Specific characters of 16S rRNA gene and 16S–23S rRNA internal transcribed spacer sequences of *Xylella fastidiosa* pear leaf scorch strains

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Abstract Pear leaf scorch, the only *Xylella fastidiosa*-induced disease reported from Taiwan, was found in area where the variety Hengshan (*Pyrus pyrifolia*) was grown. Strains of pear leaf scorch *Xyl. fastidiosa* (XF-PLS) shared similarities to strains of other host origins in the requirement of complex medium and the exhibition of rippled cell walls, however, recent serological and molecular biology studies showed difference among them. Five strains of XF-PLS were compared with 20 other strains originally isolated from almond, oleander, pecan, plum, peach, mulberry, grapes, citrus, coffee, and sycamore by sequence analyses of the 16S rRNA gene and 16S–23S rRNA internal transcribed spacer region (ITS). When sequences of 16S rRNA gene based on fragment size of 1,537–1,540 bp were compared, the similarity index among 5 XF-PLS strains was 99.3–99.8%, whereas it was 97.8–98.6% between XF-PLS strains and strains from other hosts. When sequences of 16S–23S rRNA ITS based on fragment size of 510–540 bp were compared, the similarity index among 5 XF-PLS strains was 99.0–100%, whereas it was 80.7–82% between XF-PLS strains and strains from other hosts. Multiple sequence alignments led to the identification of 5 polymorphic nucleotides in the 16S rRNA gene among the 25 *Xyl. fastidiosa* strains, and there were considerable variations in the nucleotide sequences of 16S–23S rRNA ITS between XF-PLS and the other 20 *Xyl. fastidiosa* strains. The phylogenetic trees revealed that XF-PLS strains were separated from strains of other hosts. Strains of other hosts were divided into four subgroups: strains from (1) oleander, (2) grape, almond M23 and mulberry, (3) citrus and coffee, and (4) pecan, peach, plum, sycamore and almond M12. Results indicate that XF-PLS strains were not closely related to the above-mentioned strains from other hosts and could possibly belong to a new subspecies of *Xyl. fastidiosa*.

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Introduction

Xylella fastidiosa, a Gram-negative, rod shaped cells with ripple cell walls without flagella, resides only in xylem tissues and requires specific and enriched media for in vitro growth (Wells et al. 1987). *Xyl. fastidiosa* has a wide host range: it was reportedly involved in diseases of more than 100 host plants including numerous crops and ornamentals (Hopkins and Purcell 2002) and recently emerged as economically important diseases such as citrus variegated chlorosis (Chang et al. 1993; Hartung et al. 1994), pear leaf scorch (Leu and Su 1993), and bacterial leaf scorch of blueberry (Chang et al. 2009). Based on the reciprocal inoculations (Hopkins 1989; Hopkins and Adlerz 1988), culturing characteristics (Purcell and Hopkins 1996), DNA homology (Mehta and Rosato 2001), restriction fragment length polymorphisms (RFLPs) (Chen et al. 1992; Hendson et al. 2001), and random amplified polymorphic DNA (RAPD-PCR) (Chen et al. 2002; Hendson et al. 2001; Pooler and Hartung 1995; Qin et al. 2001; Rosato et al. 1998), *Xyl. fastidiosa* was formerly separated into four groups namely Pierce's disease group, citrus variegated chlorosis group, plum leaf scald and phony peach group, and oleander group (Hopkins and Purcell 2002; Purcell and Hopkins 1996). Even though in the genus *Xylella*, there is still only one known species, *Xyl. fastidiosa*, five subspecies *fastidiosa, multiplex, pauca, sandyi*, and *tashke* have recently been proposed (Randall et al. 2009; Schaad et al. 2004; Schuenzel et al. 2005). *Xyl. fastidiosa* subsp. *fastidiosa* covers strains originated from grape, almond, alfalfa, and maple, *Xyl. fastidiosa* subsp. *multiplex* covers strains from peach, plum, almond, elm, sycamore, and pigeon grape, *Xyl. fastidiosa* subsp. *pauca* covers strains from citrus, *Xyl. fastidiosa* subsp. *sandyi* covers strains from oleander, daylily, jacaranda, and magnolia (Schuenzel et al. 2005; Hernandez-Martinez et al. 2007) and *Xyl. fastidiosa* subsp. *tashke* covers strains from *Chitalpa tashkentensis*, a common ornamental landscape plant (Randall et al. 2009). No known *Xyl. fastidiosa* strain that infects pear trees has been identified in the American Continent.

Pear leaf scorch (PLS), the only reported *Xyl. fastidiosa*-induced disease in Taiwan (Leu and Su 1993), was described and recorded around 1991 in the area where the low chilling variety Hengshan (*Pyrus pyrifolia*) was planted. Leu and Su (1993) reported that *Xyl. fastidiosa* was the causal bacterium of pear leaf scorch disease based on electron microscopic observation of the bacterium in xylem tissues, the isolation and cultivation of the bacterium, and the transmission of the disease through grafting and mechanical inoculation. Nevertheless, the strains isolated from pear were not serologically related to other *Xyl. fastidiosa* strains that were routinely characterized by a double-sandwich ELISA assay (Agdia Inc., IN, USA) (Leu and Su 1993), suggesting the PLS strains might posses unique features that were not present in the other *Xyl. fastidiosa* strains. Taxonomic and phylogenetic analyses using multiple methods, i.e. DNA-DNA hybridization, 16S rRNA gene, 16S–23S rRNA ITS, and randomly amplified DNA fingerprinting profiles (Su et al. 2008), produced inconclusive results regarding the relationships of the PLS strains and the other *Xyl. fastidiosa* strains. Two independent studies carried out by Mehta and Rosato (2001) and Su et al. (2008) revealed the pear strains distinctively separate from *Xyl. fastidiosa* subspecies *fastidiosa, multiplex, pauca*, and *sandyi*, whereas Randall et al. (2009) designated the pear strains to *Xyl. fastidiosa* subsp. *multiplex* based on the sequence analyses of the 16S rRNA gene and 16S–23S ITS. A recent report by Chen et al. (2010) on whole genome sequences of two *Xyl. fastidiosa* strains (M12 and M23) that cause almond leaf scorch disease in California revealed strain M12 as A genotype and strain M23 as G genotype (Chen et al. 2005), which was in agreement with M12 belonging to *Xyl. fastidiosa* subsp. *multiplex* and M23 belonging to *Xyl. fastidiosa* subsp. *fastidiosa*. In this study, we performed nucleotide comparison and phylogenetic analyses of 16S rRNA gene and 16S–23S rRNA ITS to determine the genetic relatedness of 5 pear leaf scorch strains to 20 strains of *Xyl. fastidiosa* that belong to 4 subspecies of *fastidiosa, multiplex, pauca*, and *sandyi* to clarify the taxonomic rankings of the pear leaf scorch strains.

