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Spatial Genetic Structure of Coffee-Associated Xylella fastidiosa Populations Indicates that Cross Infection Does Not Occur with Sympatric Citrus Orchards

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ABSTRACT

Xylella fastidiosa, an economically important plant-pathogenic bacterium, infects both coffee and citrus trees in Brazil. Although X. fastidiosa in citrus is well studied, knowledge about the population structure of this bacterium infecting coffee remains unknown. Here, we studied the population structure of X. fastidiosa infecting coffee trees in São Paulo State, Brazil, in four regions where citrus is also widely cultivated. Genotyping of over 500 isolates from coffee plants using 14 genomic microsatellite markers indicated that populations were largely geographically isolated, as previously found with populations of X. fastidiosa infecting citrus. These results were supported by a clustering analysis, which indicated three major genetic groups among the four sampled regions. Overall, approximately 38% of isolates showed significant membership coefficients not related to their original geographical populations (i.e., migrants), characterizing a significant degree of genotype flow among populations. To determine whether admixture occurred between isolates infecting citrus and coffee plants, one site with citrus and coffee orchards adjacent to each other was selected; over 100 isolates were typed from each host plant. No signal of natural admixture between citrus- and coffee-infecting isolates was found; artificial cross-infection assays with representative isolates also yielded no successful cross infection. A comparison determined that X. fastidiosa populations from coffee have higher genetic diversity and allelic richness compared with citrus. The results showed that coffee and citrus X. fastidiosa populations are effectively isolated from each other and, although coffee populations are spatially structured, migration has an important role in shaping diversity.

The economically important plant-pathogenic bacterium Xylella fastidiosa colonizes multiple hosts and requires insect vectors for dissemination. Although until recently reported only in the Americas, there have been recent reports from Italy and France (Almeida 2016) and Iran (Amanifar et al. 2014). Tentatively subdivided in five subspecies, this pathogen causes disease in a variety of crops such as citrus, grapevine, almond, and coffee; trees such as elm, oak, mulberry, and sycamore; as well as ornamental species including oleander citrus, grapevine, almond, and coffee; trees such as elm, oak, mulberry, and sycamore; as well as ornamental species including oleander...
were done to test whether the genetic separation between coffee and citrus populations was biologically significant.

**MATERIALS AND METHODS**

**Population sampling and DNA extraction.** In total, 519 isolates of *X. fastidiosa* were obtained from coffee trees (*Coffee arabica ‘Mundo Novo’*) from four geographic regions in São Paulo State, Brazil. Another 117 isolates were obtained from a citrus (*Citrus sinensis ‘Pera’*) orchard adjacent to coffee trees in the central region of São Paulo State (Tabatinga) (Supplementary Fig. S1; Supplementary Table S4). All *X. fastidiosa* populations were established throughout the year 2012 by sampling *X. fastidiosa*-symptomatic tissues from coffee and citrus trees according to symptoms as described by de Lima et al. (1998) and de Souza et al. (2009), respectively. Bacteria were isolated from both symptomatic branches (3 mm in diameter) or leaf petioles on solid buffered charcoal yeast extract (BCYE) agar medium (Wells et al. 1981), as previously described (Almeida et al. 2001; Coletta-Filho and Machado 2003). Solid media plates were incubated at 28°C for at least 15 days before appearance of colonies, which were identified as *X. fastidiosa* based on fastidious growth, coloring, and polymerase chain reaction (PCR) assays using primers specific to *X. fastidiosa* subsp. *pauca* (Pooler and Hartung 1995), followed by triple cloning on periwinkle wilt gelrite (PWG) medium (Almeida et al. 2004). A fraction of the purified strains was stored at −80°C in PW broth (Davis et al. 1981) with 40% glycerol for long-term storage. The other fraction was grown in PWG medium for an additional 5 days prior to DNA extraction and genotyping. A commercial kit was used for DNA extraction (Wizard Genomic DNA Purification Kit, Promega Corp., Madison, WI) according to the manufacturer’s instruction.

