Genetic Diversity of *Xylella fastidiosa* Strains from Costa Rica, São Paulo, Brazil, and United States

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ABSTRACT

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The diversity of 42 *Xylella fastidiosa* strains from Costa Rica, São Paulo, Brazil, and the United States were analyzed using the sequence of the 16S rRNA gene by variable number of tandem repeat (VNTR) fragment analysis and by restriction fragment length polymorphisms (RFLP) of a specific polymerase chain reaction (PCR)-amplification product using enzyme *Cfo*I. Limited variability in the sequence of the 16S rRNA gene was observed and, although the separation was not absolute, most strains from Costa Rica clustered with strains from the United States and not with strains from São Paulo. The PCR-RFLP produced different patterns of DNA bands. The same pattern was shared by strains from Costa Rica, the United States, and two coffee strains from São Paulo, but a different pattern was observed in six coffee and orange strains from Brazil. In all, 32 amplification products were scored in the VNTR fragment analysis. The total variation observed among the *X. fastidiosa* strains had significant ($P < 0.001$) contributions from both geography and host origin as inferred by Nei's values of genetic diversity and WINAMOVA statistics. The strains from Costa Rica were isolated from diseased grapevines, coffee, and sweet orange and these strains grouped together and could be distinguished from strains from grapevine from the United States or from either coffee or sweet orange from São Paulo. The strains tested from Costa Rica are most likely of local origin, although the possibility that they have been introduced along with horticultural crops cannot be excluded. In either case, they are examples of independent selection of strains of *X. fastidiosa* affecting coffee and sweet orange. Greater genetic similarity was observed between strains from Costa Rica and the United States than with those from São Paulo.

Additional keywords: citrus variegated chlorosis, coffee leaf scorch, Pierce's disease.

Xylella fastidiosa (60) is the causal agent of several critically important plant diseases in the Americas. The bacterium first was isolated from grapevines with Pierce's disease (PD) in North America (15). It also causes leaf scorch of several fruit tree, ornamental, and forest plants (26,27,45). In Brazil, it has become extremely important due to citrus variegated chlorosis (CVC) (23) and coffee leaf scorch (CLS) (16). PD has been described as a reemerging problem and serious threat to the grape industry because of the introduction of the glassy-winged sharpshooter (*Homalodiscus coagulata*) into California. The sweet orange strain of *X. fastidiosa* is a serious production problem for Brazilian sweet orange production (27).

Coffee was introduced to Costa Rica in the late 18th century and, by the 19th century, was the basis of the national economy. The introduction of European grapevines (*Vitis vinifera* L.) during the same period did not, however, lead to a substantial industry. PD was first reported in grapevine in Costa Rica in 1979 (21). At that time, Goheen et al. attributed the failure of the grape industry in Costa Rica to PD rather than to physiological maladjustment of *V. vinifera* to Costa Rica, as had been suggested by plant breeders who found that native species of grapevine, but not *V. vinifera*, could grow in Costa Rica (18), as had been found in the southeastern United States (38). Goheen et al. also suggested that the PD endemic area extended across the Gulf coast and at least as far south as Costa Rica (21). More recently, *X. fastidiosa* was confirmed in coffee (52) and sweet orange (1) plants in Costa Rica. Thus, it is likely that *X. fastidiosa* is native to Costa Rica as well as the southeastern United States and South America. The population structure and molecular variation present in the *X. fastidiosa* population from Costa Rica and its relationship with populations in the United States and São Paulo has not been reported.

Information on genetic variation and relatedness among strains of a plant pathogen are important for disease prevention and management. Strains of *X. fastidiosa* from one host may infect and produce symptoms in another host after experimental inoculation (36,37), but this is not always the case. Thus, the degree of host specificity of strains of *X. fastidiosa* and their genetic relationships are not yet sufficiently understood. This situation has implications beyond the horticultural issues. The United States government includes CVC strains of *X. fastidiosa* on the select agent list of especially threatening foreign pathogens (3). However, apart from association with citrus, there are no standardized criteria to define strains that may or may not be considered CVC strains.

The genetic diversity within *X. fastidiosa* populations from the United States and São Paulo, Brazil have been analyzed by multiple techniques, including arbitrarily primed polymerase chain reaction (PCR) (13); random amplified polymorphic DNA (RAPD)-PCR (9,24,33,42,46,54); restriction fragment length polymorphisms (RFLPs) (6); repetitive extragenic palindromic

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(REP)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR (24,46); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (61); RFLPs in the16S rRNA gene (39) or 16S-23S intergenic spacer-RFLPs (39,54); 16S rRNA gene sequence analysis (40); and 16S-23S intergenic spacer sequence analysis (24,40). Minor discrepancies found among studies may be due to the different techniques and the different populations of *X. fastidiosa* strains assayed. The data summarized above and whole-genome DNA::DNA hybridization data were used as the basis to propose three subspecies within *X. fastidiosa*: subsp. *pierce*i and *multiplex* from the United States and subsp. *pauca* from citrus in São Paulo (55). Coffee and oleander strains were not considered in this taxonomic proposal.

Analysis of variable number of tandem repeat (VNTR) fragments has been used to detect variability within and discriminate among different bacterial strains that are otherwise difficult to characterize, such as strains of *Microbacterium* spp. or *Yersinia pestis* biovars (22,25,30,44). Moreover, because the analysis of VNTR fragments is PCR based, it requires only small quantities of DNA and promises to be very useful for epidemiological studies. The creation of VNTR sequences within protein-coding genes may influence genomic evolution over short time spans (30,53).

Primers targeting short sequence repeats (SSR) for VNTR analysis have been identified and employed to study the genetic structure of *X. fastidiosa* populations in São Paulo, Brazil. These SSR primers showed a high discriminatory power, even greater than the RAPD technique, and have allowed the determination of up to five VNTR multiloci haplotypes in the same plant (10–12), demonstrating a discriminatory power that was greater than other methods used to distinguish among strains of *X. fastidiosa*.

