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FULL RESEARCH PAPER

Detection and identification of the phytoplasma associated with pear decline in Taiwan

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Abstract Pear decline (PD) is an important phytoplasmal disease that occurs mainly in Europe and North America. In 1994, pear trees exhibiting symptoms typical of PD disease were observed in orchards of central Taiwan. The sequence of 16S rDNA and 16S–23S rDNA intergenic spacer region (ISR) of the causative agent of pear decline in Taiwan (PDTW) were amplified with polymerase chain reaction (PCR) using a DNA template prepared from the diseased leaves. Sequence analysis of 16S rDNA revealed that the PDTW agent was closely related to the phytoplasmas of the apple proliferation group that cause diseases in stone fruits, pear and apple. Consistent with the result of 16S rDNA sequence analysis, sequence analysis of the 16S–23S rDNA ISR and putative restriction site analyses of 16S rDNA and 16S–23S rDNA ISR sequences provided further support for the view that the PDTW phytoplasma causing pear decline in Taiwan may represent a new subgroup of the apple proliferation group. According to the rDNA sequence of PDTW phytoplasma, two specific PCR primer pairs, APf2/L1n and fPD1/rPDS1, were designed in this study for the detection of the etiological agent in pear trees and insect vectors. Based on the sequence analyses of the PCR-amplified fragments, two species of pear psyllas, *Cacopsylla qianli* and *Cacopsylla chinensis*, were found to carry PDTW phytoplasma.

Keywords Phytoplasma vectors · rRNA

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Introduction

The Asian pear (*Pyrus pyrifolia*) is an important economical fruit crop in Taiwan. In 1994, pears with decline symptoms (pear decline-Taiwan, PDTW) were observed in Dungshr and Heping, two nearby areas in central Taiwan. In fall, the initial symptom of a premature red colour followed by early leaf fall developed in the leaves of affected trees. The leaves of diseased trees remained small and pale in the following spring, and little or no shoot was developed (Chen, Liu, Lin, & Kuo, 2001). When the affected trees encountered hot and dry weather conditions, quick decline which is the sudden wilt and death of the trees within a few week, occurred. The symptoms of diseased pear trees in Taiwan were similar to those of pear decline (PD), a disease

caused by a pear psylla-transmitted phytoplasma. Differences in symptom expression and the severity of pear decline disease have been described as being of three types, including quick decline, slow decline and leaf curl with foliar reddening (Agrios, 2005; Seemüller, 1990, 1992). Slow decline is characterized by a progressive weakening of the trees which may sometimes be associated with leaf curl symptoms. Notably, the symptom of leaf curl with thickened and crinkled veins in pear decline in Taiwan is quite different from the typical symptom of PD disease reported elsewhere. Most importantly, though the margins of the leaves rolled upward along the longitudinal axis up to a 70 degree angle, the reddish leaves of PDTW did not exhibit the characteristic downward curling symptom of pear decline (Chen et al., 2001; Seemüller, 1990, 1992).

Recently, phytoplasma detection and characterization are based predominantly on PCR (polymerase chain reaction) amplification of the ribosomal RNA gene (rDNA) (Avinent, Llácer, Almacellas, & Torá, 1997; Davies Barbara, & Clark, 1995; Garcia-Chapa, Laviña, Sanchez, Medina, & Batlle, 2003; Lorenz, Schneider, Ahrens, & Seemüller, 1995). The 16S rDNA and 16S–23S rDNA intergenic spacer region (ISR) have been widely used as targets to detect and identify many different types of phytoplasma (Lorenz et al., 1995; Seemüller, 1992; Seemüller, Marcone, Lauer, Ragozzino, & Göschl, 1998). According to rDNA molecular evidence, phytoplasmas are currently divided into 20 major phylogenetic groups (Lee, Davis, & Gundersen-Rindal, 2000; Seemüller et al., 1998). Among these 20 groups, pear decline (PD) phytoplasma and peach yellow leaf roll (PYLR) phytoplasma of 16SrX-C subgroup, together with those that infect temperate fruit trees, such as apple proliferation (AP) phytoplasma of 16SrX-A subgroup and European stone fruit yellows (ESFY) phytoplasma of 16SrX-B subgroup, all belong to the apple proliferation group (AP group, i.e. the 16SrX group) (Blomquist & Kirkpatrick, 2002; Lee et al., 2000). Currently, more and more evidence supports the view that AP, PD/PYLR and ESFY phytoplasmas are discrete taxa that can be distinguished at the putative species level, for which the names '*Candidatus* Phytoplasma mali', '*Ca*. Phytoplasma pyri' and '*Ca*. Phytoplasma prunorum' have been proposed, respectively (Blomquist & Kirkpatrick, 2002; Seemüller & Schneider, 2004; The IRPCM Phytoplasma/Spiroplasma Working Team - Phytoplasma taxonomy group, 2004). In this study, the causative agent of the pear disease with decline symptoms found in central Taiwan was determined as being a new phytoplasma, PDTW phytoplasma, and the results also implied that *Cacopsylla qianli* and *C. chinensis* may be the vectors of PDTW in Taiwan.

