Xylella taiwanensis sp. nov., causing pear leaf scorch disease

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A Gram-stain-negative, nutritionally fastidious bacterium (PLS229T) causing pear leaf scorch was identified in Taiwan and previously grouped into Xylella fastidiosa. Yet, significant variations between PLS229T and Xylella fastidiosa were noted. In this study, PLS229T was evaluated phenotypically and genotypically against representative strains of Xylella fastidiosa, including strains of the currently known subspecies of Xylella fastidiosa, Xylella fastidiosa subsp. multiplex and ‘Xylella fastidiosa subsp. pauca’. Because of the difficulty of in vitro culture characterization, emphases were made to utilize the available whole-genome sequence information. The average nucleotide identity (ANI) values, an alternative for DNA–DNA hybridization relatedness, between PLS229T and Xylella fastidiosa were 83.4–83.9%, significantly lower than the bacterial species threshold of 95%. In contrast, sequence similarity of 16S rRNA genes was greater than 98%, higher than the 97% threshold to justify if two bacterial strains belong to different species. The uniqueness of PLS229T was also evident by observing only about 87% similarity in the sequence of the 16S-23S internal transcribed spacer (ITS) between PLS229T and strains of Xylella fastidiosa, discovering significant single nucleotide polymorphisms at 18 randomly selected housekeeping gene loci, observing a distinct fatty acid profile for PLS229T compared with Xylella fastidiosa, and PLS229T having different observable phenotypes, such as different susceptibility to antibiotics. A phylogenetic tree derived from 16S rRNA gene sequences showed a distinct PLS229T phylectic lineage positioning it between Xylella fastidiosa and members of the genus Xanthomonas. On the basis of these data, a novel species, Xylella taiwanensis sp. nov. is proposed. The type strain is PLS229T (=BCRC 80915T=JCM 31187T).

Leu & Su (1993) described a Gram-stain-negative bacterium causing pear leaf scorch (PLS) disease in Taiwan in the area where the low-chilling pear cultivar Hengshan (Pyrus pyrifolia) was grown. Based on the nutritional fastidiousness, cell morphology and xylem-limited habitat, the bacterium was designated as a strain of Xylella fastidiosa. However, serological tests indicated that the PLS strain was not closely related to the alfalfa dwarf disease strain of Xylella fastidiosa in California, USA (Leu & Su, 1993). Later, a DNA–DNA hybridization (DDH) experiment showed less than 20% relatedness between a PLS strain and Xylella fastidiosa strains from citrus in Brazil (Mehta & Rosato, 2001). Analyses of sequences of the 16S rRNA gene and 16S-23S internal transcribed spacer (ITS) (Su et al., 2012) and randomly amplified polymorphic DNA (RAPD) profiles (Su et al., 2008) also indicated a PLS genomic group separated from the known Xylella fastidiosa strains. There

Abbreviations: ANI, average nucleotide identity; DDH, DNA-DNA hybridization; ITS, internal transcribed spacer; PD, Pierce's disease; PLS, pear leaf scorch.

Four supplementary tables are available with the online Supplementary Material.
has not yet been a systematic evaluation to determine the affiliation of the PLS strain to a novel subspecies or species.

The genus Xylella was established by Wells et al. (1987) and included a group of single, straight-rod-shaped, nutritionally fastidious plant pathogenic bacteria inhabiting the xylem tissue of plants. Currently, there is only one species, Xylella fastidiosa, in the genus Xylella, with strain PCE-RRT (=ATCC 35879T), causing Pierce’s disease (PD) of grapevine in Florida, as the type strain. Members of the species share greater than 70% DDH relatedness (Kamper et al., 1984; Wells et al., 1987) and greater than 97% similarity in 16S rRNA gene sequences (Chen et al., 2000; Mehta & Rosato, 2001). Based on DDH (greater than 84%) and sequence similarities of the 16S-23S ITS (greater than 97%) from 26 strains representing ten different plant hosts, Schaad et al. (2004) proposed three subspecies: Xylella fastidiosa subsp. fastidiosa (type strain ATCC 35879T, causing grape PD); Xylella fastidiosa subsp. multiplex (type strain ATCC 35871T, causing plum leaf scald disease/phony peach disease) and ‘Xylella fastidiosa subsp. pauca’ (proposed type strain ICPM 15198, causing citrus variegated chlorosis or CVC disease). The latter subspecies name has not yet been validly published. It is noted that the olive strain of Xylella fastidiosa found recently in Italy is related to ‘Xylella fastidiosa subsp. pauca’ (Gampetruzzi et al., 2015).

