Geographical Genetic Structure of *Xylella fastidiosa* from Citrus in São Paulo State, Brazil

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ABSTRACT

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A total of 360 *Xylella fastidiosa* strains were isolated from sweet orange (*Citrus sinensis*) cv. Pera plants growing in five geographic regions in the Brazilian state of São Paulo. The genetic variation of these strains was determined by 15 variable number tandem repeat (VNTR) and 58 random amplified polymorphic DNA (RAPD) markers. The mean values of genetic diversity (H) of *X. fastidiosa* strains within each geographic region determined by RAPD (H_{RAPD}) were substantially lower than H_{VNTR} values. H_{RAPD} values ranged from 0.00 to 0.095, whereas the

H_{VNTR} values ranged from 0.024 to 0.285. A highly significant value of Nei's coefficient of gene differentiation ($G_{\text{ST}} = 0.355$; $P = 0.000$) was detected among all five populations. Analysis of the molecular variance (AMOVA) also revealed significant genetic differentiation among regions or populations ($\phi_{\text{STAT}} = 0.810$; $P < 0.001$). In addition, genetic differentiation among subpopulations (plants) within the regions ($\phi_{\text{STAT}} = 0.699$; $P < 0.001$) and within each plant ($\phi_{\text{STAT}} = 0.368$; $P < 0.001$) were statistically significant. These high values of genetic differentiation among *X. fastidiosa* strains from different regions suggest a genetic structure according to region of host origin. However, no apparent correlation between genetic distance and region of origin of populations were supported statistically by Mantel analysis ($r = 0.27$; $P = 0.22$).

Xylella fastidiosa causes disease in many economically important plants, mainly in the American continent (25). This gram-negative bacterium grows fastidiously in vitro and is mostly found in nature in the xylem of infected plants (15) or in the precibarium and cibarium of sharpshooter leafhopper vectors (24). In citrus, *X. fastidiosa* is responsible for a disease known as citrus variegated chlorosis (CVC), which affects mainly cultivars of sweet orange (*Citrus sinensis* (L.) Osbeck). This disease was first described in Brazil in 1987 in the northwest of the state of São Paulo (6,31), but now this disease has spread to all citrus producing regions of São Paulo and to other Brazilian states that grow citrus. There have also been recent reports of CVC in Paraguay and Costa Rica (4). A similar disease associated with *X. fastidiosa* has been seen in citrus plants in Argentina since 1984, where the disease is known as *Pecocita* (5).

The rapid spread of CVC through orchards and the great damage it causes result in large economic losses of approximately 150 million U.S. dollars a year for the growers and juice industry of São Paulo State, the major citrus growing region in Brazil (2). Besides the economic damage, there are other social consequences such as the disappearance of small-scale citrus orchards. The economic and social importance of citrus production in Brazil and the high level of damage caused by CVC in Brazilian orchards have resulted in an extensive research program involving the sequencing of the entire genome of *X. fastidiosa* (32).

Despite the great interest in *X. fastidiosa*, relatively little is known about the population genetics of this pathogen. Previous work has suggested that strains of *X. fastidiosa* isolated from different host species, including citrus, were nonrandomly distributed with respect to the hosts from which they were isolated (22). Data from recent work based on random amplified polymorphic DNA

(RAPD), variable number of tandem repeats (VNTR), and repetitive sequence-based polymerase chain reaction (rep-PCR) such as BOX, ERIC (enterobacterial repetitive intergeneric consensus), and REP (repetitive extragenic palindromic) elements, has shown the occurrence of genetic diversity within strains of *X. fastidiosa* isolated from citrus plants (8,19,30). Although such surveys have shown genetic polymorphism in *X. fastidiosa*, the analyzed strains have come from ad hoc collections of strains of diverse geographic origin collected over time.

In a previous study, based on the analysis by RAPD and VNTR markers of a large number of *X. fastidiosa* strains from different sweet orange cultivars (heterogeneous hosts) and from the same sweet orange cultivar (homogeneous hosts) both growing in bordering areas, it was concluded that different sweet orange cultivars do not play a role in the population structure of *X. fastidiosa* (7). Data from Metha et al. (19), in contrast, have suggested that there is evidence of a relationship between molecular polymorphism and the geographic region from where CVC-causing *X. fastidiosa* strains were isolated.

