Genetic Variation Among *Xylella fastidiosa* Strains

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ABSTRACT

Chen, J., Chang, C. J., Jarret, R. L., and Gawel, N. 1992. Genetic variation among *Xylella fastidiosa* strains. Phytopathology 82:973-977.

Restriction fragment length polymorphism (RFLP) analysis was used to determine similarities and differences in DNAs among 24 strains of *Xylella fastidiosa* from eight different hosts. Two genomic libraries were constructed separately in pUC18 from two strains of *X. fastidiosa*: strain R112V2, isolated from grapevine showing Pierce's disease symptoms in Georgia; and strain PLMG83 (ATCC 35871), originally isolated from plum with leaf scald disease. The 12 probes from the R112V2 library, together with two restriction enzymes (24 probe-enzyme combinations), generated 67 scorable characters. Similarity coefficients (S_{xy}) among the 24 *X. fastidiosa* strains ranged from 0.58 to 1.0. Sixteen strains from Pierce's disease of grapevine examined had striking genetic uniformity ($S_{xy} > 0.87$), suggesting the presence of Pierce's disease RFLP group in *X. fastidiosa*. RFLP analysis, using probes from the PLMG83 library, yielded similar results.

Xylella fastidiosa is the causal agent of many economically important plant diseases (18). Pierce's disease of grapevine, for example, deleteriously affects grape production in many areas of the world and has been a limiting factor in the expansion of the winery industry in the southeastern United States (10). *X. fastidiosa* also causes diseases in trees, such as almond, elm, mulberry, oak, and sycamore (11). The potential impact of *X. fastidiosa* on urban forestry is high.

Plant diseases caused by *X. fastidiosa* were believed to be of a viral etiology until 1973, when two reports described the association of a rickettsialike organism (later more appropriately referred to as xylem-limited bacteria [XLB]) with Pierce's disease of grapevine (8,12). More than 10 strains of XLB were isolated from various host plants and classified as a single species, *X. fastidiosa* (18). Differences exist between strains for characteristics such as pathogenicity, nutritional fastidiousness, and DNA homology (11). SDS-polyacrylamide gel electrophoresis of total cellular proteins indicates that strains of *X. fastidiosa* share a number of similarities with, but can still be differentiated from, one another (1). DNA homology studies (13) indicated that Pierce's disease-inducing strains are distinct from strains that cause phony disease of peach, plum leaf scald, and periwinkle wilt.

Recently, several reports have demonstrated the use of restriction fragment length polymorphisms (RFLPs) to differentiate plant pathogenic bacteria at the pathovar level (7,9). Analysis of RFLPs offers several advantages when compared to analysis of characteristics such as pathogenicity, protein polymorphisms, DNA–DNA hybridization kinetics, and so on. RFLPs are not only insensitive to environmental or pleiotropic effects but are

TABLE 1. Strains of *Xylella fastidiosa*

Number	Strain	Host	Disease	Origin	Source
1	R112V2	Grapevine	Pierce's disease	Georgia	C. J. Chang[a]
2	MT1	Alfalfa	Alfalfa dwarf	California	M. Davis[b]
3	R30V15	Grapevine	Pierce's disease	Georgia	C. J. Chang
4	R112V14	Grapevine	Pierce's disease	Georgia	C. J. Chang
5	CGA13	Grapevine	Pierce's disease	Georgia	C. J. Chang
6	A4	Grapevine	Pierce's disease	Georgia	C. J. Chang
7	Chateau 3C	Grapevine	Pierce's disease	Georgia	C. J. Chang
8	PD82-21	Grapevine	Pierce's disease	Florida	D. Hopkins[c]
9	PD88-5A	Grapevine	Pierce's disease	Florida	D. Hopkins
10	PD-1	Grapevine	Pierce's disease	Florida	D. Hopkins
11	PD88-8	Grapevine	Pierce's disease	Florida	D. Hopkins
12	PD88-6A	Grapevine	Pierce's disease	Florida	D. Hopkins
13	PD-R	Grapevine	Pierce's disease	Florida	D. Hopkins
14	CP20	Grapevine	Pierce's disease	North Carolina	S. Fry[d]
15	FCP14	Grapevine	Pierce's disease	North Carolina	S. Fry
16	mul1	Mulberry	Leaf scorch	...	ATCC 35868[e]
17	mul3	Mulberry	Leaf scorch	...	ATCC 35869
18	ALS-BC	Almond	Leaf scorch	...	ATCC 35870
19	PLMG83	Hybrid plum	Leaf scald	...	ATCC 35871
20	ELM-1	Elm	Leaf scorch	...	ATCC 35873
21	RGW-R	Ragweed	Stunt	...	ATCC 35876
22	PCE-GG	Grapevine	Pierce's disease	...	ATCC 35877
23	PWT-22	Periwinkle	Wilt	...	ATCC 35878
24	PCE-RR	Grapevine	Pierce's disease	...	ATCC 35879

