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New natural host plants of '*Candidatus* Phytoplasma pini' in Poland and the Czech Republic

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The presence of phytoplasmas in seven coniferous plant species (*Abies procera*, *Pinus banksiana*, *P. mugo*, *P. nigra*, *P. sylvestris*, *P. tabuliformis* and *Tsuga canadensis*) was demonstrated using nested PCR with the primer pairs P1/P7 followed by R16F2n/R16R2. The phytoplasmas were detected in pine trees with witches' broom symptoms growing in natural forest ecosystems and also in plants propagated from witches' brooms. Identification of phytoplasmas was done using restriction fragment length polymorphism analysis (RFLP) of the 16S rDNA gene fragment with *Alu*I, *Mse*I and *Rsa*I endonucleases. All samples showed RFLP patterns similar to the theoretical pattern of '*Candidatus* Phytoplasma pini', based on the sequence of the reference isolate Pin127S. Nested PCR-amplified products, obtained with primers R16F2n/R16R2, were sequenced. Comparison of the 16S rDNAs obtained revealed high (99·8–100%) nucleotide sequence identity between the phytoplasma isolates. The isolates were also closely related to four other phytoplasma isolates found in pine trees previously. Based on the results of RFLP and sequence analyses, the phytoplasma isolates tested were classified as members of the '*Candidatus* Phytoplasma pini', group 16SrXXI.

Keywords: fir, hemlock, molecular detection, phytoplasma, pine, witches' broom

Introduction

Phytoplasmas are non-helical, mycoplasma-like bacteria that lack cell walls. These specialized bacteria are insect-transmitted and can cause devastating diseases in crops and natural ecosystems worldwide (Seemüller *et al.*, 1998; Lee *et al.*, 2000; Bertaccini, 2007; Hogenhout *et al.*, 2008). In infected plants, phytoplasmas almost exclusively inhabit the phloem sieve tube elements. They are transmitted from plant to plant by phloem-feeding homopteran insects, mainly leafhoppers and less frequently psyllids (Weintraub & Beanland, 2006). Phytoplasmas are implicated in causing host plant metabolic changes such as disrupted hormonal balance, impaired amino acid and carbohydrate translocation, inhibited photosynthesis and rapid senescence (Chang, 1998; Lepka *et al.*, 1999; Bertamini *et al.*, 2002; Ćurković Perica *et al.*, 2007). Plants infected by phytoplasmas exhibit a wide range of symptoms. Specific symptoms include flower virescence and distortion, flower abnormalities resulting in sterility, leaf discolouration and malformation, abnormal shoot branching and stunted growth. Symptoms of diseased plants may vary depending on the phytoplasma isolate, the host plant, stage of the disease, age of plant, time of infection and environmental conditions (Seemüller *et al.*, 1998; Lee *et al.*, 2000). Occasionally, phytoplasma-infected plants are nonsymptomatic (Lederer & Seemüller, 1991). A temporary or permanent remission of symptoms may also occur (Davies *et al.*, 1992; Kamińska & Korbin, 1999). In the last two decades the economic importance of some plant diseases associated with phytoplasma infection has increased considerably in many countries.

Diseases of coniferous forest trees that cause shoot proliferation symptoms (also known as witches' brooms), in combination with dwarfed needles and stunted growth, are widely distributed throughout the world. Liese (1933) categorized witches' brooms of coniferous plants into two groups according to their probable cause: those caused by known organisms and those that are not associated with any known organism. Several groups have researched the second category (Liese, 1933; von Tubeuf, 1933; Fordham, 1967; Waxman, 1975) and studied witches' brooms as possible somatic mutations that might be used for the production of genetic variation of coniferous plants. In Poland, coniferous plants obtained by vegetative propagation of witches' brooms are maintained in several nurseries and gardens. They are slow-growing trees and shrubs which are usually stunted, have abnormal shoot branching with very short needles and a dense growth habit.