Materials and methods

Plant and insect materials

Shoot samples from 20 diseased Asian pear trees of about 20–30 years old (*Pyrus pyrifolia*) were collected from four different orchards in Dungshr and Heping, from 2001 to 2004. In addition, two species of psylla *C. qianli* and *C. chinensis* were sweep-collected in pear orchards harbouring PDTW-infected trees. The periwinkle plant (*Catharanthus rosecus*) affected with peanut witches' broom (PnWB) was used as a control.

DNA isolation and PCR amplification

Total DNA was isolated from fresh plant material using the Plant Genomic DNA Extraction Maxiprep System (Viogene-Biotek Corporation, Taipei, Taiwan) according to the manufacturer's instructions. Insect DNA was extracted from a single psyllid using a DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The universal PCR primers f1/r1 (Lin & Lin, 1998) devised from 16S rDNA sequences of phytoplasmas were applied to amplify the phytoplasma-specific DNA fragment of about 650 bp in length. PCR was performed in a thermal cycler (Perkin-Elmer Cetus model 2700, Melbourne, Victoria, Australia) in 25 µl reaction solution containing 20 ng of DNA template, 0.4 µM of each primer, 200 µM of dNTPs, 2 U FastStart Taq DNA polymerase (Roche Molecular Biochemical, Mannheim, Germany) and 1× polymerase buffer (Roche), following the programme described previously

(Lin & Lin, 1998). At the same time, the PD phytoplasma-specific PCR primers fPD/rPDS (Lorenz et al., 1995) were used to detect the existence of PD phytoplasma in the diseased samples. In order to amplify a region consisting of 16S rDNA, the 16S–23S rDNA ISR and approximately 50 bp of the 5′end of 23S rDNA for phylogenetic analysis, the universal phytoplasma primers P1/P7 were used (Deng & Hiruki, 1991; Schneider, Seemüller, Smart, & Kirkpatrick, 1995). The modified PCR programme was as follows: 95°C for 30 s, 60°C for 60 s and 72°C for 90 s for 35 cycles, followed by an additional extension at 72°C for 10 min. Based on the sequence of PDTW phytoplasma which was obtained later in this study, two pairs of PDTW phytoplasma-specific PCR primers were designed to amplify PDTW phytoplasma rDNA specifically from DNA templates prepared from pear trees and insects. The first primer pair is APf2/L1n (APf2: GAT GAG TAC TAA GTG TTG GG; L1n: CAA GGC ATC CAC TGT). The second primer pair, which is a modification of the PD-specific primer pair fPD/rPDS, is fPD1/rPDS1 (fPD1: GAC CCG CAA GGG TAT GCT GA; rPDS1: CCA AGC CAT TAT TAA TTT TTA). The PCR programme used for the primer pair APf2/L1n was 30 s at 95°C, 30 s at 62°C and 45 s at 72°C for 35 cycles. The PCR programme for the primer pair fPD1/rPDS1 was the same as fPD/rPDS (Lorenz et al., 1995).

Cloning and sequencing of PCR products

The PCR-amplified products were purified using a QIAquick PCR Purification Kit (Qiagen) and cloned in *Escherichia coli* (TOP10F') using a TOPO TA cloning kit (Invitrogen Co., San Diego, CA) according to the manufacturer's instructions. The cloned rDNA fragments were sequenced by an automated DNA sequencer (Mission Biotech, Taipei, Taiwan).

Nucleotide sequence blasted in NCBI

Sequences obtained from the PCR products amplified from both insects and diseased plants by using primer pairs f1/r1 and P1/P7 were compared with the nucleotide-nucleotide BLAST programme in the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). The sequence of PDTW phytoplasma amplified by primer pair P1/P7 was assembled at a minimum of 2× sequencing coverage for each base position and submitted to GenBank. The GenBank accession numbers of the rDNA sequence of 39 phytoplasma strains belonging to 12 different 16S rDNA groups, and that of *Acholeplasma laidlawii* used in this study, are listed in Table 1.

