

# Phylogenetic relationships of *Xylella fastidiosa* strains from different hosts, based on 16S rDNA and 16S–23S intergenic spacer sequences

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**The phylogenetic relationships of *Xylella fastidiosa* strains isolated from different hosts, including citrus trees, coffee, grapevine, plum and pear, were inferred by sequence analysis of the 16S rDNA and 16S–23S intergenic spacer region. A high level of similarity (97.1–100%) was found in the 16S rDNA of the *Xylella fastidiosa* strains. The 16S–23S region showed a higher level of variation, with similarity values ranging from 79.8 to 100%. Two tRNAs (tRNA<sup>Ala</sup> and tRNA<sup>Ile</sup>) were encountered within the spacer sequence. The phylogenetic trees, constructed using the neighbour-joining method, showed that the citrus, coffee, peach and plum strains were closely related and separate from grapevine strains. The pear strain remained isolated from all the other *Xylella* strains in both analyses and produced values of less than 20% in DNA–DNA hybridization experiments with a citrus strain. These results show that this strain does not belong to the *Xylella fastidiosa* genomic species.**

**Keywords:** *Xylella fastidiosa*, rDNA, DNA–DNA hybridization

## INTRODUCTION

*Xylella fastidiosa* is a xylem-limited bacterium responsible for diseases in many economically important crops such as grapevine, peach, plum, pear and, more recently, citrus trees and coffee (for a review, see Hopkins, 1977). This Gram-negative, slow-growing bacterium has a wide host range and is transmitted by grafting and via leafhopper vectors (Purcell, 1990). *Xylella fastidiosa* was first identified in 1973, causing Pierce's disease of grapevine; it is responsible for heavy losses in this crop (Chen *et al.*, 1995; Hopkins, 1977). Recently, citrus variegated chlorosis (CVC) (Chang *et al.*, 1993; Rossetti *et al.*, 1990) and coffee leaf scorch (CLS) (Paradela Filho *et al.*, 1997), also caused by *Xylella fastidiosa*, have been reported in Brazil.

Many molecular techniques have been used to characterize *Xylella fastidiosa* strains and different groups have been distinguished (Chen *et al.*, 1992, 1995; Pooler & Hartung, 1995; Rosato *et al.*, 1998). However, information regarding the phylogenetic relation-

ships and genetic relatedness of strains of *Xylella fastidiosa* from recently reported diseases remains limited.

rDNA has been used widely to infer phylogenetic relationships among micro-organisms (Woese, 1987). Sequence analyses of the small subunit, 16S rRNA, has been used frequently as a powerful and accurate method for determining inter- and intraspecific relationships (Leblond-Bourget *et al.*, 1996). However, as evolutionary distances decrease, the diversity found in the 16S rRNA gene is often insufficient and thus the genetic relationships of closely related species cannot be accurately defined (Rogall *et al.*, 1990). It has been proposed that sequencing of the 16S–23S intergenic spacer region could overcome this problem because of its higher variation in length and sequence. Indeed, analysis of this region has successfully differentiated strains of many groups of bacteria, and sequences of the 16S–23S regions of many species have become available for comparison (Leblond-Bourget *et al.*, 1996; Yoon *et al.*, 1997).

In this study, we have sequenced the 16S rDNA and 16S–23S intergenic spacer of *Xylella fastidiosa* strains in an attempt to determine the phylogenetic relationships among strains from different hosts and between *Xylella fastidiosa* and related species. We have

**Abbreviations:** CLS, coffee leaf scorch; CVC, citrus variegated chlorosis; PLS, plum leaf scald; RAPD, random amplified polymorphic DNA.

The GenBank accession numbers for the 16S rDNA and 16S–23S intergenic spacer sequences of *Xylella fastidiosa* strains described in this work are AF203388–AF203397 and AF237650–AF237651.

also performed DNA–DNA hybridization in order to determine the taxonomic level of the *Xylella fastidiosa* strains isolated from different hosts.

