A Systematic Study Reveals that *Xylella fastidiosa* Strains from Pecan Are Part of *X. fastidiosa* subsp. *multiplex*

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Abstract

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Xylella fastidiosa causes disease in a number of economically important crops, ornamental plants, and shade trees, including grapevine, citrus, oleander, and sycamore. In pecan, *X. fastidiosa* causes pecan bacterial leaf scorch (PBLS), which leads to defoliation and reduces nut yield. No economically effective treatments are available for PBLS. In order to improve PBLS management practices, it is necessary to determine the subspecies of *X. fastidiosa* strains that infect pecan so that potential sources of inoculum may be identified. Multiprimer polymerase chain reaction (PCR) and phylogenetic analyses using nucleotide sequence data from the 16S-23S rRNA intergenic transcribed spacer (ITS) region and *pglA* consistently identified strains of *X. fastidiosa* isolated from pecan as *X. fastidiosa* subsp. *multiplex.* Enterobacterial repetitive intergenic consensus PCR and repetitive extragenic palindromic (REP)-PCR analyses were congruent with phylogenetic analyses. REP-PCR analyses indicated genetic variation within strains of *X. fastidiosa* from pecan. From these same analyses, *X. fastidiosa* strains from sycamore, grapevine, and oleander from Louisiana were identified as subsp. *multiplex*, subsp. *fastidiosa*, and subsp. *sandyi*, respectively. This study provides additional information about the host ranges of *X. fastidiosa* subspecies.

Xylella fastidiosa is a gram-negative, xylem-limited, fastidious bacterium that causes disease in a number of economically important crops, ornamental plants, and shade trees, including grapevine, peach, citrus, oleander, and sycamore (6,14,39,58,67). Symptoms of disease in these hosts typically include scorching of leaves, reduced growth, defoliation, plant death, or chlorosis (8,23,39, 46,58,59). Insect transmission by xylem-feeding insects in the families Cercopidae and Cicadellidae is considered the primary means of transmission of *X. fastidiosa* from infected to noninfected plant hosts (41); however, other forms of transmission, including graft transmission and seed transmission, have also been reported in some hosts (7,30,41,45).

Five subspecies of *X. fastidiosa* have been determined since the genus and species was first described by Wells et al. in 1987 (40,54,55,57,67). These include *X. fastidiosa* subsp. *fastidiosa*, which infects and causes disease in grapevine and almond, as well as in other plant hosts (54,55); *X. fastidiosa* subsp. *pauca*, which infects citrus and coffee and is the only described subspecies not present in North America (35,54); *X. fastidiosa* subsp. *multiplex*, which infects a large number of plant hosts, including almond, elm, peach, pigeon grape, plum, sycamore, and other shade trees (54); *X. fastidiosa* subsp. *sandyi*, which infects oleander and some other hosts (21,57); and the most recently described subspecies, *X. fastidiosa* subsp. *tashke*, which infects chitalpa in the southwestern United States (40). These subspecies are based on various phenotypic and genotypic factors such as DNA–DNA relatedness, DNA sequence variation, growth characteristics in vitro, and the ability of strains to grow in or infect particular hosts (40,54,57). Some degree of host specificity exists among subspecies and strains of *X. fastidiosa*; however, this specificity is not always clear and some subspecies and strains are able to infect multiple plant hosts (22).

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In Louisiana, *X. fastidiosa* strains have been isolated from various plants, including a grapevine with Pierce's disease (PD) and a sycamore tree and an oleander plant with leaf scorch (60).

X. fastidiosa 9a5c, a strain isolated from citrus (sweet orange), was the first plant-pathogenic bacterium to have its genome completely sequenced (59). Since then, additional strains of *X. fastidiosa*, including strains from almond and grapevine, have been completely sequenced; sequence information for these whole genomes as well as many DNA regions and genes of other strains is available in the National Center for Biotechnology Information (NCBI) GenBank database (9,56,65). The availability of these complete genome sequences, as well as other sequence information from the 16S rRNA, the 16S-23S rRNA internal transcribed spacer (ITS) region, and various housekeeping and pathogenicity-related genes, has enabled comparative genomic or genetic analyses among strains from different hosts and subspecies and has led to assays for the differentiation of subspecies as well as the identification of new subspecies (19,20,24,57,63,65).

In 2000, *X. fastidiosa* was reported as the causative agent of pecan bacterial leaf scorch (PBLS; 46). The symptoms of PBLS are similar to those caused by the bacterium in other plant hosts and include leaflets turning tan to brown beginning at the margin and progressing basipetally, followed by abscission of infected leaflets (46). This disease tends to occur annually and has been identified in over 20 pecan cultivars and in nongrafted trees (45,50). *X. fastidiosa* threatens the pecan industry by causing reduced nut yields in infected trees. In pecan 'Cape Fear' trees, diseased terminals had reduced kernel weights that averaged 16% less when compared with noninfected terminals in the same orchard (47). Currently, only management strategies to reduce the introduction of this pathogen into pecan orchards exist. No resistant pecan cultivar has been identified, and there is no treatment for trees that become infected.

Compared with other diseases caused by *X. fastidiosa*, PBLS is a recently recognized disease. The majority of the studies on PBLS have been focused on the transmission of the pathogen in pecan (45–49,51,52). Because pecan cultivars are clonally propagated, graft transmission of the pathogen may be a major source of pathogen spread into orchards. *X. fastidiosa* can be transmitted from

either scions or rootstocks obtained from infected pecan trees following grafting (45,49). A hot-water treatment of pecan scion wood reduced graft transmission of the pathogen; however, the pathogen continues to spread in established orchards, apparently by insect transmission (29,48,51). Most recently, insect transmission tests in pecan have shown that the pecan spittlebug, *Clastoptera achatina*, and several leafhopper species, including the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, and the Johnsongrass sharpshooter, *H. insolita*, are able to transmit *X. fastidiosa* from infected to noninfected pecan with subsequent PBLS development (52).

Prior to this work, pecan strains of *X. fastidiosa* had not been studied well. However, a paper that came out while this manuscript was in review reported that strains of *X. fastidiosa* from pecan might be grouped within *X. fastidiosa* subsp. *multiplex* (63). The taxonomical identification of the pecan strains, as well as their relationship to other strains of *X. fastidiosa* present in Louisiana and in other hosts, would aid in the development of more effective management strategies for PBLS. These strategies should take into account other plant hosts in and around pecan orchards that may harbor the same subspecies and serve as sources of inoculum for transmission by insect vectors, such as the GWSS, that feed on and transmit the pathogen in several hosts (1,4,12,28,39).

This study determined the subspecies of *X. fastidiosa* strains isolated from pecan and other hosts in Louisiana and the phylogenetic relationship among the strains using a multiprimer polymerase chain reaction (PCR) assay, DNA sequence analyses, and rep-PCR techniques. The information obtained through this study will contribute to a better understanding of PBLS epidemiology and *X. fastidiosa* population biology by helping to identify potential sources of *X. fastidiosa* inoculum for pecan and by providing new strain information that can be used to study and understand the diversity found within this species.

Materials and Methods

Bacterial strains, isolation, growth conditions, and DNA extraction. *X. fastidiosa* strains used in this study are listed in Table 1. Bacterial strains were previously isolated from plant tissue showing scorch-like symptoms by squeezing plant sap from pecan rachises and from grapevine and sycamore petioles directly onto modified periwinkle wilt medium (13) and subsequently incubating the culture plates in the dark at 28°C. Culture plates were examined periodically and individual colonies resembling *X. fastidiosa* were transferred to tubes containing 30% glycerol and stored at –60 to –70°C. The strain from oleander was isolated as previously described (60).

Strains from pecan, grapevine, and sycamore previously tested positive for *X. fastidiosa* with a commercial *X. fastidiosa* double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Agdia, Inc., Elkhart, IN). The oleander strain, XF Oleander, was previously verified as *X. fastidiosa* using a *X. fastidiosa*-specific primer set (24,60). All strains were recovered from glycerol storage and grown on modified periwinkle wilt medium at 28°C. Genomic DNA was extracted from 11-day-old cultures of all pecan, grapevine, and oleander strains and from a 1-month-old culture of the sycamore strain using the GenElute Bacterial Genomic DNA Extraction kit (Sigma-Aldrich, St. Louis, MO). DNA samples were stored at –20°C for long-term use or 4°C for short-term use.

PCR and electrophoresis conditions. All PCRs were performed in a DNA Engine Peltier Thermal Cycler (Bio-Rad, Hercules, CA). PCR reagents and DNA template solutions were used at the following concentrations: GeneAmp 10× PCR Buffer I containing 15 mM $MgCl_2$ (Applied Biosystems, Carlsbad, CA); 10× EasyA reaction buffer (Agilent Technologies, Santa Clara, CA); EasyA high-fidelity PCR cloning enzyme, 5 U/µl (Agilent Technologies); homemade *Taq* polymerase, approximately 1.0 U/µl; primers, 10 µM; $MgCl_2$, 50 mM; dNTP mix, 10 mM; and DNA template, 1 ng/µl. Sterile distilled deionized water was used to bring the volume up to the desired reaction volume. Primers used in this study were synthesized by Bioneer, Inc. (Alameda, CA). Primer sequences are listed in Table 2.