Phylogenetic and putative restriction site analyses

Sequences of both 16S rDNA and 16S–23S rDNA ISR obtained from the PCR products of PDTW phytoplasma were aligned with sequences of other phytoplasmas as shown in Table 1 by using the CLUSTAL X programme (Thompson, Plewniak, & Poch, 1999), and were further analyzed for the existence of the signature sequences shared among these phytoplasmas by using the GeneDoc programme (Nicholas & Nicholas, 1997). The similarities of rDNA sequence among phytoplasmas were evaluated by using the Meg-Align option of the DNASTAR programme (DNASTAR Inc., Madison, WI). Phylogenetic trees were constructed according to the original data set by the neighbour-joining method, as well as 1,000 boostrap data sets generated by CLUSTAL X. The tree for 16S rDNA sequence was generated using *A. laidlawii* as the outgroup sequence to allow the tree to be rooted. The tree of 16S–23S rDNA ISR sequence for the apple proliferation group was also generated using aster yellows phytoplasma (AY1) as the outgroup. The putative restriction site maps of 16S rDNA sequence and 16S–23S ISR sequence of all strains in AP group (Table 1) and PDTW phytoplasma were evaluated by using MapDraw option of the DNASTAR programme (DNASTAR Inc.), and then manually aligned to compare recognition sites for restriction endonucleases.

Grafting plant materials

Thirty 2-year-old healthy Asian pear trees grown at the Taichung District Agricultural Improvement

Table 1 List of phytoplasma strains and GenBank accession numbers of their respective 16S rDNA and 16S–23S rDNA intergenic spacer region sequences used to construct the phylogenetic tree in this study

Acronyms	Strain	16S rDNA group affiliation	GenBank accession number	
			16S rDNA	16S–23S
AsWB	Ash witches'-broom	16SrI (Aster yellows group)	AY566302	
AY1	Aster yellows	16SrI		AY557614
MuD	Mulberry dwarf	16SrI	AY685056	
CPAu	*Ca.* Phytoplasma aurantifolia	16SrII (Peanut WB group)	U15442	
SwPLLV4	Sweet potato little leaf	16SrII	AJ289193	
MiY	Milkweed yellows	16SrIII (X-disease group)	AF510724	
WXP	Western X	16SrIII	AF533231	
CoLYC2	Coconut lethal yellowing	16SrIV (Coconut lethal yellows group)	AF498309	
LDN	Nigerian Awka disease	16SrIV	Y14175	
AlmWB2	Almond witches'- broom	16SrIX (Pigeon pea witches'-broom group)	AF390137	
PiPWB	Pigeon pea witches'-broom	16SrIX	AF248957	
EY	Elm yellows	16SrV (Elm yellows group)	AF189214	
FDC	Flavescence doree	16SrV	AF176319	
CPTr	*Ca.* Phytoplasma trifolii	16SrVI (Clover proliferation group)	AY390261	
PoWB	Potato witches'-broom	16SrVI	AY500818	
ArAWB	Argentinian alfalfa witches'-broom	16SrVII (Ash yellows group)	AY147038	
ErWB	Erigeron witches'-broom	16SrVII	AY034608	
LWB	Loofah witches'-broom	16SrVIII(Loofah witches'-broom group)	AF086621	
AP	Apple proliferation	16SrX (Apple proliferation group)		U54985
AP15	*Ca.* Phytoplasma mali	16SrX	AJ542541	
ApP	Apple proliferation	16SrX	AF248958	AF248958
APS	*Ca.* Phytoplasma mali	16SrX	X76426	
AT	*Ca.* Phytoplasma mali	16SrX	X68375	X68375
AT193	*Ca.* Phytoplasma mali	16SrX	AJ542542	AJ542542
ESFY173	*Ca.* Phytoplasma prunorum	16SrX		AJ575106
ESFY215	*Ca.* Phytoplasma prunorum	16SrX		AJ575105
ESFY4	European stone fruit yellows	16SrX	Y11933	Y11933
ESFY5	European stone fruit yellows	16SrX	AY029540	AY029540
ESFY63	*Ca.* Phytoplasma prunorum	16SrX		AJ575107
ESFYG1	*Ca.* Phytoplasma prunorum	16SrX	AJ542544	AJ542544
ESFYG2	*Ca.* Phytoplasma prunorum	16SrX	AJ542545	AJ542545
ESFYs	European stone fruit yellows	16SrX		U54988
PD	Pear decline	16SrX	Y16392	
PDs	Pear decline	16SrX		U54989
PD1	*Ca.* Phytoplasma pyri	16SrX	AJ542543	AJ542543
PYLR	Peach yellow leaf roll	16SrX	Y16394	U54990
SpaWB	Spartium witches broom	16SrX	X92869	X92869
RiYD	Rice yellow dwarf	16SrXI (Rice yellow dwarf group)	D12581	
BGWL	Bermuda grass white leaf	16SrXIV (Bermuda white leaf group)	AF248961	
A.laidlawii	*Acholeplasma laidlawii*		M23932	

Station were used for grafting experiments. To transmit phytoplasma into these healthy Asian pear plants, grafting experiments had been performed by means of the whip-and-tongue method in March 2001 and March 2002. The first scions infected with PDTW phytoplasma were grafted onto the healthy rootstock in March 2001. After incubation until the following March, the second healthy scions (phytoplasma-free) were grafted onto those first scions which were still alive. In July and August 2002, the leaves grown on the second scions were collected for the detection of PDTW phytoplasma using PCR with primer pairs Apf2/L1n and fPD1/rPDS1.

