Differentiation of Strains of *Xylella fastidiosa* Infecting Grape, Almonds, and Oleander Using a Multiprimer PCR Assay

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ABSTRACT

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Xylella fastidiosa is a xylem-limited, nutritionally fastidious bacterium that causes several plant diseases including Pierce's disease (PD) in grape and leaf scorch in almond (ALS) and oleander (OLS). OLS strains belong to *X. fastidiosa* subsp. *sandyi,* PD strains belong to *X. fastidiosa* subsp. *fastidiosa,* and strains from almond designated as ALS strains are of two general types belonging either to *X. fastidiosa* subsp. *multiplex* or *X. fastidiosa* subsp. *fastidiosa*. The ALS strains assigned to *X. fastidios*a subsp. *multiplex* belong to two different genotypes (ALSI and ALSII) below the subspecies level. The OLS strains do not infect grape or almond. PD strains produce diseases in grape, alfalfa, almond, and some weeds, but they do not infect oleander, oak, peach, or citrus. ALS strains that belong to *X. fastidiosa* subsp. *multiplex* do not produce disease on grape. In this study, a relatively simple polymerase chain reaction (PCR) based method was developed to distinguish among PD, OLS, and ALS strains. PCR performed with primers XF1968-L and XF1968-R amplified a 638-bp fragment from OLS strains but not from PD strains or ALS strains that belong to *X. fastidiosa* subsp. *fastidiosa*. PCR with primers XF2542-L and XF2542-R amplified a 412-bp fragment from PD strains, but not from OLS strains. PCR with primers ALM1 and ALM2 produced a fragment of 521 bp from strains isolated from almond that belong to *X. fastidiosa* subsp. *multiplex*. The combination of the three primer sets allowed the distinction of the two ALS genotypes of *X. fastidiosa* subsp. *multiplex*. These results are in agreement with those obtained from analysis of sequences of 16S-23S rDNA intergenic spacer regions sequence analysis and with previous results based on randomly amplified polymorphic DNA analysis.

Additional keywords: glassy-winged sharpshooter, phylogeny

Xylella fastidiosa (39) is a gram-negative, xylem-limited, nutritionally fastidious, nonflagellate bacterium that infects a broad range of plants from diverse taxonomic groups. This pathogen causes several economically important diseases, including Pierce's disease of grape (PD) (15), oleander leaf scorch (OLS) (31), citrus variegated chlorosis (CVC) (26), almond leaf scorch (ALS) (17), and phony peach (14). Numerous members of the leafhopper and spittlebug families of insects transmit *X. fastidiosa* from plant to plant. In southern California, the dominant vector is the recently introduced glassy-winged sharpshooter, *Homalodisca coagulata* Say (4,37). *H. coagulata* feeds on a broad range of woody and herbaceous plants and is capable of transmitting both the PD and OLS strains of the pathogen (12,25), but shows low acquisition efficiency for some strains infecting almonds (2).

Currently, *X. fastidiosa* strains are differentiated using several approaches, including nutritional requirement profiles (2), cellular protein profiles (40), multigene coding loci analysis (34), DNA homology (33), DNA pulsed-field gel electrophoresis (DNA-PFGE) (22), repetitive extragenic palindromic polymerase chain reaction (REP-PCR) (32), randomly amplified polymorphic DNA (RAPD) (1,2,8,22), and both 16S rDNA and 16S-23S intergenic spacer region (ISR) sequences (5,7,9,28). These analyses are time-consuming, which makes them inefficient when high numbers of samples are analyzed. Using an enzyme-linked immunosorbent assay (ELISA), over 100 plant species have tested positive for *X. fastidiosa*, but the bacterium has not been cultured from most of them. Thus, strain identification from each potential host could not be done. In addition, new hosts are being discovered, and some of these are symptomless (13). As a result, the complete host range of *X. fastidiosa* and the pathogenic relationships among strains remain unclear.

Currently, all strains of *X. fastidiosa* are classified into a single species (39); however, it is probable that there are several pathotypes, because there are clear differences in plant host range and pathogenicity among strains (3,22). Using 16S-23S rDNA ISR, DNA-DNA homology, and phenotypic and serological information, Schaad et al. (33) divided *X. fastidiosa* into three subspecies (33). *X. fastidiosa* subsp. *fastidiosa* includes strains isolated from cultivated grape (*Vitis vinifera*), alfalfa *(Medicago sativa*), maple (*Acer* sp.), and almond (*Prunus dulcis*). Strains of *X. fastidiosa* subsp. *fastidiosa* can also be found infecting some weeds (13), but they do not infect oleander (25). Cross-inoculation with *X. fastidiosa* subsp. *fastidiosa* strains isolated from grape, almond, and alfalfa generally result in disease, suggesting the lack of pathovars within that subspecies (33). The subspecies *multiplex* includes strains isolated from peach (*Prunus persica*), elm (*Ulmus* sp.), plum (*Prunus domestica*), pigeon grape (*V. aestivalis*), almond (*Prunus dulcis*), sycamore (*Platanus occidentalis*), and other shade trees. Cross-inoculation with strains of *X. fastidiosa* subsp. *multiplex* from different hosts does not always result in disease (20,35), suggesting that pathovars with restricted host ranges may exist (33). The subspecies *pauca* includes strains isolated from sweet orange (*Citrus sinensis*) (33), and because the lack of genetic differences, most likely coffee (*Coffea arabica*) (18,27). There has also been a suggestion for a new subspecies called *sandyi* that includes strains isolated from oleander (*Nerium oleander*) (34).