**Genotyping and fragment analysis.** Seven variable number of tandem repeat (VNTR) loci previously described by Lin et al. (2005) and seven new ones developed for this study were used for genotyping strains (Supplementary Tables S1 and S2). Five new repeat regions on the genome of *X. fastidiosa* strain 3124 (based on the draft genome of a strain isolated from coffee plants) (unpublished data) and two from citrus reference strain 9a5c were identified with Tandem Repeat Finder, version 2.02 (Benson 1999). Primers flanking these novel repeat regions were designed with Primer3, version 0.4.0 (Untergasser et al. 2012). Multiplexed primers were used in reactions for multilocus VNTR analysis. The multiplex amplifications were performed in a final volume of 13.5 µl containing 25 to 50 ng of DNA, 6.5 µl of DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, MA), and variable primer pair concentrations (with a fluorescent-labeled forward primer). The following program was used for PCR amplification: a denaturation step at 95°C for 5 min; followed by 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s; and a final of extension at 72°C for 10 min. Before fragment analysis, reactions were diluted 20× (set 1) or 40× (sets 2, 3, 4, and 5) in sterile milli-Q water. For fragment analysis, 0.7 µl of the dilution was added to 10 µl of Hi-Di Formamide and 0.1 µl of GeneScan 500-LIZ Size Standard (both from Life Technology, Foster City, CA). The capillary electrophoresis was run in an ABI 3730 DNA sequencer (Life Technology) and peak size estimated in base pairs by the Peak Scanner software (version 1.0: Life Technology).

**VNTR profile.** The distribution of allele frequencies, the range of repeat size, and number of alleles per locus for all VNTR loci were reported in supplementary materials (Supplementary Fig. S2). Allele size was converted into number of repeat units for better visualization of VNTR evolutionary dynamics within each population. Considering the observed repeat unit increments of single-repeat changes (the insertion or deletion of one repeat motif), we assumed a stepwise mutation model (SMM) for all analysis; similar observations were made for *X. fastidiosa* infecting grapevine (Coletta-Filho et al. 2011) and citrus (Coletta-Filho et al. 2014). Parameters such as allele size, average of number of repeats, and identification of private alleles were determined using CONVERT software, version 1.3.1 (Glaubitz 2004).

**Clonal diversity.** Strains with the same multilocus microsatellite genotype (MLMG) were considered as a clone or haplotype, as determined by GENODIVE, version 2.0b23 (Meirmans and van Tienderen 2004), and only one representative of each haplotype was selected per population to construct clone-corrected data sets that were used for downstream analysis. Indicators of genotypic diversity estimated included (i) number of genotypes per population; (ii) clonal fraction, calculated as 1 − (number of different genotypes within the population)/(total number of isolates within the population) (Zhan et al. 2003); (iii) the Simpson’s diversity index (S), which was estimated as $S = 1 - \sum_{i=1}^{r} P_i^2$, where $P_i$ is the relative abundance of the rth genotype at the population (1 represents infinite diversity and 0 no diversity) (He and Hu 2005); and (iv) the genotypic distribution over populations (evenness) (Stoddart and Taylor 1988).

**Genetic diversity.** The level of genetic variation in populations was estimated by both genetic diversity ($H_{S\text{eq}}$), corrected by the number of individual (n) in a population through of the formula $H_{S\text{eq}} = [(1 - 2p_x) \times (n/2 - 1)]$, where $p_i$ is the frequency of allele i at the locus p (Nei 1978), and by the allelic richness within each geographical population. Both parameters were estimated using the software FSTAT, version 2.9.3.2 (Goudet 1995). Statistics for the significance between pairs of comparison were obtained by 1,000 randomizations. An analysis was also run to estimate genetic indices ($H_{S\text{eq}}$ and allelic richness) using available data from *X. fastidiosa* populations from citrus (Coletta-Filho et al. 2014). The goal was to compare these genetic indices between coffee and citrus groups of strains to infer population size.

**Linkage disequilibrium analysis.** The null hypothesis that all loci are in equilibrium among populations was tested using the multilocus index of association test ($I_\text{x}$) (Smith et al. 1993), as well as $rbarD$, which is less sensitive to the number of loci (Agapow and Burt 2001), with MULTILOCUS, version 1.3 (http://www.bio.ic.ac.uk/evolve/software/multi locus/). $I_\text{x}$ or $rbarD$ values significantly different from zero indicate disequilibrium, which was tested with 1,000 randomizations.