Transmission electron microscopy

The samples including the leaf midrib and minor veinlets of the systemically infected pear trees were cut into smaller pieces and fixed in 2%

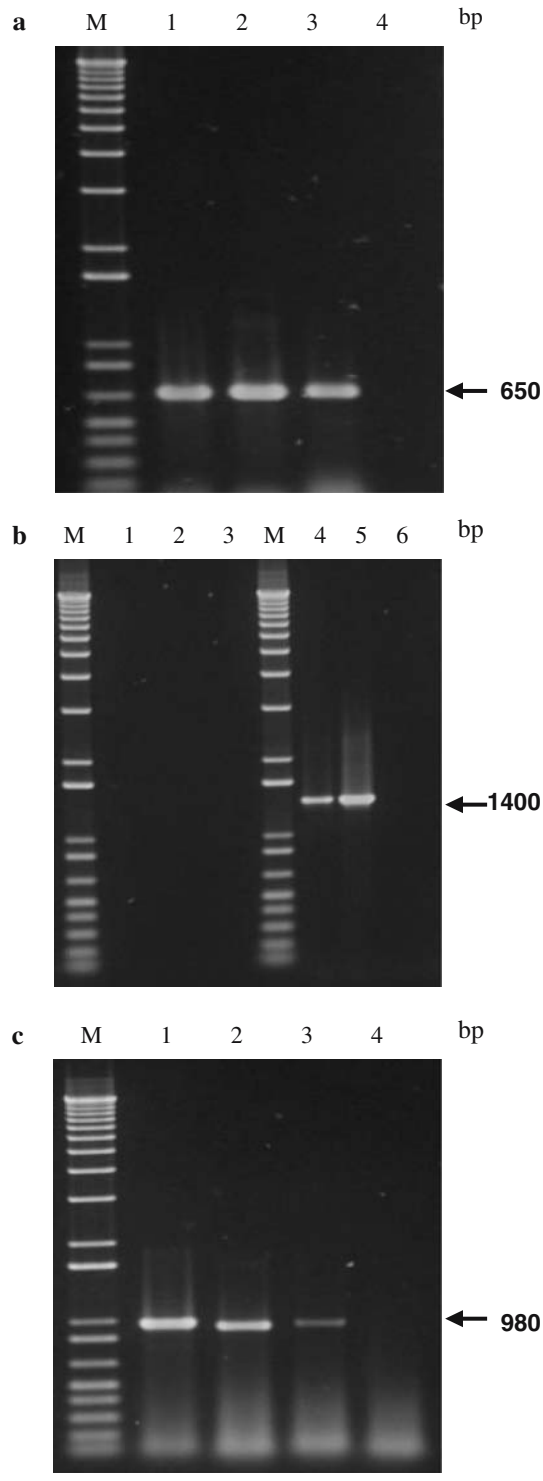

Fig. 1 Amplification of phytoplasma rDNA fragment with polymerase chain reaction (PCR) using DNA template prepared from pear trees, insect vector, and peanut witches' broom (PnWB) infected periwinkle. (**a**) PCR-products amplified with the universal primer pair f1/r1 using DNA templates prepared from diseased pear trees (lane 1), *Cacosylla qianli* (lane 2), PnWB infected periwinkle (lane 3) and healthy pear trees (lane 4). (**b**) PCR-products amplified with the PD-specific primer pair fPD/rPDS (lanes 1–3), and PDTW-specific primer pair fPD1/rPDS1 (lanes 4–6) using DNA templates prepared from diseased pear trees (lanes 1, 4), *C. qianli* (lanes 2, 5) and *C. chinensis* (lanes 3, 6). (**c**) PCR-products amplified with the PDTW-specific primer pair Apf2/L1n using DNA templates prepared from diseased pear tree (lane 1), *C. qianli* (lane 2), *C. chinensis* (lane 3) and PnWB infected periwinkle (lane 4). M, 1 kb DNA ladder as molecular weight standard (Invitrogen). Sizes of PCR products are shown on the right

glutaraldehyde prepared in 0.1 M phosphate solution (pH 7.0) overnight, then treated in 2.5% osmium tetroxide at room temperature for an additional 2 h before being dehydrated in a gradient series of ethanol. Samples were embedded in LR white resin (Agar Scientific Limited, Cambridge, UK). Ultrathin sections were stained with 2% uranyl acetate followed by 2% lead citrate and examined in the electron microscope (JEOL, JEM 1010, Philips Ltd., Eindhoven, The Netherlands).