Materials and methods

Bacterial strains and genomic DNA extraction All bacterial strains used in this study are listed in Table 1. *Xyl. fastidiosa* strains were routinely incubated at 28–30°C unless specified otherwise. *Xyl. fastidiosa* PLS strains were isolated from pear leaf petioles showing typical scorch symptoms as described (Leu and Su 1993) and cultured on PD2 medium (Davis et al. 1980). *Xyl. fastidiosa* strains of grape, mulberry, oleander,

Table 1 Strains of *Xylella fastidiosa* used in the study and GenBank accession numbers of their 16S rRNA and 16S–23S rRNA internal transcribed spacer (ITS) sequences

Species/host	Strain	GenBank accession no		Source or reference
		16S rRNA	16S–23S ITS	
Xylella fastidiosa				
Pear	PLS2[a]	DQ987473	DQ991164	This study
	PLS45[a]	DQ987474	DQ991165	This study
	PLS194[a]	DQ987475	DQ991166	This study
	PLS222[a]	DQ987476	DQ991167	This study
	PE.PLS[a]	AF203392	AF203396	Mehta and Rosato 2001
Almond	**M12**[b]	CP000941	CP000941	Chen et al. 2010
	M23	CP001011	CP001011	Chen et al. 2010
Citrus	CI.52	AF203389	AF203393	Mehta and Rosato 2001
	9a5c	AE003849	AE003849	Simpson et al. 2000
Coffee	CO.01	AF203390	AF203394	Mehta and Rosato 2001
Grape	ATCC35876	DQ991182	DQ991168	This study
	ATCC35879[c]	DQ987477	DQ991169	This study
	Temecula1	AE009442	AE009442	Van Sluys et al. 2003
	GB514	CP002165	CP002165	Schreiber et al. 2010
Mulberry	Mul 17[d]	ND[f]	DQ991171	This study
	GHS 505[d]	DQ991183	DQ991170	This study
	G9E[d]	DQ991184	ND	This study
Oleander	GH-9[d]	DQ991185	DQ991172	This study
	Ol[d]	DQ991186	DQ991173	This study
Peach	4–5[de]	DQ991187	DQ991174	This study
Plum	2–4[de]	DQ991188	DQ991175	This study
	2–5[d]	DQ991189	DQ991176	This study
Pecan	4BD2[d]	DQ991190	DQ991177	This study
	4BD7[d]	DQ991191	DQ991178	This study
Sycamore	SLS 27[d]	DQ991192	DQ991179	This study
	SLS 55[d]	DQ991193	DQ991180	This study
Xanthomonas axonopodis pv. *citri*				
Citrus	XCW	DQ991194	DQ991181	This study

[a] PLS2 and PLS45 were originally isolated from samples of *Pyrus pyrifolia* cv. Hengshan collected in 2000 from Howli and Chuchi, respectively. PLS194 and PLS222 were originally isolated from samples of *Pyrus pyrifolia* cv. Niauli collected in 2001 from Tungshih and Howli, respectively. EP. PLS was originally isolated from samples of *Pyrus pyrifolia* cv. Hengshan collected in 1995 from Tungshih

[b] Five strains shown in bold represent those whose complete genomes were completely sequenced and deposited in GenBank database under the indicated accession numbers

[c] The 16S rRNA gene and 16S–23S rRNA ITS sequences of the grape strain ATCC35879 were independently sequenced by Dr. J. Chen at Florida A&M University and deposited under the respective accession number AF192343 and AF272834

[d] Total DNAs of the indicated *Xylella fastidiosa* strains from different hosts were extracted by C. J. Chang; mulberry strains were originally isolated in 1998 from mulberry leaf scorch tissues provided by Dr. Anne Vidavar from University of Nebraska; oleander strains were originally isolated in 1999 from tissues with leaf scorch symptom collected from St. Simon Island, Georgia; peach and plum strains were originally isolated in 2000 from phony peach and plum leaf scald tissues respectively collected from Georgia; pecan strains were originally isolated in 2000 from pecan leaf scorch tissues collected from Albany, Georgia; and sycamore strains were originally isolated in 1998 from sycamore leaf scorch tissues collected from Athens, Georgia

[e] Plum strain 2–4 is previously named as 2#4 (Hendson et al. 2001), and peach strain 4–5 is also known as PP4#5 (Chen et al. 2000a) and 4#5 (Schaad et al. 2004), and the 16S–23S rRNA ITS sequence of 2#4 and 16S rRNA gene sequence of 4#5 have been deposited in the GenBank database under the accession number AF073209 and AF159580, respectively

[f] ND, not determined

peach, pecan, plum, and sycamore were isolated using the reported procedures (Chang and Walker 1988), confirmed by Double Antibody Sandwich (DAS) ELISA complete kit (Agdia Inc., IN, USA) according to the manufacturer's specifications, and grown on PW (Davis et al. 1981) or CS20 medium (Chang and Walker 1988). *Xanthomonas axonopodis* pv. *citri* strain XCW was grown on nutrient agar at 30°C. *Escherichia coli* and its derivatives were cultured at 37°C in Luria-Bertani (LB) medium or SOC broth (Bacto-tryptone 20 g/l, Bacto-yeast extract 5 g/l, NaCl 0.5 g/l, MgCl$_2$ 0.95 g/l, KCl 0.186 g/l, glucose 3.6 g/l, pH 7.0) supplemented with 50 μg/ml kanamycin when appropriate. Genomic DNA of each bacterial strain was extracted according to Sambrook et al. (1989). Bacterial cells of *Xyl. fastidiosa* were harvested from PD2, PW, or CS20 agar plates, transferred to 40 ml of the respective liquid medium, and incubated at 30°C with a rotation speed of 180 rpm for 72 h, whereas *Xan. axonopodis* pv. *citri* strain XCW was grown in 40 ml nutrient broth at 30°C for 16 h, prior to the extraction of genomic DNA. Extracted DNA was dissolved in sterile deionized water, quantified by a spectrophotometer (Pharmacia Biotech, England) at OD$_{260}$, and adjusted to a concentration of 20 ng/μl for PCR reactions.