Currently, it is thought that the OLS strains do not infect grape or almond (25,31); the PD strains have a broader host range and produce diseases in grape, alfalfa (21), almond (2), and some weeds (13), but they do not infect oleander, oak,

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peach, or citrus (25). On the other hand, ALS can be caused by two subspecies: *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *fastidiosa*. In almond, strains of *X. fastidiosa* subsp. *fastidiosa* produce typical leaf scorch symptoms, but they develop lower populations than in grape (3), whereas strains of *X. fastidiosa* subsp. *multiplex* cause scorch and multiply within grapes but do not cause typical PD symptoms (3). Both subspecies can coexist simultaneously in the same infected almond orchard and have different bacterial colony morphologies (6). Using RAPD analysis, strains of *X. fastidiosa* subsp. *multiplex* isolated from almond were subdivided into two groups, ALSI and ALSII (3). These strains could not be differentiated using 16S-23S rDNA ISR but showed differences in growth and in their ability to infect grapevines (3,22).

In southern California, where OLS, PD, and ALS strains are present and cause disease, when a new plant species tests positive for the presence of *X. fastidiosa*, it is critical to know which strain of the pathogen is present in order to determine which other host plants in the area may be threatened. Also, to identify primary sources of infectious vector populations, insects must be surveyed for the presence of the pathogen. As with plants, a method is needed to identify which strains are present in a particular population. Subsequently, a simple, fast, reproducible and inexpensive method to differentiate among strains is needed.

The objective of this research was to develop a method to differentiate among strains of *X. fastidiosa* producing PD, ALS, and OLS. For this purpose, polymerase chain reaction (PCR) primers were designed and used to test *X. fastidiosa* strains isolated from grape, oleander, and almond. To support our results, we also analyzed the 16S-23S rDNA ISR sequences of several *X. fastidiosa* strains and compared the results with those obtained using the strain-specific primer sets.

MATERIALS AND METHODS

Strains used in this study are shown in Table 1. New *X. fastidiosa* strains were isolated from almond, grape, and oleander as described before (13). *X. fastidiosa* strains were grown in PD3 (14) and PW (16) media for 7 to 14 days at 28°C. For long-term storage, bacterial cells were harvested from petri dishes and maintained in PW medium with 20% glycerol at –80°C. Genomic DNA was extracted from some *X. fastidiosa* strains used in previous studies as well as from newly isolated strains, and directly from infected and noninfected oleander, almond, and grape tissues. For bacterial cultures, DNA was extracted with a Wizard SV Genomic DNA Purification System (Promega Corp., Madison, WI) following the manufacturer instructions.

Infected plant tissue was obtained from *X. fastidiosa*–inoculated plants. Almonds were inoculated with strains Tulare-ALS and 276 (five plants with each strain) and grapes with the A05 strain (five plants). Strains were grown on PW medium for 7 days at 28°C and resuspended in 3 ml of pH 7 PBS buffer solution (10 mM phosphate buffer, pH 7.4, 150 mM NaCl) to obtain a turbid suspension of approximately 1×10^8 cells per ml or higher. Plants were inoculated as described before (23), by pipetting a small drop of the bacterial solution onto a stem and probing the drop with a no. 1 insect pin (Indigo Instruments, Tonawanda, NY) until uptake from the drop was observed. After 3 months, total DNA from infected plant tissues was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). For this, 10 petioles, leaf veins, or thin branches were selected. The tissue was transferred to a petri dish and cut into 1- to 5-mm pieces using a razor blade. Up to 3 ml of deionized sterile water was added to the cut tissue, which was incubated for 15 min. Approximately 1.5 ml of solution was pipetted into an Eppendorf microfuge tube, centrifuged for 20 min at 14,000 rpm (16,000 × *g*), and the recovered pellet resuspended in 400 µl of buffer AP1 and processed according to the instructions of the kit manufacturer. The DNA was resuspended in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) to a final concentration of 50 ng/µl quantified by absorbance (A_{260}) in a spectrophotometer.

The genome sequence annotation of 9a5c (36), a CVC strain, was used to select open reading frames (ORF) with putative roles in pathogenicity and virulence as well as others involved in nucleic acid and protein metabolism, cellular transport, and stress (10). The primers were used to amplify from the genomes of STL, a strain from grape; Ann1, a strain from oleander;

Table 1. Strains of *Xylella fastidiosa* used in this study, their host sources, and GenBank accession numbers for their 16S-23S rDNA intergenic spacer region sequences