Results

Detection of PDTW phytoplasmas in diseased pear trees and insect vector

When the phytoplasma-specific primer pair f1/r1 was applied, the expected 650 bp phytoplasma-specific PCR product was amplified using the DNA templates prepared from diseased pear plants, *C. qianli* and PnWB-phytoplasma infected periwinkle plants, which served as a positive control in the PCR reaction (Fig. 1a). The universal phytoplasma primer pair P1/P7 was further used to amplify the target rDNA region and the expected 1,800 bp PCR products were obtained when using DNA templates extracted

from the diseased pear samples and *C. qianli* (data not shown). In contrast, no PCR product was obtained with primer pair fPD/rPDS specific for pear decline phytoplasma (Lorenz et al., 1995) by using the DNA templates prepared from diseased pear trees, *C. qianli* and *C. chinensis* (lanes 1–3, Fig. 1b). On the other hand, the expected 1,400 bp PCR fragments were specifically amplified with PDTW phytoplasma-specific primer pair fPD1/rPDS1 by using the DNA templates prepared from PDTW phytoplasma infected pear plants and from *C. qianli* (lanes 4 and 5, Fig. 1b). Although no PCR product was amplified with primer pairs f1/r1, P1/P7 or fPD1/rPDS1 (lane 6, Fig. 1b) when using the DNA template prepared from *C. chinensis*, PDTW phytoplasma-specific products of about 980 bp in length were amplified with specific primer pair APf2/L1n by using the DNA templates prepared from diseased pear trees, *C. qianli* and *C. chinensis* (lanes 1–3, Fig. 1c).

Nucleotide sequence similarities blasted in NCBI

The sequences of the PCR products amplified from both insects and diseased plants are completely identical. The PCR-amplified 658 bp fragment of the PDTW-phytoplasma 16S rDNA sequence using primer pair f1/r1 were subjected to nucleotide-nucleotide BLAST analyses in the NCBI database and showed 98% identity with the sequences of *Ca*. P. mali, *Ca*. P. pyri and *Ca*. P. prunorum. Furthermore, the PCR-amplified 1784 bp fragment of the PDTW-phytoplasma sequence using primer pair P1/P7 also exhibited a great identity, ranging from 96% to 98%, to the sequences of phytoplasmas of the AP group, whereas there was less than a 90% identity to the sequences of phytoplasmas of other groups. The nucleotide sequence of this 1,784 bp fragment was submitted to GenBank and the accession number is DQ011588.

Phylogenetic analysis of 16S rDNA sequences

To reveal the relation of PDTW phytoplasma to other phytoplasmas, 16S rDNA sequences of PDTW and other 32 phytoplasma strains (Table 1) were used to conduct a phylogenetic analysis. The boostrap analysis revealed that the phylogenetic tree generated (Fig. 2) is reliable and consistent with the previous study (Seemüller et al., 1998). High boostrap values suggested that PDTW phytoplasma is most closely related to apple proliferation phytoplasmas and is a member of the AP group (group 16SrX). The phylogenetic tree also showed that all phytoplasmas of the apple proliferation group that infected the same host were grouping at one clade except for the PDTW. Furthermore, using the MegAlign of DNASTAR programme, sequence alignment also revealed that the homologies between PDTW phytoplasma and phytoplasmas of the apple proliferation group, such as AP, ESFY and PD/PYLR strains, were of 98.7–98.9%, 98.5–98.8% and 98.5–99.1%, respectively. On the other hand, sequence divergences greater than 2.5% were observed between PDTW phytoplasma and other phytoplasmas.

16S rDNA signature sequence

The 16S rDNA signature sequences unique to AP group phytoplasmas were described by Seemüller and Schneider (2004). One of the 16S rDNA signature sequence, 5′-GCG TAG GCG GTT AAA TAA GTC TAT GGT AT-3′, located at the position 560–588 of 16S rDNA sequence of PDTW phytoplasma was identical to those of the ESFY strains. Another 16S rDNA signature sequence, 5′-AAT ACC CGA AAC CAG TA-3′, located at positions 1,394 to 1,410 of the 16S rDNA sequence of PDTW phytoplasma was identical to those of PD strains. When compared with those of other AP group phytoplasmas, the 16S rDNA sequence of PDTW phytoplasma revealed six unique sequence sites at positions 77 (C to T), 180 (G to A), 396 (C to T), 399 (T to C), 791 (C to T) and 1224 (T to A).

