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Combined use of a new SNP-based assay and multilocus SSR markers to assess genetic diversity of *Xylella fastidiosa* subsp. *pauca* infecting citrus and coffee plants

Miguel Montes-Borrego,[1] Joao R. S. Lopes,[2] Rafael M. Jiménez-Díaz,[1,3] Blanca B. Landa[1]*

[1]Institute for Sustainable Agriculture (IAS), CSIC, and Agrifood Campus of International Excellence, Córdoba, Spain.
[2]Department of Entomology and Acarology, São Paulo University, Piracicaba, São Paulo, Brazil.
[3]IAS-CSIC and College of Agriculture and Forestry, University of Córdoba, Córdoba, Spain

Summary. Two haplotypes of *Xylella fastidiosa* subsp. *pauca* (*Xfp*) that correlated with their host of origin were identified in a collection of 90 isolates infecting citrus and coffee plants in Brazil, based on a single-nucleotide polymorphism in the *gyrB* sequence. A new single-nucleotide primer extension (SNuPE) protocol was designed for rapid identification of *Xfp* according to the host source. The protocol proved to be robust for the prediction of the *Xfp* host source in blind tests using DNA from cultures of the bacterium, infected plants, and insect vectors allowed to feed on *Xfp*-infected citrus plants. AMOVA and STRUCTURE analyses of microsatellite data separated most *Xfp* populations on the basis of their host source, indicating that they were genetically distinct. The combined use of the SNaPshot protocol and three previously developed multilocus SSR markers showed that two haplotypes and distinct isolates of *Xfp* infect citrus and coffee in Brazil and that multiple, genetically different isolates can be present in a single orchard or infect a single tree. This combined approach will be very useful in studies of the epidemiology of *Xfp*-induced diseases, host specificity of bacterial genotypes, the occurrence of *Xfp* host jumping, vector feeding habits, etc., in economically important cultivated plants or weed host reservoirs of *Xfp* in Brazil and elsewhere [**Int Microbiol** 2015; 18(1):13-24]

Introduction

Xylella fastidiosa is a Gram-negative, xylem-inhabiting bacterium with very slow in vitro growth. It is non-specifically transmitted by several xylem-fluid feeder insect species of sharpshooter leafhoppers (Hemiptera: Cicadellidae: Cicadel-linae) and spittlebugs or froghoppers (Hemiptera: Cercopidea) [3,6,15,19,34]. *Xylella fastidiosa* causes enormous yield losses as the etiological agent of Pierce's disease (PD) of grapevine, *Vitis vinifera*; phony peach disease in peach, *Prunus persica*; and citrus variegated chlorosis (CVC) in *Citrus* spp. It is also the cause of a number of so-called leaf scorch diseases in *Prunus* spp. (including almond leaf scorch in *Prunus amygdalus* and plum leaf scald in *Prunus domestica*), *Acer* spp., *Carya illinoinensis*, *Coffea arabica*, *Hedera helix*, *Morus rubra*, *Nerium oleander, Olea europaea*, *Platanus occidentalis*, *Quercus* spp., and *Ulmus americana* [1,6,15,19].

***Corresponding author:** B.B. Landa
Instituto de Agricultura Sostenible-CSIC
Alameda del Obispo, s/n
14080 Córdoba, Spain
Tel. + 34-957499279. Fax + 34-957499252
E-mail: blanca.landa@ias.csic.es

Several pathogenic variants of the bacterium have been described; these are often host specific and have been given the category of subspecies [37]: *Xylella fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *pauca*, and *X. fastidiosa* subsp. *sandyi*. Of these, *X. fastidiosa* subsp. *pauca* (*Xfp*) has caused significant economic losses to Brazilian agriculture since it was first reported as the etiologic agent of CVC in 1987 [3,6], and currently poses a serious potential threat to the citrus industry worldwide. This subspecies also infects coffee, another economically important crop in Brazil, causing coffee leaf scorch (CLS), a disease that was first documented in Brazil in 1995 [4]. *Xilella fastidiosa* is a quarantine bacterium in the European Union (EU) [11]. A strain of *X. fastidiosa* was recently associated with olive quick decline syndrome (OQDS), which is devastating olive trees in the Apulian province of Lecce, Salento peninsula. OQDS is a destructive disorder that developed suddenly, a few years ago, in the olive groves of a restricted area close to the city of Gallipoli, in southeast Italy. From there, the disease expanded to a wider area, currently estimated at ~10,000 ha [11,24,36]. This OQDS infestation is the first detection and establishment of this quarantine bacterium in Europe. Preliminary phylogenetic analysis using the gene encoding the β-subunit polypeptide of the DNA gyrase (*gyrB*) indicates that isolates from olive group are close to the branch comprising *X. fastidiosa* isolates that belong to the subspecies *pauca* [5]. Furthermore, multilocus sequence typing indicates that isolates of *X. fastidiosa* from olive in Apulia represent a novel strain within the subspecies *pauca* [23].