In the work presented in this paper, we studied genetic variation among strains of *X. fastidiosa* recovered from sweet orange plants with symptoms of CVC and investigated the genetic diversity within and among populations from several geographically distant locations. For this, we collected over 360 strains of *X. fastidiosa* from sweet orange plants growing in five different geographic regions of São Paulo State and characterized the strains using RAPD and VNTR markers.

MATERIALS AND METHODS

Sampling procedure and isolation of *X. fastidiosa*. The trees used as the source of the *X. fastidiosa* isolates were 4-year-old sweet orange trees cv. Pera grafted onto 'Rangpur' lime rootstock growing in orchards located in five different citrus-producing geographical regions (one orchard per geographic region) of São Paulo State, Brazil (Fig. 1). The canopies of trees were sampled by randomly collecting 3- to 5-mm-diameter branches with leaves showing the typical symptoms of CVC.

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The branches were transferred to the laboratory where they were surface disinfected with 2% bleach for 3 min, 70% ethanol for 2 min, a quick immersion in 95% ethanol, and flaming. Each branch was cut in the middle and the internal cut ends were squeezed with a pair of pliers, the sap was blotted onto plates of buffered charcoal yeast extract (BCYE) agar (35) (three plates per plant), and they were incubated at 28°C for 15 to 20 days. Two isolated colonies per plate were selected with a stereo-microscope, streaked onto fresh BCYE agar plates, and incubated at 28°C for 7 days. Identification of strains as *X. fastidiosa* was carried out based on in vitro fastidious growth, white color of colonies, and PCR assays using primers specific to CVC-causing strains of *X. fastidiosa* (23). Isolates confirmed as *X. fastidiosa* were stored in periwinkle wilt broth (10) containing 30% glycerol at -80°C.

A total of 360 *X. fastidiosa* strains were obtained, representing 60 sweet orange trees from five different geographical citrus producing regions (12 plants sampled per region and 6 strains per plant, totaling 72 strains per region). All strains from the same region were regarded as one population, whereas isolates from a given plant within the same orchard (geographic region) were regarded as one subpopulation. Thus, the 360 collected strains were divided into five populations consisting of a total of 60 subpopulations (12 subpopulations per geographic region).

DNA preparation. The genomic DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) technique (36), with minor modifications. Briefly, the cells were harvested and washed twice in pH 8.0 buffer (20 mM Tris-HCl and 10 mM EDTA) and treated with proteinase K (Sigma Chemical, St. Louis) and 10% sodium dodecyl sulfate. Proteins and other cellular components were removed with hexadecyltrimethylammonium bromide (Sigma) and two extractions with chloroform. The DNA present in the aqueous phase was precipitated with absolute ethanol followed by washing with 70% ethanol. The pellet obtained was dissolved in pH 8.0 TE buffer (10 mM Tris-HCl and 1 mM EDTA) containing 20 mM RNase A (USB Corp., Cleveland). The DNA concentration was estimated by running an aliquot of the sample in 0.8% agarose gel, and the final DNA concentration was adjusted to approximately 5 ng/μl and stored at -20°C.

RAPD amplification. RAPD analysis was performed in a total volume of 13 μl containing 10% 10× buffer, 2.0 mM MgCl₂, 0.25 mM each dNTP (Invitrogen Corp., San Diego, CA), 15 ng of template DNA, 1.5 units of *Taq* DNA polymerase (Invitrogen Corp., São Paulo, Brazil), and 15 ng of primer (Operon Technologies, Alameda, CA; OPA02, OPA03, OPD20, OPG19, OPH07, OPH12, OPH18, OPI02, OPN04, and OPU10) that had been

previously selected (7). Amplifications were performed with a thermal cycler (PTC100; MJ Research, Watertown, MA) using the following conditions: initial denaturation at 94°C for 3 min followed by 36 cycles consisting of denaturation for 1 min at 94°C, annealing for 1 min at 36°C, and extension for 2 min at 72°C, with a final 10-min extension at 72°C at the end of the 36 cycles. The reproducibility of the selected RAPD markers used in the analysis was tested by performing amplifications with different concentration (5 to 45 ng) of DNA, all of which resulted in identical RAPD selected bands. The RAPD reaction products were separated by electrophoresis in 1.3% agarose and stained with 0.4% ethidium bromide. RAPD band size was estimated by comparison with a 1-kb DNA ladder marker (Invitrogen Corp., San Diego).