Amplification, isolation, and cloning of 16S rRNA genes and 16S–23S rRNA ITS sequences The 16S rRNA gene of each bacterial strain was amplified by polymerase chain reaction using the universal primers F/R (5′-AGA GTTTGATCCTGGCTCAG-3′/5′-AAG GAGGTGATCCAGCC-3′) (Weisburg et al. 1991). A 20-μl PCR reaction mixture contained 20 ng of template DNA, 0.5 μM of each primer, 100 μM dNTP, 1X reaction buffer (10 mM Tris–HCl; pH 8.8, 1.5 mM MgCl$_2$, 50 mM KCl, 0.1% Triton X-100), and 0.8 U GenTap DNA Polymerase (GenMark Technology Co., Taiwan). PCR was performed on the PTC-200 thermal cycler (MJ Research Inc., MA, USA) using the following conditions: 1 cycle of pre-heating at 94°C for 5 min, 40 cycles of amplification at 94°C 1 min, 60°C 1 min and 72°C 1 min, followed by 1 cycle of termination at 72°C for 1 min. The 16S–23S rRNA ITS sequences were amplified by primers uni1330/uni322 (5′-GTTCCCGGGCCT TGTACACAC-3′/5′-GGTTCTTTTCGCCTTTCCCTC-3′) (Honeycutt et al. 1995) following the PCR conditions for the synthesis of 16S rRNA gene, except the amplification program was

changed to 30 cycles. Amplified products were purified by Montage™ PCR centrifugal filter devices (Millipore, MA, USA) using the procedures recommended by the manufacturer. The purified PCR products were ligated with pOSI-T vector (GeneMark, Technology Co. Ltd., Taiwan) and transformed into *E. coli* DH5α high-efficiency competent cell (GeneMark, Technology Co. Ltd., Taiwan) according to the manufacturer. The constructed plasmids harbouring the 16S rRNA gene and 16S–23S rRNA ITS were purified using a plasmid miniprep purification kit (GeneMark, Technology Co., Taiwan) and confirmed by *Eco*RI restriction endonuclease digestion (Promega Co., WI, USA).

DNA sequencing and analysis The cloned DNA fragments were sequenced on an ABI 377 automated DNA sequencer (Applied Biosystems Inc., CA, USA) at Mission Biotech Co. (Taiwan). All clones were sequenced at least twice to obtain accurate reads. The sequences of the cloned 16S rRNA gene and 16S–23S rRNA ITS have been deposited in GenBank under the accession numbers indicated in Table 1. Homology searches against GenBank database (www.ncbi.nlm.nih.gov) were done with the BLASTN program (Altschul et al. 1990). All nucleotide sequences of 16S rRNA gene and 16S–23S ITS of *Xyl. fastidiosa* strains and *Xan. axonopodis* pv. *citri* strain XCW were used as queries for the similarity search and comparison. Multiple sequence alignments were performed using the Clustal X program (Jeannmougin et al. 1998). The phylogenetic tree was constructed using the neighbour-joining method and bootstrap analyses for 1,000 replicates according to the Phylogenetic Inference Package Phylip Version 3.6 (Felsenstein 2004) and displayed by TreeView program (Page 1996). The trees were rooted using the 16S rRNA gene or 16S–23S rRNA ITS sequence of XCW as an outgroup for phylogenetic analyses.

Results

Sequence alignment and comparisons of the 16S rRNA gene

The sizes of XF-PLS 16S rRNA gene and 16S–23S ITS sequences amplified by the F/R and

uni1320/unil332 primer sets were 1,537–1,540 bp and 512–540 bp, respectively (Table 1). Pairwise comparison of the 16S rRNA gene sequences revealed high similarities (greater than 97.5%) among different *Xyl. fastidiosa* strains. The comparison also indicated the nucleotide similarities are 95.8%–96.2% between XF-PLS strains and *Xan. axonopodis* pv. *citri* XCW, which are slightly higher than the similarities of 95.1%–95.6% between XCW and the other 20 strains of *Xyl. fastidiosa* (almond, citrus, coffee, grape, mulberry, oleander, peach, plum, pecan, and sycamore) (data not shown). Multiple sequence alignment of the near-complete 16S rRNA gene of *Xyl. fastidiosa* strains showed there were 4 nucleotide differences between the positions 69 to 85 of the XF-PLS strains and the other *Xyl. fastidiosa* strains (Fig. 1a), and the region has been reported to contain divergent sequences that vary among different bacterial species (Gendel 1996). The 16S rRNA genes of the XF-PLS strains harbour additional 15 nucleotide variations throughout the 1.5-kb sequences, including 11 nucleotide transitions at positions 58 (A/G), 251 (A/G), 261 (T/C), 464–465 (TA/CG), 584 (A/G), 593 (T/C), 993–994 (TG/CA), 998 (A/G), and 1,273 (T/C), and 4 nucleotide transversions at positions 203 (A/T), 472 (A/C), 1,131 (A/T), and 1,206 (T/A), which are highly conserved among the other 20 *Xyl. fastidiosa* strains of 10 host origins (Fig. 1). Previously, Chen et al. (2000a, c) reported that C/T transition at position 143 separated *Xyl. fastidiosa* strains into one group of grape and mulberry strains and the other of citrus, coffee, oleander, peach, plum, pecan, and sycamore strains. In this study, an A/G transition at the 447th nucleotide was identified as a new feature for the group of grape and mulberry stains, whereas an additional T between positions 466–467 and 2 A/G transitions at positions 69 and 1,255 further separate these strains into four subgroups: grape and mulberry strains (69A, 143C, 447G, 1,255A), citrus and coffee strains (69G, 143T, 447A, 1,255A), oleander strains (69A, 143T, extra T between 466 and 467, 447A, 1,255A), and peach, plum, pecan, and sycamore strains (69A, 143T, 447A, 1,255G). As shown in Fig. 1, the XF-PLS strains harbour 69A, 143T, 447A, and 1,255A at the five polymorphic nucleotides, which were distinct to the above 4 subgroups of *Xyl. fastidiosa* strains.

Sequence alignment and comparisons of the 16S–23S rRNA ITS sequences

In comparison with the 16S rRNA gene sequences, the 16S–23S ITS sequences derived from XF-PLS strains shared lower similarity (ranging between 80.7% and 82%) with the ones from the other 20 strains of *Xyl. fastidiosa*, while pairwise comparisons of the 16S–23S ITS sequences among the 20 strains resulted in greater than 97% sequence similarities (data not shown). Analyses of the aligned 16S–23S ITS sequences revealed highly similar regions that reside between nucleotide positions 127–198 and 214–286, and there were also considerable nucleotide differences between positions 112–126, 199–213, and 413–481 between the XF-PLS strains and the other *Xyl. fastidiosa* strains (Fig. 2). The highly similar regions of nucleotide 127–198 (72 bp) and 214–286 (73 bp) respectively code for tRNA[ala] and tRNA[ile], and the genetic conservation has been revealed by comparing 51 16S–23S rRNA ITS sequences of *Xyl. fastidiossa* strains (Chen et al. 2000b). In the tRNA[ala] coding region, two nucleotides at the positions 170 and 189 show polymorphism between XF-PLS strains (170T, 189G) and the other 20 *Xyl. fastidiossa* strains (170G, 189A). As to the tRNA[ile] gene, XF-PLS and the other *Xyl. fastidiossa* strains share 100% identity with one exception: the plum strain 2–5 harbours G, instead of the conserved A, at the 257th nucleotide. The ITS sequences of XF-PLS strains do not harbour GGGTTTATGTTGG (Fig. 2, positions 112–126) and AAAGTAT (Fig. 2, positions 199–213) that are commonly present in the other 20 *Xyl. fastidiosa* strains. Meanwhile, there are 42 out of 69 nucleotide differences between positions 413–481, showing unique sequences that exist in the XF-PLS strains but not in the other *Xyl. fastidiosa* strains (Fig. 2). The 13 polymorphic nucleotides, as indicated by the '#' symbol in Fig. 2, were collectively identified by Schaad et al. (2004) and Hernandez-Martinez et al. (2007) to classify *Xyl. fastidiosa* subspecies *fastidiosa* (grape strains), *pauca* (citrus strain), *multiplex* (peach, plum, sycamore strains), and *sandyi* (oleander strain). The 13 SNPs group mulberry strains (GHS505 and Mul7) together with the grape strains, coffee strain CO.01 with citrus strains CI.52 and 9a5c, pecan strains 4BD2 and 4BD7 with the peach,