Strain designation	Host of origin	Place from which strain was isolated	GenBank accession no. of 16S-23S rDNA sequences	References/ source
Dixon	Almond	Solano, CA	AF073251	(22)
ALS3	Almond	San Joaquin, CA	AF073244	(22)
ALS9	Almond	San Joaquin, CA	AF073249	(22)
ALS5	Almond	San Joaquin, CA	AF073246	(22)
ALS2	Almond	San Joaquin, CA	AF073243	(22)
ALS6	Almond	San Joaquin, CA	AF073247	(22)
187	Almond	Temecula, CA	DQ011258	This study
H50	Almond	Temecula, CA	AY604730	(13)
276	Almond	Temecula, CA	AY603084	(13)
Fresno-ALS	Almond	Fresno, CA	DQ011259	A. Purcell
Stanislaus	Almond	Stanislaus, CA	DQ011260	A. Purcell
Tulare	Almond	Tulare, CA	AF073242	(22)
H10	Almond	Temecula, CA	AY603085	(13)
237	Almond	Temecula, CA	AY603086	(13)
239	Almond	Temecula, CA	AY603082	(13)
189	Almond	Temecula, CA	AY603083	(13)
H51	Almond	Temecula, CA	AY603080	(13)
PD-PDF VI	Grape	Georgia	DQ011261	C. J. Chang
PD 95-2	Grape	Florida	AF073220	(22)
A05	Grape	Temecula, CA	AY603090	(13)
STL	Grape	Napa, CA	AF073228	(22)
Conn Creek	Grape	Napa, CA	AF073225	(13)
Fetzer	Grape	Napa, CA	AF073227	(22)
Preston Ranch	Grape	Sonoma, CA	AF073232	(22)
Medeiros	Grape	Fresno, CA	AF073234	(22)
Traver	Grape	Tulare, CA	AF073235	(22)
Douglas	Grape	San Luis Obispo, CA	AF073237	(22)
Hopland	Grape	Mendocino, CA	AF073239	(22)
I03	Grape	Temecula, CA	AY603089	(13)
Ann1	Oleander	Palm Springs, CA	AF073215	(22)
Cathedral city	Oleander	Cathedral City, CA	DQ011262	A. Purcell
Texas	Oleander	Texas	DQ011263	This study
Riverside	Oleander	Riverside, CA	DQ011264	This study
H45	Oleander	Temecula, CA	AY603087	(13)
H44	Oleander	Temecula, CA	AY603088	(13)
TR2	Oleander	Orange, CA	DQ011265	A. Purcell
CI.X0	Coffee	Brazil	AF237651	(28)
N10	Spanish broom	Temecula, CA	AY603081	(13)
G10	*Brassica* spp.	Temecula, CA	AY603091	(13)
07882	*X. campestris* pv. *campestris*	California	–[a]	(11)
7882	*X . axonopodis* pv. *vesicatoria*	California	–	(11)

[a] – Means no data available.

and Dixon, a strain from almond (Table 1). The objective was to identify primer pairs that would amplify fragments from some strains but not from others. Primers RST31/RST33 were used for PCR confirmation of *X. fastidiosa* at the species level (29), to determine if the extraction methods were effective and to compare the relative efficacy of the specific primers.

PCR amplification was performed in a 25-µl reaction mixture containing 1× buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, and 1.0% Triton X-100), 2.5 mM $MgCl_2$, dNTPs at 200 µM each, 25 pmol of each primer, 50 ng of genomic DNA, and 2.5 Units of *Taq* DNA polymerase (Promega Corp.). All amplifications were done in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). For bacterial DNA, the cycle program used was 5 min at 94°C, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. After the final cycle, an additional extension step was performed at 72°C for 10 min. For RST31/RST33 primers, the cycle program used was 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min.

For DNA extracted from plants, Ready-to-go PCR beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ) were used (final reaction concentration: 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, dNTP mix at 200 µM each, and 3 Units of *Taq* DNA polymerase). Each reaction contained one Ready-to-go PCR bead, 0.25 µl of each of primer (25 pM/µl solution), 5 µl of DNA extract, with sterile water added to a total volume of 25 µl. Multiprimer PCR reactions were carried out with a mixture of six primers at 25 pmol each. Primers used were: XF1968-L (5′ GGAGGTTTACCGAAGACAGAT 3′), XF1968-R (5′ ATCCACAGTAAAACCACATGC 3′), XF2542-L (5′TTGATCGAGCTGATGATCG3′), XF2542-R (CAGTACAGCCTGCTGGAGTTA3′), ALM1 (5′-CTGCAGAAATTGGAAACTTCAG-3′) and ALM2 (5′-GCCACACGTGATCTATGAA-3′). The cycle program used was: 5 min at 94°C, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension step of 10 min at 72°C. Products were analyzed in a 1.5% agarose gel in TBE (88.9 mM Tris, 8.9 mM boric acid, 2.5 mM EDTA, pH 8.3) and stained with ethidium bromide at 0.5 µg/ml to visualize the products. To confirm *X. fastidiosa* specificity, the mixture of primers was also tested with *Xanthomonas campestris* pv. *campestris* and with *Xanthomonas axonopodis* pv. *vesicatoria* strains.