Phytoplasma infection in coniferous plants of the Pinaceae, Taxodiaceae and Cupressaceae families, with leaf yellowing, shoot proliferation and stunting symptoms,

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was first demonstrated by Koyama (1970) and Gopo *et al.* (1989) using electron microscopy. Using molecular-based techniques for detection and identification, thirty 16Sr phytoplasma groups and numerous subgroups have been identified in association with diseases of about a thousand plant species, of which almost all were angiosperms (Lee *et al.*, 2000; Bertaccini, 2007). Paltrinieri *et al.* (1998) demonstrated, by PCR-RFLP analysis, that *Cypress* species in Italy were naturally infected with a phytoplasma related to the X disease phytoplasma group 16SrIII. Shoot proliferation symptoms or ball-like structures in *Pinus sylvestris* (Scots pine) and *Pinus halepensis* (Aleppo pine) trees in Germany and Spain were associated with infection by a new taxon '*Candidatus* Phytoplasma pini' group 16SrXXI (Schneider *et al.*, 2005), identified on the basis of PCR amplification of 16S rDNA and sequence analysis. These results were confirmed by Śliwa *et al.* (2008) who identified '*Ca.* Phytoplasma pini' infection in *P. sylvestris* trees in Poland. In the following years phytoplasma infection was reported in other coniferous plant species. In Poland, shoot fasciation and proliferation symptoms in *Picea abies* (Norway spruce) were associated with phytoplasma X group 16SrIII infection (Kamińska & Śliwa, 2010). Abnormal shoot branching and rosetting was also reported in *Araucaria heterophylla* (Norfolk Island pine) in India and in *Juniperus occidentalis* (Western juniper) in Oregon, USA. The disease symptoms in Norfolk Island pine have been associated with '*Ca.* Phytoplasma trifolii' group 16SrVI infection (Gupta *et al.*, 2009), while symptoms in Western juniper have been associated with infection by the new phytoplasma subgroup 16SrIX-E ('*Ca.* Phytoplasma phoenicium'-related; Davis *et al.*, 2010).

The objective of this paper was to investigate the association of phytoplasmas with selected coniferous trees and shrubs propagated from witches' brooms (WB), as well as with pine trees with WB symptoms (Fig. 1), occurring in the nursery and the natural forest ecosystems in Poland using molecular methods. Coniferous plants originating from witches' brooms grown in a commercial nursery in Průhonice, the Czech Republic, were examined for comparison.

Materials and methods

Plant material

Samples of needles were collected in early spring, summer or autumn during 2009–2010 from plants of the *Abies*, *Pinus* and *Tsuga* species (Table 1). The samples were collected from plants growing in eight commercial nurseries, a botanical garden and natural forest ecosystems:

- One *Abies procera* (noble fir) tree of WB origin with compact habit and needle discoloration, from the nursery in the Czech Republic; *Abies concolor* (white fir) and *Abies koreana* (Korean fir) with stunted growth and needle chlorosis growing in a forest ecosystem in Poland. The trees were 15–20 years old.

Figure 1 Witches' broom on *Pinus banksiana* var. Turtle Creek tree.

- Twenty-six *Pinus* spp. plants of WB origin with retarded growth and dwarfed needles were selected. Most of the pines were grown in the nurseries in Poland and some of them were from the Czech Republic. The pine trees in the nurseries in Poland were 3–7 years old while the trees grown in the Czech Republic were approximately 10–15 years old.
- Four plants of *Pinus nigra* (Austrian pine) var. Hornibrookiana, one plant of *P. sylvestris* var. Beauvronensis. The varieties were obtained by grafting WBs at the end of the XIX century. These pines had compact growth and relatively short internodes, the trees were 30–40 years old and were maintained in four nurseries or botanical gardens.
- Two *P. sylvestris* trees with witches' brooms, approximately 70–80 years old, growing in a natural forest ecosystem in the central part of Poland.
- Four *Tsuga canadensis* (Canadian hemlock) plants of WB origin from the nurseries in Průhonice and Poland, all with very compact growth, about 15–20 years old.