VNTR amplification. Amplifications were conducted in a volume of 25 μl containing 10% 10× buffer, 2.5 mM MgCl₂, 0.25 mM each dNTP (Invitrogen Corp., San Diego), 50 ng of each primer, 50 ng of template DNA, and 1.5 units of *Taq* DNA polymerase (Invitrogen Corp., São Paulo). Amplifications were conducted using the following primers: SSR20 (5'-ATGAAGAAGCCAGGATACAT-3', 5'-GCTACACGTGCAACAAC-3'), SSR30 (5'-TACGCTGCACCTGTCTG-3', 5'-CTGTGAACCTCCATCATCC-3'), and SSR40 (5'-ACCTTGACGACGGATG-3', 5'-AGGAAGTGTGCTGCTACTGAT-3'). PCR amplification reactions were performed in a PC100 thermal cycler as follows: an initial denaturation step at 94°C for 3 min was followed by a touchdown ampli-

TABLE 1. Frequency of random amplified polymorphic DNA (RAPD) and variable number of tandem repeats (VNTR) polymorphic alleles in *Xylella fastidiosa* populations from five regions of São Paulo, Brazil

Polymorphic alleles	Frequency of the marker allele ^a					G ² test ^b
	NPA	GPO	SRQ	BAU	PLA	
RAPD locus						
OPA02 ₉₀₀	0.67	0.00	0.00	0.00	0.00	**
OPA03 ₉₂₃	0.33	1.00	0.00	1.00	1.00	**
OPD20 ₁₆₃₄	0.33	1.00	0.00	1.00	1.00	**
OPG19 ₁₂₆₈	0.35	1.00	0.00	1.00	1.00	**
OPH07 ₁₄₈₀	0.35	1.00	0.00	1.00	1.00	**
OPH12 ₁₈₉₀	0.67	0.00	0.00	0.00	0.00	**
OPH12 ₁₃₆₇	0.42	1.00	0.00	1.00	1.00	**
OPH18 ₉₂₀	0.33	1.00	0.00	1.00	1.00	**
OPH18 ₁₂₈₅	0.00	0.00	0.00	0.90	1.00	**
OPI02 ₁₇₂₀	0.43	1.00	0.58	1.00	1.00	**
OPI02 ₁₀₀₈	0.36	1.00	0.00	1.00	1.00	**
OPI02 ₈₇₀	0.01	0.00	0.00	0.00	0.00	ns
OPN04 ₁₂₂₇	0.35	1.00	0.00	1.00	1.00	**
OPN04 ₁₃₇₀	0.10	0.00	0.00	0.00	0.00	**
OPU10 ₁₃₃₀	0.08	0.07	0.03	1.00	1.00	**
OPU10 ₁₂₄₀	0.00	0.00	0.00	0.03	1.00	**
OPU10 ₉₅₀	0.08	1.00	1.00	1.00	1.00	**
VNTR locus						
SSR20 ₁₀₀	0.01	0.00	0.00	0.11	0.00	**
SSR20 ₁₃₀	0.36	0.99	0.99	0.78	1.00	**
SSR20 ₁₇₀	0.08	0.01	0.01	0.11	0.00	**
SSR20 ₁₉₀	0.33	0.00	0.00	0.00	0.00	**
SSR20 ₂₃₀	0.21	0.00	0.00	0.00	0.00	**
SSR30 ₂₀₀	0.11	0.03	0.00	0.11	0.00	**
SSR30 ₂₃₅	0.40	0.92	0.10	0.08	0.00	**
SSR30 ₂₆₀	0.12	0.05	0.71	0.07	0.26	**
SSR30 ₃₀₀	0.22	0.00	0.12	0.65	0.74	**
SSR30 ₃₂₀	0.11	0.00	0.07	0.08	0.00	**
SSR30 ₃₆₀	0.04	0.00	0.00	0.00	0.00	**
SSR40 ₁₆₀	0.35	1.00	1.00	1.00	1.00	**
SSR40 ₂₆₀	0.01	0.00	0.00	0.00	0.00	ns
SSR40 ₃₂₀	0.29	0.00	0.00	0.00	0.00	**
SSR40 ₃₅₀	0.34	0.00	0.00	0.00	0.00	**

^a NPA = Neves Paulista; GPO = Gavião Peixoto; SRQ = Sta Rita do Passa Quatro; BAU = Bauru; and PLA = Paulínia. All of these are located in São Paulo, Brazil.

^b G² = likelihood ratio G² test using a null hypothesis of no differences in allele frequencies among populations. ns = not significant; ** indicates significant at *P* < 0.01.