To compare the results obtained with the specific primer sets, the 16S-23S rDNA ISR of *X. fastidiosa* strains isolated here (Table 1) were PCR-amplified using primers G1 (5′-GAAGTCGTAACAAGG-3′) and L1 (5′-CAAGGCATCCACCGT-3′) as described previously (22). After agarose gel electrophoresis, the PCR products were cut from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. Purified fragments were cloned into pGEM-T Easy Vector Systems (Promega), and two clones per each strain were sequenced. Table 1 shows the nucleotide sequence accession numbers of the amplified DNA as well as some other sequences obtained from GenBank. For the analysis, the sequences were aligned with the CLUSTALX package (38), and the phylogenetic tree was constructed using maximum parsimony analysis with the PHYLIP software (19). After performing 1,000 bootstrap replicates with the SEQBOOT program, each replicate was analyzed with DNAPARS program using the jumble option to randomize the nucleotide sequence input order and finally a majority-rule consensus tree was generated with the CONSENSE program.

Table 2. Banding patterns of polymerase chain reaction (PCR) products obtained from *Xylella fastidiosa* strains with the designed primers sets

Host of origin	Strain name	PCR fragment sizes[a] XF1968 (638 bp)	ALM (521 bp)	XF2542 (412 bp)	Subspecies/strain genotype based on multiprimer PCR[b]
Grape	PD-PDF VI	–	–	+	*fastidiosa*/PD
Grape	Conn Creek	–	–	+	*fastidiosa*/PD
Grape	A05	–	–	+	*fastidiosa*/PD
Grape	Fetzer	–	–	+	*fastidiosa*/PD
Grape	STL	–	–	+	*fastidiosa*/PD
Grape	Preston Ranch	–	–	+	*fastidiosa*/PD
Grape	Medeiros	–	–	+	*fastidiosa*/PD
Grape	Traver	–	–	+	*fastidiosa*/PD
Grape	Douglas	–	–	+	*fastidiosa*/PD
Grape	Hopland	–	–	+	*fastidiosa*/PD
Grape	PD 95-2	–	–	+	*fastidiosa*/PD
Grape	I03	–	–	+	*fastidiosa*/PD
Spanish broom	N10	–	–	+	*fastidiosa*/PD
Brassica sp.	G10	–	–	+	*fastidiosa*/PD
Almond	237	–	–	+	*fastidiosa*/PD
Almond	239	–	–	+	*fastidiosa*/PD
Almond	189	–	–	+	*fastidiosa*/PD
Almond	H51	–	–	+	*fastidiosa*/PD
Almond	H10	–	–	+	*fastidiosa*/PD
Almond	Fresno-ALS	–	–	+	*fastidiosa*/PD
Almond	Stanislaus	–	–	+	*fastidiosa*/PD
Almond	Tulare	–	–	+	*fastidiosa*/PD
Almond	ALS5	+	+	–	*Multiplex*/ALSI
Almond	ALS2	+	+	–	*Multiplex*/ALSI
Almond	ALS3	+	+	–	*Multiplex*/ALSI
Almond	Dixon	+	+	–	*Multiplex*/ALSI
Almond	ALS9	+	+	–	*Multiplex*/ALSI
Almond	276	+	+	+	*Multiplex*/ALSII
Almond	187	+	+	+	*Multiplex*/ALSII
Almond	ALS6	+	+	+	*Multiplex*/ALSII
Almond	H50	+	+	+	*Multiplex*/ALSII
Oleander	H44	+	–	–	*sandyi*/OLS
Oleander	H45	+	–	–	*sandyi*/OLS
Oleander	Riverside	+	–	–	*sandyi*/OLS
Oleander	Ann1	+	–	–	*sandyi*/OLS
Oleander	Cathedral city	+	–	–	*sandyi*/OLS
Oleander	Texas	+	–	–	*sandyi*/OLS
Oleander	TR2	+	–	–	*sandyi*/OLS

[a] Primers used for PCR were XF1968 (GGAGGTTTACCGAAGACAGAT 3′ and 5′ ATCCACAGTAAAACCACATGC 3′); ALM (5′-CTGCAGAAATTGGAAACTTCAG-3′ and 5′-GCCACACGTGATCTATGAA-3′) and XF2542 (5′TTGATCGAGCTGATGATCG3′ and CAGTACAGCCTGCTGGAGTTA3′). + Means that a DNA product was obtained by electrophoresis. – Means that no DNA product was obtained by electrophoresis.

[b] Subspecies were assigned according to results obtained in the phylogenetic analysis (Fig. 2). Genotypes were based on multiprimer PCR reactions presented in this paper.