Phylogenetic analysis of the 16S–23S rDNA intergenic spacer region sequence

To further reveal the relationship of PDTW phytoplasma with other members of the AP group (Table 1), a phylogenetic tree was constructed according to 16S–23S rDNA ISR

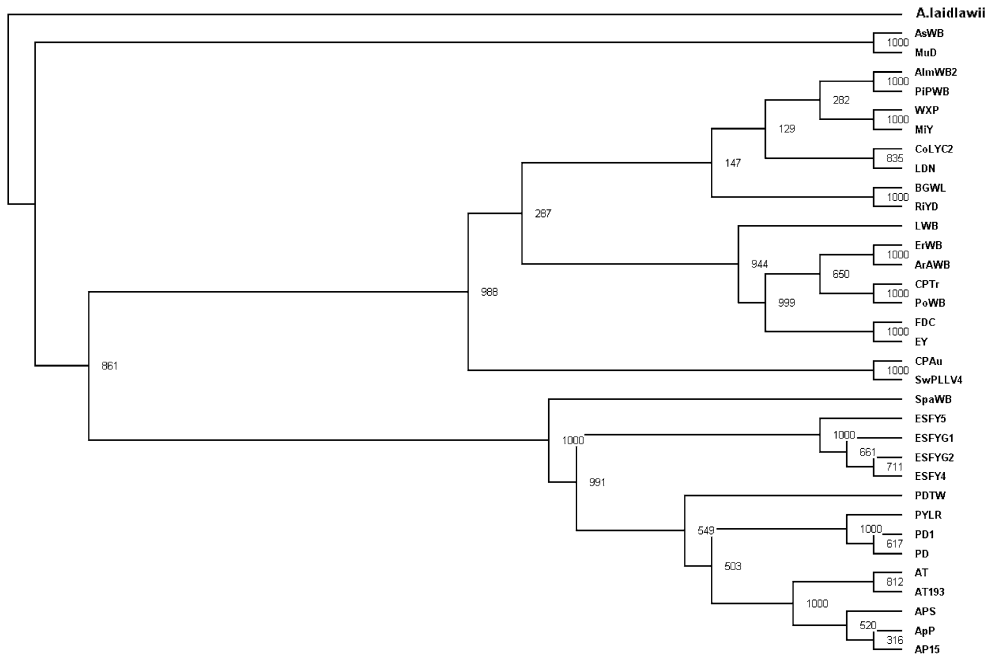

Fig. 2 Phylogenetic analysis of 16S rDNA sequences of the PDTW phytoplasma and 32 reference phytoplasmas (Table 1). The phylogenetic tree was constructed using the neighbour-joining method and using *Acholeplasma laidlawii* as the outgroup. The numbers on the branching points refer to the number of times (out of 1000) in which the given branch is supported

sequences, and a high bootstrap value supported the fact that the PDTW/ESFY clade was distinct from the PD/PYLR clade (Fig. 3). Moreover, direct pairwise sequence comparisons of the whole spacer region by MegAlign of the DNASTAR programme showed that the sequence of PDTW phytoplasma had the highest identity to those of the strains of ESFY subgroup (98.8%), 97.7–98.4% identity to those of the strains of PD/PYLR subgroup and only about 95.2–96% identity to those of the strains of the AP subgroup. On the other hand, sequence comparison also revealed that strains of each of AP, ESFY and PD/PYLR phytoplasmas were identical or nearly identical with identity values of 99.2–100%, 100%, and 99.2–99.6%, respectively.

Putative restriction site analysis

On the basis of putative restriction site analysis of 16S rDNA sequences, PDTW strain was quite different from PD/PYLR strains because of the presence of a *Dde*I, a *Bsp*MI and a *Bsp*MII site in positions near 116, 117 and 131 bp in PDTW, and the absence of PD/PYLR strains. On the other hand, an *Nla*III site and a *Dde*I site which were only present in PD/PYLR strains were absent in the PDTW strain in positions near 265 bp and 587 bp. PDTW strain was also distinguishable from other strains of the AP group based on the absence of two *Tsp*RI sites in positions near 82 bp and 84 bp and the presence of an *Mse*I site in the position near 180 bp in the PDTW strain. Besides, based on 16S–23S rDNA ISR sequence analysis with restriction enzymes (Fig. 4), PDTW strain was distinguishable from PD/PYLR and AP strains by the absence of a *Bss*KI, an *Msp*I, an *Nci*I, an *Eco*O1091 and an *Nla*IV site. PDTW strain was distinguishable from ESFY strains by the absence of a *Bsa*BI site and by the presence of a *Tsp*5091, and two additional *Mse*I sites.