For delineation of a novel bacterial species, a DDH greater than 70% to the known bacterial species is required (Wayne et al., 1987). However, DDH experiments are labour-intensive and can be impractical for fastidious bacteria where in vitro cultivation itself is challenging. Alternatively, sequence similarity of the 16S rRNA gene can be used, but with limitations: if two strains share less than 97% 16S rRNA gene sequence similarity, they belong to different species (Stackebrandt & Goebel, 1994); however, if two strains share equal to or greater than 97% 16S rRNA gene sequence similarity, they may or may not belong to different species (Ash et al., 1991; Hauben et al., 1997; Rossello-Mora & Amann, 2001). Recently, average nucleotide identity (ANI), a value from pairwise comparison of whole-genome sequences, was developed and used as a replacement for DDH (Konstantinidis & Tiedje, 2005). An ANI value of 95–96% is considered as equivalent to the 70% DDH for species delineation (Goris et al., 2007; Kim et al., 2014; Konstantinidis & Tiedje, 2005; Richter & Rossello-Mora, 2009).

PLS229T was isolated from leaf samples exhibiting scorched symptoms in a pear orchard in Houli, Taiwan, on PD2 medium (Davis et al., 1980) using the protocol described by Leu & Su (1993). Rod-shaped bacterial cells were observed through Gram staining and by transmission electron microscopy as previously reported (Leu, 1993). For scanning electron microscopy, main and lateral vein tissues of the scorched leaves were fixed with 5% F.A.A. solution (ethanol/acetic acid/formalin, 90:5:5, by vol.) and observed with a scanning electron microscope (JEOL JSM-6330F). Rod-shaped bacterial cells in host xylem tissue are shown in Fig. 1.

For bacterial growth tests, media BCYE (Wells et al., 1981), CS-20 (Chang & Walker, 1988), PD2 (Davis et al., 1980), PD3 (Davis et al., 1981b) and PW (Davis et al., 1981a) were used. The optimum pH for growth in PD2 medium was determined following a previously described procedure (Wells et al., 1981). Amylase activity was measured by culturing PLS229T on PW agar (Davis et al., 1981a) supplemented with 1% soluble starch. Plates were heavily streaked and incubated for 10 days at 28 °C. Zones of hydrolysis indicated positive responses. Lipase was detected on PW agar supplemented with 1% Tween 80 (Smibert & Krieg, 1981). Bacillus subtilis was used as a positive reference for starch hydrolysis and the lipase assay. Coagulase was tested with commercial plasma (Difco) by the tube method (MacFaddin, 1976). Staphylococcus aureus was used as a positive reference. Beta-lactamase was detected with penicillin-starch paper strips (Oberhofer & Towle, 1982). Gelatinase was tested with Kohn gelatin-charcoal discs added to 7-day-old cultures in PW broth and incubated for 7 days (MacFaddin, 1976). Serological characteristics were determined by indirect ELISA using the procedures described by Leu & Su (1993). All test results are summarized in Table 1. For fatty acid analysis, PLS229T and a Xylella fastidiosa PD strain from Taiwan, which was identical or highly similar to the PD strain from North America (Su et al., 2013), were cultured on PD2 agar at 28 °C for 7 days. Cells were collected, saponified, methylated and analysed using an Agilent 6890N Network Gas Chromatograph system (Agilent Technologies) equipped with the Sherlock Microbial Identification System (MIDI MIS software 6.0). The results are summarized in Table 1. The percentage of octadecenoic acid (18:1) was characteristically high in PLS229T as compared with that of the PD strain. Overall, PLS229T showed differences from all Xylella fastidiosa strains. However, the slow growth rate and the requirements for special