RESULTS

Specificity and sensitivity of primers using pure cultures. To identify primer pairs that amplified a product in some strains but not in others or that amplified different size products from different strains, three independent PCR reactions were done using as template DNA of strains STL, Ann1, or Dixon and 113 primer sets (10). Of the 113 primer sets used, two sets did not amplify any DNA fragment from the STL strain but amplified DNA from the Ann1 strain. Two primer sets amplified a DNA fragment from the STL strain but not from the Ann1 strain, and one primer set did not amplify a fragment from the STL or from the Ann1 strains but amplified a fragment from the Dixon strain. To test the consistency of the results, the primer sets were also tested

using additional ALS, PD, and OLS strains (data not shown). Based on the fragment size of the PCR fragments produced, three primer sets were chosen. The first pair, XF1968-L (5′ GGAGGTTTACCGAAGACAGAT 3′) and XF1968-R (5′ ATCCACAGTAAAACCACATGC 3′), designed to amplify part of the XF1968 gene that encodes a putative methyltransferase of the restriction/methylation system (36), produced an amplification product of 638 bp from OLS, but not from PD strains (Table 2). When XF1968-L/XF1968-R primers were used to amplify DNA from ALS strains, no product was obtained from the *X. fastidiosa* subsp. *fastidiosa* strains (237, 239, 189, H51, H10, Tulare, Stanislaus, and Fresno-ALS), whereas DNA from ALS strains from the *X. fastidiosa* subsp. *multiplex* (H50, 187, 276, ALS6, Dixon, ALS3, ALS2, ALS5, and ALS9) yielded a 638-bp product (Table 2).

The second primer set, XF2542-L (5′TTGATCGAGCTGATGATCG3′) and XF2542-R (CAGTACAGCCTGCTGGAGTTA3′), designed from the gene XF2542 that encodes a putative fimbrial protein (36), produced a 412-bp fragment from all PD strains tested (PD-PDF VI, 95-2, Conn Creek, A05, Fetzer, STL, Preston Ranch, Medeiros, Traver, Douglas, Hopland, PD 95-2, and I03), from ALS strains that belong to *X. fastidiosa* subsp. *fastidiosa* (237, 239, 189, H51, H10, Tulare, Stanislaus, and Fresno-ALS), and from ALSII genotype strains (276, 187, H50, and ALS6), but not from oleander strains or from the ALSI genotype strains (Dixon, ALS3, ALS5, ALS2, and ALS9) (Table 2).

Amplification with the third primer set, ALM1 (5′-CTGCAGAAATTGGAAACTTCAG-3′) and ALM2 (5′-GCCACACGTGATCTATGAA-3′), produced a fragment of 521 bp using DNA from *X. fastidiosa* subsp. *multiplex* genotypes ALSI and ALSII (H50, 187, 276, ALS6, Dixon, ALS3, ALS2, ALS5, and ALS9) but not from PD or ALS strain members if the *X. fastidiosa* subsp. *fastidiosa* or from OLS strains (Table 2).

Multiplex PCR assay using pure cultures. When *X. fastidiosa* strains isolated from grape, oleander, and almond were tested using a mixture of the three primer sets, an expected fragment of 412 bp was always amplified, and a smaller band was never noticed from PD and ALS strains of *X. fastidiosa* subsp. *fastidiosa* (Fig. 1, lanes 10 to 17). When OLS strains were tested, a 638-bp band was amplified, and again, no other band was seen (Fig. 1, lanes 18 to 22). In contrast, with ALS strains that belong to *X. fastidiosa* subsp. *multiplex*, two different banding patterns were produced. One, consisting of two fragments, one of 638 bp and another of 521 bp, was observed with the ALSI genotype strains (Fig. 1, lanes 2 to 5). A second group, with three fragments of 412, 521, and 638 bp, was observed with the ALSII genotype strains (Fig. 1, lanes 6 to 9). No PCR products were amplified from *Xanthomonas* strains (Fig. 1, lanes 23 and 24).

Consistency was again tested using DNA from additional strains of *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *fastidiosa,* and *X. fastidiosa* subsp. *sandyi.* Banding patterns were always as expected (Table 2). Since one of our objectives was to find a suitable method for identifying new inoculum sources, we tested our primers with the recently isolated *X. fastidiosa* strains from Spanish broom (N10) and *Brassica* spp. (G10) (13). Both strains were characterized as PD using transmission studies and 16S-23S ISR sequence analysis (13). As expected, when we used the multiplex PCR assay, a fragment of 412 bp was amplified from both N10 and G10 strains (Table 2).

Comparison of 16S-23S ISR sequences of *X. fastidiosa* strains. In the case of the PD, OLS, and ALS strains, the *X. fastidiosa* subsp. *multiplex*, the topology of the phylogenetic tree obtained showed that they belong to different groups (Fig. 2). It also showed that the ALS strains 239, H51, 189, Tulare, Fresno, 237, H10, and Stanislaus grouped with PD strains, demonstrating that they were members of *X. fastidiosa* subsp. *fastidiosa* (Fig. 2). We were unable to differentiate between ALSI and ALSII genotypes by 16S-23S rDNA ISR sequences comparisons.