The aim of this work was to evaluate, using the VNTR fragment technique, the genetic diversity of *Xylella fastidiosa* strains from the United States (US); Costa Rica, Central America (CR); and São Paulo, Brazil (Br). The strains tested originated from a wide range of plant hosts, including grapevine (US and CR); sweet orange (Br and CR); coffee (Br and CR); and mulberry, oak, and stone fruit (US). We were particularly interested in determining whether the *X. fastidiosa* strains isolated from grapevine, coffee, and citrus in Costa Rica were genetically similar to strains from the same hosts in other countries and, thus, the result of inadvertent introduction from abroad, or if they have evolved independently in Costa Rica.

MATERIALS AND METHODS

Bacterial strains and DNA preparation. *X. fastidiosa* strains from North America; São Paulo, Brazil; and Costa Rica were grown on solid or in liquid PW medium (14) for DNA extraction (Table 1). Strains with an ATCC prefix are from the American Type Culture Collection, Manassas, VA; other North American

TABLE 1. Strains of *Xylella fastidiosa* analyzed by *Cfo*I digestion of a specific amplification product and variable number of tandem repeat fragment analyses

Bacterial strain[a]	Host	Geographical origin
Funde-3	Sweet orange (*Citrus sinensis*)	São Paulo, Brazil
Funde-5	Sweet orange (*C. sinensis*)	São Paulo, Brazil
LAR 20/11*	Sweet orange (*C. sinensis*)	São Paulo, Brazil
Taq 30*	Sweet orange (*C. sinensis*)	Taquaratinga, Brazil
Café 20/11*	Coffee (*Coffea* sp.)	São Paulo, Brazil
Funde-2	Coffee (*Coffea* sp.)	São Paulo, Brazil
Funde-4*	Coffee (*Coffea* sp.)	Casa Branca, Brazil
122WCoffee	Coffee (*Coffea* sp.)	Brazil
C.sp.-Ic1*	*Citrus* sp.	Santa Elena, Costa Rica
Ca-Ic2*	Coffee (*Coffea arabica*)	Desamparados, Costa Rica
Ca-IIc2*	Coffee (*C. arabica*)	Grecia, Costa Rica
Ca-IIIc3*	Coffee (*C. arabica*)	Grecia, Costa Rica
Ca-IVc2	Coffee (*C. arabica*)	Curridabat, Costa Rica
Ca-Vc1*	Coffee (*C. arabica*)	Desamparados, Costa Rica
Ca-Vc2*	Coffee (*C. arabica*)	Desamparados, Costa Rica
Ca-Vc3*	Coffee (*C. arabica*)	Desamparados, Costa Rica
Ca-VIc2*	Coffee (*C. arabica*)	Curridabat, Costa Rica
Ca-VIc3*	Coffee (*C. arabica*)	Curridabat, Costa Rica
Ca-VIIc1	Coffee (*C. arabica*)	Orosí, Costa Rica
Ca-VIIc2*	Coffee (*C. arabica*)	Orosí, Costa Rica
Ca-VIIIc1	Coffee (*C. arabica*)	Grecia, Costa Rica
Vv-Ic1*	Grapevine (*Vitis* sp.)	Santa Ana, Costa Rica
Vv-Ic2*	Grapevine (*Vitis* sp.)	Santa Ana, Costa Rica
Vv-Ic3*	Grapevine (*Vitis* sp.)	Santa Ana, Costa Rica
Vv-IIc1*	Grapevine (*Vitis* sp.)	San José, Costa Rica
Vv-IIc2*	Grapevine (*Vitis* sp.)	San José, Costa Rica
Vv-IIc3*	Grapevine (*Vitis* sp.)	San José, Costa Rica
ATCC 35870	Almond (*Prunus amygdalus*)	California, United States
ALS-BCG	Almond (*P. amygdalus*)	California, United States
ATCC 35873*	Elm (*Ulmus americanus*)	Washington, D.C., United States
Elm-1	Elm (*U. americanus*)	Washington, D.C., United States
ATCC 35877	Grapevine (*Vitis* sp.)	California, United States
ATCC 35879*	Grapevine (*Vitis* sp.)	Florida, United States
ATCC 35881	Grapevine (*Vitis* sp.)	Florida, United States
PCE-FG*	Grapevine (*Vitis* sp.)	Florida, United States
ATCC 35868	Mulberry (*Morus* sp.)	Massachussets, United States
ATCC 35869	Mulberry (*Morus* sp.)	Massachussets, United States
MUL-1*	Mulberry (*Morus* sp.)	Massachussets, United States
ATCC 35878*	Periwinkle (*Catharantus roseus*)	Florida, United States
ATCC 35871*	Plum (*P. salicina*)	Georgia, United States
ATCC 35876	Ragweed (*Ambrosia artemisiifolia*)	Florida, United States
ATCC 35874*	Red Oak (*Quercus* sp.)	Georgia, United States

[a] The full nucleotide sequence of the 16S rRNA gene of strains marked with an asterisk (*) was used to calculate Figure 3. Sequences from Costa Rica and São Paulo were determined in this work; sequences of the American Type Culture Collection (ATCC) strains were obtained from GenBank.

strains were kindly provided by colleagues in the United States and are kept at the Agricultural Research Service (ARS), United States Department of Agriculture (USDA). Each of the following sample pairs represent one original strain each: MUL-1 and ATCC 35868, ALS-BCG and ATCC 35870, ELM-1 and ATCC 35873, and PCE-FG and ATCC 35881. The first name corresponds to the original identification given by the researchers who performed the original isolations; the second name corresponds to the identification code given at the ATCC when the strains were deposited. These strains have had independent subculturing and storage history since approximately 1987. Brazilian strains previously were isolated at Beltsville, MD or at Fundecitrus, São Paulo (46); and Costa Rican strains (Table 1) previously were isolated at Centro de Investigación en Biología Celular y Molecular, Universidad de Costa Rica.

DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI) following Protocol G with modifications. PW broth (500 µl) was centrifuged for 10 min at 14,000 rpm, the supernatant was removed, and the bacterial pellet was resuspended in 600 µl of nuclei lysis solution. DNA also was obtained from bacterial colonies growing on PW plates by suspending a colony in 600 µl of nuclei lysis solution. The protocol then was followed from step number seven as indicated by the manufacturer. DNA was rehydrated with 50 µl of DNA rehydration solution and incubated at 65°C for 1 h. DNA was quantified photometrically and the concentration of the DNA solution was adjusted to 50 ng/µl.