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[b]Tropical Research and Education Center, University of Florida, Homestead 30031.
[c]University of Florida, Leesburg 34748.
[d]Department of Plant Pathology, North Carolina State University, Raleigh 27695.
[e]American Type Culture Collection, Rockville, Maryland 20852-1776.

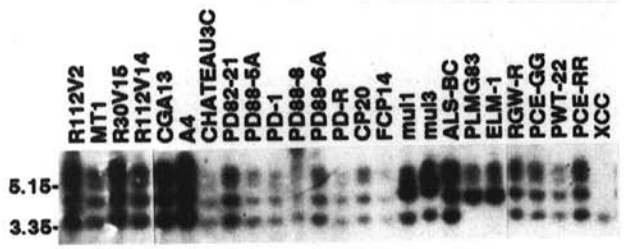

Fig. 1. Restriction fragment length polymorphisms from strains of *Xylella fastidiosa* and *Xanthomonas campestris* pv. *campestris* (XCC) probed with pXFPD10. Note the different hybridization patterns for mul1, mul3, PLMG83, and ELM-1. Numbers on the left are DNA size markers in kilobases from *Eco*RI–*Hin*dIII digestion of phage λ DNA.

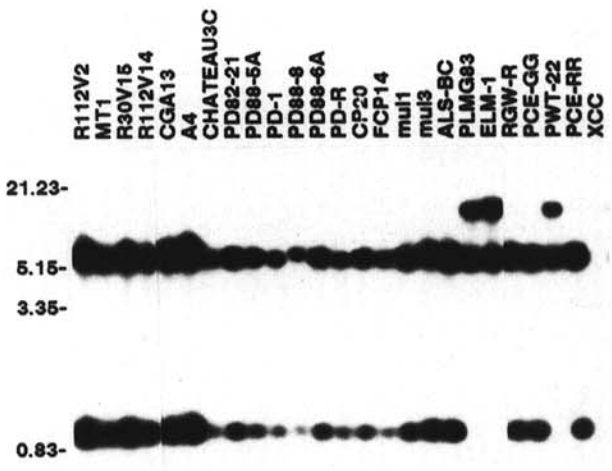

Fig. 2. Restriction fragment length polymorphisms from strains of *Xylella fastidiosa* and *Xanthomonas campestris* pv. *campestris* (XCC) probed with pXFPD49. Note the different hybridization patterns for PLMG83, ELM-1, and PWT-22. Numbers on the left are DNA size markers in kilobases from *Eco*RI–*Hin*dIII digestion of phage λ DNA.

also sensitive to detect small differences in DNA sequences (15). This makes RFLP analysis particularly suitable for differentiating closely related bacterial strains.

In this paper, we report the genetic variation among 24 strains of *X. fastidiosa* based on RFLP analysis. The genetic relationship of one strain of *Xanthomonas campestris* pv. *campestris* to *X. fastidiosa* is also studied.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. *X. fastidiosa* strains ATCC 35868, ATCC 35869, ATCC 35871, ATCC 35876, and ATCC 35878 were maintained in PW (periwinkle wilt) agar medium (4). All other *X. fastidiosa* strains were maintained in PD2 (Pierce's disease) medium (5) (Table 1). Since *X. fastidiosa* was reported to be related to *Xanthomonas* spp. (18), one Georgia strain of *Xanthomonas campestris* pv. *campestris*, causing black rot disease of cabbage, was included for comparison. The strain of *X. c. campestris* was maintained in YDC (yeast-dextrose-calcium carbonate) agar (19). All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