Multiplex PCR using infected oleander, grape, and almond tissues. The mixed primers were tested with DNA extracted from infected grape, oleander, and almond plants. First, to determine if the extraction method was effective, extracted DNA was tested in PCR reactions with the RST31/RST33 primers (29). When DNA extracted from *X. fastidiosa*–infected oleander, grapevine, or almond was used as template, a single 721-bp band was produced in all cases (Fig. 3, upper panel, lanes 2 to 9), confirming the success of the extraction method. Since the RST31/RST33 primers do not distinguish among subspecies, when infected grapevine and oleander tissues were mixed and extracted together, the reaction still amplified a single 733-bp band (Fig. 3, upper panel, lane 10). In contrast, when the multiprimer PCR method was used to amplify extracts from infected grape, a band of 412 bp was produced (Fig. 3, lower panel, lanes 2 and 3). When extracts from infected oleander were amplified with the multiprimer mixture, a 638-bp band was produced (Fig. 3, lower panel, lanes 4 and 5). When extracts from infected almond were used as template, almonds infected with strain Tulare-ALS resulted in a single band of 412 bp (Fig. 3, lower panel, lanes 6 and 7), whereas almonds infected with strain 276 resulted in the three bands of 638, 521, and 412 bp (Fig. 3, lower panel, lanes 8 and 9). When oleander and grape tissues were mixed together and extracted, amplification produced both expected fragments of

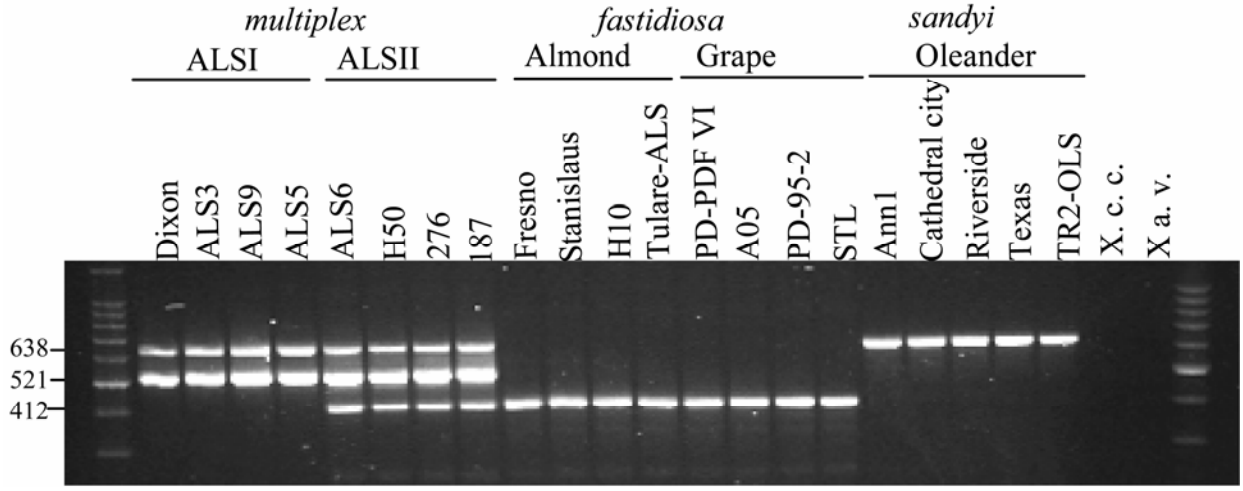

Fig. 1. Gel electrophoresis of polymerase chain reaction (PCR) amplification products from some *Xylella fastidiosa* strains using a mixture of primers (XF2542-L/XF2542-R, XF1968-L/XF1968-R, and ALM1/ALM2). Multiplex X.c.c. = *Xanthomonas campestris* pv. *campestris* and X.a.v. = *X. axonopodis* pv. *vesicatoria.* First and last lanes are 100-bp DNA molecular size ladder.