Fig. 1 Multiple alignments of the selected regions of the 16S rRNA genes of *Xylella fastidiosa* pear leaf scorch bacteria (PLS2, PLS45, PLS194, PLS222, and PE.PLS), strains of *Xyl. fastidiosa* isolated from other hosts (almond, citrus, coffee, grape, mulberry, oleander, peach, plum, pecan, and sycamore), and *Xanthomonas axonopodis* pv. *citri* strain XCW. Gray boxes indicate the consensus sequences of the 16S rRNA genes of *Xyl. fastidiosa* strains. The number listed on the top of the aligned sequences indicates the nucleotide positions of the 16S rRNA gene of the XF-PLS strains. Symbol // indicates nucleotide sequences that are omitted from the diagram. At the bottom of the alignment, symbol * signifies the variable sequence of the XF-PLS strains and the other *Xyl. fastidiosa* strains, and symbol # indicates the single nucleotide polymorphism (SNP) of the 16S rRNA genes. *Xyl. fastidiosa* strains used in the comparison and their accession numbers of the 16S rRNA sequences are listed in Table 1. **a** Sequences of 212 nucleotides between positions 55 and 266. **b** Sequences of 559 nucleotides between positions 445 and 1,003. **c** Sequences of 154 nucleotides between positions 1,127 and 1,280

plum, and sycamore strains, suggesting the strains of mulberry, coffee, and pecan might be placed in the

subspecies of *fastidiosa*, *pauca*, and *multiplex*, respectively. Taken together with the extra T between

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b             445                              476 582         594 990          1003
PE.PLS     AAAGCAATTGGTTAATACCCG-GTTGTTCTGAC// TTGTTTAAGTCCG//ATCCGCGG-ACTTTC
PLS2       AAAGCAATTGGTTAATACCCG-GTTGTTCTGAC// TTGTTTAAGTCCG//ATCCACGGGACTTTC
PLS45      AAAGCAATTGGTTAATACCCG-GTTGTTCTGAC// TTGTTTAAGTCCG//ATCCACGGGACTTTC
PLS194     AAAGCAATTGGTTAATACCCG-GTTGTTCTGAC// TTGTTTAAGTCCG//ATCCACGGGACTTTC
PLS222     AAAGCAATTGGTTAATACCCG-GTTGTTCTGAC// TTGTTTAAGTCCG//ATCCACGGGACTTTC
CI.52      AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
9a5c       AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
CO.01      AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
ATCC35876  AAGGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
ATCC35879  AAGGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
Temecula1  AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
GB514      AAGGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
M23        AAGGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
GHS505     AAGGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
G9E        AAGGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
GH-9       AAAGCAATTGGTTAATACCTATGTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
O1         AAAGCAATTGGTTAATACCTATGTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
4-5        AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
2-4        AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
2-5        AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
4BD2       AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
4BD7       AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
SLS27      AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
SLS55      AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
M12        AAAGCAATTGGTTAATACCTA-GTTGTTATGAC// TTATTTAAGTCTG//ATCTGCGGAACTTTC
XCW        AAAGCAGTCGGTTAATACCCG-ATTGTTCTGAC// TGGTTTAAGTCTG//ATCCACGGAACTTTC
              #             ***#     *       *        *     **    *