Agarose gels contained ethidium bromide for visualization of PCR products under UV light. Unless specified, gels were run under standard conditions in 1× Tris-borate EDTA buffer (44). PCR products were visualized and photographed with a KODAK Gel Logic 1500 Imaging System (Eastman Kodak Company, Rochester, NY).

***X. fastidiosa*-specific PCR.** Standard PCR was performed on all strains of *X. fastidiosa* from Louisiana with primers RST31 and RST33 (Table 2) to confirm previous ELISA and PCR results. Each reaction contained 2 µl of template DNA, 2.5 µl of GeneAmp 10× PCR Buffer I, 1.0 µl of each primer, 0.5 µl of dNTP mix, and 1 µl of *Taq* polymerase in a total reaction volume of 25 µl. The PCR program included an initial denaturation period of 95°C for 1 min; 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final extension period of 72°C for 5 min (34). PCR products were separated by gel electrophoresis in a 0.7 or 1% agarose gel. PCR was repeated at least twice for each bacterial strain.

Multiprimer PCR assay. A multiprimer PCR assay designed by Hernandez-Martinez et al. (18) was performed on all strains of *X. fastidiosa* from Louisiana. Each reaction contained 2 µl of template DNA; 2.5 µl of GeneAmp 10× PCR Buffer I; 1.0 µl each of primers XF1968-L, XF1968-R, XF2542-L, XF2542-R, ALM1, and ALM2 (Table 2); 0.5 µl of dNTP mix; and 1.0 µl of *Taq* polymerase in a total reaction volume of 25 µl. The PCR program included an initial denaturation period of 94°C for 5 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension period of 72°C for 10 min (20). Multiprimer PCR products were separated by gel electrophoresis in a 1.5% agarose gel. PCR products were visualized and photographed as previously described. PCR was repeated at least twice for each bacterial strain.

PCR and sequence analysis of the 16S-23S rRNA ITS and *pglA*. Primers G1 and L1 (Table 2) were used to amplify the 16S-

Table 1. Strains of *Xylella fastidiosa* isolated from symptomatic plant tissue and used in this study

Strain designation	Host plant (cultivar)[a]	Location of plant host[b]	Year of isolation
XF A-05	*Carya illinoinensis* pecan (Cape Fear)	Shreveport, LA[c]	2005
XF B-05	*C. illinoinensis* pecan (Cape Fear)	Shreveport, LA[c]	2005
XF C-05	*C. illinoinensis* pecan (Cape Fear)	Shreveport, LA[c]	2005
XF A-06	*C. illinoinensis* pecan (Cape Fear)	Shreveport, LA[c]	2006
XF A-07	*Platanus occidentalis* sycamore	Shreveport, LA	2007
XF D-07	*C. illinoinensis* pecan (Cape Fear)	Shreveport, LA[c]	2007
XF A-08	*Vitis vinifera* grapevine	Hessmer, LA[d]	2008
XF Oleander	*Nerium oleander* oleander	Baton Rouge, LA	2009
XF A-10	*C. illinoinensis* pecan (Oconee)	Hessmer, LA[d]	2010
XF B-10	*C. illinoinensis* pecan (Desirable)	Hessmer, LA[d]	2010

[a] Scientific name, common name, and cultivar (in parentheses) for host plant are given whenever the information is known.
[b] Shreveport, LA, is located in Caddo Parish; Hessmer, LA, is located in Avoyelles Parish; Baton Rouge, LA, is located in East Baton Rouge Parish.
[c] Strains were isolated from symptomatic orchard trees or trees in pots at the Pecan Research-Extension Station in Shreveport, LA.
[d] Strains were isolated from symptomatic trees or vines growing at the same location in Hessmer, LA.

23S rRNA ITS region of all strains of *X. fastidiosa*. Each reaction contained 15 µl of template DNA, 5.0 µl of GeneAmp 10× PCR Buffer I, 2 µl of each primer, 1.0 µl of dNTP mix, and 1.5 µl of *Taq* polymerase in a total reaction volume of 50 µl. The PCR program, modified from Hendson et al. (19), included an initial denaturation period of 94°C for 5 min; 21 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 2 min; and a final extension period of 72°C for 10 min.

Primers XFPglA_Fw and XFPglA_Rv (Table 2) were designed within homologous regions surrounding the designated *pglA* gene in the complete genome sequences of *X. fastidiosa* strains 9a5c, Temecula1, M12, and M23 available in NCBI's GenBank to amplify the *pglA* gene of strains of *X. fastidiosa*. Each reaction contained 1.0 µl of template DNA, 5.0 µl of 10× EasyA reaction buffer, 2 µl of each primer, 1.5 µl of $MgCl_2$, 1.0 µl of dNTP mix, and 0.2 µl of EasyA enzyme for a total reaction volume of 50 µl. The PCR program included an initial denaturation period of 95°C for 5 min; 21 cycles of 94°C for 40 s, 60°C for 30 s, and 72°C for 2 min; and a final extension period of 72°C for 7 min.

Aliquots of the ITS and *pglA* PCR products were separated by gel electrophoresis in a 0.7 or 1.0 % agarose gel to confirm PCR product. The remaining ITS and *pglA* PCR products were purified using the QuickClean 5M PCR Purification Kit (GenScript, Piscataway, NJ). Purified PCR products were sequenced by the Louisiana State University School of Veterinary Medicine's GeneLab (Baton Rouge) or by Macrogen Inc. (Seoul, Korea). Products from at least two independent PCR reactions were sequenced for each DNA sample to ensure correct base identification, with the exception of strain XF B-10 from pecan, whose sequence was determined from a single product.

ITS and *pglA* sequences were analyzed with Geneious Pro 5.4 (Biomatters Ltd., Auckland, New Zealand) (16). Alignment of sequences was performed with ClustalW2 (European Molecular Biology Laboratory's European Bioinformatics Institute, www.ebi.ac.uk) (10). *pglA* sequences were translated into protein sequences using ExPASy Translate (Swiss Institute of Bioinformatics, http://expasy.org/) (17).

Rep-PCR fingerprinting. Rep-PCRs were performed on the basis of two repetitive elements: enterobacterial repetitive intergenic consensus (ERIC)-PCR and repetitive extragenic palindromic (REP)-PCR (66). Each reaction contained: for ERIC-PCR, 2 µl of template DNA, 2.5 µl of GeneAmp 10× PCR Buffer I, 5.0 µl each of primers ERIC1R and ERIC2 (Table 2), 0.8 µl of dNTP mix, and 1.0 µl of *Taq* polymerase in a total reaction volume of 25 µl; and, for REP-PCR, 2 µl of template DNA, 2.5 µl of GeneAmp 10× PCR Buffer I, 5.0 µl each of primers REP1R-I and REP2-I (Table 2), 0.8 µl of dNTP mix, and 1.0 µl of *Taq* polymerase in a total reaction volume of 25 µl. PCR programs were performed as previously described and included: for ERIC-PCR, an initial denaturation period of 95°C for 7 min; 30 cycles of 94°C for 1 min, 52°C for 1 min, and 65°C for 8 min; and a final extension period of 65°C for 16 min; and, for REP-PCR, an initial denaturation period of 95°C for 6 min; 30 cycles of 94°C for 1 min, 40°C for 1 min, and 65°C for 4 min; and a final extension period of 65°C for 16 min (32).

ERIC-PCR and REP-PCR products (10 µl) were separated by gel electrophoresis in a 1.8% agarose gel at 60 V for 14 h at 4 to 6°C. Each rep-PCR was repeated at least three times. The presence or absence of bands between 100 and 1,650 bp for ERIC-PCR and between 400 and 2,000 bp for REP-PCR was recorded. A consensus dataset for distinguishable bands that regularly appeared within these ranges in rep-PCR fingerprints was used for phylogenetic reconstruction.

Phylogenetic analyses. DNA sequences of *X. fastidiosa* strains used for comparative analyses in this study were obtained from NCBI's GenBank (Table 3).

ITS sequences from 49 strains of *X. fastidiosa* and one strain of *Xanthomonas campestris* pv. *campestris* were aligned with MUSCLE and cured in Gblocks (Méthodes et Algorithmes pour la Bio-informatique LIRMM, http://www.phylogeny.fr/version2_cgi/index.cgi) (15). A maximum likelihood (ML) search for the best-scoring ML tree of the cured ITS sequences was performed in RAxML using the rapid bootstrapping algorithm and the GTRMIX model of evolution (61,62). Analyses were run with 1,000 bootstrap replicates. A Markov Chain Monte Carlo (MCMC) search for the cured ITS sequences was performed in MrBayes 3.1 (25,27,42). Four runs each with four chains were tested until the standard deviation of the split frequencies reached 0.01. A starting tree obtained from the ML analysis was used for Bayesian analysis.