**Population differentiation.** The null hypothesis of no genetic differentiation among sampled populations was tested by both $D_{est}$ (Jost 2008), using the DEMEthics package in R, and $R_{ST}$ (Slatkin 1995) using ARLEQUIN, version 3.11 (Excoffier et al. 2005). Both are analogs to the widely used population differentiation index $F_{ST}$ (Wright 1949) but more adapted to highly polymorphic markers such as microsatellites. Populations were considered significantly differentiated when the observed values of $D_{est}$ and $R_{ST}$ were larger than 95% of the values obtained with 1,000 bootstrapping of MLMG over the populations. Analysis of molecular variance (AMOVA) (implemented in ARLEQUIN) was performed to assess and quantify the differentiation among subpopulations (geographical regions). Analyzes of the sum of squared size differences ($\Phi_{ST\text{AMP}}$) between two haplotypes were used as a distance measure (Slatkin 1995), with the significance ($P \leq 0.05$) tested by 1,023 permutation tests. GENODIVE (version 2.0b23) was used to perform the Mantel’s test to estimate correlation between matrices of geographic distances (linear kilometers drawn from the latitude and longitude of populations) and genetic distances, the latter using values of both $D_{est}$ and $R_{ST}$ indexes. The null hypothesis of no linear relationship between geographic and genetic distances among all pairwise populations was tested by Markov Chain Monte Carlo (MCMC) by 1,000 randomizations, assuming $P \leq 0.05$.

The number of genetic clusters was estimated with STRUCTURE, version 2.3 (Pritchard et al. 2000), using the admixture model and assuming an unknown number of populations. The analysis was run three times using 10 replications of number of populations (K) ranging from 2 to 15. For these runs, the following conditions were adopted: burn-in period at 30,000 following 300,000 replicates of the MCMC. The K value with the highest likelihood was determined
according to Evanno et al. (2005). We also performed a principal coordinate analysis with GeneAlex, version 6.5 (Peakall and Smouse 2012), to plot major patterns within a multivariate data set (e.g., multiple loci and multiple samples). This method was used to identify population structure of individuals from citrus bordered with coffee populations in the central region of São Paulo State. We note that the data set had linkage disequilibrium, violating the STRUCTURE assumption of linkage equilibrium.

Demographic parameters and historical migration. The effective population sizes and migration rates among coffee-infecting *X. fastidiosa* populations were inferred using a Bayesian estimation based on the MCMC method implemented in MIGRATE, version 3.0.3 (Beerli and Felsenstein 2001) (Department of Biological Science, Florida State University, Tallahassee). This method allows estimation of the effective population size of populations (Θ) (which, for haploids, is equal to 2Νeμ, where Ne = effective population size and μ = mutation rate for each locus) and migration rates between population pairs (M = 2Νem, the number of migrants exchanged per generation). The data type chosen was microsatellite data with Brownian motion assuming the SMM, with 10 distinct runs realized. A single run consisted of one long Markov chain and a static heating scheme with four temperatures (1.0, 1.3, 2.6, and 3.9). The Markov chain was carried out with 5,000 samples, sampling interval of 100 (500,000 steps), burn-in period of 10,000, and 5,000 trees recorded. Prior distribution for parameter for Θ and 2Νem was uniform, with minimum = 0.0, mean = 10.0, and maximum = 20.0. The Bayesian estimates at 95% for every parameter mode were determined considering the 0.025 and the 0.975 percentiles of their a posteriori distribution.

Natural and artificial events of cross infection by *X. fastidiosa* strains. The occurrence of natural cross infection between *X. fastidiosa* coffee and citrus strains was tested with a population (226 isolates) obtained from diseased citrus (n = 99) and coffee (n = 127) trees grown at adjacent blocks in a farm at the central region (Tabatinga) of São Paulo State (Supplementary Fig. S1B). Identification of individuals in admixture between populations was done with STRUCTURE, as described above. The number of *X. fastidiosa* genetic clusters (subpopulations) within citrus and coffee hosts, as well which individuals belonged to each cluster, was estimated with BAPS v.5.2 (Corander et al. 2003), performing independent runs using the “clustering of individuals”, as recommended by Waples and Gaggiotti (2006), with subpopulations number (K) ranging from 1 to 40. Once we identified the best value of K, we reanalyzed the data with BAPS using the “fixed-K module”, which ran 1,000 times to identify the individuals that compose each genetic cluster. This value represents the number of times that each individual was analyzed using simulations of different allele frequencies. Based on clustering of individuals results, one randomly selected isolate of several genetic clusters was selected to conduct the artificial inoculation assays.