Determination of 16S rRNA gene sequences. The nucleotide sequence of the 16S rRNA gene encoding the small subunit ribosomal RNA was determined for 16 strains of *X. fastidiosa* isolated in Costa Rica and 3 strains isolated in Brazil (Table 1). Primers were designed based on the sequence of the 16S rRNA gene (XFr04) of *X. fastidiosa* strain 9a5c, isolated from sweet orange in Brazil (57). The primers, designated XFr04af (5′-TAA GTG AAG AGT TTG ATC CTG GC-3′) and XFr04ar (5′-AAA GGA GGT GAT CCA GCC-3′), directed the amplification of the entire gene, producing an amplicon of 1,545 bp. The amplification program consisted of 1 cycle of 2 min at 95°C; followed by 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min; followed by a single cycle of 72°C for 5 min. Amplification products were visualized in agarose gels and then purified directly from the amplification mixture using the PureLink kit and cloned using the TOPO TA dual promoter cloning kit (Invitrogen, Carlsbad, CA). The resulting plasmids were purified using the Miniprep Kit (Invitrogen). Nucleotide sequences were determined in both directions at the University of Maryland sequencing facility and deposited to GenBank as accessions EF433931 to EF433949.

PCR-RFLP analysis. PCR with primers 272-1-int and 272-2-int (43) specific for *X. fastidiosa* was performed in a final volume of 20 µl; each reaction contained 50 ng of DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM each primer, 1× PCR buffer, and 0.5 unit of Platinum *Taq* DNA Polymerase (Invitrogen). Reactions were carried out on a MJ Research Thermal Cycler (model PTC-200) programmed for one cycle at 95°C for 4 min; 30 cycles at 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min; followed by an extension cycle at 72°C for 10 min. Electrophoresis of products was performed in 1% agarose gels with 2 µg of ethidium bromide at 70 V for 100 min. Amplification products were visualized with UV light. All sets of PCR reactions included an *X. fastidiosa*-positive control and PCR mix negative controls. All gels included a 100-bp DNA ladder (New England Biolabs, Ipswich, MA). The PCR amplification products were analyzed by treating with 5 units of restriction endonuclease *Cfo*I (Promega Corp.) in a final volume of 15 µl containing 10 µl of PCR product, 1.5 µl of 10× BufferB, 0.5 µl of enzyme, and 3 µl of H_2O. Digestions were incubated at 37°C for 3 h.

VNTR fragment-amplification. Nine primer pairs originally designed to amplify SSRs within the *X. fastidiosa* genome of sweet orange strains of *X. fastidiosa* in São Paulo were used (12). Amplification was performed in a final volume of 25 µl (1× PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM each dNTP, 50 ng of each primer, 50 ng of DNA, and 0.5 unit of Platinum *Taq* DNA Polymerase [Invitrogen]). A touchdown amplification program (12) was carried out in a MJ Research Thermal Cycler (model PTC-200). Reactions included PCR mix without DNA as negative controls. VNTR fragment products were separated in 3% agarose gels at 70 V for ≈270 min and visualized by staining in a 0.5 µg/ml ethidium bromide solution. Fragment sizes were estimated based on migration relative to a 50-bp molecular marker (Invitrogen).

Data analysis. The 19 *X. fastidiosa* 16SrDNA sequences were combined in a dataset with 16S rRNA gene sequences from 8 additional reference strains of *X. fastidiosa* obtained from GenBank. These were the same strains used for VNTR analysis (Table 1). Sequence data from three xanthomonads and one pseudomonad also were obtained from GenBank and used as outgroups in the analysis. Phylogenetic analysis was conducted using MEGA version 3.1 (32). The sequences were aligned and phylogeny was constructed with the neighbor-joining and maximum parsimony methods with 1,500 bootstrap permutations.

VNTR fragments were scored as putative loci with two alleles, one indicating the presence of the specific fragment and the other the absence of the fragment. Only reproducible bands were scored, after three or more repetitions of the experiment, although a few strains were tested only two times. The resulting matrix of strains and VNTR fragments was analyzed in two ways. First, the three different geographic origins of the strains were treated as populations and the hosts as subpopulations within each geographic population. Conversely, the data also was analyzed treating the strains from each host as populations and the distinct geographic origins as subpopulations within a host. Individual geographic populations and subsets of the strains also were analyzed. Population genetic statistics for the different data arrangements were calculated using the POPGENE (version 1.31) software (62). The WINAMOVA (version 1.55) software was used to calculate the molecular analysis of variance (17). The genetic distance dendrogram was generated using Treecon (version 1.3) software (59) with an unweighted pair group arithmetic mean average algorithm. Repetitions (permutations) of the analysis in all programs were set to 1,000 times.

RESULTS

16S rRNA gene sequence comparisons. BLASTn was used to identify the16S rRNA gene sequences determined in this study against the RefSeq database in GenBank. The best matches (*e* value = 0) were in all cases to either *X. fastidiosa* (grapevine Temecula strain) or *X. fastidiosa* (sweet orange strain 9a5c) with >98% sequence identity. The sequence of the 16S rRNA gene from eight strains of *X. fastidiosa* from Costa Rica perfectly matched the sequence of the Temecula (PD) strain of *X. fastidiosa*, but none provided a perfect match for strain 9a5c (CVC) from São Paulo. The neighbor-joining method was used to construct a dendrogram, in which all strains of *X. fastidiosa* clustered together and were well separated from the strains of *Xanthomonas* and *Pseudomonas* spp. used as outliers. Within the cluster of *X. fastidiosa* strains, the strains were not well sorted by host of origin or by geography (Fig. 1). Most strains isolated from coffee, citrus, and grapevine in Costa Rica clustered together, and strains from grapevine or other hosts from the United States were interspersed among the Costa Rican strains. Strain CaVIc2, isolated from coffee in Costa Rica, clustered with the coffee and citrus strains from São Paulo. However, strains of *X. fastidiosa* were not well resolved due to the lack of diversity in the sequence data and, therefore, lack of parsimonious sites. A larger tree, which included 16S rRNA genes from an additional 20 strains of

X. fastidiosa obtained from GenBank, had a similar topology (not shown). In the larger tree, strains from Costa Rica also clustered by themselves or with strains from the United States or São Paulo.