## METHODS

**Bacterial strains and culture conditions.** Strains of *Xylella fastidiosa* isolated from five different hosts, including citrus species, coffee, grapevine, plum and pear, were used in this study (Table 1). Strains used in the DNA–DNA

hybridization study were selected on the basis of an earlier analysis of genetic diversity by random amplified polymorphic DNA (RAPD) analysis and rep-PCR, whereby different clusters were obtained (Rosato *et al.*, 1998; Mehta *et al.*, 1999a, b). Strains used for sequencing were chosen on the basis of different groups observed by PCR–RFLP of the 16S rDNA and the 16S–23S intergenic spacer (Rosato *et al.*, 1998; Mehta *et al.*, 1999a, c). In all analyses, the type strain of *Xylella fastidiosa*, GR.8935<sup>T</sup> (= ATCC 35879<sup>T</sup>), which corresponds to strain PCE-RR (Wells *et al.*, 1987), was used.

**Table 1** Bacterial strains used in this study and GenBank accession numbers for 16S rDNA and 16S–23S intergenic spacer sequences

Abbreviations for sources: IAPAR, Instituto Agronômico do Paraná, Londrina, PR, Brazil; IBSBF, Instituto Biológico, Seção de Bacteriologia Fitopatológica Campinas, SP, Brazil; Fundecitrus, Fundo Paulista de Defesa da Citricultura, Araraquara, SP, Brazil.

Taxon	Strain	Host	GenBank accession no.		Source or reference
			16S	16S–23S	
<i>Xylella fastidiosa</i>	CI.11380*	Citrus			IAPAR
<i>Xylella fastidiosa</i>	CI.11039*	Citrus			IAPAR
<i>Xylella fastidiosa</i>	CO.11752*	Coffee			IAPAR
<i>Xylella fastidiosa</i>	CO.12288*	Coffee			IAPAR
<i>Xylella fastidiosa</i>	GR.9713*	Grapevine			IAPAR
<i>Xylella fastidiosa</i>	PL.9746*	Plum			IAPAR
<i>Xylella fastidiosa</i>	PL.788 (ATCC 35871)*†	Plum	AF203388	AF203395	IBSBF
<i>Xylella fastidiosa</i>	GR.8935 <sup>T</sup> (ATCC 35879 <sup>T</sup> )*†	Grapevine	AF203391	AF203397	IAPAR
<i>Xylella fastidiosa</i>	PE.PLS*†	Pear	AF203392	AF203396	IBSBF (Leu & Su, 1993)
<i>Xylella fastidiosa</i>	CI.11067*†	Citrus		AF237650	IAPAR
<i>Xylella fastidiosa</i>	CI.X0*†	Citrus		AF237651	Chang <i>et al.</i> (1993)
<i>Xylella fastidiosa</i>	CI.52†	Citrus	AF203389	AF203393	Fundecitrus
<i>Xylella fastidiosa</i>	CO.01†	Coffee	AF203390	AF203394	Fundecitrus
<i>Xylella fastidiosa</i>	PP4–5	Unknown	AF159580		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	CVC93–2	Citrus	AF159575		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	PD28–5	Grapevine	AF159574		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	PWT-22	Periwinkle	AF159578		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	PLS2–9	Plum	AF159579		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	Mul-2	Mulberry	AF159576		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	PCE-FG	Grapevine	AF159572		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	r116v11	Grapevine	AF159573		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	OSL92–3	Oak	AF159577		Chen <i>et al.</i> (2000)
<i>Xylella fastidiosa</i>	5s2	Peach		AF073206	Purcell <i>et al.</i> (1999)
<i>Xylella fastidiosa</i>	88.9	Oak		AF073210	Purcell <i>et al.</i> (1999)
<i>Xylella fastidiosa</i>	am	<i>Acer macrophila</i>		AF073219	Purcell <i>et al.</i> (1999)
<i>Xylella fastidiosa</i>	ann1 (ATCC 700598)	Oleander		AF073215	Purcell <i>et al.</i> (1999)
<i>Pseudomonas boreopolis</i>			AB021391		
<i>Xanthomonas campestris</i>			AF123092	AF209755	
<i>Xanthomonas axonopodis</i>			AF123090	AF209753	
<i>Xanthomonas vesicatoria</i>			AF123089	AF123088	
<i>Xanthomonas pisi</i>			Y10758	AF209761	
<i>Xanthomonas hyacinthi</i>			Y10754	AF209759	
<i>Xanthomonas sacchari</i>			Y10766	AF209762	

\* Strains used for DNA–DNA hybridization.