The aligned *pglA* and *pglA*-homologous sequences from 13 strains of *X. fastidiosa* were analyzed in RAxML and MrBayes 3.1, as previously described, except that MCMC search runs were tested until the standard deviation of the split frequencies reached 0.02, and no starting tree was used.

Phylogenetic reconstruction using the unweighted pair-group method with arithmetic means (UPGMA) was performed in MEGA5 for the individual and combined datasets of binary data for ERIC-PCR and REP-PCR (64). Analyses were run with 1,000 bootstrap replicates. An MCMC search for the individual and combined datasets for ERIC-PCR and REP-PCR was performed in MrBayes 3.1. Four runs each with four chains were tested until the standard deviation of the split frequencies reached 0.01.

An MCMC search for the total combined dataset of 49 strains of *X. fastidiosa* and one strain of *X. campestris* pv. *campestris*, including the aligned and cured ITS sequences, the aligned *pglA* sequences, and the binary data from ERIC-PCR and REP-PCR, was performed in MrBayes 3.1. Four runs each with four chains were tested until the standard deviation of the split frequencies

Table 2. Primers used in this study

Primer name	Primer sequence (5′→3′)	Test[a]	Reference
RST31	GCGTTAATTTTCGAAGTGATTCGATTGC	Species ID	34
RST33	CACCATTCGTATCCCGGTG	Species ID	34
XF1968-L	GGAGGTTTACCGAAGACAGAT	Multiprimer PCR	20
XF1968-R	ATCCACAGTAAAACCACATGC	Multiprimer PCR	20
XF2542-L	TTGATCGAGCTGATGATCG	Multiprimer PCR	20
XF2542-R	CAGTACAGCCTGCTGGAGTTA	Multiprimer PCR	20
ALM1	CTGCAGAAATTGGAAACTTCAG	Multiprimer PCR	20
ALM2	GCCACACGTGATCTATGAA	Multiprimer PCR	20
G1	GAAGTCGTAACAAGG	ITS amplification	26
L1	CAAGGCATCCACCGT	ITS amplification	26
XFPglA_Fw	GCCTCCGGTGCGACTGCTTC	*pglA* amplification	This study
XFPglA_Rv	GCTGCGATTGGACACACATTG	*pglA* amplification	This study
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	ERIC-PCR	66
ERIC2	AAGTAAGTGATGGGGTGAGCG	ERIC-PCR	66
REP1R-I	IIIICGICGICATCIGGC	REP-PCR	66
REP2-I	ICGICTTATCIGGCCTAC	REP-PCR	66

[a] PCR = polymerase chain reaction, ITS = intergenic transcribed spacer, ERIC = enterobacterial repetitive intergenic consensus, and REP = repetitive extragenic palindromic.

Table 3. Bacterial strains and sequences used in this study for phylogenetic analysis

Strain designation	Plant host	Geographical origin	GenBank accession number	Accession number description[a]	Reference
Xylella fastidiosa					
M12	Almond	California	CP000941	Complete genome	9
M23	Almond	California	CP001011	Complete genome	9
ALS1	Almond	California	AF073240	Partial ITS	19
Tulare	Almond	California	AF073242	Partial ITS	19
Contra Costa	Almond	California	AF073250	Partial ITS	19
Dixon	Almond	California	AF073251	Partial ITS	19
Fresno	Almond	California	DQ011259	Partial ITS	20
AZ03	Chitalpa	Arizona	EU714189	Partial ITS	40
CA01	Chitalpa	California	EU714190	Partial ITS	40
NM02	Chitalpa	New Mexico	EU714192	Partial ITS	40
9a5c	Citrus	Brazil	AE003849	Complete genome	59
CI.52	Citrus	Brazil	AF203393	Partial ITS	33
CI.11067	Citrus	Brazil	AF237650	Partial ITS	33
CI.X0	Citrus	Brazil	AF237651	Partial ITS	33
CO.01	Coffee	Brazil	AF203394	Partial ITS	33
P3	Coffee	Brazil	AY388464	Partial ITS	31
ELM-1	Elm	Washington	AY388468	Partial ITS	31
Temecula 1	Grapevine	California	AE009442	Complete genome	65
GB514	Grapevine	Texas	CP002165	Complete genome	56
PD 95-2	Grapevine	Florida	AF073220	Partial ITS	19
PD 95-4	Grapevine	Florida	AF073221	Partial ITS	19
P 95-9	Grapevine	Florida	AF073222	Partial ITS	19
R116V3	Grapevine	Florida	AF073223	Partial ITS	19
R116V3-4	Grapevine	Florida	AF073224	Partial ITS	19
Conn Creek	Grapevine	California	AF073225	Partial ITS	19
Santa Cruz	Grapevine	California	AF073229	Partial ITS	19
Preston Ranch	Grapevine	California	AF073232	Partial ITS	19
Moore Park	Grapevine	California	AF073236	Partial ITS	19
XF A-08	Grapevine	Louisiana	JN092384	Partial ITS	This study
	…	…	JN092393	*pglA*	This study
Acer macrophyla	Maple	California	AF073219	Partial ITS	19
MUL-1	Mulberry	Massachusetts	AY388467	Partial ITS	31
88-9	Oak	Florida	AF073210	Partial ITS	19
92-3	Oak	Florida	AF073211	Partial ITS	19
92-10	Oak	Florida	AF073212	Partial ITS	19
OLS#2	Oak	Georgia	AF073213	Partial ITS	19
Stucky	Oak	Georgia	AF073214	Partial ITS	19
Ann1	Oleander	California	AF073215	Partial ITS	19
PF1	Oleander	California	AF073216	Partial ITS	19
H44	Oleander	California	AY603088	Partial ITS	11
Riverside	Oleander	California	DQ011263	Partial ITS	20
Texas	Oleander	Texas	DQ011264	Partial ITS	20
XF Oleander	Oleander	Louisiana	JN092385	Partial ITS	This study
	…	…	JN092394	*pglA*	This study
5S2	Peach	Georgia	AF073206	Partial ITS	19
5R1	Peach	Georgia	AF073207	Partial ITS	19
4S3	Peach	Georgia	AF073208	Partial ITS	19
PE.PLS	Pear	Taiwan	AF203396	Partial ITS	33
XF A-05	Pecan	Louisiana	JN092378	Partial ITS	This study
	…	…	JN092387	*pglA*	This study
XF B-05	Pecan	Louisiana	JN092379	Partial ITS	This study
	…	…	JN092388	*pglA*	This study
XF C-05	Pecan	Louisiana	JN092380	Partial ITS	This study
	…	…	JN092389	*pglA*	This study
XF A-06	Pecan	Louisiana	JN092381	Partial ITS	This study
	…	…	JN092390	*pglA*	This study
XF D-07	Pecan	Louisiana	JN092383	Partial ITS	This study
	…	…	JN092392	*pglA*	This study
XF B-10	Pecan	Louisiana	JN092386	Partial ITS	This study
PWT-22	Periwinkle	Florida	AY388470	Partial ITS	31
2#4	Plum	Georgia	AF073209	Partial ITS	19
PL.788	Plum	Georgia	AF203395	Partial ITS	33
RGW-R	Ragweed	Florida	AY388469	Partial	31
XF A-07	Sycamore	Louisiana	JN092382	Partial ITS	This study
	…	…	JN092391	*pglA*	This study
Xanthomonas campestris pv. *campestris*					
LMG 568	…	…	AF209755	Complete ITS	18

[a] Subspecies designations for the complete genomes of strains are as follows: M12, an almond leaf scorch (ALS) strain, is a *Xylella fastidiosa* subsp. *multiplex* strain; M23, an ALS strain, and Temecula1 and GB514, Pierce's disease strains, are *X. fastidiosa* subsp. *fastidiosa* strains; and 9a5c, a citrus variegated chlorosis strain, is a *X. fastidiosa* subsp. *pauca* strain. ITS = intergenic transcribed spacer region between the 16S and 23S ribosomal subunits described in the National Center for Biotechnology Information (NCBI) GenBank as the 16S-23S ribosomal RNA intergenic spacer. *pglA* was designated as the sequence description for the strains used in this study based on the homology with the polygalacturonase gene in the complete genome sequences of *X. fastidiosa* strains 9a5c, Temecula1, M12, M23, and GB514 available in NCBI's GenBank.

reached 0.02. A starting tree obtained from the ML analysis for the aligned and cured ITS sequences was used for the MCMC search. Because *pglA* sequences were only available for strains whose complete genomes have been published, these sequences were included with the ITS sequences in the combined dataset so that a *pglA* sequence from a particular subspecies and host was included with the ITS sequence of a strain from the same subspecies and host. These sequences were included as follows: 9a5c *pglA* sequence included with strain CI.X0, Temecula1 *pglA* sequence included with strain PD 95-2, GB514 *pglA* sequence included with strain PD 95-4, M12 *pglA* sequence included with strain Dixon, and M23 *pglA* sequence included with strain ALS1.