In addition, leaf samples were collected from symptomless *Abies concolor*, *Pinus banksiana* (Jack pine, two plants), *P. sylvestris* (two plants), *Pinus tabuliformis* (Chinese pine), healthy *Catharanthus roseus* and periwinkle plants infected by grafting with the reference strains of aster yellows phytoplasma (AY1, 16SrI-B, kindly supplied by Dr I.-M. Lee, Beltsville, USA). In total, 66 needle samples from 40 plants were sampled and analysed.

DNA extraction and PCR amplification

Total DNA was extracted from frozen needles using the DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's recommendations.

Table 1 Phytoplasma detection in *Abies* spp., *Pinus* spp. and *Tsuga canadensis* plants by polymerase chain reaction (PCR) in 2009 and 2010

		Sampling date							
Plant species and variety	Prominent symptoms	6 May 2009	16 Jun 2009	9 Aug 2009	30 Sep 2009	17 Nov 2009	26 May 2010	20 Jun 2010	26 Oct 2010
Abies procera var. Procumbens Formanek	Stunted growth, needle discolouration		N[a]					nd[b]	
Abies concolor 1	Chlorosis						nd		
A. concolor 2	No symptoms						nd		
Abies koreana var. Kopiczko	Compact habit, dwarf needles						nd		nd
Pinus banksiana var. Turtle Creek	WB[c], stunted growth,		DN[d]			nd		N	
P. banksiana var. Schneverdingen	Stunted growth				nd				
P. banksiana var. Gala	Stunted growth				nd				
Pinus heldreichii var. Schmidtii	Stunted growth					nd			
Pinus mugo var. Mop	Stunted growth	DN	nd	N					
P. mugo var. Pudełek	Stunted growth	DN	N						
P. mugo ssp. *uncinata*	Stunted growth	DN			nd				
Pinus nigra	Stunted growth	N	nd	nd	N	nd			
P. nigra var. Hornibrookiana (4 trees)	Compact habit						nd		
Pinus sylvestris B	Stunted growth	N	DN	DN					
P. sylvestris C	Stunted growth		nd	nd					
P. sylvestris ssp. *lapponica*	WB, stunted growth		DN			DN		N	
P. sylvestris ssp. *lapponica* (3 hybrid trees)	Poor foliage						N		
P. sylvestris var. Rogów	Stunted growth				nd				
P. sylvestris 157	Inhibited growth			nd					
P. sylvestris var. Chybie	Stunted growth				N				
P. sylvestris var. Beauvronensis	Compact habit						nd		
P. sylvestris var. Globosa viridis	Stunted growth							nd	
P. sylvestris YANG	Stunted growth							N	
P. sylvestris YIN	Stunted growth							N	
P. sylvestris (from Paprotnia, Rogów)	Stunted growth							nd	
P. sylvestris 157	Inhibited growth							nd	N
P. sylvestris 1319	WB								nd
P. sylvestris 1319 (asymptomatic part)	WB								N
P. sylvestris 1519	WB								nd
P. sylvestris 1519 (asymptomatic part)	WB								N
Pinus tabuliformis	No symptoms						DN		DN
Tsuga canadensis var. Jervis	Stunted growth		N				nd		
T. canadensis var. Jervis JW							nd		
T. canadensis var. Vercade recurved	Stunted growth		nd					nd	
T. canadensis var. Minima	Stunted growth		nd						
T. canadensis var. Everitt Golden	Stunted growth		nd						
T. canadensis var. Pendula	Stunted growth		nd						

[a]Phytoplasma detected by nested PCR.
[b]Phytoplasma not detected.
[c]Witches' broom.
[d]Phytoplasma detected by direct and nested PCR.

Extracted nucleic acids were used as templates for direct PCR with the primers P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995). Products from the first PCR were diluted 25-fold and then used in nested reactions as templates for amplification with the primers R16F2n/R16R2 (Lee *et al.*, 1993; Gundersen & Lee, 1996). All the PCR assays were run using the parameters described previously (Śliwa *et al.*, 2008).