For DNA extraction, each *X. fastidiosa* strain was aerobically cultured in 200 ml of CS20 (Chang-Schaad) medium (2) with agitation on a gyrotory shaker (100 rpm; model G2, New Brunswick Scientific Co., Inc. Edison, NJ) for 7–14 days at 30 C. Bacteria were harvested by centrifugation at 15,300 *g* for 20 min at 4 C. The bacterial pellet was resuspended in 15 ml of extraction buffer (50 mM Tris-HCl, pH 7.5; 62.5 mM EDTA; and 0.4% Triton X-100) and incubated at 37 C overnight after addition of 200 μl (10 mg/ml) of lysozyme. Protein was removed by extraction with an equal volume of a chloroform–isoamyl alcohol mixture (24:1, v/v). DNA was precipitated with sodium acetate-ethanol (14) and collected by spooling or centrifugation at 4,350 *g* for 5 min. DNA was dissolved in 0.5 ml of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0) containing RNase A (200 g/ml) and further purified by extraction with chloroform–isoamyl alcohol, phenol, and ether (14). DNA

	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	1	0.95	0.96	0.96	0.96	1	0.94	0.96	0.92	0.96	0.96	0.94	0.96	0.96	0.98	0.77	0.83	0.93	0.67	0.67	0.94	0.93	0.68	0.94	0.07
2		1	0.98	0.94	0.98	0.95	0.94	0.96	0.96	0.96	0.96	0.98	0.96	0.98	0.96	0.78	0.80	0.97	0.68	0.68	0.93	0.92	0.69	0.94	0.08
3			1	0.93	1	0.96	0.93	0.97	0.95	0.95	0.95	0.97	0.95	1	0.97	0.77	0.82	0.96	0.69	0.69	0.94	0.93	0.68	0.95	0.08
4				1	0.93	0.96	0.95	0.93	0.91	0.97	0.97	0.95	0.97	0.93	0.95	0.77	0.82	0.94	0.65	0.65	0.90	0.89	0.68	0.91	0.08
5					1	0.96	0.93	0.97	0.95	0.95	0.95	0.97	0.95	1	0.97	0.77	0.82	0.96	0.69	0.69	0.94	0.93	0.68	0.95	0.08
6						1	0.94	0.96	0.92	0.96	0.96	0.94	0.96	0.96	0.98	0.77	0.83	0.93	0.67	0.67	0.94	0.93	0.68	0.94	0.07
7							1	0.91	0.91	0.95	0.95	0.95	0.95	0.93	0.93	0.77	0.82	0.94	0.69	0.69	0.88	0.87	0.71	0.89	0.08
8								1	0.93	0.95	0.95	0.95	0.95	0.97	0.97	0.78	0.82	0.94	0.68	0.68	0.96	0.96	0.69	0.97	0.07
9									1	0.93	0.93	0.95	0.93	0.95	0.93	0.75	0.77	0.94	0.69	0.69	0.90	0.89	0.71	0.91	0.08
10										1	1	0.97	1	0.95	0.97	0.80	0.84	0.96	0.67	0.67	0.92	0.91	0.71	0.93	0.08
11											1	0.97	1	0.95	0.97	0.80	0.84	0.96	0.67	0.67	0.92	0.91	0.71	0.93	0.08
12												1	0.97	0.97	0.95	0.79	0.81	0.98	0.69	0.69	0.92	0.91	0.70	0.93	0.08
13													1	0.95	0.97	0.80	0.84	0.96	0.67	0.67	0.92	0.91	0.71	0.93	0.08
14														1	0.97	0.77	0.82	0.96	0.69	0.69	0.94	0.93	0.68	0.95	0.08
15															1	0.78	0.84	0.94	0.68	0.68	0.94	0.94	0.69	0.95	0.07
16																1	0.92	0.78	0.58	0.58	0.81	0.82	0.69	0.80	0.08
17																	1	0.82	0.61	0.61	0.83	0.84	0.68	0.84	0.08
18																		1	0.68	0.68	0.91	0.90	0.69	0.92	0.08
19																			1	1	0.67	0.66	0.84	0.65	0
20																				1	0.67	0.66	0.84	0.65	0
21																					1	0.99	0.73	0.96	0.07
22																						1	0.74	0.98	0.07
23																							1	0.71	0
24																								1	0.07
25																									1

*Numbers encode the names of bacteria as shown in Table 1; Xanthomonas campestris pv. campestris strain XCC = 25.