c             1127    1136 1201    1210 1250                        1280
PE.PLS     ACGTTATGGT//GGCCCATACG// GCTGCAATCTCGCGAGGGTGAGCCAATCCCA
PLS2       ACGTTATGGT//GGCCCATACG// GCTGCAATCTCGCGAGGGTGAGCCAATCCCA
PLS45      ACGTTATGGT//GGCCCATACG// GCTGCAATCTCGCGAGGGTGAGCCAATCCCA
PLS194     ACGTTATGGT//GGCCCATACG// GCTGCAATCTCGCGAGGGTGAGCCAATCCCA
PLS222     ACGTTATGGT//GGCCCATACG// GCTGCAATCTCGCGAGGGTGAGCCAATCCCA
CI.52      ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
9a5c       ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
CO.01      ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
ATCC35876  ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
ATCC35879  ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
Temecula1  ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
GB514      ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
M23        ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
GHS505     ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
G9E        ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
GH-9       ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
O1         ACGTAATGGT//GGCCCTTACG// GCTGCAATCTCGCGAGGGTGAGCTAATCCCA
4-5        ACGTAATGGT//GGCCCTTACG// GCTGCGATCTCGCGAGGGTGAGCTAATCCCA
2-4        ACGTAATGGT//GGCCCTTACG// GCTGCGATCTCGCGAGGGTGAGCTAATCCCA
2-5        ACGTAATGGT//GGCCCTTACG// GCTGCGATCTCGCGGGGGTGAGCTAATCCCA
4BD2       ACGTAATGGT//GGCCCTTACG// GCTGCGATCTCGCGAGGGTGAGCTAATCCCA
4BD7       ACGTAATGGT//GGCCCTTACG// GCTGCGATCTCGCGAGGGTGAGCTAATCCCA
SLS27      ACGTAATGGT//GGCCCTTACG// GCTGCGATCTCGCGAGGGTGAGCTAATCCCA
SLS55      ACGTAATGGT//GGCCCTTACG// GCTGCGATCTCGCGAGGGTGAGCTAATCCCA
M12        ACGTAATGGT//GGCCCTTACG// GCTGCGATCTCGCGAGGGTGAGCTAATCCCA
XCW        ACGTAATGGT//GGCCCTTACG// GCTGCAAACCCGCGAGGGCAAGCCAATCCCA
              *          *           #              *
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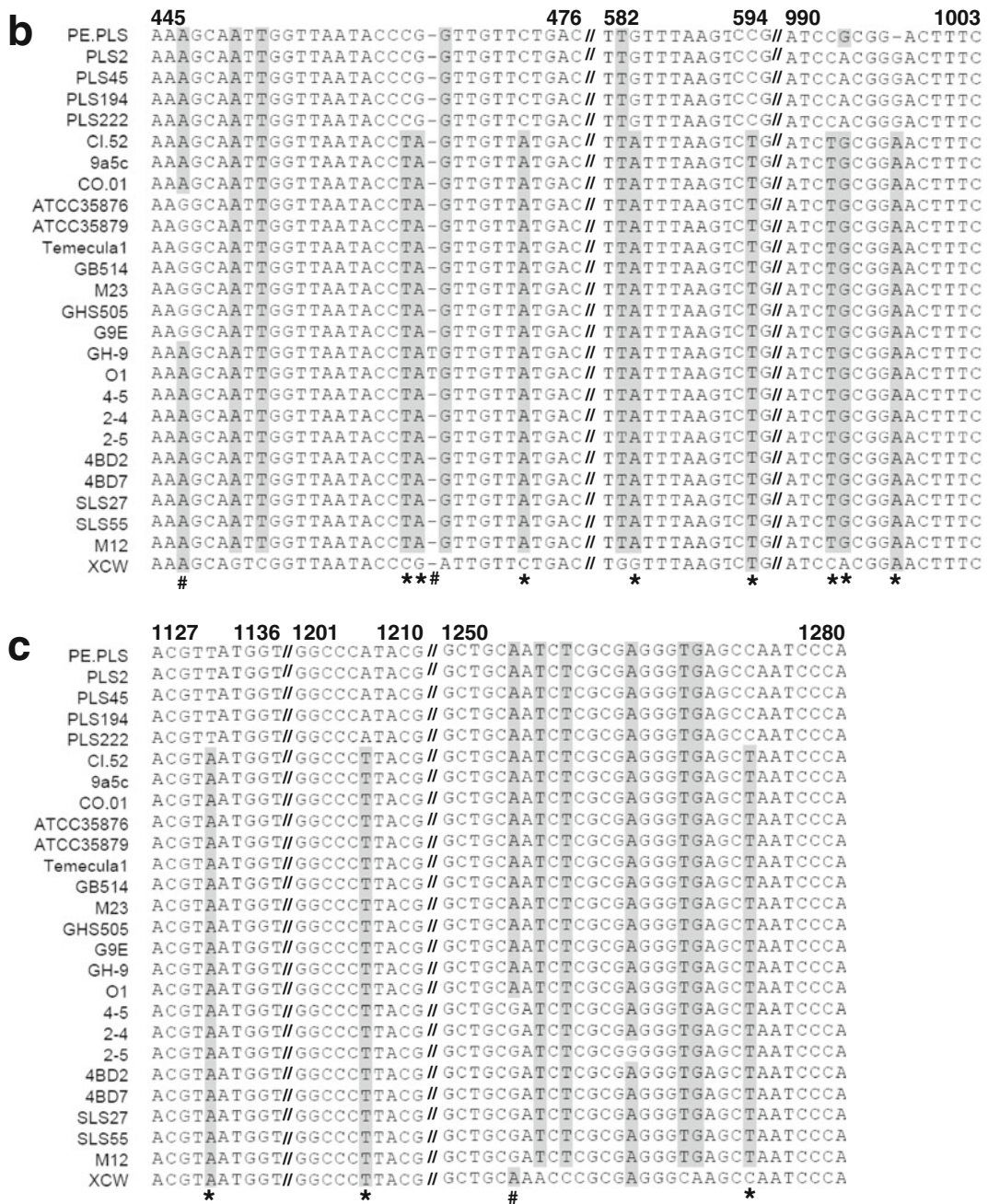**Fig. 1** (Continued)

the positions 466–467 of the 16S rRNA gene of oleander strains, the SNPs at positions 203 (G→C) and 316 (G→T) in the 16S–23S ITS can be applied as informative characters for classifying the subspecies *sandyi*.

Phylogenetic analyses of 16S rRNA gene and 16S–23S rRNA ITS sequences

Neighbour-joining (NJ) method and bootstrap probability were used for constructing and validating the

topology of phylogenetic trees of the 16S rRNA genes and 16S–23S rRNA ITS sequences of 25 *Xyl. fastidiosa* strains. The resulting NJ trees showed 2 distinct monophyletic groups of the 25 strains (Figs. 3 and 4): Group 1 contained 5 XF-PLS strains and group 2 contained the other 20 strains. The strains in group 1 and group 2 were closely related to each other with bootstrap probabilities of 96.6% and 100% for the 16S rRNA gene tree and 100% and 86.4% for the 16S–23S rRNA ITS tree, respectively. Additionally, the 16S–23S ITS tree clearly distinguished the group 1 and 2 with 100% bootstrap probability, suggesting the XF-PLS strains in group 1 were divergently evolved from the other 20 *Xyl. fastidiosa* strains in group 2. The 20 operational taxonomic units (OTUs) in group 2 can be further classified into 4 subgroups: O (oleander), GM (grape, mulberry and almond M23), C (coffee and citrus), and PS (peach, pecan, plum, sycamore, and almond M12). The subgroups of group 2 identified from the constructed NJ trees agree with previously classified subspecies of *Xyl. fastidiosa*: the C subgroup corresponds with subsp. *pauca*, the GM subgroup with subsp. *fastidiosa*, the PS subgroup with subsp. *multiplex* (Schaad et al. 2004), and the O subgroup with subsp. *sandyi* (Schuenzel et al. 2005).

Discussion

We report here the comparison of strains that caused pear leaf scorch disease to strains of other host origins from north and south Americas using the 16S rRNA gene and 16S–23S rRNA ITS sequences. The similarities and/or differences generated from this report may provide important information for the epidemiology of the *Xyl. fastidiosa*-induced diseases worldwide. Sequence analyses reveal the total 25 strains of *Xyl. fastidiosa* with different host origins can be separated into five subgroups, which give rise to similar grouping results using DNA-DNA related-ness, 16S–23S rRNA ITS sequences, and multigene phylogenetic analyses, and the XF-PLS strains were genetically distinct from the other *Xyl. fastidiosa* strains. Multiple sequence alignment of the 16S rRNA gene of *Xyl. fastidiosa* strains showed a few nucleo-tide differences between positions 69–85 (Fig. 1) that are located in the V1 variable region of the predicted SSU rRNA secondary structure (Neefs et al. 1991).