PCR-RFLP. All DNA samples amplified repeatedly the expected fragment specific for *X. fastidiosa* (43). The digestion with restriction endonuclease *Cfo*I generated two basic patterns (Fig. 2; some not shown), as expected (46). All strains from the United States and Costa Rica presented the same pattern. All citrus strains and two of four coffee strains from São Paulo presented the pattern expected for CVC strains. The remaining coffee strains from São Paulo presented patterns shared by the strains from the United States and Costa Rica (Fig. 2).

VNTR fragment amplification. Amplification was successful with primer pairs SSR20, SSR21, SSR28, SSR30, SSR36, and SSR40, and useful data was readily obtained. However, primers SSR26, SSR32, and SSR34 produced only inconsistent, faint, or large amplification products; therefore, useful data could not be obtained with these primers. Lowering the annealing temperature 5°C (touchdown program range 59 to 50°C rather than 64 to 55°C) did not resolve the problem. In this study, primer SSR36 generated no amplification products with the Brazilian strains but did generate polymorphic products for strains from the United States or Costa Rica.

A polymorphism was observed when mulberry strains ATTC 35868 and MUL-1 were compared with primer SSR30. These

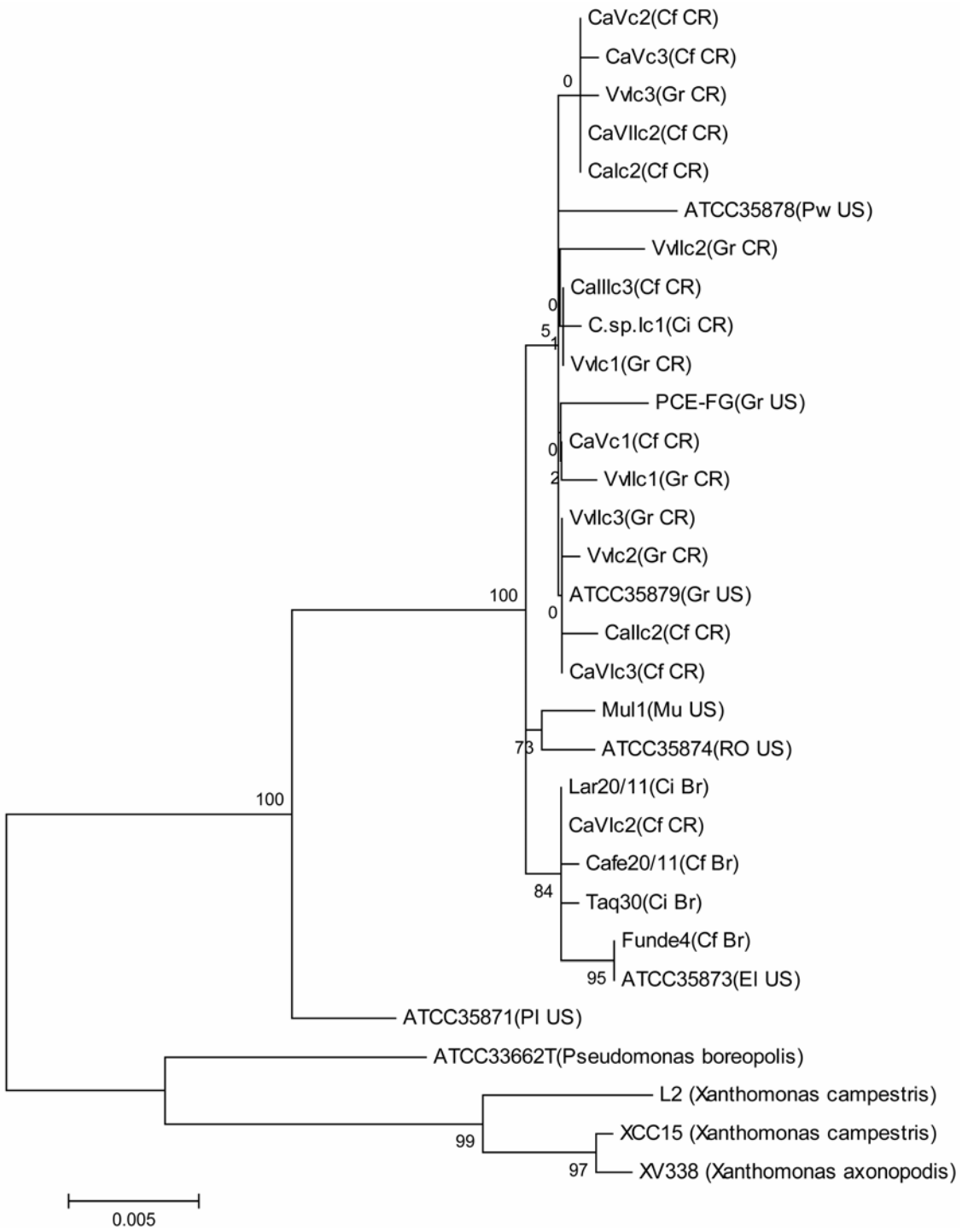

Fig. 1. Dendrogram based on full 16S rDNA sequence data for strains of *Xylella fastidiosa* from Costa Rica, Brazil, and the United States. Abbreviations: US, United States; CR, Costa Rica; Br, Brazil; Ci, sweet orange; Cf, coffee; and Gr, grapevine.

strains, which were derived from the same original strain, have an independent history of subculturing and storage at least since 1987 (31,60), and the genome has changed over time. This was not observed for other SSR markers, or with the paired samples ATTC 35870–ALS-BCG, ATTC 35873–ELM-1, and ATCC 35881–PCE-FG, which present a similar historical situation.

VNTR fragment analysis. In all, 32 VNTR products were scored for the 42 strains of *X. fastidiosa* analyzed. All loci were polymorphic, and a minimum of two and a maximum of nine amplification products were generated per locus. The frequencies of particular amplification products varied considerably among the three geographic origins (Table 2).