Fig. 1. Map of São Paulo State, Brazil, showing the location of the orchards from which the *Xylella fastidiosa* populations were collected. Neves Paulista (NPA, northwest), Gavião Peixoto (GPO, center), Sta Rita do Passa Quatro (SRQ, east), Paulínia (PLA, southeast), and Bauru (BAU, west).

fication program (TAP) during which the DNA was denatured at 94°C for 1 min, and primers were annealed for 30 s and extended at 72°C for 1 min. The initial TAP annealing temperature was 64°C for 1 cycle, dropping 1°C every cycle until a final annealing temperature of 55°C was reached. This was followed by an additional 22 cycles consisting of denaturation for 1 min at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C. A final extension at 72°C for 4 min was followed by a soak at 4°C for 30 min. VNTR products were separated by gel electrophoresis in 3% agarose gels and stained with ethidium bromide. The fragment size was estimated based on migration relative to a 100-bp size marker (Invitrogen Corp., San Diego) loaded with the samples in the gel.

Data analysis. RAPD and VNTR fragments were scored as putative loci with two alleles, one allele indicating the presence of a fragment and the other the absence of a fragment. The data sets were compiled as a matrix of strains and molecular fragments. Standard population genetics statistics were calculated using the POPGENE population genetic software (POPGENE, version 1.32; Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada). The mean genetic diversity, H , was calculated as $H = (1 - \sum p_i^2)$, where p_i is the frequency of allele i at the locus (21). For each locus, heterogeneity of the marker frequency across populations was calculated by the likelihood ratio (G^2), based on a null hypothesis of no differences in allele frequencies among populations. The proportion of the total genetic diversity attributable to population differentiation was measured using Nei's coefficient of gene differentiation (G_{ST}) among populations (21). The null hypothesis of nondifferentiation among geographic areas ($G_{ST} = 0$) was tested by the Monte Carlo randomization approach (1,000 times) of strains to geographic areas using the software provide by T. L. Peever (Department of Plant Pathology, Washington State University). The null hypothesis was rejected if fewer than 50 of the simulated G_{ST} values ($P = 0.05$) exceed the observed G_{ST} .

The genetic distance phenogram was generated using the numerical taxonomy and multivariate analysis system (NTSYS-pc) software package (version 1.70, Exeter Software, State University of New York, Setauket). The matrix of maximum genetic distances between populations was estimated from allele frequencies by the SIMGEND (similarity for genetic data) program using Nei's coefficient (20). For this procedure, each *X. fastidiosa* population from a specific region was treated as an operational taxonomic unit. Clustering was performed using the sequential

agglomerative hierarchical nested (SAHN) program with an unweighted pair group arithmetic mean averages (UPGMA) algorithm. The phenogram form of the tree matrix data was constructed using the TREE (tree display) program. Statistical support for phenogram branches was provided by PHYLIP (version 3.5c, J. Felsenstein, Department of Genetics, University of Washington, Seattle) software package using the SEQBOOT, GENDIST, NEIGHBOR, and CONSENSE programs with 1,000 bootstrapped samples.

Molecular analysis of variance (AMOVA) (11) was carried out using a matrix of Euclidian distances between all pairs of haplotypes to estimate how much of the total variation among strains was attributable to a population effect. The AMOVA statistic is based on analysis of variance in which the estimation of variance components is expressed as the proportion of the total variance attributable to the population level effect (ϕ_{STAT}). The variance components and ϕ_{STAT} values were statistically tested by nonparametric randomization tests with 1,000 repetitions based on a null hypothesis of variation due to random sampling in the construction of the populations. The AMOVA was performed with the WINAMOVA 1.55 software provided by L. Excoffier (Department of Anthropology and Ecology, University of Geneva, Switzerland).

Genetic and geographic distance matrices and RAPD and VNTR maximum Nei's genetic distance matrices were compared using the Mantel test. The tools for population genetics analysis (TFPGA) software (version 1.3; M. P. Miller, Northern Arizona University, Flagstaff) package was used to calculate the product-moment correlation coefficient, r , which is monotonically related to the Mantel Z value. The statistical significance of the resulting correlation coefficient was tested by performing 1,000 random permutations of the data set and calculating the proportion yielding values that were equal to or greater than the observed coefficient correlation, a significant positive correlation between the two matrices.