† Strains sequenced in this study.

Citrus, coffee and plum strains from different geographical regions were included in this study. Strains CI.11067, CO.12288 and PL.9746 were isolated in the state of Paraná (in the south of Brazil), strain CI.11380 was from the state of Santa Catarina (in the south of Brazil) and strains CI.11039, CI.X0, CI.52, CO.01 and CO.11752 were from the state of São Paulo (in the south-east of Brazil). The plum strain PL.788, which corresponds to strain PLM-G83 (Wells *et al.*, 1987), and the grapevine strains GR.8935<sup>T</sup> and GR.9713 were isolated in the USA, and the pear strain PE.PLS was isolated in Taiwan. The strains were cultivated on solid buffered cysteine/yeast extract (BCYE) medium (Wells *et al.*, 1981a) for a period of approximately 10 d at 28 °C. For long-term storage, bacterial cells were harvested from Petri plates and maintained at -70 °C in Pierce's disease medium no. 3 (PD3) (Davis *et al.*, 1980a) containing 30% glycerol.

**DNA extraction.** Cells were scraped from the BCYE plates, washed with TAS buffer (50 mM Tris/HCl, 50 mM EDTA, 150 mM NaCl, pH 8.0) and resuspended in 450 µl of the same buffer. SDS (final concentration 1%) and proteinase K (150 µg ml<sup>-1</sup>) were added and the tubes were incubated for 1 h at 50 °C. Cell debris was removed by phenol/chloroform extraction. The suspension was dialysed against TE buffer (40 mM Tris/HCl, 1 mM EDTA, pH 7.8) for 48 h.

**DNA-DNA hybridization.** The hybridization was performed using the Hybri slot blot manifold, as described by the manufacturer (Bio-Rad), with nylon membrane (Amersham). The probes were labelled with digoxigenin, using the DIG DNA labelling kit (Boehringer Mannheim). A hybridization buffer containing 50% formamide, 5 × SSC (0.15 M sodium citrate; 1.5 M NaCl, pH 7.0), *N*-laurylsarcosine (0.1%), SDS (0.02%) and blocking reagent (2%) was used and the hybridization was performed overnight at 42 °C. The membranes were washed at 68 °C in a solution containing 0.1 × SSC and 0.1% SDS. The detection of bands was carried out using the chemiluminescent substrate CSPD [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2''-(5''-chloro)tricyclo[3.3.1.1<sup>3,7</sup>]decan}-4-yl) phenyl phosphate] as recommended by the manufacturer (Boehringer Mannheim). The signal, detected by exposure to X-ray film (Kodak), was measured by using an Ultrascan XL-Enhancer laser densitometer (LKB).

**Amplification of the 16S rDNA and 16S-23S spacer.** The 16S rRNA gene was amplified by using primers 27f (5'-AGAG-TTTGATCMTGGCTCAG-3') and 1525r (5'-AAGGAG-GTGWTCARCC-3') (Lane, 1991). Primers 16S uni1330 (5'-GTTCCCGGGCCTTGACACAC-3') and 23S uni322 anti (5'-GGTCTTTTCGCCTTTCCTC-3') from conserved regions of *Xanthomonas* spp. (Honeycutt *et al.*, 1995), which are phylogenetically related to *Xylella fastidiosa* (Wells *et al.*, 1987), were used to amplify the 16S-23S region.

Amplification of the 16S rDNA genes and 16S-23S spacers of *Xylella fastidiosa* strains from different hosts was performed in a Thermal Cycler 4800 (Perkin Elmer) in a total volume of 25 µl containing 30-50 ng DNA, 0.5 µM of each primer, 100 µM dNTP, 2.5 mM MgCl<sub>2</sub> and 0.5 U *Taq* DNA polymerase (Amersham-Pharmacia). PCR was performed under the following conditions: for 16S rDNA, 1 min at 94 °C and then 40 cycles of 1 min at 94 °C, 1 min at 65 °C and 2 min at 72 °C; for the 16S-23S region, 4 min at 94 °C and then 40 cycles of 30 s at 94 °C, 30 s at 65 °C and 1 min at 72 °C.