Xfp isolates from citrus and coffee are generally reciprocally host specific [2,25], although artificial inoculation assays have shown that isolates from citrus can sometimes infect coffee plants [21,31]. Additionally, molecular studies using a limited number of *Xfp* isolates have shown that they are genetically distinct [1,28,33,38]. The main objective of this study was to develop a simple molecular protocol to *Xfp* strains from coffee and citrus that could be used in epidemiological studies. Following the detection of a single nucleotide polymorphism in the *gyr*B gene that differentiates between citrus and coffee strains of *Xfp*, we developed a simple, reliable, and fast single nucleotide primer extension (SNuPE), or SNaPshot, protocol that can be used to differentiate between these strains (haplotypes) in samples of total DNA extracted from bacteria, infected plants, and insect vectors. Additionally, we analyzed the genetic structure of a large collection of *Xfp* strains from infected citrus and coffee plants grown in different regions in Brazil by using multilocus simple sequence repeat markers (SSR) (microsatellite).

Materials and methods

***Xylella fastidiosa* isolates.** *Xylella fastidiosa* (*Xfp*) is a difficult-to-grow (i.e., fastidious, exigent) Gram-negative xylem-limited gammaproteobacterium in the family Xanthomonadaceae. It is rod-shaped with distinctive rippled cell walls, non-flagellate, does not form spores and measures 0.1–0.5 × 1–5 μm. Ninety *Xfp* isolates from Brazil were analyzed in this study (Table 1), including 28 from citrus plants showing CVC and 62 from coffee plants showing CLS symptoms. All isolates were obtained using standard isolation and triple-cloning protocols [1,2]. Some of the isolates were obtained from different trees within the same orchard in different years. The orchards were located in 19 counties in four states of Brazil (Table 1). Of the 90 isolates, 27 were used in a previous study (Table 1) [2,28], as it was the reference strain 9a5c (first *X. fastidiosa* isolate sequenced).

Molecular characterization of *Xylella fastidiosa* isolates. All bacterial isolates were confirmed as *X. fastidiosa* based on their in vitro fastidious growth and PCR assays using the primer pairs RST 33/RST 31 [27] and S-S-X.fas-0067-a-S-19/S-S-X.fas-0838-a-A-21 [35], which are universal for all *X. fastidiosa* subspecies, as well as primer pair CVC-1/272-2-int, which is specific for *Xfp* causing CVC and CLS [30] (Table 2).

An in silico analysis was first performed using the *X. fastidiosa* multilocus database [http://pubmlst.org/xfastidiosa/] hosted on the PubMLST-Public databases for molecular typing and microbial genome diversity. The *X. fastidiosa* database includes data from seven loci (*leuA, petC, malF, cysG, holC, nuoL, gltT*). We also used information contained in Nunney et al. [28], which included some of the isolates listed in Table 1. The seven loci as well as other sequences (16S rRNA and *gyrB*) harbored in the GenBank database were used to test for the presence of candidate SNPs that could serve as markers in the differentiation of citrus and coffee isolates by a simple approach.

Sequencing and phylogenetic analysis. The *gyrB* gene was selected as the candidate gene for SNP validation. Sequences were amplified using the primer pair FXYgyr499/FXYgyr907 as described by Rodrigues et al. [35] (Table 2), purified with a gel extraction kit (Geneclean turbo; Q-BIOgene, Illkirch, France), quantified as described for genomic DNA, and used for direct partial sequencing, carried out at the University of Córdoba sequencing facilities. Sequencing was done with the primer FXYgyr499 with a terminator cycle sequencing ready reaction kit (BigDye; Perkin-Elmer Applied Biosystems, Madrid, Spain) according to the manufacturer's instructions and using a DNA multicapillary sequencer (model 3100 genetic analyzer; Applied Biosystems). The sequences were deposited in the Genbank database under accession numbers DQ223435–DQ223506. The *gyrB* sequences from this study were edited and aligned with all GenBank published *gyrB* sequences of *X. fastidiosa* from different hosts and geographic origins. Phylogenetic analysis was performed using Bionumerics 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium).