Grafting experiment

After grafting, only 10 of the 30 Asian pear trees survived, and the PDTW phytoplasma-specific

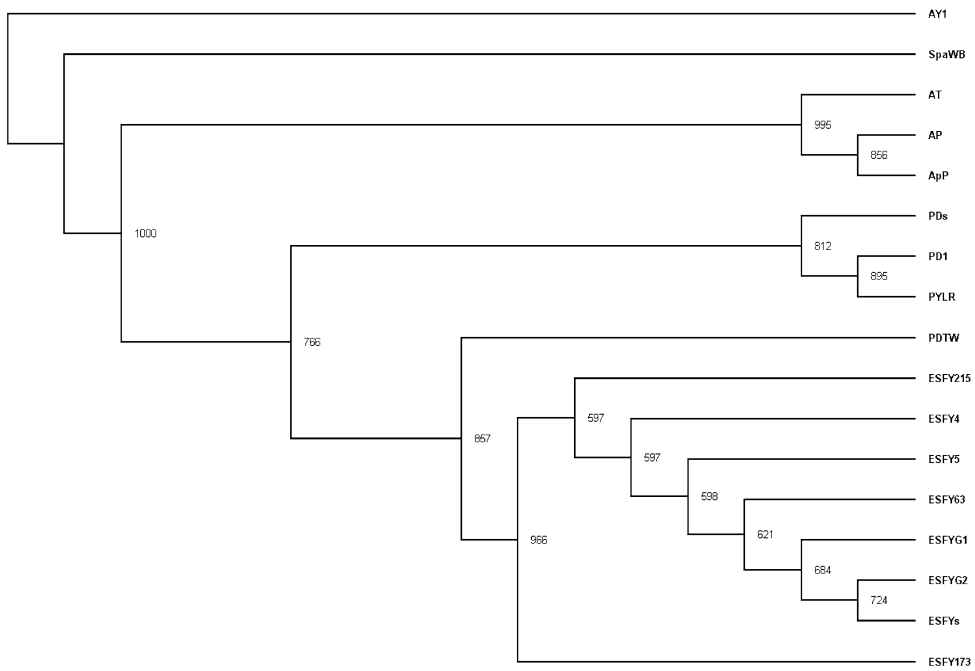

Fig. 3 Phylogram showing the relationship of the PDTW phytoplasma and the 15 phytoplasma strains (Table 1) of the apple proliferation group based on the sequences of the 16S–23S rDNA intergenic spacer region. Strain AY1 of aster yellows phytoplasma was used as the outgroup. The numbers on the branching points refer to the number of times (out of 1000) in which the given branch is supported

rDNA sequences could be amplified by PCR from five of those that survived. The results show that PDTW phytoplasma can be transmitted by grafting.

Transmission electron microscopy (TEM)

In the transmission electron microscopic examination, wall-less, single-unit membrane, irregular or round-shaped phytoplasma bodies were found in the sieve elements of phloem tissues of the diseased pears and grafted pear trees but not in the healthy control. The observation is consistent with the PCR results.

Discussion

According to the molecular evidence and the results of electron microscopic examination, the pear decline disease observed in the orchards of central Taiwan was proven to be caused by phytoplasma. In the beginning of this study, the phytoplasma-specific rDNA sequence was successfully amplified using PCR with DNA templates prepared from the PDTW-affected pear trees when using the phytoplasma-specific primer pair f1/r1 (Lin & Lin, 1998), but no PCR product was amplified when using the PD phytoplasma-specific primer pair fPD/rPDS (Lorenz et al., 1995). Since it is known that these primers did not amplify all European strains of the PD agent (Lorenz et al., 1995; Martín et al., 2001), it was not surprising that the PD-specific primer pair fPD/rPDS did not amplify PDTW phytoplasma.

In this study, phytoplasma particles were successfully observed by TEM in pears with decline symptoms when using the samples that gave positive PCR results. However, only a low titer of PD phytoplasmas was observed in the TEM sections. Previous studies have also indicated that the population of phytoplasma was low and the distribution was probably not uniform in the plants, thus resulting in difficulty in the visualization of phytoplasma in the transverse sections of sieve tubes (Schneider & Gibb, 1997; Seemüller,