Convergence of all Bayesian analyses was tested using the cumulative and compare functions in AWTY (http://king2.scs.fsu.edu/CEBProjects/awty/awty_start.php) (36). Phylogenetic trees were obtained from RAxML for ITS sequence, *pglA* sequence, and total combined analyses and from MEGA5 for ERIC-PCR and REP-PCR data analyses. Bootstrap values from ML analyses or UPGMA analyses greater than 70% and posterior probabilities from MCMC analyses greater than 0.95 were included on phylogenetic trees. *X. fastidiosa* strain XF A-10 was not included in the ML searches or in the MCMC analysis of the total combined dataset due to a lack of sequence data.

Results

Genetic verification of *X. fastidiosa* strains isolated from pecan and other plant hosts in Louisiana. A DNA band of approximately 733 bp, the expected product size for confirmation of *X. fastidiosa* strains when using *X. fastidiosa*-specific primers RST31 and RST33, was produced from all bacterial strains isolated from pecan and other plants (grapevine, oleander, and sycamore) in Louisiana and used in this study.

Multiprimer PCR assay of *X. fastidiosa* strains isolated in Louisiana. Three DNA bands of approximately 412, 521, and 638 bp, the expected sizes of the products amplified from the multiprimer PCR assay for *X. fastidiosa* subsp. *multiplex* almond leaf scorch (ALS) II genotype, were amplified from each of the pecan and sycamore strains from Louisiana (Fig. 1; *data not shown* for XF A-10 and XF B-10) (20). A single band of approximately 412 bp, the expected size of the product amplified from the multiprimer PCR assay for *X. fastidiosa* subsp. *fastidiosa* PD genotype, was amplified from the Louisiana grapevine strain, XF A-08 (Fig. 1) (20). A single band of approximately 638 bp, the expected size of the product amplified from the multiprimer PCR assay for *X. fastidiosa* subsp. *sandyi* oleander leaf scorch (OLS) genotype, was amplified from the Louisiana oleander strain, XF Oleander (Fig. 1) (20).

Sequence analyses of the 16S-23S rRNA ITS region of *X. fastidiosa* strains isolated in Louisiana. ITS sequences were obtained from nine strains of *X. fastidiosa* used in this study. ClustalW2 alignment of the ITS sequences trimmed to the same starting and ending points showed that the ITS sequences from all strains of *X. fastidiosa* from pecan and sycamore from Louisiana were identical. These strains had a sequence identity of 98% when compared with the grapevine (XF A-08) and oleander (XF Oleander) strains from Louisiana. A basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the pecan and sycamore ITS sequence against the complete genomes of *X. fastidiosa* (Table 3) showed the highest sequence identity to *X. fastidiosa* subsp. *multiplex* strain M12 from almond (100%) followed by *X. fastidiosa* subsp. *pauca* strain 9a5c from citrus (99%) and *X. fastidiosa* subsp. *fastidiosa* strains Temecula1 and GB514 from grapevine and M23 from almond (98%) (5). A BLAST search of the ITS sequence from grapevine strain XF A-08 showed the highest sequence identity (100%) to *X. fastidiosa* subsp. *fastidiosa* strains Temecula1 and GB514 from grapevine and M23 from almond. A BLAST search of the ITS sequence from oleander strain XF Oleander showed the highest sequence identity (99%) to *X. fastidiosa* subsp. *fastidiosa* strains Temecula1 and GB514 from grapevine and M23 from almond. ITS sequences obtained for strains of *X. fastidiosa* in this study were deposited in GenBank under accession numbers JN092378 through JN092386 (Table 3).

Sequence analyses of *pglA* of *X. fastidiosa* strains isolated in Louisiana. DNA sequences of *pglA* encoding a polygalacturonase, a major virulence factor of *X. fastidiosa* (43), were obtained from eight strains of *X. fastidiosa* used in this study. ClustalW2 alignment of the *pglA* sequences showed that the *pglA* sequences from all *X. fastidiosa* strains from pecan and sycamore from Louisiana were identical. These strains had a sequence identity of 98% when compared with the grapevine (XF A-08) and oleander (XF Oleander) strains from Louisiana. A BLAST search of the pecan and sycamore *pglA* sequence against the complete genome sequences of *X. fastidiosa* (Table 3) showed the highest sequence identity (99%) to *X. fastidiosa* subsp. *multiplex* strain M12 from almond, followed by *X. fastidiosa* subsp. *fastidiosa* strains Temecula1 and GB514 from grapevine and M23 from almond (98%), and *X. fastidiosa* subsp. *pauca* strain 9a5c from citrus (97%). A BLAST search of the *pglA* sequence from grapevine strain XF A-08 showed the highest sequence identity (100%) to *X. fastidiosa* subsp. *fastidiosa* strains Temecula1 and GB514 from grapevine and M23 from almond. A BLAST search of the *pglA* sequence from oleander strain XF Oleander showed the highest sequence identity (98%) to *X. fastidiosa* subsp. *fastidiosa* strains Temecula1 and GB514 from grapevine and M23 from almond and to *X. fastidiosa* subsp. *multiplex* strain M12 from almond. Early termination, which is present in the *pglA* gene of strain 9a5c from citrus (65), was not found in any of the *pglA* sequences from strains of *X. fastidiosa* from Louisiana. *pglA* sequences obtained from strains of *X. fastidiosa* in this study were deposited in GenBank under accession numbers JN092387 through JN092394 (Table 3).

Rep-PCR analyses of *X. fastidiosa* strains isolated in Louisiana. Bands in 10 band classes between 100 and 1,650 bp for ERIC-PCR and in 9 band classes between 400 and 2,000 bp for REP-PCR were scored as being present or absent for each *X. fastidiosa* strain tested.

All pecan strains, regardless of the cultivar from which they were isolated, and the sycamore strain produced the same ERIC-PCR fingerprint (Fig. 2A). Obvious band differences were present in the ERIC-PCR fingerprints between the grapevine and oleander strains and between these strains and those from pecan and sycamore (Fig. 2A). Namely, bands approximately 155, 550, and 880 bp in size were present only in grapevine strain XF A-08 and oleander strain XF Oleander but not in any of the other strains; bands approximately 160 and 950 bp in size were present in all pecan strains and in sycamore strain XF A-07 but not in grapevine strain XF A-08 or oleander strain XF Oleander; bands approximately 300 and 420 bp in size were present in all strains except oleander strain XF Oleander; and a band approximately 1,275 bp in size was present in oleander strain XF Oleander but not in any of the other strains (Fig. 2A).

Obvious band differences were present in the REP-PCR fingerprints between the grapevine and oleander strains and between

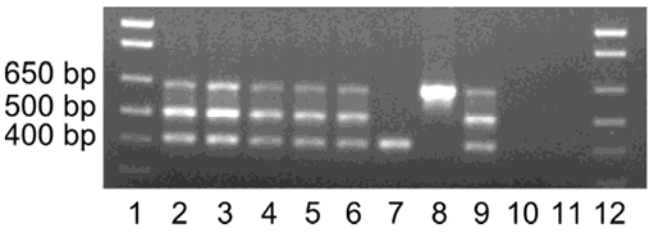

Fig. 1. Multiprimer polymerase chain reaction products of strains of *Xylella fastidiosa* isolated from diseased grapevine, oleander, pecan, and sycamore from Louisiana. A strain of *Burkholderia glumae* and sterile distilled deionized water (ddH_2O) were used as negative controls. Samples were arranged as follows: lane 1: 1-kb-plus ladder (Invitrogen); lane 2: XF A-05 (pecan); lane 3: XF B-05 (pecan); lane 4: XF C-05 (pecan); lane 5: XF A-06 (pecan); lane 6: XF D-07 (pecan); lane 7: XF A-08 (grapevine); lane 8: XF Oleander (oleander); lane 9: XF A-07 (sycamore); lane 10: *B. glumae* 336gr-1; lane 11: sterile ddH_2O; and lane 12: 1-kb-plus ladder.

these strains and those from pecan and sycamore (Fig. 3A). Namely, a band approximately 460 bp in size was present in all strains except grapevine strain XF A-08; a band approximately 600 bp in size was present only in grapevine strain XF A-08; and bands approximately 750 and 1,600 bp were present only in oleander strain XF Oleander (Fig. 3A). A band approximately 520 bp in size was present in all strains except pecan strain XF A-06, grapevine strain XF A-08, oleander strain XF Oleander, and sycamore strain