**Cloning and sequencing methods.** The amplified 16S rDNA and 16S-23S intergenic spacer of *Xylella fastidiosa* strains

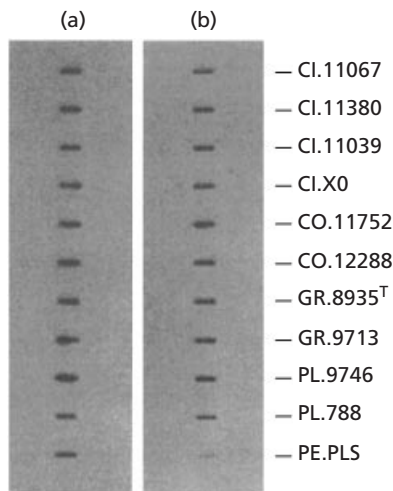
were cloned into pBluescript II KS(+) (Stratagene) or pGEM (Promega) cloning vectors. Subcloning was performed when necessary. Plasmid DNA of the clones was extracted using the Nucleospin nucleic acid purification kit (Clontech) and used in sequencing reactions. The PCRs for sequencing were performed in a total volume of 10 µl containing 300-500 ng DNA, 5 pmol primer (M13 forward or M13 reverse), 3 µl buffer (20 mM Tris/HCl, pH 9.0, 5 mM MgCl<sub>2</sub>) and 1 µl of the ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer). The reactions were conducted with an initial denaturation for 5 min at 95 °C, followed by 25 cycles of denaturation for 1 min at 95 °C, annealing for 10 s at 55 °C and extension for 4 min at 65 °C. The products were precipitated with 80 µl 2-propanol (75%) and washed with ethanol (70%). Sequencing was performed in an automatic sequencer (ABI PRISM 377; Perkin Elmer) and repeated twice for all clones.

**Sequence analysis.** The 16S rDNA and 16S-23S spacer of *Xylella fastidiosa* strains sequenced in this study, as well as other *Xylella fastidiosa* sequences available in the GenBank database, were used for comparison. Sequences of *Xanthomonas* species representing the different cores obtained in the analysis of the 16S rDNA (Hauben *et al.*, 1997) and 16S-23S spacer (E. R. Gonçalves and Y. B. Rosato, unpublished) were also included in the analysis. The accession numbers are given in Table 1. The sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) and phylogenetic trees were constructed using the p-distance with the neighbour-joining method with the MEGA package (Kumar *et al.*, 1993). Bootstrap analysis with 1000 replications was performed to provide support for the branches of the phylogenetic trees obtained.

## RESULTS AND DISCUSSION

### DNA-DNA hybridization

Eleven *Xylella fastidiosa* strains (CI.11067, CI.11380, CI.11039, CI.X0, CO.11752, CO.12288, GR.8935<sup>T</sup>, GR.9713, PL.9746, PL.788 and PE.PLS) isolated from five different hosts (Table 1), representing the different clusters obtained by rep-PCR and RAPD analysis (Mehta *et al.*, 1999a, b), were investigated by DNA-DNA hybridization. A presumed DNA amount of 100 ng, estimated by agarose gel electrophoresis, was placed onto nylon membranes and the final amount was calculated through DNA hybridization with digoxigenin-labelled 16S rDNA of *Xanthomonas* as a probe (Fig. 1a). It was assumed that the homology of the 16S rDNA was equivalent in all *Xylella* strains (this was later confirmed by sequencing the 16S rDNA of four of the 11 strains used in this method). Therefore, observed differences in the intensity of the hybridization signal with the 16S rDNA of *Xanthomonas* as probe would indicate different amounts of DNA. The intensity of the hybridization bands obtained was read in a densitometer and a band with a defined amount of DNA was considered as a control. For example, a reading of 10.3 in the densitometer, corresponding to the band of strain CO.12288, was assumed to represent 100 ng DNA. All other readings were corrected accordingly. The membranes were washed and subjected to another



**Fig. 1.** DNA-DNA hybridization using genomic DNA of *Xanthomonas fastidiosa* strains from different hosts and *Xanthomonas* 16S rDNA (a) and DNA of the *Xylella fastidiosa* citrus strain CI.11067 (b) as probes.

hybridization using DNA of the citrus strain CI.11067 as a probe (Fig. 1b).

