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Research Article

'Candidatus Phytoplasma pini' affecting *Taxodium distichum* var. *imbricarium* in China

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Abstract

Suspected phytoplasma symptoms were observed in *Taxodium distichum* var. *imbricarium* at the Nanyang Normal University campus, Nanyang City, Henan, China. The major symptoms consisted of little necrotic leaves, abnormal proliferation of twigs and overall necrotic appearance of the whole tree. Nested PCR was performed to confirm the association of phytoplasmas with symptomatic *T. distichum* var. *imbricarium*. PCR amplification using universal phytoplasma primers P1/P7 followed by R16F2n/R2, amplifying 16S ribosomal gene, yielded positive results. Amplified products were cloned and sequenced. Blast analysis showed 99% sequence identity with several '*Candidatus* phytoplasma pini'. Phylogenetic analysis confirmed the close relationship of the phytoplasma from *T. distichum* var. *imbricarium* with '*Ca.* P. pini' belonging to 16SrXXI group. Hence this is the first report of association of '*Ca.* P. pini' with a disease of *T. distichum* var. *imbricarium* tree in China.

Keywords : '*Ca.* P. pini', *Taxodium distichum* var. *imbricarium*, 16SrXXI group, phytoplasma, PCR assay, China

Introduction

Taxodium distichum var. *imbricarium* (Nutt.) Sarg. (Synonym: *Taxodium ascendens* Brongn.), also known as pond cypress, is a conifer ornamental species of *Taxodium* native to the southeastern United States, from coastal North Carolina to southeastern Louisiana. The species was introduced to Shanghai, China in the early 20th century, subsequently to the other cities or areas of the country such as Nanjing, Nantong, Wuhan, Jigong Mountain of Henan province (Hangzhou City Arboretum Adventitious Plant Group, 1977), and have become widespread throughout the Yangtze River valleys at present (Li *et al.*, 2010). *T. distichum* var. *imbricarium* is also a water-tolerant bottomland species and frequently used for reforestation at many wetlands and riparian zones in China.

Phytoplasmas are gaining international importance because of their association with serious economic losses and severe epidemic outbreaks throughout the world. Epidemics of these diseases have compelled withdrawal of many economical important plant species from cultivation. They are transmitted by phloem-sucking leafhoppers and are introduced directly inside the sieve tubes of plants via homopterous insect vectors, primarily belonging to the family Cicadellidea (Lee *et al.*, 2000).

So far, four different groups of phytoplasmas are reported from conifers all over the world. These are '*Candidatus* Phytoplasma phoenicum', 16SrIX group (infecting *Juniperus occidentalis*), '*Ca.* P. pini', 16SrXXI group (infecting *Abeis procera, Pinus banksiana, P. halepensis, P. nigra, P. sylvestris, P. tabuliformis, Tsuga canadensis*), '*Ca.* P. trifolii', 16SrVI group (affecting

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Araucaria heterophylla), and '*Ca.* P. asteris', 16SrI group (infecting *Thuja* species) that have been reported from different parts of the world (Schneider *et al.*, 2005; Gupta *et al.*, 2009; Zhao *et al.*, 2009; Davis *et al.*, 2010; Kamińska *et al.*, 2011). Among identified tree phytoplasmas, only '*Ca.* P. tamaricis', 16Sr XXX group was recently reported to infect *Tamarix chinensis* (salt cedar tree, family Tamaricaeae) in China (Zhao *et al.*, 2009). No report is available on phytoplasma affecting conifers in China. Hence, in this study, detection, characterization and classification of an unknown phytoplasma causing little necrotic leaves followed by stunting and necrotic appearance of *T. distichum* var. *imbricarium* tree in China is described.

Materials and Methods

During a survey in November 2010 at Nanyang Normal University Campus, China a phytoplasma associated symptom (described above) on leaves and twigs of *T. distichum* var. *imbricarium* was observed and samples were collected for verification of phytoplasma presence.

Procedure of Ahrens and Seemuller (1992) was followed for isolation of DNA from samples from symptomatic branches and leaves. Samples consisted of 500 mg young symptomatic twigs and were grinded in pre-cool motor and pestle in 6 ml of grinding buffer. The homogenate was centrifuged at 4°C for 4 min at 5,000 rpm. The pellet was discarded. The supernatant was again centrifuged at 4°C for 25 min at 10,000 rpm. The pellet was resuspended in 1.5 ml of warm (60°C) extraction buffer and incubated at 60°C for 30 min in water bath followed by RNase (50 µg/ml) treatment at 37°C for 30 min. It was again extracted with equal volume of phenol: chloroform (1:1) followed by equal volume of chloroform/ isoamyl alcohal (24:1). The supernatant was precipitated with 2/3 volume of isopropanol (-20°C) and centrifuged at 10,000 rpm for 15 min. The pellet was washed with 70% alcohol and centrifuged for 10,000 rpm for 3 min. The DNA pellet was dissolved in 100 µl of double distilled water and used for molecular identification of phytoplasmas through nested PCR assays.