Mechanical infections of coffee and citrus were done to test whether strains were able to maintain successful infections over time in homologous and heterologous host combinations. All the strains used for inoculation in coffee and citrus were also inoculated in *Nicotiana tabacum* as a control for cell viability during inoculation and persistence of infection over time, because tobacco is highly susceptible to *X. fastidiosa* (Lopes et al. 2000). Seedlings of all tested hosts—*C. sinensis* Pera, *Coffea arabica* (L.), Mundo Novo, and *Nicotiana tabacum* ‘Havana’—were inoculated in two different points of the main stem with 10 μl of *X. fastidiosa* suspensions at 10⁸ CFU/ml, following methods previously described (Almeida et al. 2001). The plants were maintained inside an insect-free greenhouse. For each genotype (seven from coffee and four from citrus genetic groups, one strain per group), five homologous and five heterologous plants (coffee and citrus) were selected, and three tobacco seedlings were inoculated. Plants inoculated with phosphate-buffered saline buffer and with strain 9a5c (*X. fastidiosa* reference isolate, originally from citrus) were kept as negative and positive controls, respectively. The list of haplotypes used in this biological assay and the number of plants tested are provided in Supplementary Table S3; haplotypes were selected based in the clustering analysis described above. The presence of *X. fastidiosa* in the coffee and citrus plants as well as the *X. fastidiosa*-associated symptoms were evaluated at 120, 210, 300, 390, and 510 days after inoculation, and the tobacco plants only during the first three sampling dates. Leaves at inoculation point or closer were sampled and 250 mg of petiole was disrupted and homogenized using the TissueLyser II (Qiagen, Venlo, Netherlands) for 2 min at 30 Hz (1,800 oscillations/min), following the DNA extraction by cetyl-trimethylammonium bromide methodology (Murray and Thompson 1980). All DNA were checked for quality and standardized to 100 ng/µl before the amplifications by using the TaqMan Real-time quantitative PCR (qPCR) chemistry (Applied Biosystems, Foster, CA) adopting primers and probe specific to *X. fastidiosa* (Oliveira et al. 2002). DNA of *X. fastidiosa* strain 9a5c grown on PWG medium was added to healthy citrus and coffee tissues and used as a positive control for both DNA extraction and qPCR amplifications.

RESULTS

Gene and genotypic diversity. The number of alleles for the VNTR loci ranged from 4 to 17 (totaling 153 alleles). The average number of alleles per locus amplified by primers based on the genome of an *X. fastidiosa* strain from coffee (COSSR) was higher (14.8) than from primers based on oleander (OSSR = 9.7), grape (GSSR = 9.0), or citrus (CSSR = 7.7) strains. Private alleles (n = 63) were randomly distributed throughout populations analyzed. Strains from eastern São Paulo State hosted 56% of the total of private alleles, compared with the population from central São Paulo State that hosted only 5%. From a total of 519 isolates of *X. fastidiosa* infecting coffee plants, 185 MLMG (35.6%) were identified as genetically distinct haplotypes (Table 1). The four different regions had distinct indexes of genotypic diversity (Table 1). The clonal fraction varied from 0.56 (eastern São Paulo State) to 0.72 (northwestern São Paulo State), while Simpson’s genotypic diversity index was significantly lower for northwestern São Paulo State (0.86) in comparison with the other populations. The highest gene diversity indexes (Hnei and allelic richness) were observed for the *X. fastidiosa* population from eastern São Paulo State (Table 1). The lower gene diversity indexes were observed for both northwestern and central São Paulo State populations, which were not statistically different from each other (Table 1).