The amplification products (5 μL) were analysed by 1% agarose gel electrophoresis in 0·5 × TBE (45 mM Tris–borate, 1 mM EDTA, pH 8·3) buffer followed by staining with ethidium bromide (0·5 μg mL^{-1}) and visualized with a UV transilluminator (Syngen Biotech).

RFLP analysis of PCR products

Restriction fragment length polymorphism analysis of nested PCR products was performed using the restriction endonucleases *Alu*I, *Mse*I or *Rsa*I (Fermentas) according to the manufacturer's instructions. The digested DNA was resolved by electrophoresis through an 8% polyacrylamide gel, stained with ethidium bromide and

observed under UV light. The lengths of DNA fragments were estimated by comparison to the position of DNA bands with those of the size marker GeneRuler 100 bp DNA Ladder Plus (Fermentas).

Sequencing and computer analysis

Nested PCR-amplified products obtained for samples from *A. procera*, *Pinus* spp. and *T. canadensis* were resolved by agarose gel electrophoresis, cut from the gel and purified using the QIAquick PCR Purification kit (QIAGEN). Purified rDNAs were directly sequenced at the Maria Skłodowska Memorial Cancer Center and Institute of Oncology, Warsaw, Poland, using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequencing was performed with the same primers as for the original PCR.

Sequences were analysed using the LASERGENE v7·1 software package (DNASTAR). Consensus sequences were produced using the SEQMAN program; the similarity level of 16S rDNA gene fragments was determined with the MEGALIGN program and GENEDOC program (http://www.psc.edu/biomed/genedoc; Nicholas *et al.*, 1997). Sequences were compared with analogous 16S rDNA gene sequence fragments already available in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Comparisons were made with the following phytoplasma strains: '*Candidatus* Phytoplasma pini' (GenBank accession numbers AJ310849, AJ632155, AJ632156 and EF128037), '*Ca.* Phytoplasma cynodontis' (AF248961), '*Ca.* Phytoplasma palmae (AF434961), '*Ca.* Phytoplasma trifolii' (AY270156), '*Ca.* Phytoplasma ulmi' Y16387, '*Ca.* Phytoplasma phoenicium' (AF248957), '*Ca.* Phytoplasma pruni' (AY034090), '*Ca.* Phytoplasma asteris' (M86340), '*Ca.* Phytoplasma solani' (AF248959), '*Ca.* Phytoplasma mali' (AF248958) and '*Ca.* Phytoplasma pyri' (Y16392).

Phylogenetic relationships were estimated with the neighbour-joining method and subsequent bootstrap analysis using MEGA software v4·0·2 (Tamura *et al.*, 2007).

Results

Using the universal primer pairs in PCR, products of the expected size were obtained in the samples from 20 out of 40 coniferous trees or shrubs of seven species.

In 2009, direct and nested PCR products (~1800 and ~1250 bp, respectively) were obtained for DNA samples isolated from six out of 14 symptomatic *Pinus* spp. trees: *P. banksiana*, *P. mugo* (mugo pine, var. Mop and Pudełek), *P. mugo* ssp. *uncinata* and *P. sylvestris* (plant B and ssp. *lapponica*), and for the reference AY1 strain (Table 1). Nested PCR products (~1250 bp) were obtained for DNA samples isolated in the same year from *A. procera*, *P. sylvestris* var. Chybie and from one out of five tested *T. canadensis* shrubs, all derived from witches' brooms.

In 2010, direct and nested PCR products were obtained only for DNA samples extracted from a *P. tabuliformis* tree and for the control samples of the reference strain AY1. In nested PCR, phytoplasmal DNA was detected in four WB-derived pine trees, in four *P. sylvestris* hybrid trees and two *P. sylvestris* trees with witches' brooms growing in the forest ecosystem. For the latter two *P. sylvestris* trees, products were amplified from DNA samples obtained from the symptomless part of trees but not from needles collected from witches' brooms. No visible PCR products were obtained from the DNA isolated from needles of the symptomatic *A. procera* and *T. canadensis* var. Jervis plants, from which PCR products were obtained in 2009.