The levels of homology presented by strains from citrus, coffee, grapevine and plum were considered high, ranging from 85 to 140%. Unexpectedly, homology levels above 100% were obtained for some strains and may be due to limitations in the quantification process. The procedure was performed in a densitometer, which detects a restricted region of the slot and thus could produce results that may not be completely accurate. Similar results were obtained by Gonçalves & Rosato (2000), who observed homology values between 73 and 160% when analysing *Xanthomonas axonopodis* pv. *passiflorae*. It seems reasonable to assume that the method used in this study produces more accurate results when distantly related organisms are compared. Although variation was observed in this study, it did not seem to interfere with the final results, which were consistent in both repetitions performed.

The results obtained in this study show that *Xylella fastidiosa* strains isolated from citrus, coffee, grapevine and plum belong to the same DNA-homology group and therefore to the same genomic species. The present findings confirm the homology levels (above 85%) obtained by Wells *et al.* (1987) in the analysis of *Xylella fastidiosa* strains isolated from different hosts (grapevine, peach, periwinkle, almond, plum, elm, sycamore, oak, ragweed and mulberry). Using the same approach, Kamper *et al.* (1985) analysed five strains of *Xylella fastidiosa* from plum, peach, periwinkle (*Vinca* sp.) and grapevine, and the level of DNA homology ranged from 75 to 100%.

A striking result, however, was obtained for the pear strain PE.PLS, which presented low homology with

citrus strain CI.11067 (below 20%). This result was consistent in all repetitions, even when DNA of the grapevine strain GR.8935<sup>T</sup> was used as a probe (data not shown). Pear strains were identified as *Xylella fastidiosa* by several characteristics such as the presence of rippled cell walls, rod-shaped cells and growth fastidiousness (Leu & Su, 1993); however, the homology results suggest strongly that strain PE.PLS does not belong to the species *Xylella fastidiosa*. Previous reports also described a low level of similarity between strain PE.PLS and other *Xylella fastidiosa* strains in genetic diversity studies using rep-PCR and RAPD fingerprinting (Mehta *et al.*, 1999a, b) and in serological analyses (Leu & Su, 1993). Pear leaf scorch is the second disease reported, outside America, to be caused by *Xylella fastidiosa*, following the report of almond leaf scorch in India (Jindal & Sharma, 1987). Strains from this host have not been isolated elsewhere. Further studies need to be performed with a larger number of strains in order to confirm the existence of a second homology group within the *Xylella* genus and to confirm the relationships between strains isolated from pear and *Xylella fastidiosa* strains from other hosts.

#### Comparison of the 16S rDNA sequences

The 16S rRNA genes of five *Xylella fastidiosa* strains isolated from citrus (CI.52), coffee (CO.01), plum (PL.788), grapevine (GR.8935<sup>T</sup>) and pear (PE.PLS) were sequenced and all the strains yielded sequences comprising 1536 bases. The citrus and coffee strains showed identical sequences, whereas small numbers of nucleotide substitutions (two and four, respectively) were detected in the plum and grapevine *Xylella fastidiosa* sequences in relation to the citrus *Xylella fastidiosa* sequence. A larger number of alterations (20) was revealed in the sequence from the pear strain, however. Most of these alterations consisted of base substitutions (13 transitions and six transversions).

The five *Xylella fastidiosa* 16S rDNA sequences were also aligned with nine other *Xylella fastidiosa* sequences available in GenBank (Table 1). Signature sequences corresponding to the *Escherichia coli* positions 170 (CTAATACCG), 315 (YCACAYYG), 510 (CTAACTYYG) and 1410 (TCACACCATG) (Stackebrandt *et al.*, 1988) were found in the *Xylella fastidiosa* 16S rDNA sequences analysed in this study, confirming that the strains belong to the  $\gamma$ -*Proteobacteria*, as did the other *Xylella fastidiosa* strains studied previously by Wells *et al.* (1987).