<table>
<thead>
<tr>
<th>Populations</th>
<th>Sample size (n)</th>
<th>Number of genotypes</th>
<th>Clonal fraction</th>
<th>Evenness</th>
<th>Simpson’s index</th>
<th>Hnei</th>
<th>Allelic richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>127</td>
<td>44</td>
<td>0.65</td>
<td>0.49 a</td>
<td>0.96 a</td>
<td>0.54 c</td>
<td>5.06 c</td>
</tr>
<tr>
<td>Northwest</td>
<td>60</td>
<td>17</td>
<td>0.72</td>
<td>0.39 c</td>
<td>0.86 b</td>
<td>0.57 c</td>
<td>5.07 c</td>
</tr>
<tr>
<td>Center-West</td>
<td>178</td>
<td>61</td>
<td>0.64</td>
<td>0.49 a</td>
<td>0.97 a</td>
<td>0.60 b</td>
<td>5.52 b</td>
</tr>
<tr>
<td>East</td>
<td>154</td>
<td>63</td>
<td>0.56</td>
<td>0.41 b</td>
<td>0.97 a</td>
<td>0.69 a</td>
<td>5.77 a</td>
</tr>
<tr>
<td>Overall</td>
<td>519</td>
<td>185</td>
<td>0.64</td>
<td>0.44</td>
<td>0.94</td>
<td>0.60</td>
<td>5.35</td>
</tr>
</tbody>
</table>

* Values of indexes followed by different letter were significantly different (P ≤ 0.05) based on 1,000 pairwise bootstraps.

* For details how this index was estimated, please see Materials and Methods.
Linkage disequilibrium analysis. A nonrandom association between different alleles was detected for all coffee-infecting populations \((P \leq 0.01)\) (Table 2). Values for the less-sensitive index \(r_{barD}\) were 0.08 to 0.33, and were confirmed by \(I_{A}\), with values of 0.91 to 4.29. Both indexes indicate strong linkage disequilibrium among loci of coffee populations from \(X. fastidiosa\). As a consequence, a significant percentage of allele pairs at significant disequilibrium were observed in all populations (15.4 to 84.6%). The lowest values were observed for northwestern São Paulo State and the highest for the eastern São Paulo State populations (Table 2).

Genetic differentiation between populations. The null hypothesis of no genetic differentiation among coffee populations was tested using two statistical parameters \((D_{est} and R_{ST})\). Both \(D_{est}\) and \(R_{ST}\) indexes yielded similar results (Table 3). Except for the population pair from central São Paulo State and northwestern São Paulo State \((D_{est} = 0.02; R_{ST} = 0.01; P > 0.05)\), all other population pairs were significantly distinct \((D_{est} = 0.36 to 0.63; R_{ST} = 0.31 to 0.50, P \leq 0.05)\). AMOVA showed that 56% of the variance was attributed to within-population and 44% to among-population variability, reflecting a high value of genetic differentiation at the population level \((\Phi_{ST} = 0.44, P < 0.01)\). Similarly to others studies with \(X. fastidiosa\) in citrus (Coletta-Filho and Machado 2003; Coletta-Filho et al. 2014), and grapevine (Coletta-Filho et al. 2011), no correlation was found between genetic differentiation index \((D_{est}\) and \(R_{ST}\)) and geographical distances among populations using the Mantel test \((r = 0.012, P = 0.50)\). Based on a Bayesian clustering analysis used to infer the number of genetically homogeneous groups, three subpopulations \((K = 3)\) were identified (Fig. 1), despite the fact that the number of geographic populations was four. This result is in agreement with the \(D_{est}\) and \(R_{ST}\) values that indicated no subdivision between the central and northwestern São Paulo State populations of \(X. fastidiosa\), also inferring three subpopulations in the data set. In fact, all genotypes from these two populations were clustered in K-1, together with some genotypes from center-west \((37.7%)\) and eastern São Paulo State \((46%)\) populations. The cluster K-2 was unique for the eastern São Paulo State genotypes while the K-3 cluster grouped genotypes from the center-west São Paulo State population \((62.3%)\) (Fig. 1).

Migration. The population size estimates of coffee-infecting \(X. fastidiosa\) \((\Theta = 0.18 to 0.55)\) were not significantly different. However, the migration rates between pairs of geographical populations were asymmetrical. For example, while the central São Paulo State population contributed with a high number of migrants to the eastern São Paulo State population \((2N_m_{Central→Northwest} = 13.0\) migrants/generation; 95% interval = 12.3 to 14.7), the opposite migration (eastern São Paulo State population to central São Paulo State population) was six times lower \((2N_m_{Northwest→Central} = 2.2\) migrants/generation; 95% interval = 1.2 to 3.5). Despite an asymmetrical trend, we observed that central and northwestern São Paulo State populations had exchanged the highest amount of migrants among any of the populations pairs \((2N_m_{Northwest→Central} = 14.4\) migrants/generation; \(2N_m_{Northwest→Central} = 8.3\) ), which might explain the genotype flow detected between these two populations. On average, the coffee-infecting central São Paulo State population contributed the highest number of migrants per generation \((M = 10.7;\) Bayesian 95 percentile = 8.7 to 13.4). In contrast, the northwestern São Paulo State population received the higher number of migrants \((M = 10.5;\) Bayesian 95 percentile = 7.6 to 13.4) (Fig. 2).