During the 2 year period, no visible PCR products were obtained from the DNA extracted from needle samples taken from the following plants: *Abies concolor*, *Abies koreana*, *P. banksiana* (var. Gala, Schmidtii and Schneverdingen), *Pinus heldreichii* (Bosnian pine), four *P. nigra* var. Hornibrookiana plants, *P. sylvestris* (var. Beauvronensis, Globosa viridis, Paprotnia, Rogów and plant C) and five *T. canadensis* shrubs. No PCR products were obtained from the five symptomless control plants (one *A. concolor*, two *P. banksiana* and two *P. sylvestris* trees) or from healthy periwinkle plants.

The specificity of amplified PCR products was confirmed by RFLP analysis. After enzymatic digestion, all samples showed a restriction pattern similar to the theoretical pattern calculated for the sequence of the reference isolate '*Ca.* phytoplasma pini' Pin127S (Schneider *et al.*, 2005). Samples of the results are shown in Figure 2.

Nested PCR-amplified 16S rDNA fragments were sequenced and deposited in GenBank (Table 2). Sequence analysis revealed that the phytoplasma ribosomal gene fragments derived from *A. procera*, *P. banksiana* var. Turtle Creek, *P. mugo* var. Pudełek, *P. mugo* subsp. *uncinata*, *P. sylvestris* ssp. *lapponica* (from Průhonice), *P. sylvestris* YIN, *P. sylvestris* 1319 *P. sylvestris* 1519 and *T. canadensis* var. Jervis (all plants obtained by grafting from witches' brooms), as well as from the naturally infected seedlings of *P. sylvestris* ssp. *lapponica* (from Powsin), *P. sylvestris* 157 and *P. tabuliformis*, were very similar to each other with identity values of 99·8% or higher. The phytoplasma strains were closely related to four other '*Ca.* Phytoplasma pini' strains (GenBank accession numbers AJ310849, AJ632155, AJ632156, EF128037) and formed a separate cluster together on a phylogenetic tree (Fig. 3). They were distantly related to other phytoplasma species used for comparison. Based on the results of RFLP and sequence analyses, the phytoplasma isolates in this study were classified as members of the '*Ca.* Phytoplasma pini' group 16SrXXI.

Discussion

The present study provides evidence that *A. procera* and *T. canadensis* plants and trees of five species of *Pinus* (*P. banksiana*, *P. mugo*, *P. nigra*, *P. sylvestris* and *P. tabuliformis*) were infected with phytoplasmas. Results of sequence analysis showed that all the phytoplasmas detected showed high 16S rDNA gene sequence identity

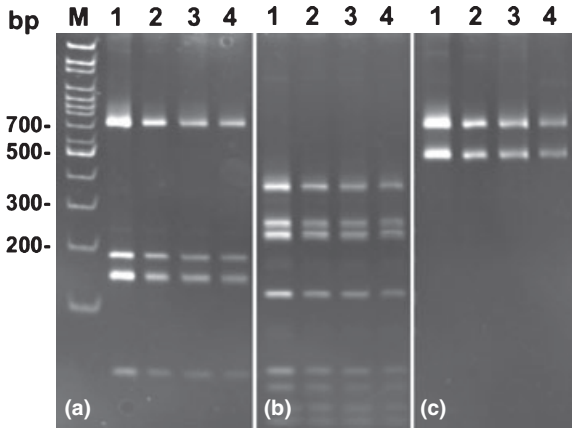

Figure 2 Restriction profiles of the nested PCR products from '*Candidatus* Phytoplasma pini' obtained after digestion with (a) *Alu*I, (b) *Mse*I and (c) *Rsa*I. Lane M: Gene Ruler 100 bp DNA Ladder Plus (Fermentas). Lanes 1–4: isolates from *Pinus sylvestris* ssp. *lapponica* (from Powsin), *Abies procera*, *Pinus banksiana* and *Tsuga canadensis* var. Jervis, respectively.