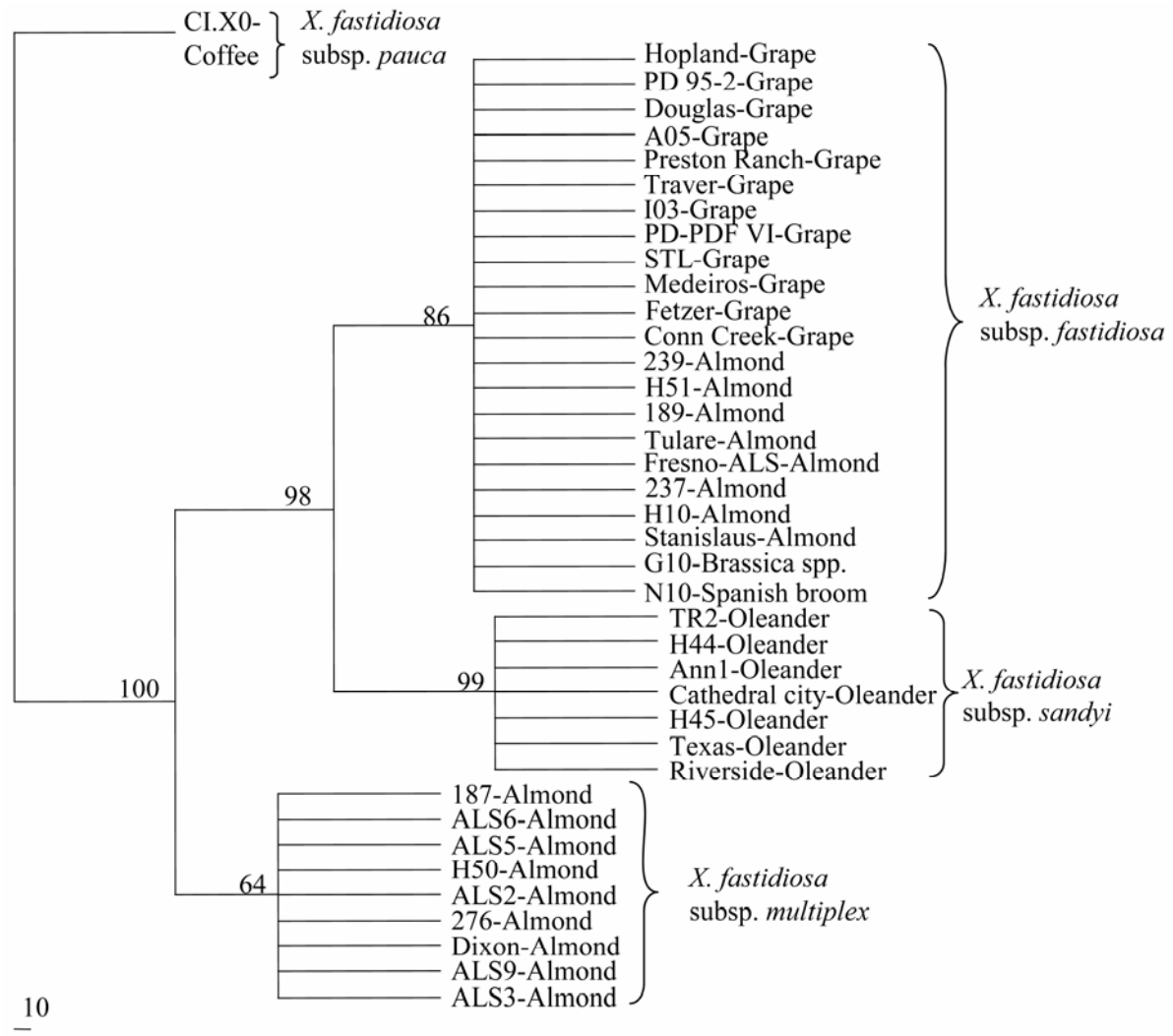

Fig. 2. Consensus phylogenetic tree of the 16S-23S rDNA intergenic sequences region of *Xylella fastidiosa* strains. The DNAPARS program from the PHYLIP software (19) was used to construct the maximum parsimony tree. Numbers above the branches represent bootstrap percentage values obtained for 1,000 replicates. See Table 1 for strain details.

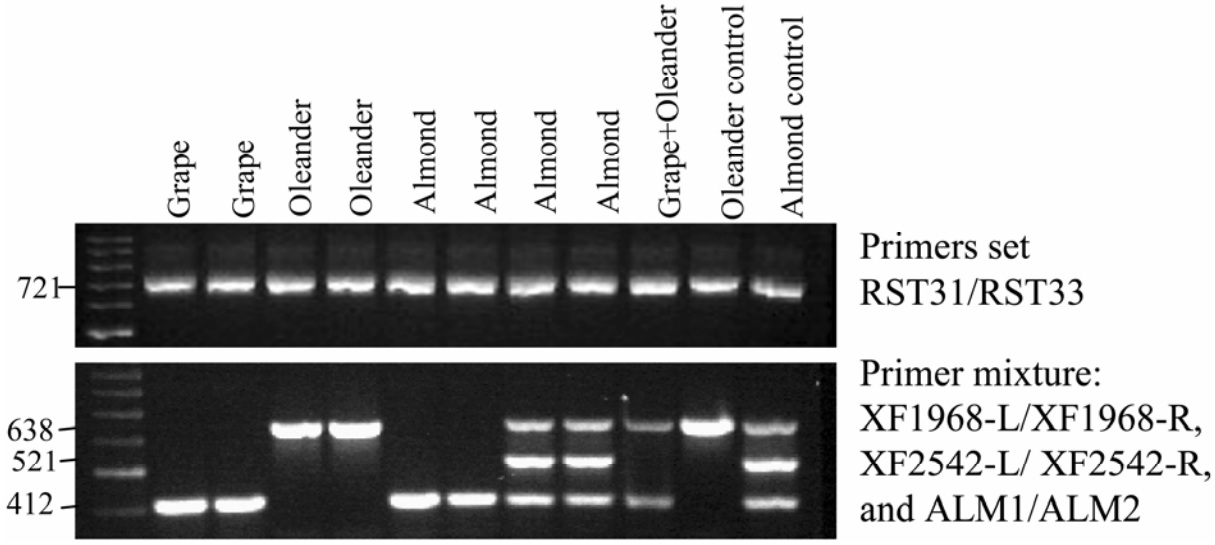

Fig. 3. Gel electrophoresis of amplification products of DNA extracted from *Xylella fastidiosa*–infected plants. Primer set RST31-33 was used in the upper panel and a mixture of strain-specific primers: XF2542-L/XF2542-R, XF1968-L/XF1968-R, and ALM1/ALM2 in the lower. Lanes 1 and 2, DNA extracted from infected grapevines; lanes 3 and 4, DNA extracted from infected oleander; lanes 5 to 8, DNA extracted from infected almond; lane 9, a mixture of DNA extracted from infected grape and oleander tissue; lanes 10 and 11 are controls: 10, 50 ng of Ann1, extracted DNA, and 11, 50 ng of 276 extracted DNA. First and last lanes are 100-bp DNA molecular size ladder.