RESULTS

VNTR and RAPD variation. A total of 58 RAPD and 15 VNTR products were scored for all of the 360 strains of *X. fastidiosa* from sweet orange plants with CVC symptoms. The 10 RAPD primers that were used generated a maximum of 17 polymorphic alleles. On the other hand, all of the 15 alleles generated by the three VNTR primers were polymorphic (Table 1). The majority of the frequencies of 17 polymorphic RAPD alleles varied

TABLE 2. Mean values of Nei's genetic diversity (H) for random amplified polymorphic DNA (RAPD) and variable number of tandem repeats (VNTR) locus in *Xylella fastidiosa* populations from five regions of São Paulo, Brazil

Locus	H value for <i>X. fastidiosa</i> strains from ^a				
	NPA	GPO	SRQ	BAU	PLA
RAPD locus					
OPA02	0.07 (1/6)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)
OPA03	0.07 (1/6)	0.00 (0/6)	0.00 (0/5)	0.00 (0/6)	0.00 (0/6)
OPD20	0.11 (1/4)	0.00 (0/4)	0.00 (0/4)	0.00 (0/4)	0.00 (0/4)
OPG19	0.09 (1/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)	0.00 (0/5)
OPH07	0.11 (1/4)	0.00 (0/4)	0.00 (0/4)	0.00 (0/4)	0.00 (0/4)
OPH12	0.15 (2/6)	0.00 (0/5)	0.00 (0/4)	0.00 (0/5)	0.00 (0/5)
OPH18	0.09 (1/5)	0.00 (0/5)	0.00 (0/5)	0.03 (0/5)	0.00 (1/5)
OPI02	0.14 (3/7)	0.00 (0/6)	0.07 (1/6)	0.00 (0/6)	0.00 (0/6)
OPN04	0.08 (2/8)	0.00 (0/8)	0.00 (0/7)	0.00 (0/6)	0.00 (0/7)
OPU10	0.04 (2/7)	0.02 (1/7)	0.008 (1/7)	0.08 (1/8)	0.00 (0/7)
Average	0.095 (15/58)	0.002 (1/55)	0.009 (2/56)	0.004 (2/55)	0.000 (0/54)
VNTR locus					
SSR20	0.28 (5/5)	0.01 (2/5)	0.01 (2/5)	0.15 (3/5)	0.00 (0/5)
SSR30	0.25 (6/6)	0.05 (3/6)	0.15 (4/6)	0.18 (5/6)	0.12 (2/6)
SSR40	0.34 (4/4)	0.00 (0/4)	0.00 (0/4)	0.00 (0/4)	0.00 (0/4)
Average	0.285 (15/15)	0.024 (6/15)	0.066 (6/15)	0.121 (8/15)	0.051 (2/15)

^a NPA = Neves Paulista; GPO = Gavião Peixoto; SRQ = Sta Rita do Passa Quatro; BAU = Bauru; and PLA = Paulinia. RAPD and VNTR primers. Number of polymorphic alleles / number of total alleles in parentheses.

significantly ($P < 0.01$) among the *X. fastidiosa* populations (Table 1). As expected, the frequencies of the other 41 monomorphic alleles did not vary intra- or interpopulations (data not shown). The frequencies of all VNTR makers varied significantly among the five populations for 14 of the 15 alleles as indicated by the significant G^2 values (Table 1).

A minimum of one and a maximum of three polymorphic alleles per locus were identified when amplifying DNA from the Neves Paulista (NPA) population with the RAPD primers. Amplifications using strains from the Gavião Peixoto (GPO), Sta Rita do Passa Quatro (SRQ), and Bauru (BAU) populations generated only one polymorphic allele (primers OPH18, OPI02, and OPU10) and no polymorphism was observed for the other seven RAPD primers (Table 2). No polymorphism was observed in the Paulinia (PLA) population for any RAPD primers (Table 2). The level of polymorphism shown by the VNTR markers was high and ranged from 2 alleles per locus (15.5%) for the PLA population to 15 alleles per locus (100%) for the NPA population. Although the number of polymorphic VNTR alleles was lower for GPO, SRQ, BAU, and PLA populations when compared with NPA, it was higher than that obtained for RAPD in these populations (Table 2).

The genetic diversity values (H), based on the total number of alleles and their frequency, were greater for the VNTR loci for all populations when compared with RAPD loci (Table 2). The maximum value for H_{RAPD} (0.095) was obtained for NPA population, whereas the other populations showed values even lower or zero. On the other hand, the H_{VNTR} value for the NPA population reached a maximum value of 0.285 and the H_{VNTR} values obtained for GPO, SRQ, BAU, and PLA were greater than those obtained for RAPD (Table 2). Nei's maximum genetic distances from VNTR data between *X. fastidiosa* populations gave distances ranging from ≈ 2.5 to $\approx 50\%$ (Fig. 2), whereas the maximum genetic distance by RAPD data was 8% (data not shown).