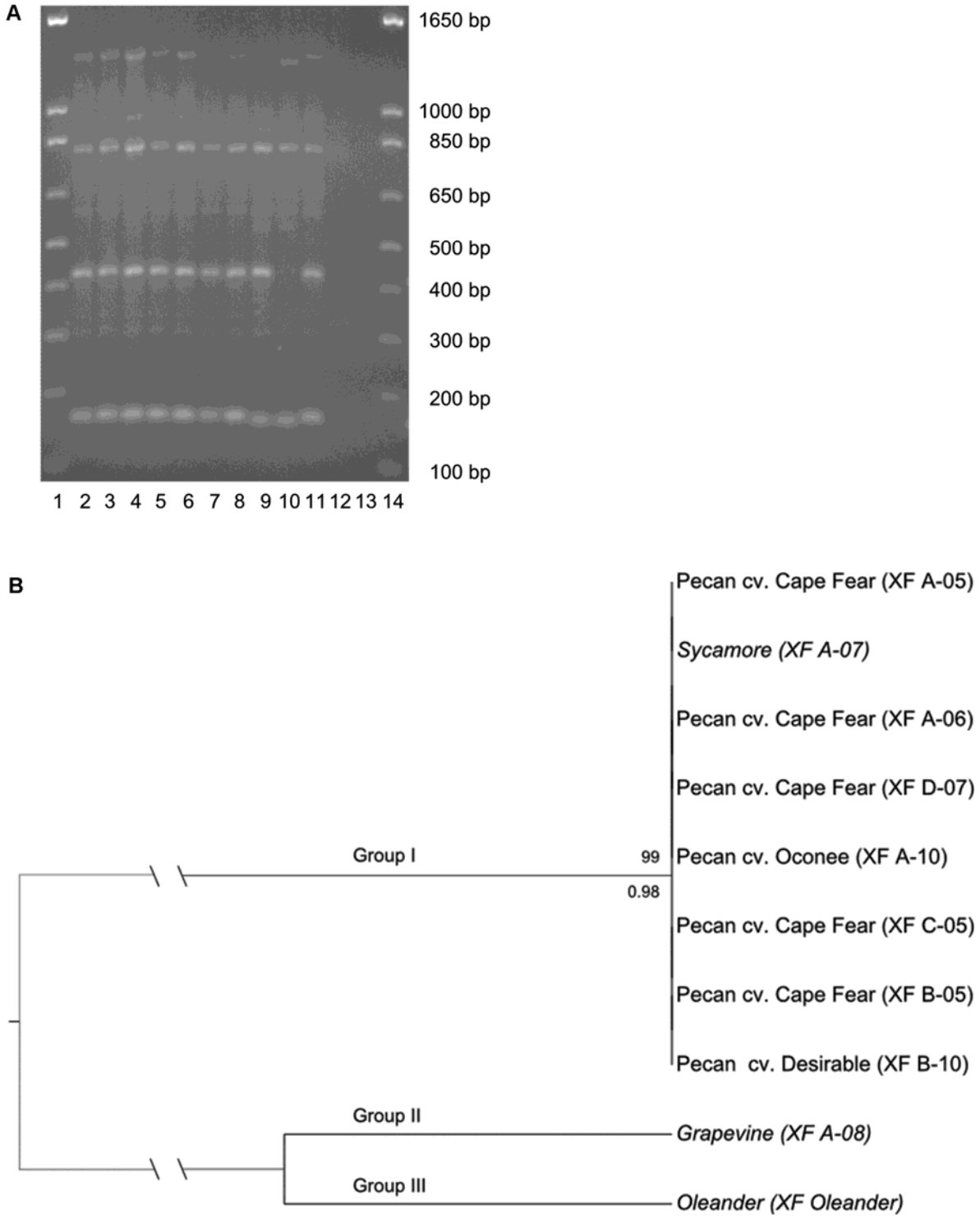

Fig. 2. A, Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) product fingerprints from 10 strains of *Xylella fastidiosa* isolated from diseased grapevine, oleander, pecan, and sycamore from Louisiana. Sterile distilled deionized water (ddH_2O) was used as a negative control. Samples were arranged as follows: lane 1: 1-kb-plus ladder (Invitrogen); lane 2: XF A-05 (pecan, CF); lane 3: XF B-05 (pecan, CF); lane 4: XF C-05 (pecan, CF); lane 5: XF A-06 (pecan, CF); lane 6: XF D-07 (pecan, CF); lane 7: XF A-10 (pecan, O); lane 8: XF B-10 (pecan, D); lane 9: XF A-08 (grapevine); lane 10: XF Oleander (oleander); lane 11: XF A-07 (sycamore); lane 12: *Burkholderia glumae* 336gr-1; lane 13: sterile ddH_2O; and lane 14: 1-kb-plus ladder. Pecan cultivar designations: CF = Cape Fear; D = Desirable; O = Oconee. **B,** Phylogram constructed from the ERIC-PCR fingerprints shown in A. Branch tip labels include the plant host and cultivar, when applicable, from which the strain of *X. fastidiosa* was obtained and the strain name (parentheses). Phylogram was obtained from an unweighted pair-group method with arithmetic means analysis (1,000 bootstrap replicates) using MEGA5. Bootstrap values (>70%) are shown above branch nodes; posterior probabilities (>0.95) obtained from Bayesian inference in MrBayes 3.1 are shown below branch nodes.

XF A-07. In addition, REP-PCR fingerprints of pecan strains showed variation among some pecan strains (Fig. 3A). Pecan Cape Fear strain XF A-06 and sycamore strain XF A-07 produced a band approximately 900 bp in size that was not present in any other strains tested. Pecan 'Oconee' strain XF A-10 failed to produce a band approximately 1,900 bp in size that was present in all other pecan strains and in the sycamore strain but produced a slightly larger band, approximately 1,950 bp in size, that was not present in any of the other strains tested.

Phylogenetic analyses of *X. fastidiosa* strains isolated in Louisiana. Three main groups were recovered from Bayesian inference and UPGMA using the ERIC-PCR dataset (Fig. 2B).