**X. fastidiosa from adjacent citrus and coffee orchards: admixture of strains and genetic diversity.** For the population of 226 \(X. fastidiosa\) strains established from diseased coffee \((n = 127)\) and citrus \((n = 99)\) trees in adjacent orchards, no evidence of admixture between populations was obtained (Fig. 3A). A defined number of populations as \(K = 2\) (coffee and citrus) was obtained with strains associated with their respective host plant of origin (Fig. 3A). Furthermore, analyses were performed to verify the number of genetic clusters of coffee and citrus strains. In total, 14 genetic clusters were detected, whereas 10 clusters \((71.42%)\) were composed exclusively of \(X. fastidiosa\) from coffee and 4 clusters \((28.57%)\) only of isolates from citrus (Fig. 3B). The \(D_{est}\) and \(R_{ST}\) values between \(X. fastidiosa\) populations infecting citrus and coffee plants \((D_{est citrus × coffee} = 0.56; R_{ST citrus × coffee} = 0.91; P \leq 0.05)\) showed high genetic differentiation between both populations (Supplementary Fig. S3).

Based on genotypic richness of \(X. fastidiosa\) from coffee \((10\) genetic clusters) compared with citrus \((4\) genetic clusters) (Fig. 3B), a broad comparison of gene diversity indexes between populations from coffee-infecting (data set obtained in this article) and citrus-infecting \(X. fastidiosa\) (previously published by our group, similar but not identical sampling design with 320 strains genotyped) (Coletta-Filho et al. 2014) was performed. Genotypic diversity indexes of coffee-infecting \(X. fastidiosa\) populations \((mean = 0.60 and 5.82 for H_{NEI} and allelic richness, respectively)\) were higher than those described for citrus populations \((mean = 0.43 and 2.82 for H_{NEI} and allelic richness, respectively)\). The values of the \(t\) \((coffee versus citrus)\) for differences in \(H_{NEI}\) \((t = 2.34 ± 0.13, P = 0.038)\) and allelic richness \((t = 7.00 ± 0.64, P = 0.001)\) were both significant (Supplementary Fig. S4).

**Host colonization assays.** Overall artificial inoculation rates reached 40 and 45% for coffee and citrus plants, respectively, as estimated at 510 days after inoculation with respective homologous \(X. fastidiosa\) isolates. In total, 17% of citrus plants exhibited typical

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### Table 2. Test for random association of alleles between pairwise loci in Xylella fastidiosa populations infecting coffee plants in four regions of São Paulo State, Brazil

<table>
<thead>
<tr>
<th>Populations</th>
<th>Central</th>
<th>Northwest</th>
<th>Center-West</th>
<th>East</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I_{A})</td>
<td>0.9109</td>
<td>0.0793</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(r_{barD})</td>
<td>2.6070</td>
<td>0.2253</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n)</td>
<td>2.6225</td>
<td>0.2188</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n) estimation</td>
<td>4.2992</td>
<td>0.3563</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| \(P^\alpha\)       | <0.001  | 0.0001    | <0.001      | <0.001|
| Pairs in disequilibrium \((n)\) | 24 in 66 | 12 in 78 | 52 in 66 | 77 in 91 |
| Pairs (%)\gamma\   | 36.4    | 15.4      | 78.8        | 84.6   |

\(P^\alpha\) \(r_{barD}\) are indexes of multilocus gametic disequilibrium. \(r_{barD}\) is a modification of \(I_{A}\) that removes the dependency on number of loci.

\(P^\alpha\) Testing \(H_0\) (complete panmixia) based on 1,000 randomizations, where the values of \(I_{A}\) and \(r_{barD}\) significantly different from zero indicate that loci are in disequilibrium.

\(P^\alpha\) Locus pairs in significant disequilibrium.