The initial PCR was performed with P1/P7 universal primers amplifying the 16S rRNA gene, the spacer region and the beginning of 23S gene (Deng and Hiruki, 1991; Schneider *et al.*, 1995). The reactions were carried out in a thermal cycler with an initial denaturation at 94°C for 5 min, followed by 35 cycles (94°C for 1 min, 55°C for 60 s, and 72°C for 90 s) and a final extension at 72°C for 5 min. Further, nested PCR assay was carried out with primers R16F2n/R16R2 (Gundersen and Lee, 1996) employing the initial PCR product (1:10 dilution) as template. The cycling protocol used for the nested PCR with primer pair (R16F2n/R2) was (34 cycles): 94°C: 5min (1 cycle), 94°C: 1 min, 55°C: 1 min, 72°C: 1.5 min (33 cycles), 72°C:5 min (final extension). The presence of phytoplasmas was demonstrated by electrophoresing the PCR products on 1% agarose gels at 70 volts using 0.5 × TAE running buffer. The gels were stained with ethidium bromide (10 mg/ml) and observed on UV transilluminator.

For sequencing, second round PCR products were separated by electrophoresis in 1.5% agarose gel. Fragments with sizes corresponding to the expected amplified sequences were excised from the gel and eluted using the QIAquick gel extraction kit (Qiagen). Nested PCR products (1.2 kb amplicon) amplified from *T. distichum* var. *imbricarium* phytoplasma was cloned in *Eco*RI restriction sites of pGEM-T easy vector (Promega, USA). The ligation mixture was used to transform competent cells of *E. coli* strain DH5α. Recombinants were screened by blue and white screening method (Sambrook *et al.*, 1989). Selected recombinant clones were screened for phytoplasma rRNA inserts by PCR using the primer pair R16F2n/ R16R2. Plasmid DNA was purified using the NucleoSpin system kit (Macherey-Nagel). Sequencing of both strands was performed by ABI's AmpliTaq FS dye terminator cycle sequencing chemistry in an automated ABI 3100 Genetic Analyzer. Primers for sequencing PCR products were the same as for PCR amplification, whereas the standard primers SP6 and T7 were used for sequencing the cloned fragments. The 16S rRNA gene sequences of phytoplasma from *T. distichum* var. *imbricarium* were aligned using CLUSTAL Method of DNASTAR software (DNASTAR, Madison, WI, USA).

Aligned sequences were used as query sequences in a BLAST 2.0 search (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) and were trimmed to include the 16S sequences only. The sequence generated from the present study and reference phytoplasma strains sequences retrieved from GenBank were used to construct phylogeny through MEGA version 4.0 software (Tamura *et al.*, 2007).

Results and Discussion

The major symptoms associated with *T. distichum* var.

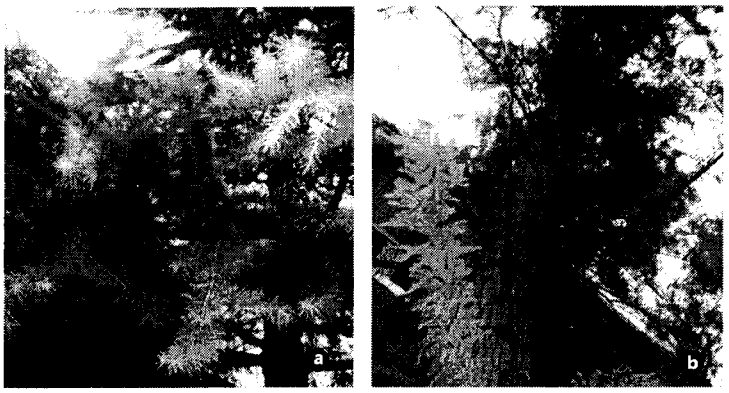

Figure 1. (a) Closer view of healthy twigs of *Taxodium distichum* var. *imbricarium* showing green healthy leaves; **(b)** closer view of symptomatic tree with browning and necrosis of leaves.

imbricarium trees were little necrotic leaves and twig necrosis (Figure 1b). The entire tree showed necrosis with stunting symptoms. To confirm the association of phytoplasma with the symptomatic plants, universal primers pair (P1/P7) was used which amplified the 1.8 kb DNA fragment of phytoplasma 16SrRNA from nucleic acid extracted from symptomatic twigs of *T. distichum* var. *imbricarium*. In the nested PCR analysis ~1.2 kp fragment was amplified with R16F2n/R2 (Figure 2). No amplification was observed in any of the healthy looking plant samples neither in direct nor in nested PCR. This confirmed the association of a phytoplasma with symptomatic *T. distichum* var. *imbricarium* trees in China. DNA fragment of 1.2 kbp amplified from the nested PCR analysis was sequenced. Sequence of 1,246 bp was submitted to GenBank (Accession No. JQ419756). Consensus sequence was used for the BLASTn query and phylogenetic analysis.