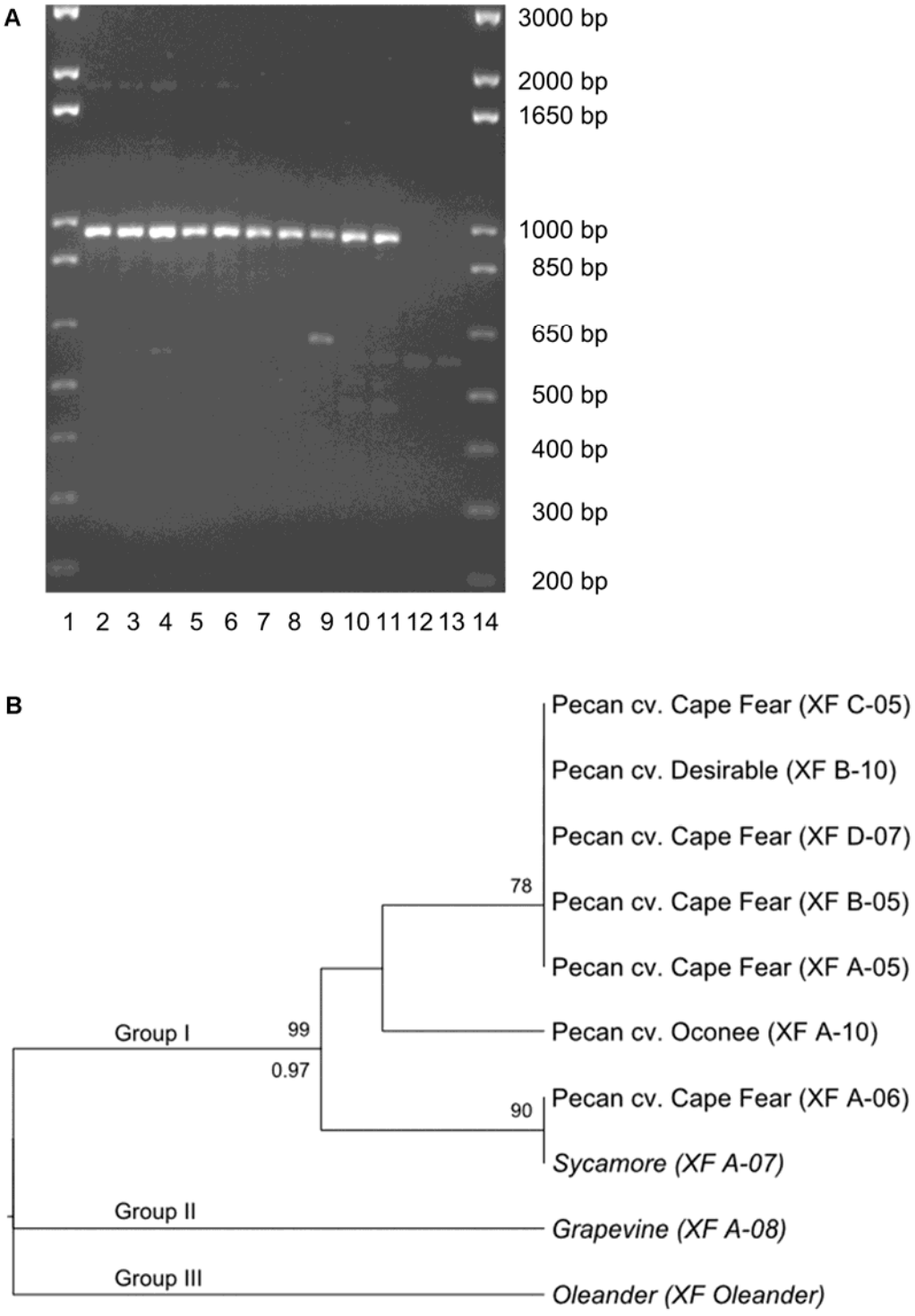

Fig. 3. A, Repetitive extragenic palindromic polymerase chain reaction (REP-PCR) product fingerprints from 10 strains of *Xylella fastidiosa* isolated from diseased grapevine, oleander, pecan, and sycamore from Louisiana. Sterile distilled deionized water (ddH_2O) was used as a negative control. Samples were arranged as follows: lane 1: 1-kb-plus ladder (Invitrogen); lane 2: XF A-05 (pecan, CF); lane 3: XF B-05 (pecan, CF); lane 4: XF C-05 (pecan, CF); lane 5: XF A-06 (pecan, CF); lane 6: XF D-07 (pecan, CF); lane 7: XF A-10 (pecan, O); lane 8: XF B-10 (pecan, D); lane 9: XF A-08 (grapevine); lane 10: XF Oleander (oleander); lane 11: XF A-07 (sycamore); lane 12: *Burkholderia glumae* 336gr-1; lane 13: sterile ddH_2O; and lane 14: 1-kb-plus ladder. Pecan cultivar designations: CF = Cape Fear; D = Desirable; O = Oconee. **B,** Phylogram constructed from the REP-PCR fingerprints shown in A. Branch tip labels include the plant host and cultivar, when applicable, from which the strain of *X. fastidiosa* was obtained and the strain name (parentheses). Phylogram was obtained from an unweighted pair-group method with arithmetic means analysis (1,000 bootstrap replicates) using MEGA5. Bootstrap values (>70%) are shown above branch nodes; posterior probabilities (>0.95) obtained from Bayesian inference in MrBayes 3.1 are shown below branch nodes.

Group I, which was well supported by Bayesian posterior probability (0.98) and UPGMA bootstrap (99%), was monophyletic and included all strains of *X. fastidiosa* from pecan and sycamore (Fig. 2B). Group II and group III included only the grapevine strain and the oleander strain, respectively, separating each of them from all other strains.

Three main groups were recovered from Bayesian inference and UPGMA using the REP-PCR dataset (Fig. 3B). Group I, which was well supported by Bayesian posterior probability (0.97) and UPGMA bootstrap (99%), was monophyletic and included all strains of *X. fastidiosa* from pecan and sycamore (Fig. 3B). Three subgroups, however, were present within group I: one subgroup included strain XF A-06 from pecan Cape Fear and strain XF A-07 from sycamore (UPGMA bootstrap = 90%); the second subgroup included only strain XF A-10 from pecan Oconee; and the third subgroup included four strains from pecan Cape Fear (XF A-05, XF B-05, XF C-05, and XF D-07) and strain XF B-10 from the pecan 'Desirable' (UPGMA bootstrap = 78%; Fig. 3B). Group II and group III included only the grapevine strain and the oleander strain, respectively, separating each of them from all other strains.

A UPGMA phylogram recovered from the combined ERIC-PCR and REP-PCR datasets was similar to the REP-PCR UPGMA phylogram, with similar support from bootstrap values and posterior probabilities, except that it showed the pecan-sycamore subgroup to be more closely related to the larger pecan subgroup (*data not shown*).

The ML phylogram of aligned and cured 16S-23S ITS regions from 49 strains of *X. fastidiosa* and one strain of *X. campestris* pv. *campestris* included as an outgroup placed the majority of the *X. fastidiosa* strains into six major monophyletic or paraphyletic groups, with bootstrap values greater than 84% (Fig. 4). The majority of these groups were also strongly supported by posterior probabilities greater than 0.95. Group I was monophyletic and included strains from citrus and coffee. Group II was paraphyletic, with unresolved relationships among strains from a variety of hosts, including the pecan and sycamore strains from Louisiana and strains from almond and peach. Group III was a monophyletic group within group II and included strains of *X. fastidiosa* from chitalpa (40). Group IV was a monophyletic group also recovered within group II that included strains of *X. fastidiosa* from oak. Group V was monophyletic and included strains of *X. fastidiosa* from oleander, including the Louisiana oleander strain. Group VI was monophyletic and included strains of *X. fastidiosa* from almond and grapevine, including the Louisiana grapevine strain.

The ML analysis of the aligned *pglA* sequences from 13 strains of *X. fastidiosa*, including citrus strain 9a5c used as an outgroup, separated the strains into four groups (Fig. 5). The pecan and sycamore strains formed a monophyletic group with *X. fastidiosa* subsp. *multiplex* strain M12 from almond. The Louisiana grapevine strain formed a monophyletic group with strains of *X. fastidiosa* subsp. *fastidiosa* from grapevine and almond. The Louisiana oleander strain formed a separate group distinct from all other strains of *X. fastidiosa*. *X. fastidiosa* subsp. *pauca* strain 9a5c from citrus also formed a group distinct from all other strains of *X. fastidiosa*. Most of the major groups were strongly supported, with bootstrap values greater than 85% and posterior probabilities of 0.99 (Fig. 5).

The MCMC analysis of the total combined dataset of 49 strains of *X. fastidiosa* and one strain of *X. campestris* pv. *campestris*, including the aligned and cured ITS sequences, the aligned *pglA* sequences, and the binary data from ERIC-PCR and REP-PCR, placed the majority of the *X. fastidiosa* strains into six major monophyletic or paraphyletic groups, with bootstrap values greater than 84% (Fig. 6). Though the location of groups on the phylogram differed in comparison with the phylogram recovered from the cured ITS sequences (Fig. 4), the different monophyletic and paraphyletic groups in this analysis comprised the same strains of

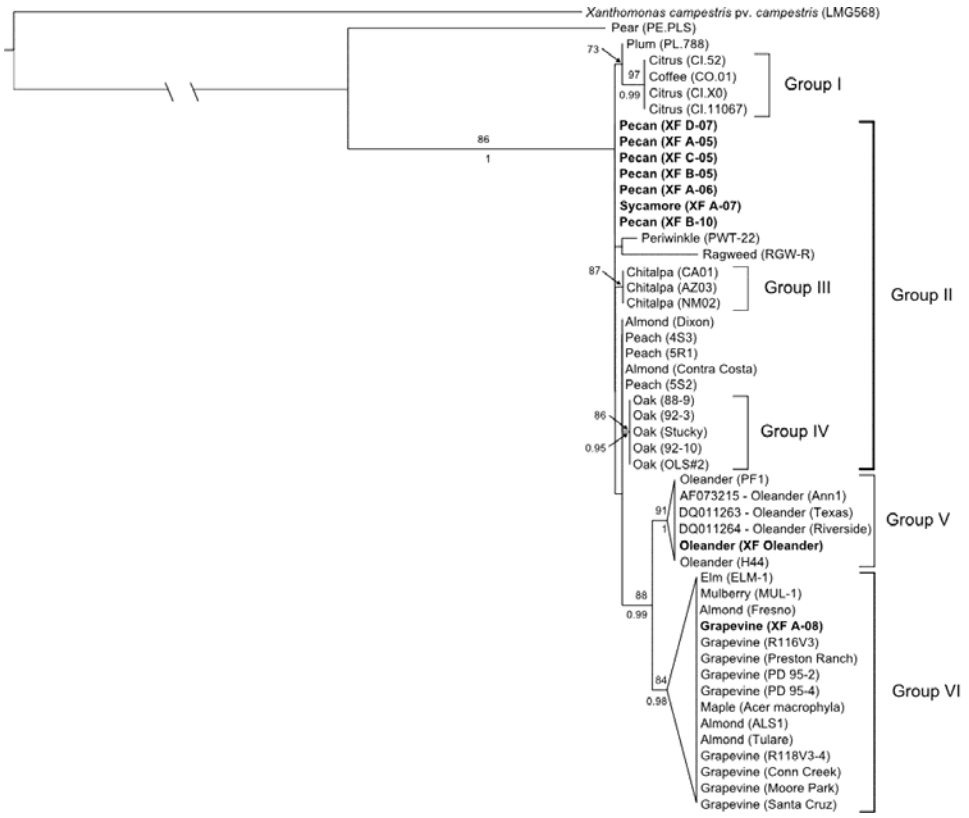

Fig. 4. Phylogram of the 16S-23S rRNA intergenic transcribed spacer (ITS) dataset from 49 strains of *Xylella fastidiosa* and one strain of *Xanthomonas campestris* pv. *campestris* included as an outgroup. Branch tip labels include the host and strain name (parentheses) of *X. fastidiosa*. ITS sequences obtained from this study are indicated in bold. Sequences from all other strains were obtained from the National Center for Biotechnology Information GenBank. Phylogram was obtained from a maximum likelihood (ML) search using RAxML. Bootstrap values (>70%) from 1,000 bootstrap replicates from the ML search are shown above branch nodes; posterior probabilities (>0.95) obtained from Bayesian inference in MrBayes 3.1 are shown below branch nodes.