SNuPE protocol development and validation. Based on the identification of a single nucleotide polymorphism in the *gyrB* sequence, a SNuPE assay was developed that differentiates between *Xfp* genotypes according to the host source, i.e., citrus (G genotype) and coffee (A genotype). The SNuPE protocol was designed with reference to the single-base extension (SBE) protocol of the ABI SNaPshot multiplex kit (Applied Biosystems). SNaPshot is a commercially available kit for the multiplex detection of SNPs that relies on the extension of a primer annealed immediately adjacent to the SNP of interest. Detection is possible via the use of fluorescently labeled dideoxynucleotides, each of which emits a different wavelength such that each base is identified by a specific color. The fluorescently labeled extension products can be visualized by electrophoresis using a capillary automated sequencer [12].

Table 1. *Xylella fastidiosa* subsp. *pauca* isolates from Brazil used in the study: host source, isolation information, code number, and haplotype as defined by the SNuPE protocol developed in this study

Host plant	State[a]	County	Tree[b]	Prefix or strain	Isolate number[c]	Haploype	Year of isolation[d]
Coffea arabica							
	MG	Ervália	A	COF-	E1, E2, E15, E17, E18	A	2003
			B	COF-	E3, E19	A	2003
			C	COF-	E4, E5, E8, E11, E22	A	2003
			D	COF-	E1N, E2N, E3N	A	2005
			----		E10N, E13N, E15N	A	2005
	MG	São Gotardo	E	COF-	E10*, E13*, E14*, E21*	G	2003
			----	COF-	E22N, E23N, E25N, E26N,	A	2005
			----	COF-	E24N*, E27N*	G	2005
			----	COF-	J68*, J69*,	G	2006
			----	COF-	J70, J71, J72, J73, J74, J75, J76,	A	2006
			----	COF-	J78*,	G	2006
			----	COF-	J77, J79, J80, J81, J82, J83	A	2006
	MG	Lavras	----	COF-	J48*, J49*	G	2005
			----	COF-	J50, J51	A	2005
	MG	Varginha	----	COF-	J33	A	2002
	SP	Cravinhos	----	COF-	J4	A	2002
	SP	Garça	----	COF-	J29	A	2002
	SP	Matao		6756		A	1999
	SP	Muritinga do Sul	----	COF-	J32	A	2002
	SP	Neves Paulista	----	COF-	J17	A	2002
	DF	Planaltina	----	COF-	J56, J57, J58, J59, J60, J61, J62, J63	A	2005
Citrus sinensis							
	SP	Bebedouro	----	CIT-	J1, J2, J5, J6, J7, J8	G	2003
				6570		G	1997
	SP	Gaviao Peixoto	----	CIT-	J45, J46, J47	G	2005
	SP	Macaubal		9a5c		G	1993
	SP	São Carlos	----	CIT-	J11, J12, J15, J16, J18, J19, J20	G	2003
	SP	Taquaritinga	----	CIT-	J41, J42, J43	G	2005
	SP	Ubirajara	----	CIT-	J44	G	2005
	BA	Itapirucu	----	CIT-	J65	G	2005
	MG	Comendador Gomes	----	CIT-	J35	G	2002
					J52, J53		2005
	MG	Frutal	----	CIT-	J54, J55	G	2005

[a]BA = Bahia, MG = Minas Gerais, SP = Sao Paulo, DF = Distrito Federal. [b]Same letters identified single trees from which different isolates were obtained. ---- = Indicate that each strain was isolated from a different tree.[c]Underlined isolates were used in a previous study [2]. (*) Indicates the coffee isolates that showed the G haplotype.

Two different primers were designed in the reverse and two in the forward direction, so that they exactly adjoined to stop just 5′ of the SNP found in the *gyrB* sequence. The criteria for primer design were the avoidance of 3′ self-priming as well as 3′ dimerization and a size of 20–30 nucleotides. The *gyrB* PCR products were purified by incubating 15 μl of each one at 37ºC for 1 h in a PCR tube containing 10 μl of ExoSAP-IT (U.S. Biochemical, Cleveland, OH). An additional incubation of 15 min at 75ºC resulted in deactivation of the enzyme. Primer extension minisequencing reactions were performed according to the protocol of the SNaPshot multiplex kit in a total volume of 10 μl: 3 μl of the PCR purified product was mixed with 2.5 μl of SNaPshot multiplex ready reaction mix, 3.5 μl of ultrapure water, and 1 μl of the SNP-gyrB primer diluted to 0.6 pmol/μl. This 10-μl mixture was placed in a thermal cycler under the following cycle conditions: 25 cycles of 96ºC for 10 s, 50ºC for 5 s, and 60ºC for 30 s. Samples were analysed with a DNA 3130 genetic analyzer (Applied Biosystems) at the University of Córdoba sequencing facilities according to the SNaPshot reaction mix protocol. The results were read using the GeneScan software (Applied Biosystems).

