, accession number AJ632155, Pin190S, accession number AJ632156 and PinG, accession number AJ310849). PCR products obtained using R16F2n/R16R2 primer pair for samples CL2, NR1 and OB1 and were sequenced from both strands. Nucleotide sequence alignment revealed that fragments of 16S rDNA amplified from three *P. sylvestris* trees with ball-like structures were identical. The sequence obtained for phytoplasma isolated from pine sample OB1 was deposited in the GenBank database under accession number EF128037 (Fig. 4). Sequences of three phytoplasmas from pine plants were also submitted to BLAST analysis which showed more than 99% similarity with the sequences of phytoplasmas belong-

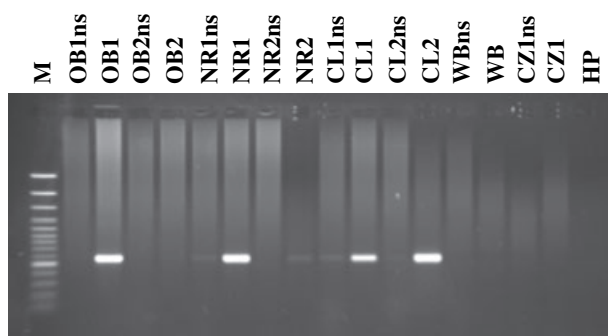


Fig. 2 Electrophoresis of nested-polymerase chain reaction products obtained using fA/rA primers pair. OB1, OB2, NR1, NR2, CL1, CL2, WB, CZ1 – samples isolated from symptomatic part of *Pinus sylvestris*; OB1 ns, OB2 ns, NR1 ns, NR2 ns, CL1 ns, CL2 ns, WBns, CZ1 ns – samples isolated from non-symptomatic part of pine; HP – healthy periwinkle plant; M – Gene Ruler 100 bp DNA Ladder Plus (Fermentas, Lithuania)

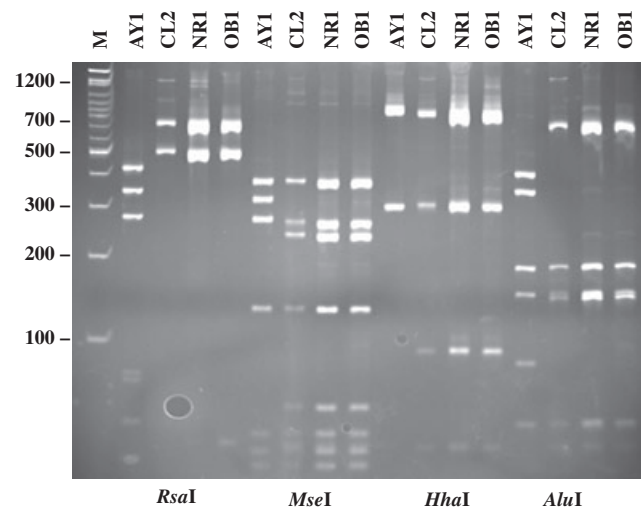


Fig. 3 Restriction profiles after *AluI*, *HhaI*, *MseI* and *RsaI* digestion of the nested-polymerase chain reaction products (primed by R16F2n/R16R2 primers pair) from *Pinus sylvestris* plants (CL2, OB1 and NR1); AY1 – reference strain of aster yellows phytoplasma; M – Gene Ruler 100 bp DNA Ladder Plus (Fermentas, Lithuania)

ing to the species 'Candidatus Phytoplasma pini' (Fig. 5) by Schneider et al. (2005).