Fig. 3. Similarity coefficients of 24 strains of *Xylella fastidiosa* and one strain of *Xanthomonas campestris* pv. *campestris* produced by *Hin*dIII and *Bam*HI digestions using 12 probes from the R112V2 library.

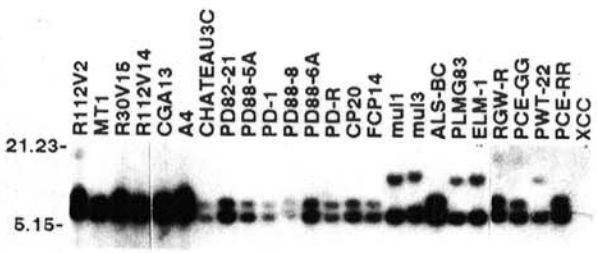

Fig. 4. Restriction fragment length polymorphisms from strains of *Xylella fastidiosa* and *Xanthomonas campestris* pv. *campestris* (XCC) probed with pXFPLS2. Note the different hybridization patterns from mul1, mul3, PLMG83, ELM-1, and PWT-22. Numbers on the left are DNA size markers in kilobases from *Eco*RI–*Hin*dIII digestion of phage λ DNA.

was precipitated with sodium acetate-ethanol and dissolved in TE. The last two steps (precipitation with sodium acetate-ethanol and dissolving in TE) were repeated twice. DNA was quantified to yield approximately 100μg of DNA from 200 ml of cell culture. The same protocol was used to isolate DNA from *X. c. campestris* cells that were grown in Difco nutrient broth.

Genomic library construction. A Pierce's disease strain (R112V2) and a plum leaf scald disease strain (PLMG83) of *X. fastidiosa* were used for the construction of genomic libraries. DNA was isolated as described above, digested with *Eco*RI and *Hin*dIII, ligated into pUC18 and used to transform *Escherichia coli* TB1 (provided by T. P. Denny, Department of Plant Pathology, University of Georgia, Athens) (14). Recombinant plasmids were isolated by the boiling method (14) with slight modification (3). Twelve recombinant plasmids from the R112V2 library and eight from the PLMG83 library were randomly selected for use as probes in detecting RFLPs; insert sizes ranged from 0.5 to 2.6 kb.

Detection of RFLPs. DNA (1–3 μg) of *X. fastidiosa* and *X. c. campestris* were completely digested with *Bam*HI or *Hin*dIII and electrophoresed in 0.8% agarose at 3 V/cm for 3 h. Gels were stained with ethidium bromide and viewed under UV light. DNA was Southern-blotted onto nylon membranes (Bio-Trans, ICN Biochemicals, Cleveland, OH) (14). Hybridizations were performed according to Sambrook et al (16). DNA probes were random primer-labeled (BRL, Gaithersburg, MD) with [^{32}P]dCTP (50 μCi; E. I. du Pont de Nemours & Company, Wilmington, DE). Membranes were prehybridized at 65 C for 4 h. Following addition of the probe (approximately 200 ng DNA), hybridizations were performed at 65 C for 18 h. Membranes were washed once in 2× SSC (sodium chloride–sodium citrate) + 0.1% SDS (sodium dodecyl sulfate) for 30 min (at 65 C), and twice in 0.1× SSC + 0.1% SDS for 30 min (at 65 C). Kodak X-Omat AR films were exposed to membranes at −130 C for 24–48 h with intensifying screens.

Similarity analysis. Banding patterns of each probe-enzyme combination were recorded for each strain. Data of all probe–enzyme combinations from the same library were combined for analysis. Similarity coefficients (S_{xy}) (15) between every two strains were calculated as $S_{xy} = 2N_{xy}/(N_x + N_y)$, where N_x and N_y are the number of bands in strains x and y, respectively, and N_{xy} is the number of bands shared by both strain x and strain y. Calculations were performed using a program in GWBASIC (available upon request).

To more clearly visualize bacterial strain relationships, principal component analysis was performed using the SAS/ETS system (17) to reduce the data dimensions from similarity coefficient data.

RESULTS AND DISCUSSION

Twenty-four probe–enzyme combinations were evaluated for their ability to detect RFLPs using probes from the R112V2 library. Hybridization of *Hin*dIII- and *Bam*HI-cleaved DNA with 12 probes from the R112V2 library produced 42 *Hin*dIII and 25 *Bam*HI scorable characters. Polymorphisms were not detected with two *Bam*HI digests. One to five bands were detected with other probe–enzyme combinations in each bacterial strain (Figs. 1 and 2), and so no highly repetitive DNA sequence was cloned and used in this study. Similarity coefficients are presented in Figure 3.