Similarity percentages within 16S rDNA sequences of *Xylella fastidiosa* ranged from 97.1 to 100%, which shows the high degree of conservation in this sequence. The lowest level of similarity (97.1%) was found between the pear strain PE.PLS and the oak strain OLS92-3. These findings agree with other reports, which describe a low level of divergence in the 16S rRNA gene, even among distinct species. Leblond-Bourget *et al.* (1996) analysed the 16S rDNA sequences

of 18 different species of *Bifidobacterium* and encountered a similarity level of 92–99%. Similarly, a mean similarity value of 98.2% was found within the genus *Xanthomonas* (Hauben *et al.*, 1997).

The high level of similarity to other strains of the 16S rDNA sequence of the pear strain (97.1–98.5%) and the low DNA–DNA homology (below 20%) found between the pear strain and a citrus strain obtained in this study resemble the results found for the genus *Xanthomonas*, in which some strains showing extremely low levels of DNA–DNA homology did not necessarily exhibit low levels of 16S rDNA similarity (Hauben *et al.*, 1997).

A striking similarity of 95% was found between the *Xylella fastidiosa* 16S rDNA sequence and that of *Pseudomonas boreopolis*, a bacterium isolated from the soil. There is, however, some uncertainty about the taxonomic position of *P. boreopolis*, which could be assigned to the genus *Xanthomonas* on the basis of DNA–rDNA hybridization (De Vos *et al.*, 1989). The similarity levels between *Xanthomonas* species and *Xylella fastidiosa* strains ranged from 94.3 to 96.1% and the highest similarity value was found between *Xanthomonas campestris* and strain PE.PLS.

#### Analysis of the 16S–23S spacer region

The sequences of the 16S–23S intergenic spacer regions of strains CI.52, CI.X0, CI.11067, CO.01, GR.8935<sup>T</sup>, PL.788 and PE.PLS of *Xylella fastidiosa* were also determined from the 1.1 kb fragment yielded in the PCR. It was expected that a higher level of variation would be found among the 16S–23S sequences and thus the spacer sequences of two additional citrus strains were determined, including that from strain CI.X0 (used for the sequencing of the genome). The entire fragment was sequenced in both directions and the ends, corresponding to parts of the 16S and 23S rRNA genes, were eliminated according to other similar 16S–23S sequences deposited in GenBank. After the exclusion of the flanking ends, a stretch of 511–523 bases was obtained. Comparisons of the seven spacer sequences obtained in this study with other *Xylella fastidiosa* sequences deposited in GenBank revealed similarity levels ranging from 79.8 to 100%. Most strains showed similarities of 97–100%, but the values were greatly reduced when the pear *Xylella fastidiosa* sequence was compared (81.3–79.8%). The lowest similarity was observed between pear (PE.PLS) and oak (88.9) strains. Analysis of most *Xylella* sequences revealed some variable sites, 17 in the entire sequence, which included base additions/deletions or substitutions. The most striking differences were observed in relation to the pear *Xylella fastidiosa* sequence (29 base additions and 18 deletions, both occurring in blocks of nucleotides or as single alterations). In addition, 42 substitutions specific to this sequence were found.

A high level of variation has been reported in the 16S–23S spacers of closely related taxa (Normand *et*

*al.*, 1996). However, the results obtained in the sequence analysis of *Xylella fastidiosa* showed that, for most strains, the number of isolated substitutions found in the 16S rRNA gene did not differ appreciably from that found in the 16S–23S spacer, as would be expected. Similar results were obtained by Luz *et al.* (1998) while analysing *Salmonella enterica* subspecies. The same authors reported that the main differences consisted of large insertions and/or deletions, as detected in this study for the pear *Xylella fastidiosa* sequence. All these alterations show the divergence of the pear strain from *Xylella* strains from other hosts and thus support the classification of this strain into a different species.