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**Fig. 1.** Micrograph from scanning electron microscopy of cells of Xylella taiwanensis sp. nov. in the xylem of an infected pear tree in Taiwan. Bar, 1 µm.
**Table 1. Differential characteristics of strain PLS229^T and Xylella fastidiosa**

Strains: 1, *Xylella taiwanensis* sp. nov. PLS229^T causing PLS disease; 2, *Xylella fastidiosa* ATCC 35879^T (=RCE-RR^T) causing PD of grapevine. +, Positive; −, negative; †, trace amount. Data in parentheses: variations among related strains of the species. All strains were negative for growth on nutrient agar, biochemical tests to detect amylase, coagulase and lipase activity, and resistance to gentamicin (80 µg ml^{-1}). All strains were positive for growth on BCYE, PW, CS-20 and PD2 media, and the biochemical test for gelatinase activity.

<table>
<thead>
<tr>
<th>Characteristic</th>
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<tr>
<td>Growth on PD3</td>
<td>-</td>
<td>(+)</td>
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<td>Antibiotic resistance</td>
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<tr>
<td>Carbenicillin (12.5 µg ml^{-1})</td>
<td>-</td>
<td>(-)</td>
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<tr>
<td>Penicillin (6.25 µg ml^{-1})</td>
<td>+</td>
<td>(+)</td>
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<td>Beta-lactamase activity</td>
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<td>Intensity of ELISA reaction with antisera:</td>
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<td>PD</td>
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<td>+++</td>
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<td>PLS</td>
<td>++</td>
<td>+</td>
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<td>Fatty acids†</td>
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<tr>
<td>10:0 iso</td>
<td>0.86</td>
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<tr>
<td>10:0</td>
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<tr>
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<td>0.75</td>
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<tr>
<td>15:0 anteiso</td>
<td>1.01</td>
<td>11.94</td>
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<tr>
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<td>39.13</td>
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<td>17:1 9c</td>
<td>TR</td>
<td>2.65</td>
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<tr>
<td>17:1 8c</td>
<td>1.79</td>
<td>2.66</td>
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<tr>
<td>17:0 iso 3-OH</td>
<td>0.82</td>
<td>9.83</td>
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*Growth, physiological and biochemical test results were based on the results of Saddler & Bradbury (2005) and Hopkins (2001).
†The identity of the fatty acids is shown as the number of carbon atoms: number of double bonds. Fatty acids amounting to more than 0.75 \% of the total cellular fatty acids are listed.

media limited the extensive use of phenotypic tests as reflected in a previous taxonomic description (Saddler & Bradbury, 2005) and suggesting the importance of genome sequence analyses.

Whole-genome sequences of strain PLS229^T (Su et al., 2014), 17 selected *Xylella fastidiosa* strains representing the three subspecies and *Xanthomonas campestris* pv. *campestris* ATCC 33913^T (Alencar et al., 2014; Chen et al., 2010, 2013; da Silva et al., 2002; Giampetruzzi et al., 2015; Guan et al., 2014a; Guan et al., 2014b; Nunney et al., 2014; Schreiber et al., 2010; Schuenzel et al., 2005; Simpson et al., 2000; Van Sluys et al., 2003; Zhang et al., 2011; Table S1, available in the online Supplementary Material) were downloaded from the GenBank database (www.ncbi.nlm.nih.gov). The *Xanthomonas* strain was selected because of its close phylogenetic relatedness to *Xylella* (Wells et al., 1987). Related information on the bacterial genome sequences is listed in Table S1. Pairwise ANI values were calculated using JSpecies software (version 1.2.1) (Richter & Rossello-Mora, 2009) with the ANIb method (Goris et al., 2007; Konstantinidis & Tiedje, 2005). As shown in Table S2, ANI values among *Xylella fastidiosa* strains were greater than 95 \%, in agreement with their current taxonomic status as one single species, *Xylella fastidiosa*, based on the greater than 70 \% DDH relatedness (Wells et al., 1987). However, ANI values between PLS229^T and *Xylella fastidiosa* strains ranged from 83.3 to 83.9 \%, significantly below the species threshold of 95 \%. ANI values lower than 95 \% between PLS229^T and *Xylella fastidiosa* strains were also observed recently by Marcelletti & Scortichini (2016). To further confirm the low ANI values, another PLS strain, PLS15, was partially sequenced on a 454 GS-FLX system using Titanium chemistry (Roche). The partial PLS15 genome sequence had 502 252 bp in 959 contigs ranging from 250 to 1421 bp. This covered 18.4 \% of the PLS229^T genome, which was close to the suggested minimum threshold of 20 \% for taxonomic analyses (Richter & Rossello-Mora, 2009). ANI values were 99.9 \% between strains PLS15 and PLS229^T, and 85.5 to 86.1 \% between strain PLS15 and those of *Xylella fastidiosa*. ANI values between all strains representing the genus *Xylella* and *Xanthomonas campestris* pv. *campestris* ATCC 33913^T were less than 67 \%.