Fig. 2 Multiple alignments of the complete 16S–23S rRNA internal transcribed spacer sequences (16S–23S ITS) of *Xylella fastidiosa* pear leaf scorch bacteria (PLS2, PLS45, PLS194, PLS222, and PE.PLS) and strains of *Xyl. fastidiosa* isolated from hosts of almond, citrus, coffee, grape, mulberry, oleander, peach, plum, pecan, and sycamore. Gray boxes indicate the consensus sequences of the 16S–23S ITS of *Xyl. fastidiosa* strains. The number listed on the top of the aligned sequences indicates the nucleotide positions of the 16S–23S ITS sequence of the XF-PLS strains. Symbol // indicates nucleotide sequences omitted from the diagram. At the bottom of the alignment, symbol * signifies the variable sequence of the PLS strains and the other *Xyl. fastidiosa* strains, and symbol # indicates the previously identified single nucleotide polymorphisms (SNPs) that separate *Xyl. fastidiosa* strains into 4 subspecies (Schaad et al. 2004; Schuenzel et al. 2005). Symbol 'v' indicates the specific characters of the 16S–23S ITS which are present in the XF-PLS strains but absent from the other *Xyl. fastidiosa* strains. *Xyl. fastidiosa* strains used in the comparison and their accession numbers of the 16S–23S rRNA ITS sequences are shown in Table 1.

The sequence variability of the V1 region was considered as a specific signature among different bacterial species (Gendel 1996), which is also reported here. The V1 sequences derived from *Xyl. fastidiosa* strains are highly different from the ones of *Xan. axonopodis* and *Xan. campestris* (Chen et al. 2000a). The variable sequences in the 16S rRNA gene and 16S–23S ITS sequences are considered as informative characters in the phylogenetic analyses that separate the XF-PLS strains, all in one single taxon, from the other 20 strains. Results from this study, contrary to the previous reports that placed a XF-PLS strain (PE.PLS) in the subsp. *multiplex* (Randall et al. 2009), strongly suggest that XF-PLS strains may belong to a new subspecies that warrants further investigation. Randall et al. (2009) indicated that the sequences for the subgroups *piercei* (*fastidiosa*), *multiplex*, and *pauca* were taken from Schaad et al. (2004). It was, however, a misquote because Schaad et al. (2004) did not include the XF-PLS in their study. Even though the XF-PLS strain (PE.PLS-pear) was listed in (Fig. 2 of) Hernandez-Martinez et al. (2007) report, the authors however did not mention its taxonomic position in the phylogenetic tree constructed using the neighbour-joining method based on 16S–23S rRNA ITS data for *Xyl. fastidiosa*. In this study, 5 *Xyl. fastidiosa* strains, i. e. citrus strain 9a5c, grape strains Temecula1 and GB514, almond strain M23 and almond strain M12 that were formerly classified to the subspecies *pauca, fastidiosa*, and *multiplex* respectively were included for comparison. The whole-genome sequences of the above-

31 ... **108**

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PE.PLS     TTGAGTATGGCAGCATATCATCGTCCTGTTAGGCGTCCTCACAAGTTACCTGCATTCAGAGTTTTATGTTGGCATTGG
PLS2       TTGAGTATGGCAGCATATCATCGTCCTGTTAGGCGTCCTCACAAGTTACCTGCATTCAGAGTTTTATGTTGGCATAGG
PLS45      TTGAGTATGGCAGCATATCATCGTCCTGTTAGGCGTCCTCACAAGTTACCTGCATTCAGAGTTTTATGTTGGCATAGG
PLS194     TTGAGTATGGCAGCATATCATCGTCCTGTTAGGCGTCCTCACAAGTTACCTGCATTCAGAGTTTTATGTTGGGCATAGG
PLS222     TTGAGTATGGCAGCATATCATCGTCCTGTTAGGCGTCCTCACAAGTTACCTGCATTCAGAGTTTTATGTTGGCATAGG
Cl.52      TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
9a5c       TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
CO.01      TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
ATCC35876  TTGAGTATGGT-GAATATAATTGTCTTACCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
ATCC35879  TTGAGTATGGT-GAATATAATTGTCTTACCAGGCGTCATCACAAGTTACCTGCATTCAGGGTTTGATGTTGGCATAGG
Temecula1  TTGAGTATGGT-GAATATAATTGTCTTACCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
GB514      TTGAGTATGGT-GAATATAATTGTCTTACCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
M23        TTGAGTATGGT-GAATATAATTGTCTTACCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
GHS505     TTGAGTATGGT-GAATATAATTGTCTTACCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
Mul7       TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGTCATAGG
GH-9       TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
O1         TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
4-5        TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
2-4        TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
2-5        TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
4BD2       TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
4BD7       TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
SLS27      TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
SLS55      TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCATCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
M12        TTGAGTATGGT-GAATATAATTGTCTTATCAGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGG
            *v *      *   *   * *#* *               *        *   *
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109 ... **126** ... tRNA^{ala} (bp127-198) ... **199** ... **213** ... tRNA^{ile} (bp214-286) ... **287** **294**

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PE.PLS     GTT-------------CGAGTTCAGTTCGGG     CCAGG---GTGTCTTATG     CCAATGTG
PLS2       GTT-------------CGAGTTCAGTTCGGG     CCAGG---GTGTCTTATG     CCAATGTG
PLS45      GTT-------------CGAGTTCAGTTCGGG     CCAGG---GTGTCTTATG     CCAATGTG
PLS194     GTT-------------CGAGTTCAGTTCGGG     CCAGG---GTGTCTTATG     CCAATGTG
PLS222     GTT-------------CGAGTTCAGTTCGGG     CCAGG---GTGTCTTATG     CCAATGTG
Cl.52      TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
9a5c       TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
CO.01      TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
ATCC35876  TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
ATCC35879  TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
Temecula1  TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
GB514      TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
M23        TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
GHS505     TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
Mul7       TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
GH-9       TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATCAAAGTAT-TTATG     CCAATGTT
O1         TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATCAAAGTAT-TTATG     CCAATGTT
4-5        TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
2-4        TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
2-5        TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
4BD2       TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
4BD7       TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
SLS27      TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
SLS55      TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
M12        TTTGGGTTTATGTTGGCGATTTTTGTTCTGG     CCATGAAAGTAT-TTATG     CCAATGTT
           * *************  *  **   *        *#***  * v                *
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295 ... **362** **371** ... **379**