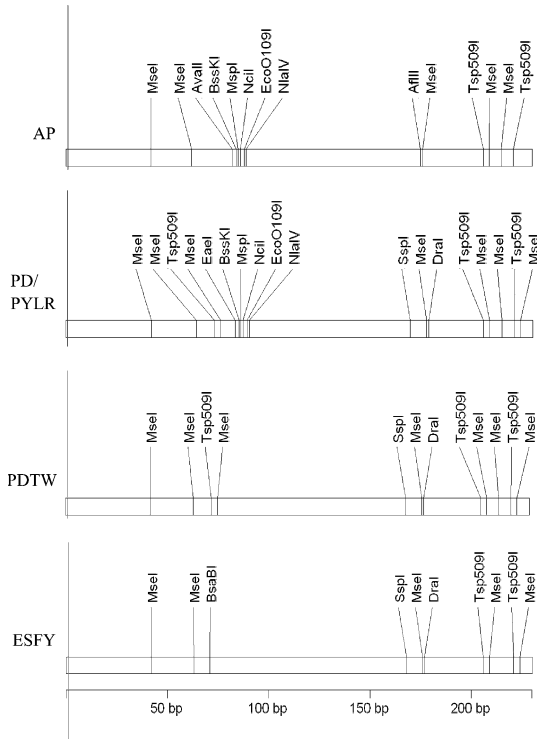

Fig. 4 Analysis of putative restriction sites of 16S–23S rDNA intergenic spacer region sequences of apple proliferation strain (AP, U54985), pear decline/peach yellow leaf roll strains (PD (U54989)/PYLR (U54990)), pear decline Taiwan strain (PDTW, DQ011588) and European stone fruit yellows strain (ESFY, U54988); maps were generated using the MapDraw option of DNASTAR programme

1992). Some collapsed sieve elements with wall thickenings and reduced lumen were observed in the declining pear trees by TEM. The morphological changes of plant tissue were much the same as those described in the previous study (Schaper & Seemüller, 1982; Schneider, 1977). This study is the first report concerning the phytoplasma associated with pear decline disease in Taiwan.

Phylogenetic analysis of 16S rDNA sequences indicated clearly that PDTW phytoplasma should be placed in the AP group. Previous studies on AP group phytoplasmas concluded that AP, PD/PYLR and ESFY phytoplasmas are coherent, but discrete, taxa that can be distinguished at the putative species level, for which the names '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum' were proposed (Seemüller & Schneider, 2004). In the descriptions of three species above, two regions inside the 16S rDNA sequence were identified as the signature sequences unique to those species (Seemüller & Schneider, 2004). In our studies, PDTW phytoplasma has been shown to contain both 16S rDNA signature sequences reported in '*Ca*. P. pyri' and '*Ca*. P. prunorum'. When compared with the 16S rDNA sequence, the sequence of the 16S–23S rDNA ISR is less conserved and can be used to differentiate members of a particular group (Marcone, Lee, Davis, Ragozzino, & Seemüller, 2000; Marcone, Schneider, & Seemüller, 2004; Regassa et al., 2004). The phylogenetic tree constructed on the basis of 16S–23S rDNA ISR sequence indicated clearly that PDTW phytoplasma is more closely related to the ESFY strain and the high bootstrap values supported the finding that PDTW/ESFY clade is distinct from PD/PYLR clade. Further comparison of the putative restriction site of 16S–23S rDNA ISR sequence showed that PDTW phytoplasma is distinguishable from AP, ESFY and PD/PYLR strains. Taken together, these putative restriction site and phylogenetic analyses of rDNA sequences revealed that PDTW phytoplasma may represent a new phytoplasma subgroup of AP group. Such analyses had been adopted in the characterization of a novel phytoplasma taxon (Jung et al., 2003; Salehi, Izadpanah, & Heydarnejad, 2006).

In Europe and North America *C. pyricola* is the main vector of pear decline, which is responsible for disease transmission (Davies, Guise, Clark, & Adams, 1992; Hibino, Kaloostian, & Schneider, 1971). Two species of pear psyllas have been identified in pear orchards in central Taiwan: *C. qianli* was first found in 1994 and maintains a low population every year in the field (Chou & Fang, 1994); *C. chinensis* was first found in 2002 and had been detected in very high populations in pear orchards in 2003 (Yang, Huang, & Li, 2004). In our studies, the PCR technique was able to amplify the PDTW phytoplasma rDNA from *C. qianli* and *C. chinensis*, and further sequencing studies also confirmed that the sequences of these PCR-amplified products are identical to the sequence of PDTW phytoplasma. Therefore, we suggest that both *C. qianli* and *C. chinensis* carry PDTW

phytoplasma and are candidates for transmitting PDTW phytoplasma. However, the much higher titers of the PDTW phytoplasma detected in *C. qianli* in comparison with *C. chinensis* indicated that the occurrence of PDTW disease may be mainly transmitted by *C. qianli* at present. On the other hand, the influence of *C. chinensis* on disease spreading in the future still needs to be evaluated closely. Further transmission experiments are necessary to demonstrate the vectorship of *C. qianli* and *C. chinensis* with PDTW disease.

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