\(P^\alpha\) Percent locus pairs in significant disequilibrium.
symptoms of CVC at the end of the experiment (inoculated with CiC03 and CiC09 haplotypes) and no CLS symptoms were observed in coffee plants. On the other hand, 88 and 91.6% of N. tabacum plants were infected with X. fastidiosa strains from coffee and citrus trees, respectively. All X. fastidiosa-positive tobacco plants showed symptoms similar to those previously described (Alves et al. 2003; Lopes et al. 2000). Regarding heterologous inoculations, strains from CVC-diseased plants (CiC03, CiC05, CiC08, and CiC09) were detected in coffee plants until 300 days but did not persist any longer. CiC08 was the only isolate originally from citrus to poorly colonize that host plant species. Only one X. fastidiosa strain from CLS-diseased plants (CoC10) was able to persistently infect citrus plants (one of five replicates), albeit with low bacterial population estimates at 390 days after inoculation. This isolate, as well as isolate CoC14, also performed poorly in coffee.

**DISCUSSION**

The genetic diversity and population structure of coffee-infecting X. fastidiosa in Brazil is poorly understood. In this study, we aimed to address this knowledge gap by using a large data set of strains collected from representative coffee-growing regions of São Paulo State in Brazil. First, we tested the hypothesis that X. fastidiosa populations from coffee trees are geographically structured, with no admixture of isolates among regions. Most of the geographical populations were genetically isolated, albeit with the presence of historical migration. The significant isolation observed among five of the six population pairs served as an indication that the pathogen was not widely spread among the sampled regions by insect vectors or contaminated plant material. These data support the hypothesis that locally well-adapted genotypes may outcompete invaders, maintaining the spatial structure of this pathogen (Coletta-Filho et al. 2014). Alternatively, noncrop hosts can serve as local pathogen reservoirs (Lopes et al. 2003) and may influence population structure, although this hypothesis was not tested. The central and northwestern São Paulo State populations were the only population pair that was not genetically independent; it was also the pair that exchanged the highest number of migrants. We propose three hypothesis to explain this observation. First, the cause may be the acquisition of asymptomatic nursery tree material infected with X. fastidiosa from the central region and subsequent introduction onto the northwestern region for establishment of new coffee plantations. Another explanation is that these two geographical populations shared migrants from a common inoculum reservoir selective to host-specific X. fastidiosa genotypes, and that did not occur in other regions (Hernandez-Martinez et al. 2007; Purcell and Saunders 1999; Randall et al. 2009). Finally, it is plausible that the high levels of X. fastidiosa migration detected between central and northwestern São Paulo State populations may be a consequence of migrants exchanged with a third population not sampled in our study. We only studied a small number of populations, whereas coffee production in São Paulo State covers a large geographic area.
with both large and small plantations, which we expect harbor 
*X. fastidiosa* genetic diversity not sampled in this study.

The structuring of populations was also observed in previous 
studies of citrus-infesting *X. fastidiosa* (Coletta-Filho and Machado 2003; Coletta-Filho et al. 2014). We postulate that similar patterns of 
geometric structure observed for both coffee- and citrus-infesting 
*X. fastidiosa* populations are a result of shared ecological traits. 
Those include vector dispersal of inoculum by the same sharpshooter leafhopper species (Marucci et al. 2008; Silva et al. 2007), which are 
potentially important but apparently inefficient for long-distance 
dispersal of the pathogen (Coletta-Filho et al. 2014). Another factor is 
that genetic structuring of bacterial populations can lead to linkage 
disequilibrium even in the presence of recombination (Feil 2010). 
Because *X. fastidiosa* is naturally competent (Kung and Almeida 2011) 
and *X. fastidiosa* subsp. *pauca* infecting coffee and citrus has been 
suggested to be of recombinogenic origin (Nunney et al. 2012), the 
departure from linkage equilibrium observed here may be a 
consequence of geographic structuring, admixture events, and limited 
genotype flow among the regions. In this case, linkage disequilibri-
um would occur due to barriers to gene exchange (e.g., geographic 
or ecological) or by an epidemic expansion in which case linkage 
disequilibrium would be temporary. Alternatively, as mentioned above, 
local alternative pathogen reservoirs may preferentially harbor distinct 
genetic variants.