#### Discussion

The present study provides evidence that pine trees with growth aberrations were naturally infected with phytoplasma. Based on sequencing of the fragments of 16S rDNA, pine plants with shoot and leaf symptoms were affected with a phytoplasma nearly identical (more than 99% similarity) with the sequences of 'Candidatus Phytoplasma pini' deposited in the GenBank under accession numbers AJ632155, AJ632156 and AJ10849 (Schneider et al., 2005). The data obtained by PCR amplification and sequence analysis confirm the report of Schneider et al. (2005) that this novel taxon is implicated in the growth abnormalities in pine trees. The phytoplasma apparently occurred in such low titres in pines that detection by direct PCR amplification using universal primers was not possible. However, phytoplasma could be detected through nested-PCR assays of the product obtained by direct PCR. This result is in agreement with that of Schneider et al. (2005), who were unable to detect 'Candidatus Phytoplasma pini' by electron microscopy or direct PCR. The examples of yellows diseases in which detection of the casual agent is very difficult are decline disease of *Quercus robur* trees and shoot proliferation and stunting of *Carpinus betulus* trees (Berges et al., 2000). Some difficulties in phytoplasma detection in *Rosa* spp., *Magnolia* spp. and *Acer negundo* plants with stunting, shoot proliferation and dieback was experienced by Kamińska et al. (2001, 2003) and Kamińska and Śliwa (2006).

The ball-like structures observed in *P. sylvestris* trees in Poland are similar to those reported in Scots pine from Germany. However, they differ from shoot

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1      TAAGACTGGA TAGGAAATAA AAAGGCATCT TTTTATTTTT AAAAGACCTT
51     TTTTCGAAAGG TATGCTTAAA GAGGGGCTTG CGCCATATTA GTTAGTTGGT
101    AGGGTAAAAG  CCTACCAAGA CGATGATATG TAGCTGGACT GAGAGGTTGA
151    ACGGCCACAT  TGGGACTGAG ACACGGCCCA AACTCCCACG GGAGGCAGCA
201    GTAGGGAATT  TTCGGCAATG GAGGAACTC  TGACCGAGCA ACGTCGCGTG
251    AACGATGAAG  TATTTCCGTA TGTAAGTTC  TTTTATTGAA GAAGAAAAAA
301    TAGTGGAAAA  ACTATCTTGA CGCTATTCAA TGAATAAGCC CTGGCTAATT
351    ATGTGCCAGC  AGCCGCGGTA ATACATAAAG GCGGAGCGTT ATCCGGAATT
401    ATTGGGCGTA  AAGGGTGCGT AGGCGGTTTG TTAAGTCTTT AGTTTAACTT
451    CAGTGCTTAA  CGCTGTTCTG CTAGAGAAAC TGACTTACTA GAGTGAGATA
501    GAGGCAAGTG  GAATTCCATG TGTCAGCGGT AAAATGCGTA AATATATGGA
551    GGAACACCAG  AGGCGTAGGC GGCTTGCTGG GTCTTTACTG ACGCTGAGGC
601    ACGAAAGCGT  GGGGAGCAA  CAGGATTAGA TACCCTGGTA GTCCACGCCG
651    TAAACGATGA  GTACTAAGTG TCGGGGAAAC TCGTACTGA  AGTTAACACA
701    TTAAGTACTC  CGCCTGAGTA GTACGTACGC AAGTATGAAA CTTAAAGGAA
751    TTGACGGGAT  CCCGCACAAG CGGTGGATCA TGTGTTTAA  TTCGAAGATA
801    CACGAAAAAC  CTTACCAGGT CTTGACATAC TCTGCAAAGC TGTAGAAATA
851    CAGTGGAGGT  TATCAGGGAT ACAGGTGGTG CATGTTTGTG  GTCAGCTCGT
901    GTCGTGAGAT  GTTAGGTTAA GTCCTAGAAC GAGCGCAACC CTTGTCTGTTA
951    GTTACCAGCA  CGTTATGGTG GGCACTTTAA CGAGACTGCC AATGAAATAT
1001   TGGAGGAAGG  TGAGGATTAC GTCAAATCAT CATGCCCTTT  ATGATATGGG
1051   CTACAAACGT  GATACAATGG CTGTTACAAA GAGTAGCTGA AACGTGAGTT
1101   TTTGGCCAAT  CTAACAAAAA CAGCCTCAGT TCGGATTGAA GTCTGCAACT
1151   CGACTTCATG  AAGTTGGAAT CGTAGTAAT  CGCAATCAG  CATGTTGCGG
1201   TGAATACG
    