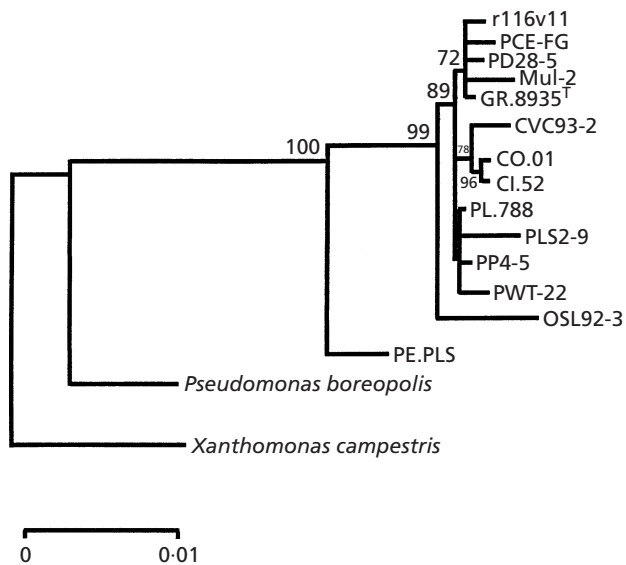
When *Xylella fastidiosa* sequences were compared with those from *Xanthomonas* spp. (Table 1), similarity levels ranging from 59.3 to 70.6% were obtained (data not shown). *Xanthomonas campestris* was again the species closest to *Xylella fastidiosa*. Regions specific to *Xylella* were revealed along the sequence at positions 47–50, 121–123, 137 and 304–318 of the citrus *Xylella fastidiosa* sequence. These sequences were present in all of the *Xylella* strains and absent in the *Xanthomonas* strains used, showing the specificity of these sequences within the genus.

The 16S–23S sequences of all the *Xylella* strains analysed revealed two tRNAs (tRNA<sup>Ala</sup> and tRNA<sup>Ile</sup>) found in most spacer regions in Gram-negative bacteria (Gürtler & Stanisich, 1996). The tRNA<sup>Ala</sup> and tRNA<sup>Ile</sup> were 76 and 77 bp long (76 bp for strain GR.8935<sup>T</sup>) and used the UGC and GAU anticodons, respectively. Except in the case of the pear sequence, tRNA<sup>Ala</sup> was highly conserved in most *Xylella* sequences, whereas a few changes were detected in the tRNA<sup>Ile</sup>, including one deletion in the grapevine sequence and one substitution in the coffee sequence.

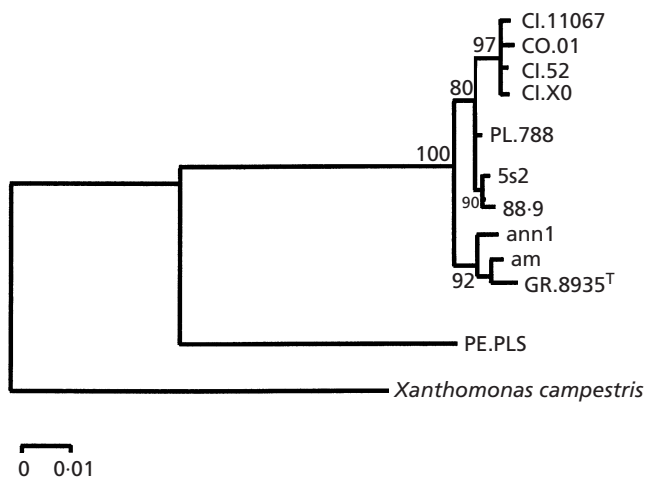
#### Phylogenetic analysis

A phylogenetic tree was constructed using the 16S rDNA of the *Xylella fastidiosa* strains sequenced in this study and those available in GenBank, as well as the 16S rDNA sequence of *P. boreopolis*. *Xanthomonas campestris* was used as the outgroup strain (Fig. 2). The tree obtained revealed two major clusters, if bootstrap values higher than 70% were considered. Cluster I comprised grapevine strains (GR.8935<sup>T</sup>, PCE-FG and r116v11) and the mulberry strain Mul-2, whereas cluster II included strains isolated from citrus (CI.52, CVC93-2), coffee (CO.01), plum (PL.788 and PLS2-9), periwinkle (PWT-22) and peach (PP4-5). Two other minor clusters were formed by single strains from oak (OSL92-3) and pear (PE.PLS). The *Xylella fastidiosa* group was close to *P. boreopolis*, which is possibly a *Xanthomonas* species, and to *Xanthomonas campestris*, confirming earlier results (Wells *et al.*, 1987) that showed that *Xanthomonas* was the genus closest to *Xylella*.