Polymorphisms were detected with all probes from the PLMG83 library in both the *Hin*dIII and *Bam*HI digests. Numbers of scorable characters generated for each probe–enzyme combination are similar to those from the R112V2 library. A representative autoradiogram is presented in Figure 4. A total of 44 scorable characters were generated—29 from *Hin*dIII digests and 15 from the *Bam*HI digests. The distributions of similarity coefficients among the 25 bacterial strains are very similar when using probes from either R112V2 or PLMG83 libraries (Figs. 3 and 5), confirming the utility of randomly selected probes in this study.

The similarity coefficients between strains of *X. fastidiosa* and *X. c. campestris* were less than 0.08 (Figs. 3 and 5), indicating little genetic relatedness between the two species at the level of discrimination conferred by RFLP analysis with randomly selected genomic probes. In contrast, similarity coefficients among strains of *X. fastidiosa* were greater than 0.58 (Figs. 3 and 5). These results suggest that all *X. fastidiosa* strains examined are closely related, and they support the previous proposal that XLB constitute a homogenous group at the species level (18).

Analysis of similarity coefficients (Figs. 3 and 5) indicates that the strains from Pierce's disease of grapevine, alfalfa dwarf, and

	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	1	0.98	1	1	1	1	0.96	0.93	0.88	0.96	1	0.96	1	1	0.98	0.80	0.80	0.98	0.65	0.65	0.96	0.96	0.71	0.96	0.06
2		1	0.98	0.98	0.98	0.98	0.94	0.91	0.86	0.94	0.98	0.98	0.98	0.98	0.96	0.79	0.79	1	0.64	0.64	0.94	0.94	0.73	0.94	0.06
3			1	1	1	1	0.96	0.93	0.88	0.96	1	0.96	1	1	0.98	0.80	0.80	0.98	0.65	0.65	0.96	0.96	0.71	0.96	0.06
4				1	1	1	0.96	0.93	0.88	0.96	1	0.96	1	1	0.98	0.80	0.80	0.98	0.65	0.65	0.96	0.96	0.71	0.96	0.06
5					1	1	0.96	0.93	0.88	0.96	1	0.96	1	1	0.98	0.80	0.80	0.98	0.65	0.65	0.96	0.96	0.71	0.96	0.06
6						1	0.96	0.93	0.88	0.96	1	0.96	1	1	0.98	0.80	0.80	0.98	0.65	0.65	0.96	0.96	0.71	0.96	0.06
7							1	0.89	0.84	0.92	0.96	0.93	0.96	0.96	0.94	0.76	0.76	0.94	0.64	0.64	0.92	0.92	0.70	0.92	0.07
8								1	0.81	0.93	0.93	0.90	0.93	0.93	0.91	0.87	0.87	0.91	0.69	0.69	0.89	0.89	0.67	0.89	0.06
9									1	0.88	0.88	0.85	0.88	0.88	0.86	0.72	0.72	0.86	0.64	0.64	0.84	0.84	0.70	0.84	0.06
10										1	0.96	0.93	0.96	0.93	0.94	0.80	0.80	0.94	0.69	0.69	0.93	0.93	0.71	0.93	0.06
11											1	0.96	1	1	0.98	0.80	0.80	0.98	0.65	0.65	0.96	0.96	0.71	0.96	0.06
12												1	0.96	0.96	0.94	0.77	0.77	0.98	0.66	0.66	0.93	0.93	0.75	0.93	0.06
13													1	1	0.98	0.80	0.80	0.98	0.65	0.65	0.96	0.96	0.71	0.96	0.06
14														1	0.98	0.80	0.80	0.98	0.65	0.65	0.96	0.96	0.71	0.96	0.06
15															1	0.82	0.82	0.96	0.62	0.62	0.94	0.94	0.69	0.94	0.06
16																1	1	0.79	0.70	0.70	0.84	0.84	0.72	0.84	0.06
17																	1	0.79	0.70	0.70	0.84	0.84	0.72	0.84	0.06
18																		1	0.64	0.64	0.94	0.94	0.73	0.94	0.06
19																			1	1	0.61	0.61	0.79	0.61	0
20																				1	0.61	0.61	0.79	0.61	0
21																					1	1	0.75	1	0.06
22																						1	0.75	1	0.06
23																							1	0.75	0.07
24																								1	0.06
25																									1

*Numbers encode the names of bacteria as shown in Table 1; Xanthomonas campestris pv. campestris strain XCC = 25.