412 and 638 bp (Fig. 3, lower panel, lane 10). To confirm *X. fastidiosa* identity, selected amplified fragments were cloned and sequenced. A BLASTn (Basic Local Alignment Search Tool) search identified the sequence as identical to *X. fastidiosa* strains.

DISCUSSION

The mixture of three primer sets in a single PCR amplification step allowed the differentiation of strains of *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *fastidiosa,* and *X. fastidiosa* subsp. *sandyi*. Our method also allowed the distinction of two groups that infect almond, genotypes ALSI and ALSII, within the *multiplex* subspecies. These genotypes were previously differentiated using RAPD analysis (3).

Epidemiological studies of diseases produced by *X. fastidiosa* require methods that allow simple and rapid distinction of different strains. Natural mixed infection by several subspecies of *X. fastidiosa* in a single location has been demonstrated (6,13), as well as in a single plant (6). Previously, strains were differentiated by their ability to grow on PD3 medium (3) or by PCR amplification of specific fragments followed by restriction enzyme analysis (3,22). Another system used single nucleotide polymorphisms in the 16S rDNA sequence of *X. fastidiosa* to distinguish between PD and ALS strains (6). The multiprimer-PCR detection method described here proved to be more convenient than previous ones, since it only required a single step, and distinguished among three subspecies (*fastidiosa, sandyi,* and *multiplex*) as well as between the ALSI and ALSII genotypes within the *multiplex* subspecies. Therefore, the method provides a tool for rapid screening that can be used for field surveys to distinguish the three subspecies in plant samples or to determine the relative ability of an individual insect vector to simultaneously retain and transmit different *X. fastidiosa* strains.

Strains of *X. fastidiosa* from grape and almond were once considered to be genetically indistinguishable (17,30), but more recent data showed that there are three distinctive genetic groupings among strains from almond, with only one of those groups being closely related to PD strains (3). The ALS strains that grouped with PD strains genetically have in vitro growth characteristics similar to PD strains and cause symptoms in grapes (3). In our previous work, almond strains 237, 239, 189, H51, H10, Fresno-ALS, Stanislaus, and Tulare were shown to belong to this PD genotype (13,22,24). In this study, we were able to differentiate these members of *X. fastidiosa* subsp. *fastidiosa* from the other ALS strains in *X. fastidiosa* subsp. *multiplex* and from *X. fastidiosa* subsp. *sandyi* by a simple PCR analysis. A second genotype of strains infecting almond is ALSI. Strains from this genotype of *X. fastidiosa* subsp. *multiplex* are unable to cause disease in grape, are more fastidious in culture, and have a distinctive colony morphology (3,6). The strains Dixon, ALS2, ALS3, ALS9, and ALS5 belong to this genotype (3,22). In our study, those are the strains from which two PCR amplification products were obtained (638 and 521 bp). The last genotypic group from almond, ALSII, comprises less fastidious strains, which infect almonds and seem to produce mild PD symptoms in grape (3). Our PCR amplification from strains of this genotype produced three bands (638, 521, and 412 bp).

The subdivision of strains from almond into those belonging to *X. fastidiosa* subsp. *fastidiosa* and those belonging to two groups within *X. fastidiosa* subsp. *multiplex* was also supported by a recent multigene phylogenetic analysis, in which 10 coding regions were amplified by PCR, sequenced, and analyzed (34). In that study, the strains 276 and ALS6 formed a group apart from the other *X. fastidiosa* subsp. *multiplex* strains, apparently due to recombination (34). This group corresponds with the ALSII genotype previously described (3) and identified in our multiprimer PCR assay through the production of three amplification products. However, our assay identified an additional almond strain, 187, as ALSII by the production of three PCR amplification products, but in the multi-gene analysis by Schuenzel et al. (34), this strain grouped with ALSI. Further genetic analysis of strain 187 is underway to evaluate this discrepancy.

Another previous method based on single nucleotide polymorphisms (SNP) in the 16S rDNA gene of *X. fastidiosa* was used to distinguish two different genotypes of strains affecting almonds (6). Our method is different from that work, since we were able to distinguish three ALS genotypes and distinguished OLS strains from PD and ALS strains.

Culturing *X. fastidiosa* from an infected plant is often difficult and time-consuming. The method used here for extracting DNA from infected tissue provided clean and sufficient DNA for PCR amplification without the need for culturing the bacterium. Plant DNA did not present a problem since the primer mixture specifically amplifies DNA sequences from *X. fastidiosa*. The mixing of strain-specific primers in a single reaction and the use of Ready-to-go PCR beads minimizes the number of samples needed, the amount of handling required for identification, and the cost.

The complete host range of *X. fastidiosa* has probably not been fully defined, and its description is limited by our ability to isolate bacteria from new hosts (25). The arrival of a highly effective vector, the glassy-winged sharpshooter, has increased the spread of *X. fastidiosa* in southern California (4). Currently, we are conducting studies using the method developed here to detect *X. fastidiosa* in various landscape plants, and to determine the ability of the glassy-winged sharpshooter to retain and transmit multiple strains of *X. fastidiosa.*

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