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PE.PLS     AGGTCGTCGATTCTGAATGTAGTTTGCGCATTG-TTTTATGCTGATCGGCGTTGTAGCTGTGAAGCGT//AACTTGATG
PLS2       AGGTCGTCGATTCTGAATGTAGTTTGCGCATTGTTTTTATGCTGATCGGCGTTGTAGCTGTGAAGCGT//AACTTGATG
PLS45      AGGTCGTCGATTCTGAATGTAGTTTGCGCATTGTTTTTATGCTGATCGGCGTTGTAGCTGTGAAGCGT//AACTTGATG
PLS194     AGGTCGTCGATTCTGAATGTAGTTTGCGCATTGTTTTTATGCTGATCGGCGTTGTAGCTGTGAAGCGT//AACTTGATG
PLS222     AGGTCGTCGATTCTGAATGTAGTTTGCGCATTGTTTTTATGCTGATCGGCGTTGTAGCTGTGAAGCGT//AACTTGATG
Cl.52      ATATCAATTATTCTGAATGTGGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
9a5c       ATATCAATTATTCTGAATGTGGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
CO.01      ATATCAATTATTCTGAATGTGGT-GCGCATT....TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
ATCC35876  ATATCAATTATTCTGAATGTAGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
ATCC35879  ATATCAATTATTCTGAATGTAGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
Temecula1  ATATCAATTATTCTGAATGTAGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
GB514      ATATCAATTATTCTGAATGTAGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
M23        ATATCAATTATTCTGAATGTAGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
GHS505     ATATCAATTATTCTGAATGTAGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
Mul7       ATATCAATTATTCTGAATGTAGTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
GH-9       ATATCAATTATTCTGAATGTATTTTGCGCATT---TTTATGCTTATCAGCCTTGGAGCAGTGAAGCGT//AATTTGATG
O1         ATATCAATTATTCTGAATGTGGTTTGCGCATT---TTTGTTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
4-5        ATATCAATTATTCTGAATGTGGTTTGCGCATT---TTTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
2-4        ATATCAATTATTCTGAATGTGGTTTGCGCATT--TTTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
2-5        ATATCAATTATTCTGAATGTGGTTTGCGCATT--TTTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
4BD2       ATATCAATTATTCTGAATGTGGTTTGCGCATT--TTTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
4BD7       ATATCAATTATTCTGAATGTGGTTTGCGCATT--TTTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
SLS27      ATATCAATTATTCTGAATGTGGTTTGCGCATT---TTTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
SLS55      ATATCAATTATTCTGAATGTGGTTTGCGCATT---TTTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
M12        ATATCAATTATTCTGAATGTGGTTTGCGCATT--TTTTATGCTTATCAGCCTTGGAGCTGTGAAGCGT//AATTTGATG
           **  ****          ##          VV#      *   *   *              *
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Fig. 2 (Continued)

mentioned 5 strains can be retrieved from the GenBank database, which would be a resource for determining the taxonomic status of XF-PLS when the XF-PLS genomic sequence becomes available.

The ribosomal RNA gene sequences including 5S, 23S, and 16S rRNA genes in eubacteria are highly conserved genes that maintain the biological evolution process and the accumulated genetic variation information (Hillis and Dixon 1991; Stackebrandt and Goebel 1994; Vandamme et al. 1996). Among them the subunit 16S ribosome and its gene sequences simply described as 16S rRNA gene were compared

through PCR reaction for the phylogenetic analysis of various eubacteria at genus levels (Vandamme et al. 1996), while the ITS between the 16S and 23S rRNA genetic loci are frequently used in PCR fingerprinting to discriminate bacterial strains at the species and intraspecies levels (Chen et al. 2000a, c; Goncalves and Rosato 2002; Hendson et al. 2001; Honeycutt et al. 1995; Mehta and Rosato 2001; Nathalie et al. 1996; Qin et al. 2001; Rosato et al. 1998; Smart et al. 1996; Toth et al. 2001). For example, Hauben et al. (1997) used 16S rRNA gene sequence analyses to differentiate the genus *Xanthomonas* and genus

Fig. 3 A neighbour-joining (NJ) tree expressing the evolution of the *Xylella fastidiosa* strains based on the 16S rRNA gene sequences. The tree was rooted using 16S rRNA gene of *Xanthomonas axonopodis* pv. *citri* strain XCW as an outgroup. Horizontal branch length is proportional to the estimated number of nucleotide substitutions, and the probabilities of bootstrap analyses (as percentage) for 1,000 resamplings that are greater than 60% are indicated above or below the internal branches. The bacterial strains and their 16S rRNA sequences used for the phylogenetic analysis are listed in Table 1. Pear = strains of pear leaf scorch; O = strains of oleander leaf scorch; C = strains of citrus variegated chlorosis and coffee leaf scorch; GM = strains of Pierce's disease of grapes, mulberry leaf scorch and almond leaf scorch M23; PS = strains of phony peach, plum leaf scald, pecan leaf scorch, sycamore leaf scorch and almond leaf scorch M12. The scale bar represents 0.01 substitutions/site
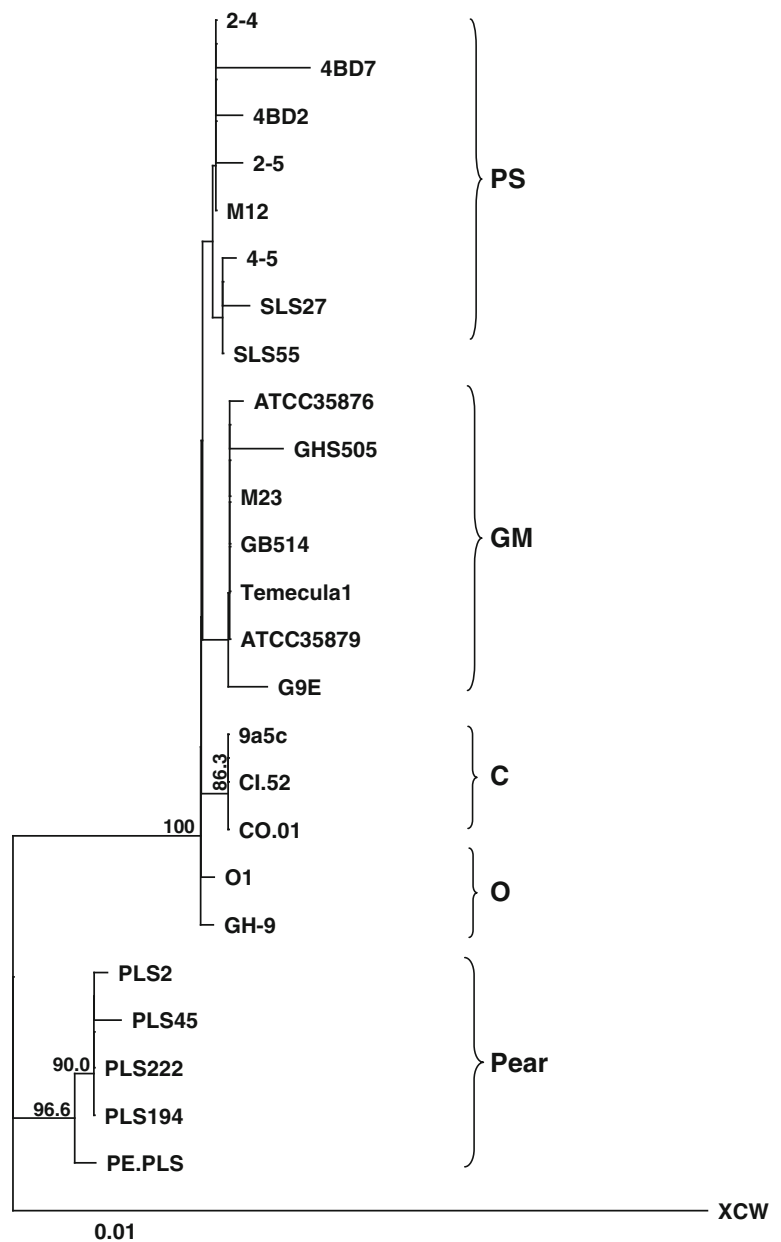
Stenotrophomonas and further divided genus *Xanthomonas* into three subgroups. Both 16S rRNA gene sequences and 16S–23S rRNA ITS sequences have been used for the classification of various strains of *Xyl. fastidiosa* (Chen et al. 2000b; Hendson et al. 2001; Schuenzel et al. 2005; Mehta and Rosato 2001; Randall et al. 2009; Schaad et al. 2004). In this work, we identify 5 single nucleotide polymorphisms (SNPs) in the 16S rRNA gene that could divide