For 16S rRNA gene sequence analyses, sequences were extracted from the whole genome sequences of members of the genus *Xylella* (Table S1) and referenced to the annotation of *Xylella fastidiosa* M23 (identical to type strain ATCC 35879^T). Sequences were first aligned using CLUSTAL-W algorithm (Thompson et al., 1994), and pairwise p-distance (proportion difference) values were calculated through MEGAS6 software (Tamura et al., 2013). In MEGAS6, a p-distance was obtained by dividing the number of nucleotide differences over the total number of nucleotides compared. Pairwise percentage similarity (Sxy) was calculated with the formula: Sxy=1\text{–}p\text{-distance}. Similarities of 16S rRNA gene sequences between strain PLS229^T and strains of *Xylella fastidiosa* were 98.5 to 98.7 \% (Table S3), similar to a previous analysis of similarities of 98.8 to 99.0 \% between strains of PLS strains and *Xylella fastidiosa* (Su et al., 2012). At the similarity level of greater than 97 \%, two strains may or may not belong to two different species (Stackebrandt & Goebel, 1994), but could be justified to be in the same genus. An example can be found in the genus *Xanthomonas*. Although no sequence differences in 16S rRNA genes was observed between the type strains of *Xanthomonas campestris* and *Xanthomonas arboricola*, DDH values clearly indicated that these taxa were distinct species (Hauben et al., 1997).

To further demonstrate the unique lineage of PLS229^T, other genomic loci were examined. In the first experiment, the sequences of the 16S-23S ITS were extracted and compared following the procedure of 16S rRNA gene sequence...
analysis described above. As shown in Table S3, similarities between PLS229 and Xylella fastidiosa strains were 86.7 to 87.4%, whereas similarities among Xylella fastidiosa strains were greater than 97%. This was in agreement with a previous report on the significant variations (80–82%) in the 510–540 bp ITS region between five PLS strains and 20 Xylella fastidiosa strains of American origin (Su et al., 2012).

In the second experiment, DNA samples were extracted from pure cultures of nine additional PLS strains, strain M12, representing Xylella fastidiosa subsp. fastidiosa, and strain M23, only eight primer sets generated amplicons from DNA of strains M12 and M23, representing Xylella fastidiosa subsp. multiplex. Based on the complete genome sequence of strain M23, 18 housekeeping genes were randomly selected, and PCR primer sets were designed with Primer 3 software (Koressaar & Remm, 2007) (Table S4). While all 18 primer sets generated the amplicons expected from DNA of strains M12 and M23, only eight primer sets generated amplicons from DNA of PLS strains. Sequences at each primer locus were hybridized the amplicons expected from DNA of strains M12 and M23. Single nucleotide polymorphisms (SNPs) were found in the primer sequences. Failures in PCR amplifications of ten primer sets were due to the presence of two or more SNPs within the last three nucleotides at the 3’ ends of at least one primer sequence.