Population structure. The following analyses have been based on only the VNTR data for several reasons. First, the VNTR markers proved to have a higher level of discrimination between the populations of strains and among the strains than RAPD markers (Tables 1 and 2). Second, the VNTR loci used in this study are selectively neutral and the VNTR mutation rates are smaller than 10^{-5} , as seen for *Bacillus anthracis* (16), which are important characteristics required for the molecular markers used for population genetic analysis. Finally, most previous works concerning epidemiological studies involving bacteria are based on VNTR data (1,7,8,16).

All the pairwise comparisons of *X. fastidiosa* populations showed highly significant G_{ST} values ($P = 0.000$), suggesting strong differentiation between *X. fastidiosa* populations from different geographical regions (Table 3). UPGMA cluster analyses using Nei's maximum genetic distances between *X. fastidiosa* populations gave distances ranging from ≈ 2.5 to $\approx 50\%$, and the clustering obtained in the phenogram was statistically well supported by 1,000 bootstrappings (Fig. 2).

Fig. 2. Phenogram of Nei's maximum genetic distance between *Xylella fastidiosa* populations isolated from orchards of sweet orange trees from five different geographical regions of São Paulo State, Brazil: Neves Paulista (NPA), Gavião Peixoto (GPO), Sta Rita do Passa Quatro (SRQ), Paulinia (PLA), and Bauru (BAU). Numbers at branches indicate the percentage of occurrence of the cluster to the right of the branch in 1,000 bootstrapped phenograms.

AMOVA was performed by partitioning the data in three different ways; according to the geographic region of the host, among plants from the same geographic regions, and within each plant. AMOVA also revealed a strong and statistically significant genetic differentiation among populations (regions) ($\phi_{\text{STAT}} = 0.810$; $P < 0.001$), explaining $\approx 37\%$ of total variance (Table 4). Furthermore, statistically significant genetic variations were observed among plants ($\phi_{\text{STAT}} = 0.699$; $P < 0.001$) and within each plant ($\phi_{\text{STAT}} = 0.368$; $P < 0.001$), which account for approximately 44 and 19% of total genetic variation observed for *X. fastidiosa*, respectively (Table 4). The significant genetic variation within plants indicates that strains genetically different were found in the same plant (Table 5). Data from Table 5 show that four multilocus VNTR haplotypes (i.e., four strains genetically different) of *X. fastidiosa* were observed within plant number 08 from the NPA population.

The comparison of the geographic and Nei's maximum genetic distances matrices for the *X. fastidiosa* populations did reveal a positive but not significant correlation between the two data sets (Fig. 3). The value of the Mantel product-moment correlation coefficient was positive ($r = 0.27$) but not significantly different from zero ($P = 0.22$).

DISCUSSION

In a previous study, we introduced the VNTR method for typing *X. fastidiosa* and showed that, compared with the RAPD method, this PCR-based methodology provided a high-resolution technique for genetic diversity studies of citrus-specific *X. fastidiosa* strains (8). In the present paper, the VNTR markers were more efficient for discrimination of *X. fastidiosa* strains isolated from citrus compared with RAPD markers, as shown by the H values and the number of polymorphic alleles. The average H_{VNTR} values are 3 (NPA) to 30 (BAU)-fold higher than that determined by RAPD data (Table 2). Changes in the number of repeats in a given VNTR

TABLE 3. Pairwise comparison of Nei's coefficient of gene differentiation (G_{ST}) values for five *Xylella fastidiosa* populations isolated from citrus plants from Brazilian state of São Paulo determined by variable number of tandem repeats (VNTR) markers^a

Populations ^b	NPA	GPO	SRQ	BAU	PLA
NPA	...	...	...	...	...
GPO	0.246**	...	...	...	...
SRQ	0.235**	0.451**	...	...	...
BAU	0.169**	0.249**	0.212**	...	...
PLA	0.245**	0.555**	0.248**	0.053**	...

^a Values indicate the genetic diversity between populations as proportion of total diversity: $G_{\text{ST}} = (H_T - H_S)/H_T$. Null hypothesis of no population differentiation. ** indicates $P = 0.000$ according to Monte Carlo simulations.

^b NPA = Neves Paulista; GPO = Gavião Peixoto; SRQ = Sta Rita do Passa Quatro; BAU = Bauru; and PLA = Paulinia.