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Fig. 4 The nucleotide sequence of 16S rRNA gene fragment of phytoplasma OBI isolate

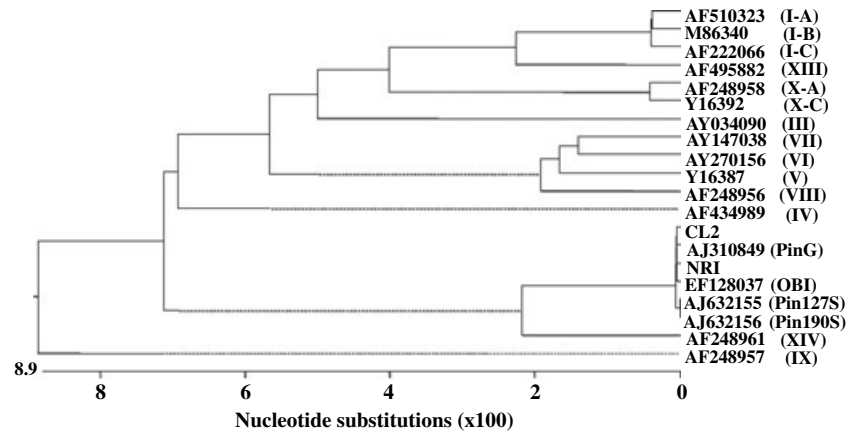


Fig. 5 Phylogenetic tree based on similarity data obtained from comparison of 16S rRNA gene fragment sequence of phytoplasma isolated from *Pinus sylvestris* and sequences from GenBank. Sequences were compared using CLUSTALW program, and they were grouped in a consensus tree by the module MegAlign (DNASTAR, LASERGENE). Roman numerals represent 16S rDNA groups according to Lee et al. (1998)

proliferation observed in *P. halepensis* from Spain (Schneider et al., 2005) or witches' brooms, observed in many species of deciduous trees, e.g. *Populus* (Berges et al., 1997), *Alnus* (Lederer and Seemüller, 1991), *Fraxinus* (Ferris et al., 1989) or *Robinia* (Chapman et al., 2001), associated with phytoplasma infection. The German and Spain observations that 'Candidatus Phytoplasma pini' was present in symptomatic as well as symptomless pines trees, indicate that there was no association between the presence of symptoms and the finding of positive PCR. The results of our study are also inconclusive; three of eight old tested pines and six of eight regenerates, all rated as having typical symptoms, showed a negative reaction. Failure to detect phytoplasma in these samples could be due to several causes. The titre of the phytoplasma in the negative samples was possibly too low for detection or the symptoms of ball-like structures may not be diagnostic of this phytoplasma and there may be other causes of those growth abnormalities in pines.

The phytoplasma so far detected in conifer trees have been established in the forest ecosystems for a very long time, so the woody plants provide a long-lived phytoplasma reservoir. To date little is known about the incidence and potential significance of 'Candidatus Phytoplasma pini' and other phytoplasmas in conifer plants. It has been hypothesized that viruses and similar pathogens are the main causes of forest damage in Central Europe (Kandler, 1985). Research to date has demonstrated that viruses and phytoplasmas contribute to disease symptomatology within a causal complex, but their potential impact on the growth and yield of conifer trees is unknown. Phytoplasmas may predispose trees to other damaging factors and lead to premature senescence, but this is only hypothetical. Phytoplasmas may seem less important than other stresses in this regard, but this may be due to our lack of understanding of phytoplasma impact in conifer trees. The clonally propagated species in managed plantations and nurseries may allow

investigating all potential impacts on the growth and other factors of these valuable, renewable resources.

This is the first report of '*Candidatus Phytoplasma pini*' in Poland.

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