Table 2. List of PCR primers used to amplify the different *Xylella fastidiosa* loci

Primer name	Sequence (5′→3′)	Reference
272-2-int	GCCGCTTCGGAGAGCATTCCT	[30]
CVC-1	AGATGAAAACAATCATGCAAA	[30]
S-S-X.fas-0067-a-S-19	CGGCAGCACATTGGTAGTA	[35]
S-S-X.fas-0838-a-A-21	CGATACTGAGTGCCAATTTGC	[35]
FXYgyr499	CAGTTAGGGGTGTCAGCG	[35]
FXYgyr907	CTCAATGTAATTACCCAAGGT	[35]
RST31	GCGTTAATTTTCGAAGTGATTCGATTGC	[27]
RST33	CACCATTCGTATCCCGGTG	[27]
CSSR-6 Fw	CGCACTGTCATCCATTTAATC	[22]
CSSR-6 Rv	GCTGCTTCATCTAGACGTG	[22]
CSSR-20 Fw	GGTATCGCCTTTGGTTCTGG	[22]
CSSR-20 Rv	GACAACCGACATCCTCATGG	[22]
OSSR-17 Fw	AGTACAGCGAACAGGCATTG	[22]
OSSR-17 Rv	AGCAACCAGGACGGGAAC	[22]

The reproducibility of the primer selected for the SNuPE protocol was validated by means of two approaches: (1) the SNaPshot protocol was first applied to a collection of eight *Xfp* isolates belonging either to the A or to the G haplotype, as determined by sequencing of the *gyrB* before primer design and the SNaPshot protocol; (2) a blind test was performed to determine the utility of the technique for genotyping *Xfp* isolates or determining the host of origin. For that purpose we used a collection of DNA from 20 isolates (J33, J35, J63, J65, and J68–J83; Table 1) obtained by J. S. Lopes and provided to B. B. Landa, but with information regarding the host of origin withheld (Table 1).

Implementation of the SNuPE protocol in epidemiological studies. The utility of the SNuPE protocol for epidemiological studies was validated using leaf and insect vector samples. Leaves were sampled from four CVC-affected plants from a citrus orchard at São Carlos (São Paulo, SP) and ten CLS-affected plants from a coffee orchard at São Gotardo (Minas Gerais, MG) that also were used to isolate bacterial strains. The leaves were placed inside labeled, dark plastic bags under moist conditions, transported on ice to the laboratory (2–4 h until delivery time), and kept in a cold room (10°C) for 1 day before DNA extraction. Total DNA was extracted from sampled leaves using cetyltrimethylammonium bromide buffer as described previously [35]. Additionally, DNA was extracted from adults of the sharpshooter vector *Bucephalogonia xanthophis* Berg (Hemiptera: Cicadellidae: Cicadellinae) that were fed for 48 h on citrus plants infected with *Xfp* belonging to the G genotype. The extraction protocol was that described by Ciapina et al. [8]. The *gyrB* gene from leaf and insect DNA was amplified using one or two rounds of the same PCR protocol [35], in which 1 µl of the first PCR run was used as template in the second run. The aim of this approach was to counteract the low concentration of the bacterium in some of the sampled leaves and insects. When a positive PCR amplification was achieved, the SNuPE protocol was performed as described above.

Microsatellite data analyses. Multilocus SSR PCR assays using three primer pairs (CSSR-6, CSSR-20, and OSSR-17) were used to assess the genetic diversity within *Xfp* strains from citrus and coffee. Primers were selected among the 34 SSR primer sets designed by Lin et al. [22] that maximize the number of haplotypes that could be differentiated according to the host of origin. PCR was performed as described by Lin et al. [22], with the forward primer labeled with HEX fluorescent dye. PCR amplification was first tested by agarose gel electrophoresis, after which allele sizes were determined with a LIZ500 size standard and a DNA 3130XL genetic analyzer (Applied Biosystems) at the University of Córdoba sequencing facilities and by using Genemapper version 3.7 (Applied Biosystems). All SSR fragments were scored as one putative locus with two alleles; one allele indicated the presence of a fragment and the other its absence. The datasets were compiled as a matrix of isolates and SSR fragments. Only reproducible bands were scored. Approximately half of the assayed isolates were tested twice, with identical results. The resulting matrix of isolates and SSR fragments was analyzed in two ways.