TABLE 4. Analysis of molecular variance of the variable number of tandem repeats haplotypes for five *Xylella fastidiosa* populations isolated from sweet orange plants growing in São Paulo, Brazil

Source of variation ^a	df	% Total variance	ϕ_{STAT}	P value ^b
Among regions	4	36.79	0.810	<0.001
Among plants within regions	55	44.19	0.699	<0.001
Within the plants	300	19.02	0.368	<0.001

^a The total population of *X. fastidiosa* was partitioned into hierarchical components; among the five different geographic regions (orchards), among plants (i.e., strains from the plants) within regions, and within plants (i.e., strains from the same plant).

^b Probability of having larger values than those observed based on 1,000 randomizations of the treatments.

locus are also an important source of DNA variability in genetically homogeneous pathogens such as *B. anthracis* (16) and *Yersinia pestis* (1).

The estimated values of Nei's genetic diversity (*H*) from the *X. fastidiosa* populations analyzed in our study (Table 2) were smaller than those reported for other *X. fastidiosa* isolates from citrus (*H* = 0.45) (8) and also smaller than most of the values obtained with other bacteria such as *Escherichia coli* (33), *B. anthracis* (16), and *Y. pestis* (1). The *X. fastidiosa* strains analyzed in our previous study came from the southern and southeastern regions of Brazil, which are very different geographic locations and environments compared with those described in this study. Besides that, these strains were isolated in different periods over years. These factors could account for the different *H* values obtained.

The low genetic diversity observed among citrus *X. fastidiosa* strains, mainly those from the GPO, SRQ, BAU, and PLA populations, is consistent with the diversity values observed by Rosato et al. (30) and Qin et al. (26), who also studied *X. fastidiosa* strains from São Paulo State. In contrast, NPA population from the northwestern region of São Paulo State showed the highest *H* value when compared with other populations (Table 2). The north and northwest regions of São Paulo State were the primary focus of CVC, and these regions now (early 2002) have the citrus trees most severely affected with CVC. As discussed by Rosato et al. (30), the northwest region was previously a coffee-growing area, and it may be that the remaining coffee plants could have been a source of *X. fastidiosa* for citrus contamination. Other important factors in the dissemination of *X. fastidiosa* are the populations of wild *X. fastidiosa*-hosts plants and insect vectors (leafhoppers). In California, grapevines were severely infected by *Xylella* spp. when grown in restricted locations ("hot spots") adjacent to permanent water sources where weeds harboring Pierce's disease strains occurred along with insect vectors (27). It is interesting to note that our NPA population was isolated from plants growing adjacent to a permanent water source. Although we have no information on the presence of potential wild host plants for *X. fastidiosa* in the NPA area, the three most important sharpshooter vectors of CVC-causing strains of *X. fastidiosa* (*Acrogonia* sp., *Dilobopterus costalimai*, and *Oncometopia facialis*) were more prevalent at the northwest citrus region (NPA area) than in any other citrus producing region (29).

The diversity of hosts from which the bacteria were isolated and the geographic origin of the host have both been pointed out as being responsible for the high values of genetic diversity found in some species of bacteria, e.g., *E. coli* (33), enteric bacteria from some Australian mammals (13), and *Xanthomonas axonopodis* pv. *manihotis* (28). In *X. fastidiosa* from sweet orange plants, the

diversity of host did not play a role in the population structure and consequently in the bacteria diversity (7). Evidence for a subdivision between *X. fastidiosa* populations consistent with geographic origin of the host was shown by da Costa et al. (9). Also, RAPD and rep-PCR patterns from CVC-causing *X. fastidiosa* strains revealed apparent geographic patterns (19). The *G_{ST}* values (Table 3) that we found agree with the hypothesis of genetic differentiation among *X. fastidiosa* populations in relation to the geographic origin of the host, and Nei's maximum genetic distance coefficients (Fig. 2) show that *X. fastidiosa* populations are genetically quite different.