Table 2 GenBank accession numbers of the nucleotide sequences of '*Candidatus* Phytoplasma pini' nested PCR-amplified 16S rDNA fragments detected in *Abies procera*, *Pinus* spp. and *Tsuga canadensis* plants in Poland and the Czech Republic

Plant species and variety	GenBank Acc. No.
Abies procera var. Procumbens Formanek	FJ409228
Pinus banksiana var. Turtle Creek	FJ409230
Pinus mugo var. Pudełek	FJ409231
P. mugo ssp. *uncinata*	FJ409234
Pinus sylvestris ssp. *lapponica* (from Průhonice)	FJ409232
P. sylvestris ssp. *lapponica* (from Powsin)	GQ290113
P. sylvestris 157	FJ409233
P. sylvestris YIN	HM190300
P. sylvestris 1319	HM190301
P. sylvestris 1519	HM190302
Pinus tabuliformis	GQ290115
Tsuga canadensis var. Jervis	FJ409235

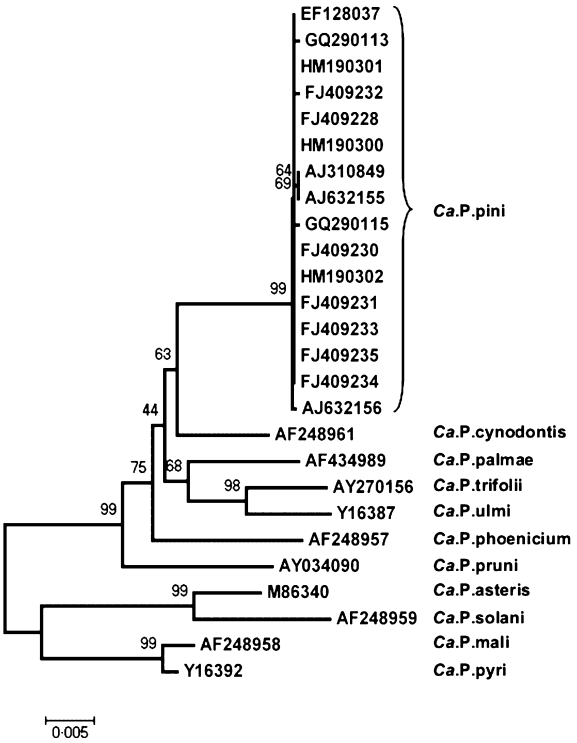

Figure 3 Phylogenetic analysis of the 16S rDNA gene sequences of the phytoplasma isolates in this study (GenBank accession numbers: FJ409228, FJ409230, FJ409235, GQ290113, GQ290115, HM190300, HM190301, HM190302). Sequences were compared with '*Candidatus* Phytoplasma pini' (AJ310849, AJ632155, AJ632156, EF128037), '*Ca.* Phytoplasma cynodontis' (AF248961), '*Ca.* Phytoplasma palmae' (AF434961), '*Ca.* Phytoplasma trifolii' (AY270156), '*Ca.* Phytoplasma ulmi' (Y16387), '*Ca.* Phytoplasma phoenicium' (AF248957), '*Ca.* Phytoplasma pruni' (AY034090), '*Ca.* Phytoplasma asteris' (M86340), '*Ca.* Phytoplasma solani' (AF248959), '*Ca.* Phytoplasma mali' (AF248958) and '*Ca.* Phytoplasma pyri' (Y16392). Evolutionary history was inferred using the neighbour-joining method in MEGA software v4·0·2. The bar represents 0·005 nucleotide substitutions per position.

with previously reported sequences of '*Ca.* Phytoplasma pini' (Schneider *et al.*, 2005; Śliwa *et al.*, 2008).