During the BLAST analysis, the *T. distichum* var. *imbricarium* phytoplasma detected in the present study showed 97% to 99% identity with '*Ca.* P. pini' from Lithuania (GU289676), Poland (HM190302, FJ409231), Czech Republic (FJ409228, FJ409232, FJ409230), Germany (AJ310849), Spain (AJ632156) and detected in different species of *Pinus*, *Abies* and *Tsuga* species (Table 1). However the *T. distichum* var. *imbricarium* phytoplasma showed only 72% to 92% identity with other reported phytoplasma from conifers on *Thuja* species, *Araucaria heterophylla* and *Juniperus occidentalis* and 93% to 94% identity with '*Ca.* P. cynodontis', golden beard grass and sunshine tree witches' broom phytoplasmas (Table 1).

In phylogenetic analysis, the *T. distichum* var. *imbricarium* phytoplasma clustered with '*Ca.* P. pini' from different species of *Pinus, Abeies, Tsuga* belonging to 16SrXXI group (Figure 3). These results confirms that *T. distichum* var. *imbricarium* phytoplasma is as

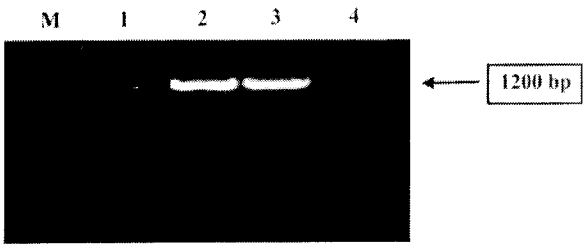

Figure 2. Amplified fragments corresponding to 16SrDNA of phytoplasma detected in *Taxodium distichum* var. *imbricarium* with nested PCR with primer pair R16F2n/R2. Lanes 1 and 4: healthy samples and lanes 2 and 3: infected samples showing amplification of 1,200 bp.

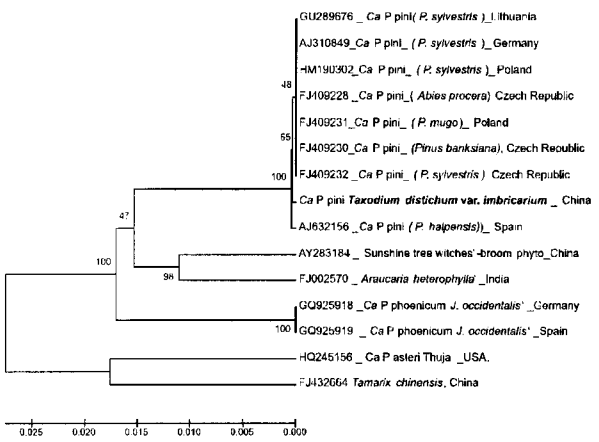

Figure 3. Phylogenetic relationship of *Taxodium distichum* var. *imbricarium* phytoplasma with other selected conifer phytoplasmas from conifer trees and from various ribosomal groups using MEGA 4.0 program. The tree was generated using 1000 bootstrap replicates.

Table 1. Comparison of 16Sr DNA of '*Ca.* P. pini' from *T. distichum* var. *imbricarium* with one of selected phytoplasmas from GenBank.

Accession No.	Phytoplasma	Host	Country	% identity
GU289676	Pine bunchy top	*Pinus sylvestris*	Lithuania	99
FJ409230	'*Ca.* P. pini'	*Pinus banksiana*	Czech Republic	99
AJ310849	'*Ca.* P. pini'	*Pinus sylvestris*	Germany	99
HM190302	'*Ca.* P. pini'	*Pinus sylvestris*	Poland	99
FJ409231	'*Ca.* P. pini'	*Pinus mugo*	Poland	99
FJ409228	'*Ca.* P. pini'	*Abies procera*	Czech Republic	99
FJ409232	'*Ca.* P. pini'	*Pinus sylvestris*	Czech Republic	97
AJ632156	'*Ca.* P. pini'	*Pinus halepensis*	Spain	97
AB052871	'*Ca.* P. cynodontis'	Bermuda grass	Thailand	94
AB642601	Golden beard grass white leaf	Golden beard grass	Myanmar	94
AF248961	'*Ca.* P. cynodontis'	Bermuda grass	Thailand	94
AY283184	Sunshine tree witches' broom	*Erythhrina variegata*	China	93
FJ002570	*Araucaria heterophylla* little leaf	*Araucaria heterophylla*	India	89
EU498727	Malaysian coconut yellow dwarf	Coconut palm	Myanmar	93
GQ925918	*Juniperus occidentalis* witches' broom	*Juniperus occidentalis*	Germany	92
GQ925919	*Juniperus occidentalis* witches' broom	*Juniperus occidentalis*	Spain	92
AY081817	Chinaberry yellows	China tree decline	China	92
HQ245156	*Thuja* aster yellows	Thuja	USA	72
FJ432664	Salt cedar witches' broom	*Tamarix chinensis*	China	88

member of '*Ca.* P. pini' and belongs to 16SrXXI group. This is the first report of phytoplasma association with conifer tree species *T. distichum* var. *imbricarium* in the world.

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