X. fastidiosa as the previous analysis. The pecan and sycamore strains were still part of a large paraphyletic group that included other strains of *X. fastidiosa* from almond and peach. This analysis showed increased posterior probability support for a number of groups (Fig. 6).

Convergence of all Bayesian analyses was verified using the cumulative and compare functions in AWTY.

Discussion

X. fastidiosa is a complex plant pathogen that infects and causes disease in a wide range of hosts (38). Subspecies classifications have been based largely on molecular techniques and host plant infectivity. However, genetic differences within subspecies and even within strains from the same host have also been observed (2,32,53). Recombination has also been shown to occur between subspecies and between strains from different hosts (2,53,57). The presence of multiple subspecies and strains of *X. fastidiosa* in the same geographic region with common insect vectors may increase the opportunity for continuing diversification within this species and the possibility of the occurrence of new diseases in hosts that have yet to be infected by *X. fastidiosa* (35).

Pecan is the only nut grown commercially in the United States that is also native to the United States (37). It is produced in 15 states (Agricultural Statistics 2010, United States Department of Agriculture NASS), including California, New Mexico, Texas, Louisiana, Georgia, and Florida, where diseases caused by *X. fastidiosa* have been reported and are known to occur (23,40). However, pecan strains of *X. fastidiosa* have rarely been studied and, consequently, their genetic and taxonomic information is very limited. In this investigation, various molecular techniques and phylogenetic methods were used to study the taxonomy of *X. fastidiosa* strains isolated from pecan and other plant hosts in Louisiana at a subspecific level. To our knowledge, this is the first phylogenetic study focused on *X. fastidiosa* strains from pecan and from Louisiana.

The multiprimer PCR assay identified strains of *X. fastidiosa* from Louisiana by genotype and subspecies (Fig. 1). The amplification of three products from the multiprimer PCR assay indicated that strains of *X. fastidiosa* from pecan and sycamore are of the ALSII genotype from *X. fastidiosa* subsp. *multiplex* (Fig. 1) (20). ALSII strains from almond can infect and produce mild symptoms in grapevine, whereas ALSI strains from almond, identified by the amplification of two products in the multiprimer PCR assay, cannot cause disease in grapevine (20). A limitation of the multiprimer PCR assay concerning *X. fastidiosa* subsp. *multiplex* is that it was designed using only strains of *X. fastidiosa* from almond, grapevine, and oleander. Even though the pecan and sycamore strains are of the ALSII genotype, this does not necessarily mean that these strains can infect grapevine. Host-plant inoculations that should determine the relevance of the pecan strains as an ALSII genotype are currently being conducted. The multiprimer PCR assay identified the grapevine strain from Louisiana as the PD genotype of *X. fastidiosa* subsp. *fastidiosa* and the oleander strain from Louisiana as the OLS genotype of *X. fastidiosa* subsp. *sandyi* (Fig. 1).

Sequence analysis of the 16S-23S rRNA ITS region has been used in previous studies to identify strains of *X. fastidiosa* below the species level and was applied to the strains in this study (26,31,33). The pecan and sycamore ITS sequences were identical and most similar to other *X. fastidiosa* subsp. *multiplex* strains. The ITS sequence of the Louisiana grapevine strain was identical to that of strains Temecula1 and GB514 from grapevine and strain M23 from almond, which are all strains of *X. fastidiosa* subsp. *fastidiosa*. Our observation that the ITS sequences of pecan strains were identical to each other and the ITS sequence of the grapevine strain was identical to that of the grapevine strains from the completely sequenced strains was not surprising because a previous study showed that, with the exception of almond strains, all strains from the same host had the same ITS sequence (19). Phylogenetic analysis of the ITS region supported the subspecies groups determined from the multiprimer PCR assay (Figs. 1 and 4). In addition, the major groups of *X. fastidiosa* strains identified in this study were consistent with groups from previous phylogenetic analyses based on ITS sequences, with the exception that the strains from chitalpa formed a monophyletic group within the unresolved subspecies *multiplex* group (Fig. 4) (19,31,33).

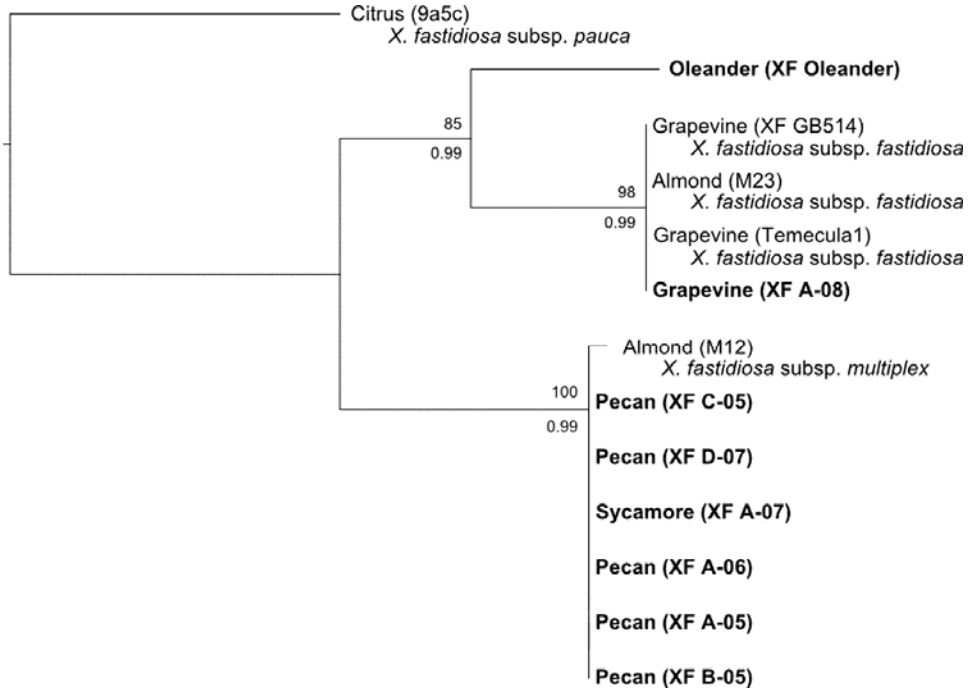

Fig. 5. Phylogram of *pglA* sequences from 13 strains of *Xylella fastidiosa* with the citrus strain 9a5c used as an outgroup. Branch tip labels include the host strain name (parentheses) of *X. fastidiosa*; the known subspecies of *X. fastidiosa* are indicated below each branch tip label. *pglA* sequences obtained from this study are indicated in bold. Sequences from all other strains were obtained from the National Center for Biotechnology Information GenBank. Phylogram was obtained from a maximum likelihood (ML) search using RAxML. Bootstrap values (>70%) from 1,000 bootstrap replicates from the ML search are shown above branch nodes; posterior probabilities (>0.95) obtained from Bayesian inference in MrBayes 3.1 are shown below branch nodes.

A frameshift causing early termination in the polygalacturonase precursor gene, *pglA*, that is present in citrus and coffee strains of *X. fastidiosa* (65) was not found in *pglA* of pecan, grapevine, oleander, or sycamore strains from Louisiana. This frameshift was also not present in almond, grapevine, mulberry, or oleander strains in the study that reported the frameshift from citrus and coffee strains (65). It was suggested that this frameshift possibly made *pglA* nonfunctional and accounted for the less-aggressive nature of citrus variegated chlorosis strains compared with PD strains of *X. fastidiosa* (3,65). Polygalacturonase has been shown to be required for colonization and pathogenicity in grapevine but is not necessary for the development of disease in citrus (43,65). Despite the absence of a frameshift in *pglA* of *X. fastidiosa* pecan, oleander, and sycamore strains, it is unknown whether this gene is required for colonization and pathogenicity of these strains. As with the ITS sequences, all *pglA* sequences from pecan and sycamore were identical. These sequences were more closely related to the *X. fastidiosa* subsp. *multiplex* strain than to any other subspecies. The *pglA* sequence from grapevine in Louisiana was identical to *pglA* sequences from the *X. fastidiosa* subsp. *fastidiosa* Temecula1 and GB514 grapevine strains. Sequence analysis of *pglA* separated strains of *X. fastidiosa* into the same subspecies groups as the multiprimer PCR assay, providing further support for these groups (Figs. 1 and 5).