The phytoplasmas apparently occurred in low titres in the tested coniferous plants and their detection in direct PCR was possible only in the case of seven pine trees, mainly in the first year of testing. However, in the following year, the concentration of phytoplasmas in those trees decreased and it was only possible to detect '*Ca.* Phytoplasma pini' by nested PCR. Phytoplasma infection was detected in about 15% of the tested trees by direct PCR and in 30% by nested PCR. These results contrast with those of Schneider *et al.* (2005) and Śliwa *et al.* (2008), who were not able to detect '*Ca.* Phytoplasma pini' by direct PCR. Similar difficulties in phytoplasma detection in woody plant species were experienced by Berges *et al.* (2000).

This work confirms the results of Schneider *et al.* (2005) who found that '*Ca.* Phytoplasma pini' was present in naturally infected *Pinus sylvestris* and *P. halepensis* trees. Additionally, this study provides evidence for the first time of phytoplasma infection in *P. banksiana*, *P. mugo*, *P. nigra*, *P. tabuliformis*, as well as *A. procera* and *T. canadensis*, which appeared to be new natural hosts of '*Ca.* Phytoplasma pini'. The results support the data of Śliwa *et al.* (2008) who found phytoplasma in *P. sylvestris* trees with witches' broom symptoms, as well as in young WB grafts.

However, the results of this study are also inconclusive. The presence of phytoplasma was found in only about 42% of the tested plants which originated from the witches' brooms and showed pronounced symptoms, as well as in *P. tabuliformis* of unknown origin without any disease symptoms. It was not possible to detect the presence of phytoplasma within witches' brooms of two

forest-grown Scots pines, despite the pathogen being detected in the symptomless surrounding part of these trees. It was also not possible to detect phytoplasma in plants of the traditional pine varieties *P. nigra* var. Hornibrookiana and *P. sylvestris* var. Beauvronensis, which originated from witches' brooms, suggesting that phytoplasmas were absent or present at very low concentrations in these plants. Another possible reason for the PCR-negative results is that the symptoms of witches' brooms in coniferous plants may not be diagnostic of this phytoplasma and there may be other causes of those growth abnormalities in pines, fir and hemlock.

Because of the relatively short period of observation reported here, it is not possible to determine if failure to detect phytoplasma in samples of pine WBs and some WB-derived and symptomatic trees and shrubs was associated with recovery from infection by these plants. It is likely that low titres and seasonal fluctuations of phytoplasma quantities within a host plant may have influenced the negative results obtained from some symptomatic as well as WB-derived symptomless trees. The results of this study indicate differences in the detectability of the pathogen depending on the year and season: in the first year '*Ca*. Phytoplasma pini' was detected in six pine trees by direct PCR; in the second year the pathogen was detected in those trees only by nested PCR. Some plants that were PCR-positive in one season (spring or summer) were PCR-negative at the end of the same year.

The phytoplasmas detected in coniferous trees have probably been established in the forest ecosystems for a very long time, with the woody plants providing a long-lived phytoplasma reservoir. Little is known about the incidence and potential significance of '*Ca*. Phytoplasma pini' and other phytoplasmas in conifer plants. Research to date has demonstrated that phytoplasmas may contribute to a complex of disease symptoms but their potential impact on the growth and yield of conifer trees is unknown.

'*Candidatus* Phytoplasma pini' infection in coniferous plants is a newly described disease (Schneider *et al.*, 2005), so preliminary measures should be taken to prevent possible spread of this pathogen. Occurrence of this phytoplasma in trees or shrubs of eight plant species in Europe suggests that '*Ca*. Phytoplasma pini' may be more widespread than was previously envisioned. The detection of this phytoplasma in *Pinus* spp. trees grown in different natural forest ecosytems in Europe, as well as in *A. procera* and *T. canadensis* plants, suggests the existence of natural vectors. The identification of the vectors and the improvement of phytoplasma diagnostics would be very desirable, especially for producing healthy plants.

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