the 20 *Xyl. fastidiosa* strains into GM, PS, C, and O subgroups that correspond to the proposed *Xyl. fastidiosa* subspecies *fastidiosa, multiplex, pauca* (Schaad et al. 2004) and *sandyi* (Schuenzel et al. 2005), indicating that the SNPs residing in the 16S rRNA gene can serve as specific characters to determine the taxonomic level of *Xyl. fastidiosa*. Nucleotide positions 413–481 in the 16S–23S ITS of the XF-PLS strains contain specific characters that do

Fig. 4 A neighbour-joining (NJ) tree expressing the evolution of the *Xylella fastidiosa* strains based on the 16S–23S rRNA internal transcribed spacer sequences (16S–23S ITS). The tree was rooted using the 16S–23S ITS of *Xanthomonas axonopodis* pv. *citri* strain XCW as an outgroup. Horizontal branch length is proportional to the estimated number of nucleotide substitutions, and the bootstrap probabilities (as percentage) for determining the grouping branching order are calculated by 1,000 resamplings, and the values that are greater than 60% are indicated above or below the internal branches. The bacterial strains and their 16S–23S ITS sequences used for the phylogenetic analysis are listed in Table 1. Pear = strains of pear leaf scorch; O = strains of oleander leaf scorch; C = strains of citrus variegated chlorosis and coffee leaf scorch; GM = strains of Pierce's disease of grapes, mulberry leaf scorch, and almond leaf scorch M23; PS = strains of phony peach, plum leaf scald, pecan leaf scorch, sycamore leaf scorch, and almond leaf scorch M12. The scale bar represents 0.1 substitutions/site
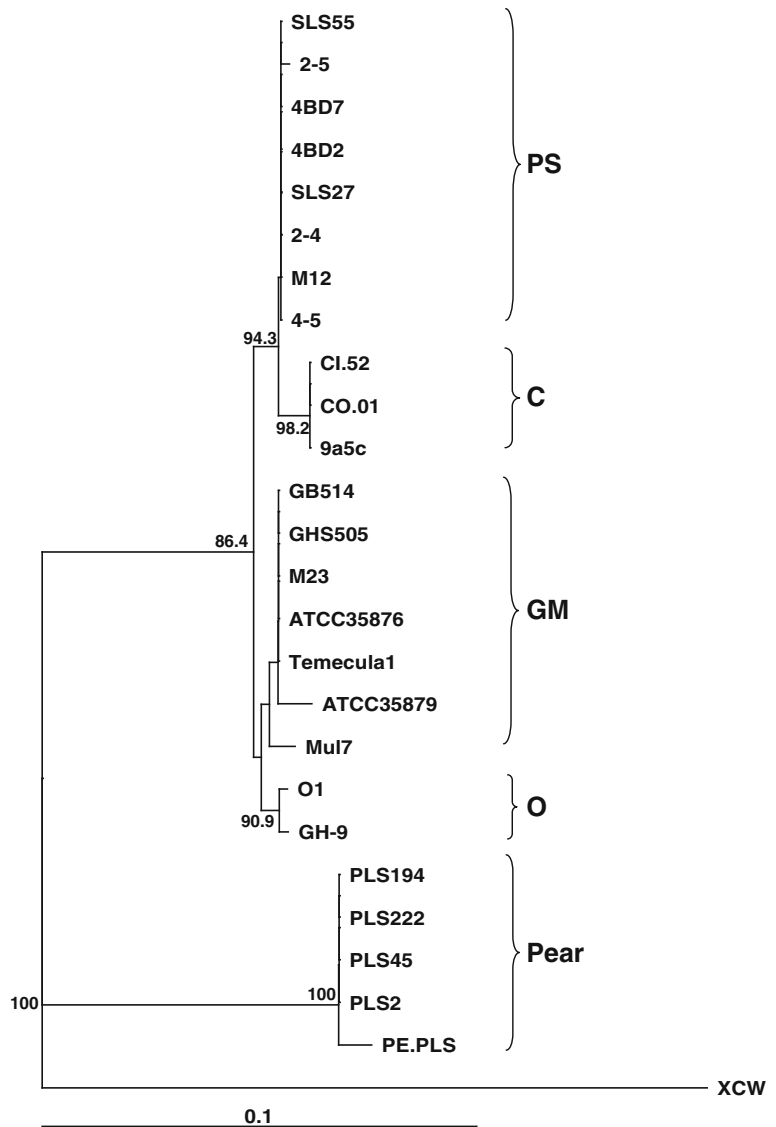
not exist in other *Xyl. fastidiosa* strains (Fig. 2), providing information for further development of specific primer(s) for the detection of pear leaf scorch strains which will be essential for the study of epidemiology of pear leaf scorch disease in Taiwan.

Xyl. fastidiosa has a wide host range (Hernandez-Martinez et al. 2007; Randall et al. 2009; Schaad et al. 2004). Most hosts were found to be infected across state lines or across oceans. For example, Pierce's disease of grapes was reported from the Americas including North, Central, and South America. There was one report describing the Pierce's disease in Kosovo (Berisha et al. 1998). It seemed the diseases

will spread if there are suitable insect vectors. It was however not the case with pear leaf scorch disease. The disease has so far been reported from Taiwan only. With work currently under investigation including the identification of the insect vectors for the transmission of the disease, the development of specific primers for the detection of XF-PLS strains, and the identification of the alternate hosts for the disease, the status of pear leaf scorch disease being the first and only caused by *Xyl. fastidiosa* in Taiwan as well as in the whole Asian Continent may change even though no major change in its status would be preferred by the pear industry in Taiwan.

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