The AMOVA data showed that a significant amount of the genetic differentiation observed in *X. fastidiosa* strains could be explained by a population effect, i.e., the geographic origin of the host was able to explain a significant amount of the total variation obtained (Table 5). There are few other studies of phytopathogenic bacteria available for comparison, but genetic fingerprinting analysis of *X. fastidiosa* strains suggest a certain degree of clustering according to the geographical region of the host (18,19), although in these studies the proportion of genetic variation explainable by the geographic effect was not determined. In *E. coli*, the geographic effect explained from 5 to 10% of the total genetic variation (12,13,33). In addition to significant genetic variation among populations, in our study, AMOVA showed significant genetic variation within the *X. fastidiosa* populations, indicating that different strains of *X. fastidiosa* are present within the orchards or even within a single plant. The analysis of six strains per plant allowed us to find up to four VNTR multilocus haplotypes colonizing a single citrus plant (Table 5). In a previous study, we were able to find up to five VNTR multilocus haplotypes colonizing a simple citrus plant in an orchard composed of different sweet orange cultivars (7).

The data also show a high and significant level of genetic differentiation between the populations, which is probably the consequence of the populations being genetically different (Tables 3 and 4; Fig. 2). However, the comparison of the genetic and geographic distance matrices by the Mantel test did not support the hypothesis of isolation-by-distance among the five populations studied. Therefore, concerning the genetic differentiation in the population, there is no evidence that there may be a significant barrier to gene flow operating in *X. fastidiosa* in São Paulo, Brazil. Until recently in São Paulo State, the majority of sweet orange seedlings were

TABLE 5. Number of *Xylella fastidiosa* haplotypes within sweet orange plants determined by variable number of tandem repeats markers^a

Plant identification	NPA	GPO	SRQ	PAL	BAU
01	2	1	2	2	1
02	2	1	1	2	1
03	2	2	2	2	2
04	3	2	2	1	1
05	1	1	2	1	1
06	2	1	3	1	1
07	1	1	2	1	1
08	4	2	2	1	1
09	2	1	1	1	1
10	2	1	1	1	2
11	1	2	1	1	1
12	2	2	2	1	2

^a NPA = Neves Paulista; GPO = Gavião Peixoto; SRQ = Sta Rita do Passa Quatro; BAU = Bauru; and PLA = Paulínia. All of these are located in São Paulo, Brazil.

Fig. 3. Plot of geographic distances and Nei's maximum genetic distances for five *Xylella fastidiosa* populations from São Paulo State, Brazil.

produced in nurseries without protection against the *X. fastidiosa* vectors, and health quality of budwood used was uncertain, moreover there was an uncontrollable seedling movement throughout the state. Therefore, the nursery distribution could be an important factor in the epidemiology because genotypes of *X. fastidiosa* could be spread by infected seedlings. On the other hand, specific regional source of the *X. fastidiosa* strains infecting citrus plants probably has contributed for the intensification of variation among populations of this pathogen. According to Spiers et al. (34), diversity arises and is maintained through the interplay between a variety of ecological (ecological opportunity and competition) and genetic (mutation and recombination) factors. Up to 11 species of sharpshooter leafhoppers (3), which can naturally transmit *X. fastidiosa* to citrus trees, and variation in leafhopper populations have been observed in different citrus growing regions, with populations of this vector being influenced by climatic conditions (29). In addition, Lopes et al. (17) showed that up to 10 species of wild plants present in citrus orchards in São Paulo State were natural hosts of *X. fastidiosa*. However, the combination of diverse sources of *X. fastidiosa* and different population dynamics of sharpshooter vectors might produce conditions that result in genetic differentiation of populations of this pathogen according to geographic location of the host plant. As discussed, there are two possible sources of infection by *X. fastidiosa*, one being alternative hosts within the orchard and the other being seedlings from infected nurseries. Studies of *X. fastidiosa* strains from wild host plants and from vectors present in these regions will be necessary for better understanding of the origin of genetic variation observed in these regions. On the other hand, although *X. fastidiosa* is recent in citrus plants, it has genetic elements (plasmids, transposons, integrons, and phages) known to have evolved for genetic exchange (14,32).

The analysis of 15 VNTR loci in 360 *X. fastidiosa* strains from different regions of the state of São Paulo has allowed us to estimate that the mean values of genetic diversity (*H*) of *X. fastidiosa* strains within each of the geographic regions studied were low, ranging from 0.024 to 0.285. However, a significant genetic differentiation among these populations was observed. The AMOVA and *G_{ST}* data show that the total genetic variation was significantly distributed among and within the subpopulations, and these were genetically divergent. Despite the questionable occurrence of pathotypes in *X. fastidiosa* of CVC (26), a controlled inoculation test may determine if different degrees of virulence exist among haplotypes of this pathogen. Based on data of the present work, we suggest that representative strains from each geographic region must be used for inoculating hybrids or lines obtained from sweet orange breeding programs oriented to find CVC resistance.

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