The 16S–23S intergenic spacers of the seven *Xylella fastidiosa* strains sequenced and those available in



**Fig. 2.** Phylogenetic tree constructed using the neighbour-joining method, based on 16S rDNA sequence data for *Xylella fastidiosa* and *Pseudomonas boreopolis*, with *Xanthomonas campestris* as the outgroup. Gaps and missing information were excluded from the analysis. The numbers above the branches are bootstrap values obtained for 1000 replications (expressed as percentages; only values greater than 70% are shown). Bar, 1% sequence divergence.



**Fig. 3.** Phylogenetic tree constructed using the neighbour-joining method, based on 16S–23S intergenic spacer sequence data for *Xylella fastidiosa*, with *Xanthomonas campestris* as the outgroup. Gaps and missing information were excluded from the analysis. The numbers above the branches are bootstrap values obtained for 1000 replications (expressed as percentages; only values greater than 70% are shown). Bar, 1% sequence divergence.

GenBank were used to construct a phylogenetic tree (Fig. 3). *Xanthomonas campestris* was used as the outgroup strain. The *Xylella fastidiosa* strains were grouped into two major clusters. Cluster I comprised strains from citrus (CI.52, CI.11067 and CI.X0), coffee

(CO.01), plum (PL.788), peach (5S2) and oak (88.9). Strains from *Acer macrophila* (am), oleander (ann1) and grapevine (GR.8935<sup>T</sup>) formed cluster II. The pear strain remained separate from the other *Xylella* strains.

The topology of the phylogenetic tree obtained with the 16S–23S spacer was comparable to that obtained using the 16S rDNA data, although different strains were used in each case. The citrus, coffee, peach and plum strains were related and separate from the grapevine strains. The pear strain was remarkably separate from other *Xylella* strains in both trees. An inconsistency, however, was found for the oak strains. In the 16S rDNA tree, the oak strain OSL92-3 was well separated from the other strains (bootstrap value of 98%), whereas, in the 16S–23S spacer tree, oak strain 88.9 was included in cluster I among strains from other hosts. At this stage, we do not have sufficient information about these strains to confirm the degree of divergence among oak strains and *Xylella fastidiosa* from other hosts, since these sequences were accessed from GenBank.

It has been suggested that enough variability exists among strains of *Xylella fastidiosa* to justify a taxonomic separation at the subspecies or pathovar level (Hopkins, 1989). In this study, we have shown that such variation exists even when highly conserved genes such as 16S rDNA are compared. The distinction between plum/peach strains and grapevine strains on the basis of 16S rDNA and 16S–23S sequences seems consistent with other studies using growth characteristics (Wells *et al.*, 1987) and pathogenicity (Hopkins, 1989). In addition, Kamper *et al.* (1985), using DNA hybridization studies, considered that the level of homology (75%) found between Pierce's disease and plum leaf scald (PLS) strains was low enough to support distinction at the varietal and, possibly, subspecies level. The relatedness of the plum strain to citrus and coffee strains found in both phylogenetic trees was, however, unknown. PLS was the first disease caused by *Xylella fastidiosa* to be reported in the southern part of Brazil (French & Kitajima, 1978). The similarity between the plum strains and the citrus/coffee strains may indicate a common origin for the CVC, CLS and PLS bacteria. Although this hypothesis is speculative, it cannot be ruled out, since single strains of *Xylella fastidiosa* have been reported to cause diseases in various hosts (Davis *et al.*, 1980b; Wells *et al.*, 1981b). Cross-inoculation tests need to be performed in order to obtain more information regarding the specificity of pathogenicity among the plum, citrus and coffee strains and to confirm the relatedness among these strains.

A consistent result found with all three techniques used was that the pear strain does not belong to the species *Xylella fastidiosa*. Although striking differences were found for this strain with all methods, the results suggest that it belongs to the genus *Xylella*. Unfortunately, only one strain was available for this study and thus an extension of these conclusions to all pear strains would be premature. However, the results

obtained herein constitute a basis for future studies relating to the proposal of a new *Xylella* species.

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