In São Paulo State, farming of coffee had a boom cycle in the 
1880s (Font 2010), while the intensification of citrus farming only 
began 80 years later, reaching a commercial large scale in the 1960s. 
It is possible that endemic *X. fastidiosa* first adapted to coffee plants, 
increasing the effective population size until it adapted more 
recently and infected citrus (Nunney et al. 2012). CVC was first 
reported in 1987 (Rossetti et al. 1990) and is still under geographical 
expansion in a fairly restricted host range (only four *Citrus sinensis* 
cultivars which are clonally propagated, representing up to 190 
million trees in São Paulo State), while the disease in coffee was 
first reported a decade later (de Lima et al. 1998). Symptoms of 

*X. fastidiosa* in coffee plants may have been attributed to other 
abiotic and biotic disorders (de Lima et al. 1998). Regardless, 
available data now suggest that *X. fastidiosa* first adapted to coffee 
and only later to citrus plants (Nunney et al. 2012). The longer 
exposure of coffee plants to *X. fastidiosa* infection would lead to 
higher genetic and genotypic diversity in coffee than in populations 
that infect citrus, explaining the lower diversity indexes found in 
*X. fastidiosa* from citrus compared with coffee in this study. Higher 
diversity indexes are generally associated with older populations 
with equilibrium between mutation and genetic drift forces (Haag 
et al. 2005). In contrast, the lack of gene diversity has been 
associated with younger, recently founded populations (Balloux 
2010; Haag et al. 2005). Nunney et al. (2012) hypothesized that 
strains of *X. fastidiosa* infecting citrus and coffee plants originated 
from a common ancestor, not yet identified. In turn, Nunes et al. 
(2003) presented evidence of recombination between *X. fastidiosa* 
subsp. *pauca* and subsp. *multiplex* in Brazilian strains, which was 
corroborated later with a larger number of strains obtained from 
Although the results presented here do not address this broader 
question, larger genetic diversity from coffee strains suggests that 
*X. fastidiosa* has been colonizing this host longer than citrus.

Despite of the similar patterns of geographic structure for *X. fastidiosa* 
causing CVC and CLS, these populations are genetically and 
biologically different, as previously reported (Almeida et al. 2008). 
Based on cross-infection assays, citrus strains were able to persistently 
infest coffee plants but no coffee strain was detected infecting citrus 
plants. Almeida et al. (2008) and Prado et al. (2008) also did not detect 
strain diversity in host plants that is not followed by systemic and persistent infection 
is common for this pathogen (Purcell and Saunders 1999). Plant 
colonization factors required for *X. fastidiosa* multiplying and 
moving within hosts appears to be conserved among *X. fastidiosa* 
strains, and the mechanisms driving host specificity remain to be

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**Fig. 3.** Bayesian inference of the structure of *Xylella fastidiosa* populations sampled from adjacent citrus and coffee orchards. **A.** STRUCTURE clustering (admixture), where each isolate is represented by a single vertical line, which is partitioned into the number of populations (K) as shaded segments that represent the individual’s estimated membership fractions in K clusters. The figure shown for K = 2 is based on the highest probability run at that. The length of the segment shows the strain’s proportion of membership (Q) in that cluster (y-axes). No multiple shades were observed in each K cluster, which means no admixed genotypes from the prior-defined multiple populations (i.e., no isolates of coffee leaf scorch [CLS] into citrus variegated chlorosis [CVC] population and vice versa). **B.** BAPS clustering of individuals. Each isolate has a shade corresponding to the cluster in which it was placed. CVC: n = 4 clusters (bars 1 to 4) and CLS: n = 10 clusters (bars 5 to 14). The width of each bar is proportional for the number of isolates for each cluster.
determined, although it has been suggested that the core machinery is shared among different genotypes (Killiny and Almeida 2011).

The results obtained here by using a large number of isolates support previous work showing that cross infection by citrus and coffee X. fastidiosa strains does not occur in natural or artificial conditions. These findings reinforce the unnecessary control of vectors in coffee trees adjacent to citrus orchards aiming to block X. fastidiosa cross infection. In addition, the genetic structure of X. fastidiosa populations causing disease in coffee trees was genetically and geographically structured, similar to that previously found for X. fastidiosa populations from citrus. However, coffee isolates of this pathogen showed genetic diversity and allelic richness values higher than those found in populations infecting citrus, suggesting that these represent older populations. Future work with genome sequences linked to plant-pathogenicity assays will be necessary to address pending questions about the evolution of host specificity of X. fastidiosa subsp. paucia in Brazil.

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LITERATURE CITED