The genetic diversity values (*H*) based on total number of bands per loci and their frequency also varied among the geographic sample sets (Table 3). In addition to the polymorphic bands, primers for the loci SSR21, SSR28, SSR36, and SSR40 also produced no amplification product (null haplotype) for certain strains. This null haplotype was included in the calculations of the values of genetic diversity obtained for SSR loci. The São Paulo strains possessed the greatest proportion of the 32 total bands obtained compared with the other two sample sets, as expected, because the SSR loci originally were identified in São Paulo (12). Several of the amplification products were shared by strains from two or three geographic origins (Table 2) and few amplification products were unique to a specific geographic region or host; however, primer SSR30 generated several amplification products unique to single strains from São Paulo (Table 2).

The statistics obtained with WINAMOVA showed that the genetic diversity found in the strains assayed has significant ($P < 0.001$) components from both geographic and host origin (Table 4). The percentage of genetic diversity due to geographic origin is less than the percentage due to variation among hosts within the same geographic origin or the variation within each host. The percentage of variation contributed by geographic origin (21.40%) scarcely changed if either the number of strains was changed or hosts from the United States (almond, elm, mulberry, periwinkle, ragweed, and red oak) were eliminated. Conversely, the percentage of variation contributed by hosts within a geographic population to the general genetic diversity of the *X. fastidiosa* strains decreased as sample numbers were reduced or hosts were eliminated (data not shown). Thus, the geographic region of origin of the strains has a significant effect on the variation of the strains and is not an artifact of the composition of the sample set used for the analyses.

Estimation of genetic diversity (Table 5) indicated that the diversity within each geographic origin (H_S) accounted for a great proportion of the total genetic diversity (H_T). Thus, with this set

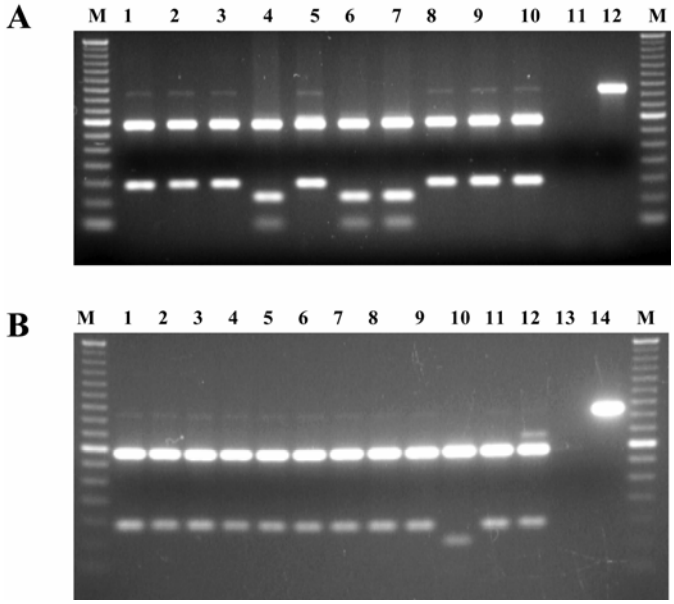

Fig. 2. Polymerase chain reaction (PCR) restriction fragment length polymorphism assay of strains of *Xylella fastidiosa* from different hosts. Products of amplification with primers 272-1-int and 272-2-int, digested with endonuclease *Cfo*I, and separated by gel electrophoresis in 2% agarose at 90 V for 150 min. **A,** Lanes 1 to 10: strains Elm-1 (elm, United States), ALS-BCG (almond, United States), PCE-FG (grapevine, United States), 122WCoffee and Café 20/11 (coffee, São Paulo), LAR 20/11 and Taq 30 (citrus, São Paulo), Vv-IIc3 (grapevine, Costa Rica), and Ca-Vc2 and Ca-Vc3 (coffee, Costa Rica); lane 11: PCR mix negative control; lane 12, undigested amplification product from strain Funde-4 (coffee, São Paulo). **B,** Lanes 1 to 9, Costa Rican strains Vv-IIc1, Vv-IIc2, and Vv-IIc3 (grapevine); Ca-IIc2, Ca-IIIc3, Ca-IVc2, Ca-VIc3, and Ca-VIIc1 (coffee); and C.sp.-Ic1 (citrus); lanes 10 to 12: LAR 20/11 (citrus, São Paulo), ATCC 35881 (grapevine, United States), and Café 20/11 (coffee, São Paulo); lane 13: PCR mix negative control; lane 14, undigested amplification product from strain Taq 30 (citrus, São Paulo). Lane M, 50-bp molecular marker.

TABLE 2. Frequency of variable number of tandem repeat polymorphic bands in *Xylella fastidiosa* strains from different hosts from three geographically distinct areas

	Frequency of band		
Polymorphic allele	São Paulo	Costa Rica	United States
SSR20a	0.000	1.000	0.667
SSR20b	0.000	0.000	0.200
SSR20c	0.375	0.000	0.066
SSR20d	0.500	0.000	0.000
SSR20e	0.125	0.000	0.066
SSR21a	0.750	0.315	0.533
SSR21b	0.250	0.000	0.000
SSR28a	0.375	0.052	0.333
SSR28c	0.000	0.000	0.133
SSR28d	0.125	0.105	0.000
SSR28e	0.250	0.421	0.000
SSR28g	0.125	0.000	0.000
SSR28h	0.125	0.105	0.000
SSR30a	0.000	0.315	0.733
SSR30b	0.125	0.000	0.200
SSR30c	0.250	0.000	0.066
SSR30d	0.125	0.000	0.000
SSR30e	0.125	0.000	0.000
SSR30f	0.125	0.000	0.000
SSR30g	0.125	0.000	0.000
SSR30h	0.125	0.000	0.000
SSR30i	0.000	0.684	0.000
SSR36a	0.000	0.000	0.133
SSR36b	0.000	0.000	0.400
SSR36c	0.000	0.000	0.066
SSR36d	0.000	0.578	0.000
SSR36e	0.000	0.210	0.000
SSR36f	0.000	0.052	0.000
SSR36g	0.000	0.157	0.000
SSR36h	0.000	0.000	0.133
SSR40a	0.375	0.000	0.466
SSR40b	0.625	0.736	0.000

TABLE 3. Mean values of Nei's genetic diversity (*H*) for variable number of tandem repeats (VNTR) loci and number of polymorphic alleles per number of total alleles (in parentheses) in *Xylella fastidiosa* strains isolated from different hosts from three geographically distinct areas