Fig. 5. Similarity coefficients of 24 strains of *Xylella fastidiosa* and one strain of *Xanthomonas campestris* pv. *campestris* produced by *Hin*dIII and *Bam*HI digestions using eight probes from the PLMG83 library.

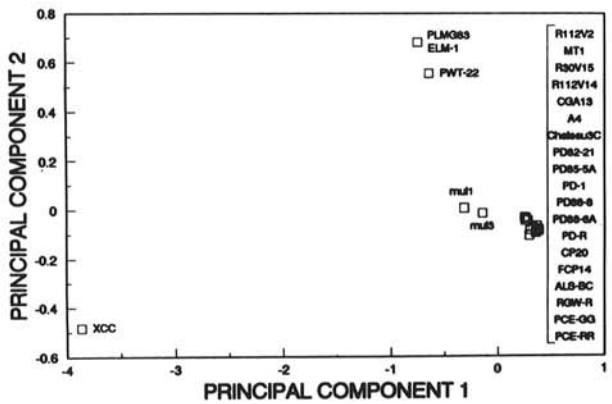

Fig. 6. A two-dimensional visualization of genetic relationships among bacterial strains based on the data from Figure 3 by using principal component analysis.

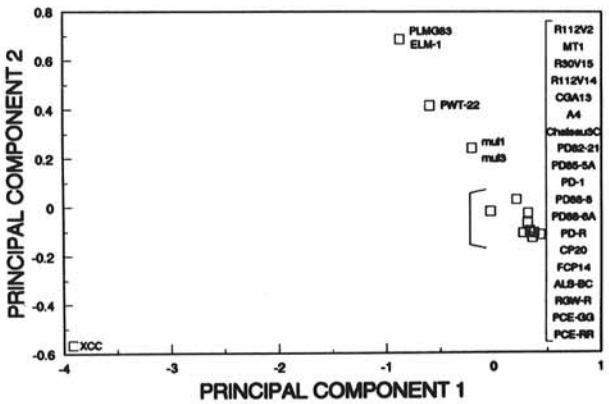

Fig. 7. A two-dimensional visualization of genetic relationships among bacterial strains based on the data from Figure 5 by using principal component analysis.

almond leaf scorch diseases, which have S_{xy} greater than 0.80, comprise a Pierce's disease RFLP group. This supports a previous report suggesting that these three diseases were caused by the same strain of *X. fastidiosa* (6). All bacterial strains in the Pierce's disease group have lower similarity coefficients (0.61–0.69) to PLMG83 and ELM-1 strains. The mul1 and mul3 strains are close to the strains in Pierce's disease group (0.72–0.87) but removed from the PLMG83 and ELM-1 strains (0.58-0.70). In contrast, PWT-22 is nearer to the PLMG83 and ELM-1 strains (0.79–0.84) than to the strains in Pierce's disease group (0.67–0.75). Figures 6 and 7 depict the relationships among different strains as determined by principal component analysis. The first two principal components accounted for 97% of the total variance.

In most instances, our findings concur with results reported previously, such as the characteristics of protein profiles, pathogenicity, and DNA–DNA hybridizations (1,11,13,18). However, our data showed that a strain associated with the ragweed stunt disease was included in the Pierce's disease group, rather than in the closely related plum leaf scald strain, as suggested previously (11). Our data indicate that ELM-1 is very similar to PLMG83, which contrasts with an earlier report (18) that strains causing elm leaf scorch disease are significantly different from those causing plum leaf scald disease based on their growth rate in PW broth medium. Additional research is required to clarify these contradictions.

In summary, we have demonstrated the occurrence of genetic variation among strains of *X. fastidiosa* using RFLP analysis. *X. fastidiosa* strains of Pierce's disease pathotype show striking genetic uniformity, indicating the presence of a Pierce's disease RFLP group. However, to confirm the presence of other RFLP group(s) in *X. fastidiosa*, more strains from other hosts or pathotypes need to be studied.

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