ERIC-PCR and REP-PCR separated the Louisiana pecan and sycamore strains from the Louisiana grapevine strain and the Louisiana oleander strain (Figs. 2 and 3). REP-PCR detected genetic differences within the strains of *X. fastidiosa* from pecan while ERIC-PCR failed to detect any differences (Figs. 2 and 3). Similar results were reported for *X. fastidiosa* strains from citrus, in which REP-PCR banding patterns were more discriminative than the ERIC-PCR banding patterns (32). Although the differences in the citrus strain REP-PCR profiles corresponded to differences in geographic location in a previous study (32), differences in the strains of *X. fastidiosa* from pecan were observed within the same geographic location and the same cultivar in this study (Table 1; Fig. 3). In the REP-PCR, one of the five pecan strains from Cape Fear, XF A-06, clustered with and was more closely related to the sycamore strain, XF A-07, than to the other pecan strains (Fig. 3). This subgroup was more related to the pecan strain from Oconee, XF A-10, than to the subgroup containing four other pecan strains from Cape Fear (XF A-05, XF B-05, XF C-05, and XF D-07) and the pecan strain from Desirable, XF B-10 (Fig. 3). Although we do not know whether the genetic differences observed here relate to any biological traits, these results suggest that there might be some level of specialization within *X. fastidiosa* subsp. *multiplex* related to pecan cultivar.

The phylogenetic analysis of the combined dataset of the ITS sequences, *pglA* sequences, and ERIC-PCR and REP-PCR data strengthened support for a number of groups identified in this study but did not place the pecan strains in a separate monophyletic group within the *X. fastidiosa* subsp. *multiplex* group (Fig. 6). Additional strains from pecan and other hosts and additional genetic information

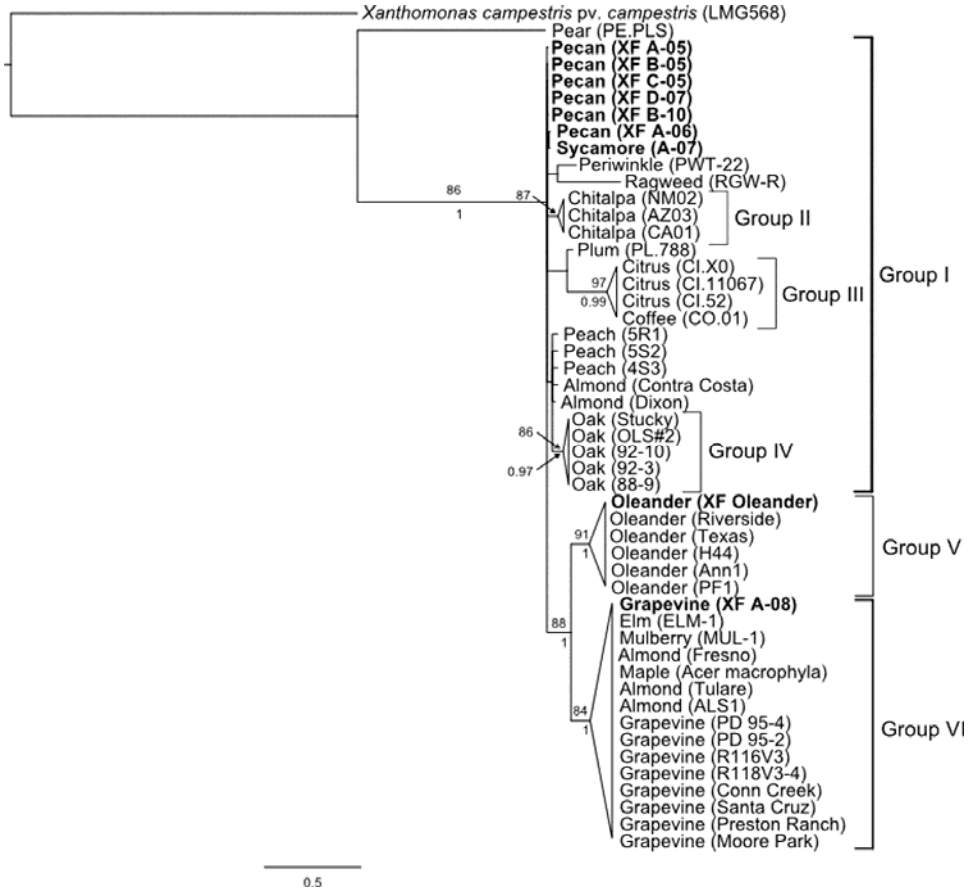

Fig. 6. Phylogram of the combined dataset constructed from available 16S-23S rRNA intergenic transcribed spacer (ITS) sequences, *pglA* sequences, and enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) and repetitive extragenic palindromic (REP)-PCR product fingerprints from 49 strains of *Xylella fastidiosa* and one strain of *Xanthomonas campestris* pv. *campestris* included as an outgroup. Pecan strain XF A-10 was not included in the analysis due to a lack of sequence data. Because *pglA* sequences were only available for strains whose complete genomes have been published, these sequences were included with the ITS sequences in the combined dataset as follows: 9a5c *pglA* sequence included with strain CI.X0; Temecula1 *pglA* sequence included with strain PD 95-2; GB514 *pglA* sequence included with strain PD 95-4; M12 *pglA* sequence included with strain Dixon; and M23 *pglA* sequence included with strain ALS1. Branch tip labels include the plant host from which the strain of *X. fastidiosa* was obtained and the strain name (parentheses). ITS sequences, ERIC-PCR, and REP-PCR data obtained from this study are indicated in bold. Sequences from all other strains were obtained from GenBank. Phylogram was obtained from Bayesian analysis in MrBayes 3.1. Bootstrap values (>70%) from 1,000 bootstrap replicates from the maximum likelihood search in RAxML of the 16S-23S ITS sequences cured in Gblocks are shown above branch nodes; posterior probabilities (>0.95) obtained from the Bayesian inference of the combined data set in MrBayes 3.1 are shown below branch nodes.

may be necessary to distinguish the pecan strains as a separate monophyletic group. A multilocus sequence typing analysis, which identified different clonal complexes within *X. fastidiosa* subsp. *multiplex* (53), may be able to identify a new clonal complex for pecan strains or may group pecan strains with other similar strains, possibly sycamore strains, based on this study (Fig. 6).

Results from the various experiments conducted in this study consistently grouped the strains of *X. fastidiosa* from pecan together with each other and with the strain of *X. fastidiosa* from sycamore from Louisiana and as part of a larger group that included strains of *X. fastidiosa* subsp. *multiplex* from other host plants. For this reason, strains of *X. fastidiosa* from pecan should be considered a part of *X. fastidiosa* subsp. *multiplex*. This placement is also supported by the findings of Su et al. (63). In addition, the analyses conducted in this study placed the Louisiana grapevine strain with other grapevine strains from *X. fastidiosa* subsp. *fastidiosa* and the Louisiana oleander strain with other oleander strains from *X. fastidiosa* subsp. *sandyi*.

Studies on PBLS incidence in the research orchards at the Pecan Research-Extension Station have shown consistent disease spread within some cultivars (48). Disease severity and rate of spread also differ between pecan cultivars. Even though the majority of the pecan strains utilized in this study came from a single location and from a single cultivar, genetic variation was detected within these strains. The genetic differences observed between strains of *X. fastidiosa* from pecan from the same location and from the same cultivar in the REP-PCR suggest that these strains may not be clones originating from a single source of infection. However, it cannot be ruled out that some mutation occurred to cause the genetic variation seen in the REP-PCR while a single strain spread throughout the orchards. To determine the biological meaning of these observed genetic variations within the pecan strains of *X. fastidiosa*, additional studies including host specificity tests with different plant hosts and pecan cultivars are needed.

One of the difficulties in working with *X. fastidiosa* is that it is very fastidious and slow growing. Isolation attempts from pecan over several years have had low success rates and yielded few strains. Concentrated efforts should be made to build a collection of *X. fastidiosa* pecan strains suitable for future studies.

This work provides a foundation for studies with additional strains of *X. fastidiosa* from Louisiana to determine variation that exists in this widespread pathogen within Louisiana and other states of the southeastern United States. Continuation of this study for examining new diseases caused by *X. fastidiosa* and the genetic diversification within *X. fastidiosa* will provide a better understanding of the population dynamics of this pathogen and a useful insight into the development of effective management strategies for plant diseases caused by this pathogen. Potential future research areas that are important for developing new disease control strategies would include identification of alternative hosts that could serve as reservoirs of inoculum for different *X. fastidiosa* subspecies and determination of genetic factors that contribute to host specificity, pathogenicity, and virulence. With the knowledge obtained from studying these research areas, disease management guidelines in terms of what other plants can be grown in the vicinity of crop growing areas without cross-infection between different hosts could be established. In addition, diagnosis and quarantine systems for plant diseases caused by various subgroups of *X. fastidiosa* could be significantly improved by the development of more reliable and specific molecular markers based on better knowledge of genetic diversity within *X. fastidiosa*.

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