	H value and no. of polymorphic alleles/total alleles[a]			
Locus	São Paulo	Costa Rica	United States	General
SSR20	0.238 (3/5)	0.000 (1/5)	0.203 (4/5)	0.199
SSR21	0.375 (2/2)	0.216 (1/2)	0.249 (1/2)	0.295
SSR28	0.250 (5/6)	0.161 (4/6)	0.113 (2/6)	0.184
SSR30	0.188 (7/9)	0.096 (2/9)	0.093 (3/9)	0.161
SSR36	0.000 (0/8)	0.148 (4/8)	0.133 (4/8)	0.151
SSR40	0.469 (2/2)	0.194 (1/2)	0.249 (1/2)	0.429
Average	0.189 (19/32)	0.119 (13/32)	0.143 (15/32)	0.194

[a] Number of polymorphic alleles/total number of alleles.

of strains, the data suggest that the diversity among strains attributable to host of origin within a geographic area contributes a greater proportion of the total genetic variation present than does the geographic origin of the 42 *X. fastidiosa* strains evaluated (Tables 4 and 5). When strains from each geographic origin were analyzed as subgroups, the total variation within the São Paulo strains was a major component of the variation attributable to differences within each host (H_S); meanwhile, total variation for Costa Rica and the United States (H_T) seems to have provided a relatively minor contribution to the variation within hosts (H_S) (Table 5). This is consistent with the origin of the SSR markers based on sweet orange strains of *X. fastidiosa* from São Paulo.

A dendrogram was generated to visually summarize the variability contained in this set of strains at this set of VNTR loci (Fig. 3). This figure contains three principal branches, and separates the strains from São Paulo from the strains from both the United States and Costa Rica, which form two branches. The smaller of these branches is composed entirely of strains from Costa Rica and the larger branch contains strains from the United States as well as six strains from Costa Rican coffee that grouped most closely with mulberry strains from the United States.

DISCUSSION

A set of *X. fastidiosa* strains comprising 42 strains from São Paulo, Costa Rica, and the United States was assembled. The identity of the strains as *X. fastidiosa* was confirmed by sequence analysis of the 16S rRNA gene (5,8) and by amplification of the expected product with primers 272-1-int and 272-2-int (43). This product was digested with endonuclease *Cfo*I and the strains were analyzed to assess genetic diversity.

Six of eight strains from sweet orange and coffee plants from São Paulo presented the pattern expected for CVC strains with enzyme *Cfo*I (46). CVC strains of *X. fastidiosa* may occur naturally in coffee plants because it was experimentally demonstrated that *X. fastidiosa* from citrus can cause CLS (36). Moreover, it has been suggested that the bacterium moved initially from coffee plants to citrus in São Paulo (7,39,41,46). The amplification products from coffee, sweet orange, and grapevine from Costa Rica presented a different restriction pattern (not the São Paulo CVC pattern), consistent with different origins for the Costa Rica and São Paulo populations of *X. fastidiosa* in sweet orange.

The 16S rRNA gene sequence from the strains of *X. fastidiosa* studied was highly conserved. Therefore, due to the relative lack of parsimonious sites in the sequence data, the discriminatory power of the phylogenetic analysis was not great and the 16S rRNA gene sequence analysis was not able to generate dendrograms with well-resolved branches. Strains were not sorted by either host or region of origin by using the 16SrDNA data (Fig. 1). Some strains from Costa Rica were indistinguishable from the Temecula strain of *X. fastidiosa* based on the 16S rRNA sequence data, and these included strains from both coffee and sweet orange. However, the 16S rRNA sequence data from other strains from Costa Rica did not match the Temecula strain. It is likely that a strain ancestral to both Temecula and the strains in Costa Rica is naturally endemic in the Americas, as proposed by Goheen et al. in 1979 (21). It is also possible that a recent ancestor of the Temecula strain was introduced into Costa Rica and has since become endemic among the native strains. In any case, the 16S rRNA gene sequence analysis was not informative regarding either the origin or diversity present among the strains of *X. fastidiosa* found in Costa Rica.

VNTR analysis was carried out on the same set of strains (Table 1). It is important to consider the genetic diversity (H) for the VNTR loci in this study in comparison with data from other studies. The frequencies of the VNTR-based polymorphic bands for each geographic origin (Table 2) and the mean value of Nei's genetic diversity index (H) for each VNTR loci (Table 3) showed that different polymorphisms were detected in the different geographic regions sampled. This also was observed among populations of *X. fastidiosa* isolated from citrus from five regions of São Paulo (11). The values of genetic diversity obtained in this study for the VNTR loci are lower than obtained in a previous study comprised mainly of São Paulo citrus strains of *X. fastidiosa* (11). The average H value of all loci for all strains or only for the São Paulo strains in this study is lower than the general average (H = 0.51) reported previously (12). Moreover, the average value of H for the São Paulo sample set is greater than that obtained for the strains sampled from either Costa Rica or the United States. The data suggest that this set of VNTR loci is more informative within Brazilian samples than within strains obtained from other regions, which is expected because the VNTR loci were identified originally in sweet orange strains from São Paulo. Conversely, locus SSR36, which was not polymorphic in the Brazilian analysis (12), displayed a high degree of polymorphism among geographic subpopulations and within Costa Rican strains. Such differences in the degree of resolution among VNTR data sets and differential polymorphism depending on the sample set assayed have been noted previously (25,53). The primers corresponding to loci SSR20, SSR30, and SSR40 were chosen as highly polymorphic primers and were used in further research in São Paulo (10,11). The data obtained herein supports loci SSR40 as highly polymorphic, showing the greatest H value in this study. Loci for SSR21 and SSR28 showed the second and third greatest values of H, respectively, in this study.