For the eight primer sets that amplified both PLS and Xylella fastidiosa strains, amplicons were sequenced in both orientations using a BigDye Terminator v3.1 Cycle Sequencing kit in a 3130xl Genetic Analyzer (Applied Biosystems) with the same primers used for PCR. Values of p-distance among PLS strains and between PLS, M23 and M12 strains were calculated using MEGA6 software. Table S4 summarizes the p-distance values among nine PLS strains and their comparisons with M12 (Xylella fastidiosa subsp. multiplex) and M23 (Xylella fastidiosa subsp. fastidiosa) at eight selected loci. Although varying from locus to locus, the p-distances among PLS strains were either equal or much smaller than those between strains of PLS and M12 or those between strains of PLS and M23.

Interestingly, as shown in Table S2, using the threshold of ANI greater than 99%, six groups of Xylella fastidiosa strains were identified. Referenced to type strains, Group 1 belonged to Xylella fastidiosa subsp. fastidiosa (ATCC 35879) and Group 4 was Xylella fastidiosa subsp. multiplex (ATCC 35871). Groups 2 and 3 fell in between but slightly closer to Xylella fastidiosa subsp. fastidiosa. The same trends were seen with 16S rRNA gene and 16S-23S ITS data using neighbour-joining method. Bootstrap values are listed at nodes. Number of bootstrap replications is 1000. Bar, 0.005 substitutions per nucleotide position.
‘Xylella fastidiosa subsp. pauca’, the sequence of ICPM 15198 (proposed type strain of ‘Xylella fastidiosa subsp. pauca’) was not available. If strain 9a5c was considered, Group 5 with two Brazilian strains was ‘Xylella fastidiosa subsp. pauca’. Group 6 was related to Group 5 but different. Of particular interest was that strain CoDiRO in Group 6 was an olive strain from Italy (Giampetruzzi et al., 2015). Unlike ANI data, similarities of 16S rRNA gene sequences showed that Groups 5 and 6 were identical, although 16S-235 ITS data showed a variation in strain 6c. Regardless, all these seem to suggest that ANI value and sequence similarity of the 16S rRNA gene and 16S-235 ITS could be useful for subspecies analysis. Yet, this was beyond the scope of this study.

A phylogenetic tree was reconstructed through MEGA6 software with 16S rRNA gene sequences using the neighbour-joining method with Xanthomonas campestris pv. campestris strain ATCC 33913T as an outgroup. The results showed that PLS229T formed a distinct phyletic lineage between Xylella fastidiosa and Xanthomonas campestris pv. campestris (Fig. 2). It was concluded that because of the high similarity in 16S rRNA gene sequence, PLS229T should be proposed to be retained in the genus Xylella. Because of its unique genomic and phenotypic features to Xylella fastidiosa strains, however, PLS229T is proposed to belong to a novel species, Xylella taiwanensis sp. nov.

Description of Xylella taiwanensis sp. nov.

Xylella taiwanensis (Taiwan. en’sis. N. L. fem. adj. taiwanensis pertaining to Taiwan, where the type strain was isolated). The species characteristics include those of the genus Xylella (Wells et al., 1987). Strains of Xylella taiwanensis can be cultured on BCYE, CS-20, PD2 and PW media but not on PD3 or general-purpose bacterial media. The bacterium is rod-shaped, and its cell size measures 0.2 to 0.5×1.1 to 3.4 µm. Colonies are convex, roundish and creamy-white with a smooth margin, and can reach 0.1–0.2 mm in diameter after incubation for 14 days at 30 °C. Resistant to penicillin (6.25 µg ml⁻¹) but sensitive to carbenicillin (12.5 µg ml⁻¹) and gentamicin (80 µg ml⁻¹). Gelatinase and beta-lactamase activities are positive. Coagulase, amylase and lipase activities are negative. Phylogenetically, Xylella taiwanensis positions between Xylella fastidiosa and the genus Xanthomonas (Fig. 2).

The type strain is PLS229T (=BCRC 80915T=JCM 31187T) and was isolated from tissue of pear (Pyrus pyrifolia) cultivar Hengshan showing leaf scorch symptoms in Houli, Taiwan.

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