A polymorphism was observed between strains ATCC 35868 and MUL-1 in this study with primer SSR30. This is interesting because these strains were one set of four pairs of strains, each derived from a single strain with separate passage histories over the past 20 years and included in this study. In a similar case, allelic variation was detected at two of eight VNTR loci tested on

TABLE 5. Estimators of genetic diversity for the total set of *X. fastidiosa* strains analyzed and subsets (among and within geographic regions or host species) determined from variable number of tandem repeats markers[a]

Comparison	n	H_T	H_S
Among geographic regions	42	0.2062	0.1509
Among host species	42	0.1789	0.0507
Among hosts within São Paulo	8	0.1895	0.1523
Among hosts within Costa Rica	19	0.1168	0.0554
Among hosts within the United States	15	0.1472	0.0113

[a] Values calculated in POPGENE (62); n = number of strains in the population, H_T = overall genetic diversity of the population, and H_S = genetic diversity within each component of the global population.

TABLE 4. Analysis of molecular variance among and within sample sets of *Xylella fastidiosa* strains from São Paulo, Costa Rica, and the United States

Source of variance[a]	df	Φ_{STAT}	Variance component (%)	P value[b]
Among geographic regions	2	0.214	21.40	<0.001
Among hosts within geographic regions	10	0.406	31.88	<0.001
Within the same host	29	0.533	46.71	<0.001

[a] The global set of samples of *Xylella fastidiosa* strains analyzed (n = 42) was partitioned into hierarchical components: (i) geographic regions, (ii) host species within each geographic region, and (iii) hosts across regions.
[b] Probability calculated by WINAMOVA with 1,000 permutations.

13 clonally derived *Microbacterium bovis* BCG daughter strains, which had been passaged in vitro hundreds of times since 1921 (53). The polymorphism at the SSR30 locus is a further indication of the power of the VNTR markers used in this study, which were able to distinguish up to five unique haplotypes of *X. fastidiosa* within single trees in Brazil. Depending on the position of the SSR loci in the genome and on the repeat size, such variation may or may not affect the expression of the gene and its final product (30). Spontaneous genetic variations such as these are not in response to a specific need of an organism in its environment but instead are random (4).

The VNTR technique has been suggested as a very powerful technique for typing analysis due to the stability and portability of the DNA markers. The results correlate well with other molecular typing methods (25,44). A limitation of the present study is that the VNTR loci identified in sweet orange strains in São Paulo were not equally informative in strains from other hosts and regions. In future studies, it is advisable to search for more VNTR loci and to define the optimal combination of heterogenic loci for the general *X. fastidiosa* population or specific geographic subpopulations, as has been suggested for *Mycobacterium* spp. or *Y. pestis* strains (25,44,53). Such a common set of primers would be advantageous for epidemiological analysis, to evaluate differences and similarities between strains and comparisons between populations in different countries (25,44,53). Different subsets of VNTR loci based on the size of the repeat unit (micro, mini, or satellite) are suitable for addressing different genetic or molecular issues (30,53). This factor should be taken into account when designing such a common set of markers.

Statistically significant contributions of geographic and host variables to the genetic diversity of plant-pathogenic bacteria have been found in populations of *Xanthomonas* spp. (29,48–50), a bacterial genus related to *X. fastidiosa* (40,60). A study with *Escherichia coli* showed that the observed differentiation by host could be due, in part, to the type and range of sugars available to the bacterium in different orders of animal hosts (58). However, media supplemented with the major and most common amino acids and other substances found in plants are able to support the growth of different *X. fastidiosa* strains (2,35), suggesting that other factors beyond sap content may be exerting a selective pressure. No avirulence genes have been detected in *X. fastidiosa* (57) and a wide range of potential plant hosts has been demonstrated (27). It may be that the host effect on genetic differentiation may be by physical aspects of the xylem vessels or by vector dynamics (47,54) rather than by gene-for-gene interactions.

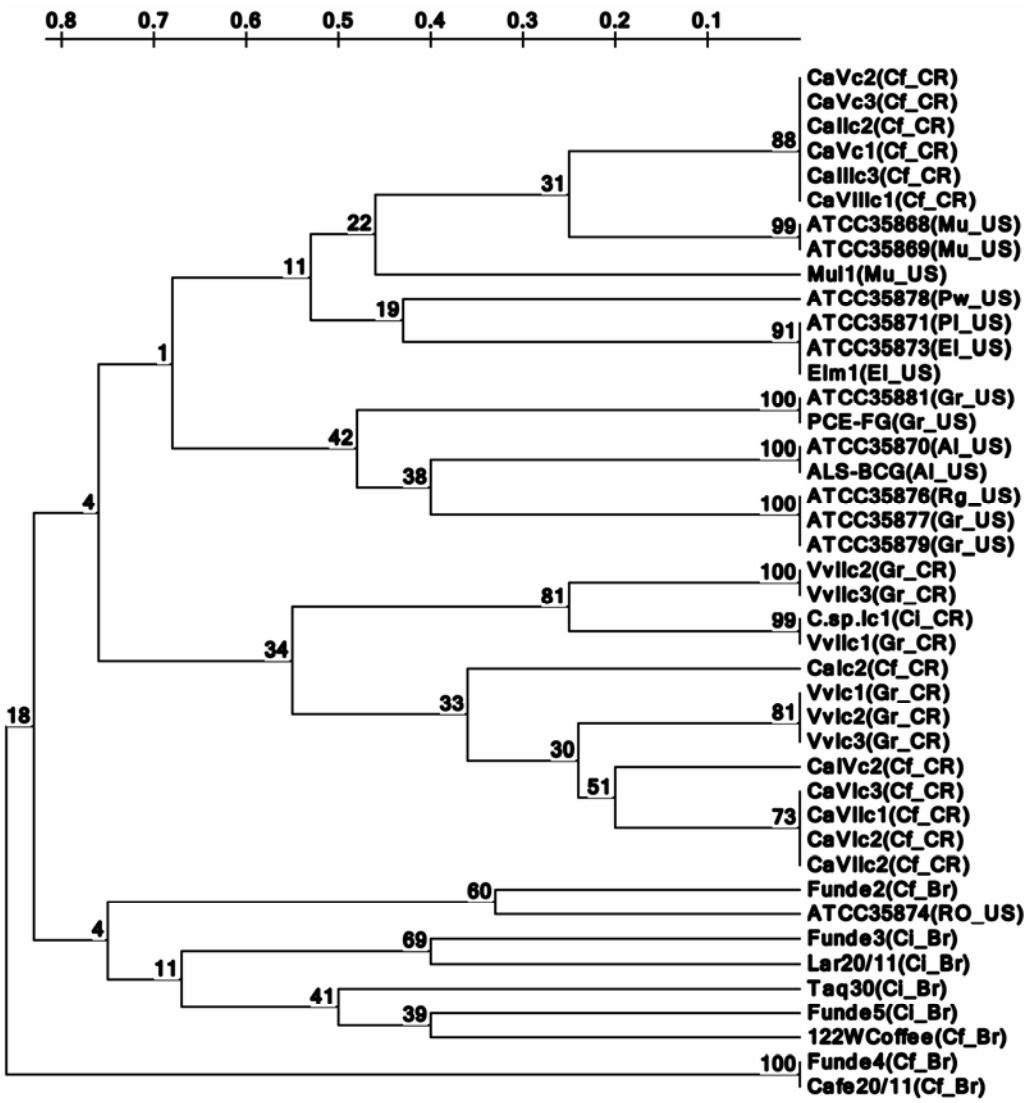

Fig. 3. Dendrogram based on computed similarity data from variable number of tandem repeat fragment analysis with the unweighted pair group arithmetic mean average algorithm for all *Xylella fastidiosa* strains used in this study. Al, almond; Ci, citrus; Cf, coffee; El, elm; Gr, grapevine, Mu, mulberry; Pl, plum; Pw, periwinkle, Rg, ragweed; RO, red oak; Br, São Paulo (Brazil); CR, Costa Rica; and US, United States. Numbers at each node indicate the bootstrap percentages (1,000 permutations).

The statistically significant geographic contribution to the total genetic variation among the strains analyzed was corroborated by obtaining similar values in the percentage of variance contributed by each component by WINAMOVA with different subsets of samples (Table 4). This data demonstrates diversity within *X. fastidiosa* consistent with geography as proposed previously for subpopulations from the United States and São Paulo (13,46). A significant effect of geography on genetic diversity within *X. fastidiosa* also was noted among populations from different regions in São Paulo using these VNTR markers (11). There also was highly significant variation found by analysis of molecular variance among the strains from different hosts within each geographic region and significant variation within hosts across geographic regions (Table 4).

A dendrogram was generated to visually summarize the variability contained in this set of strains at this set off SSR loci. The relatively low bootstrap values shown in the summary dendrogram (Fig. 3) suggest that the discriminatory power of the VNTR markers is better for the strains from São Paulo than for the other strains (Table 3). In spite of this relatively small polymorphic data set, two clusters were observed, and the strains from Costa Rica were more closely associated with the strains from the United States than with the strains from São Paulo. The overall pattern of separation and clustering of the strains was maintained in dendrograms generated with several subsets of strains, including one generated with only one member of each of the four pairs of strains with common origin (not shown). Dendrograms were separately generated with data only from the SSR20, SSR30, SSR40, SSR21, SSR28, and SSR36 loci. The dendrogram generated with all VNTR data from all strains (Fig. 3) had higher bootstrap values than the dendrograms produced with more limited data sets and is the best visual summarization of the VNTR polymorphism data. This interpretation of the data is also consistent with the statistical analysis, which showed that geographic origin of the strains provided the greatest contribution to the genetic diversity observed (Table 5). In the future, it will be interesting to confirm the results reported here using other measures of genetic variability or a larger number of VNTR loci.

The grouping of North American strains within the dendrogram (Fig. 3) shows, in general terms, a clustering topology or relationship among strains as observed in previous studies using other methods (9,42,46). We note that the VNTR data places the mulberry strains with the elm, plum, and periwinkle strains, as found previously by nucleotide sequences of gene *gyrB* (51), rather than in the cluster of grape strains (7,28,40,42,46,51). Data presented here suggest the oak strain (ATCC 35874) as more related to São Paulo citrus strains than to the other U.S. strains, in contrast to previous results based on other methods (7,9,24,28,40,46,56). Genetic diversity found within the São Paulo sweet orange and coffee strains support previous studies correlating these VNTR results with other techniques (33,34,39,51,54). Coffee and sweet orange strains from São Paulo were not separated in independent subclusters related to host origin, indicating genetic proximity. However, Costa Rican coffee strains were divided into two independent groups. Moreover, the Costa Rican sweet orange strain is more closely related to Costa Rican strains from grape rather than coffee, contrary to what would be expected based on the relationships among coffee and citrus strains in São Paulo.

Recently, it has been postulated that major North American clades of *X. fastidiosa*, subsp. *piercei*, *multiplex* (55), and *sandyi* (56), diverged ≈16,000 years ago, before the introduction of their horticulturally important host plants; thus, these taxons must have evolved within native plant hosts (56). This study of *X. fastidiosa* strains throughout the Americas—in the United States (North America), Costa Rica (Central America), and São Paulo (South America)—reveals genetic diversity among and within the distinct geographical origins. The Costa Rican sample set of *X. fastidiosa* also is genetically diverse among and within host plants. Taken together, the data shows greater genetic similarity between the strains studied from Costa Rica and the United States than between either of these with the São Paulo strains.

With the description of these strains of *X. fastidiosa* from Costa Rica, *X. fastidiosa* appears to be indigenous throughout the Americas, and its appearance as a pathogen of various crops in distinct geographical regions probably is due to the movement of the bacterium from native hosts to introduced crops and ornamentals. Such transmission likely would be done by native insects, which have been described and recently shown to be contaminated with *X. fastidiosa* (19,20). The alternative hypothesis, that *X. fastidiosa* was introduced into Costa Rica in historic times with horticultural imports from North America, cannot be excluded. Neither the 16S rRNA (Fig. 1) nor the VNTR data (Fig. 3) support clear separation of three geographic groups or multiple host-specific groups. The alternative origins for the strains of *X. fastidiosa* in Costa Rica are not mutually exclusive and, in fact, both may be correct. The 16S rRNA data are consistent with the idea that members of *X. fastidiosa* subsp. *piercei* have developed the ability to infect and incite disease in coffee and sweet orange in Costa Rica. However, the subspecies of *X. fastidiosa* previously were recognized based primarily on total DNA::DNA hybridization experiments (55). Likewise, in the case of these strains from Costa Rica, final assignment to subspecies will require total DNA::DNA hybridization analysis.

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