First, a distance dendrogram was calculated with the simple matching coefficient and the Ward algorithm using Bionumerics 6.6 software. Second, an approach similar to that recently described by Almeida et al. [2] was used, in which the hypothesis tested was whether citrus and coffee *Xfp* isolates were genetically clustered in different groups based on the host plant. GenAlEx Version 6.5 [29] was used to calculate the average number of alleles (Na), average number of effective alleles (Ne), and haploid genetic diversity (H) at each locus and across all loci for the different groups of strains according to their host of origin. Analyses of molecular variance (AMOVAs), as implemented in GenAlEx 6.5, were used to determine the covariance between groups by grouping populations by host plant. The significance of the AMOVA and of the results of the population pairwise *Fst* comparisons was tested with 1000 permutations. The number of genetic clusters in the microsatellite dataset was determined using the software package STRUCTURE 2.2 [32]. The posterior likelihood of the samples

being divided into between one and six genetic clusters (*k*) was tested by resampling the dataset ten times (burn-in, 10,000 steps; run, 100,000 steps) as described before [2].

Results and Discussion

Selection of candidate locus and phylogenetic analysis. In silico analysis of the seven loci (*leuA, petC, malF, cysG, holC, nuoL, gltT*) included in the public *X. fastidiosa* MLST database, the study from Nunney et al. [28], and the 16S rRNA sequences indicated that they were less appropriate than the *gyrB* gene in the differentiation between citrus and coffee strains of *Xfp* based on the potential presence of a single SNP (data not shown). Indeed, some of the loci (*leuA, petC, nuoL, gltT*) showed no variation between a subset of the coffee or citrus isolates from Brazil while others contained variable positions within Brazilian coffee (*cysG, holC, malF, leuA, petC, nuoL, gltT,* from 8 to 13 nucleotides) or citrus (e.g., *petC, holC, nuoL,* from 1 to 10 nucleotides) isolates. However, the analysis of 90 *gyrB* sequences from all citrus and coffee isolates of *Xfp* used in the present study showed that the sequences of the isolates were identical, with the exception of a SNP (a purine transition A→G) at position 831 with respect to the *gyrB* sequence (AE003849, locus tag XF0005) of strain 9a5c. Phylogenetic analysis of all *X. fastidiosa* isolates yielded a topology similar to those reported in other studies [5,28,35], with all *Xfp* isolates differentiated in two groups (data not shown). Within these two groups, 100% of the citrus isolates showed the same G haplotype, and most (50 out of 62) of the coffee isolates showed the A haplotype, whereas only a small proportion (12 out of 62) of the isolates from coffee shared the G haplotype of the citrus isolates. Those coffee isolates with the G haplotype were isolated from two coffee orchards in São Gotardo, MG, and Lavras, MG (Table 1).

These data indicated that two different haplotypes would infect citrus and coffee plants in Brazil, that they could co-infect a single coffee orchard, and that host jumping between haplotypes in certain regions would be possible. This result is consistent with reports that *Xfp* isolates causing CLS in coffee do not colonize citrus plants, whereas those pathogenic to citrus and causing CVC can infect and multiply in coffee plants [2,21,31]. Chen et al. [7] were able to differentiate *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *fastidiosa* based on a SNP in the 16S rRNA gene and a multiplex PCR assay, which showed the simultaneous presence of the two *X. fastidiosa* subspecies in the same infected almond orchard. However, to the best of our knowledge, mixed infections by different genotypes belonging to the same *X. fastidiosa* subspecies have not been previously demonstrated. The potential for mixed genotype infection ocurrence could affect current epidemiological studies and, therefore, *X. fastidiosa* disease management strategies.

Previous attempts to analyze the genetic relatedness of *Xfp* strains through sequencing of the 16S rRNA gene and 16S-23S intergenic space region [13,26] were hampered by the limited variability, which did not consistently cluster with the host [35]. Similar limited variability was found in the sequence analysis of some of the seven loci referred to above. In contrast, using *gyrB*, a gene that evolves much faster than rRNAs, we were able to differentiate between *Xfp* isolates according to the host of origin, i.e., coffee or citrus. Other studies have reported the utility of SNP molecular markers in the analysis of *X. fastidiosa* genetic diversity in isolates from coffee and citrus plants. However, those studies found a larger number of SNPs (10–24 and 2–12 for coffee citrus isolates, respectively), which, unlike the *gyrB* SNP, must be used together to distinguish between strains according to the host of origin of the isolates [38].

SNuPE protocol development and validation. Out of the four primers designed, SNP-gyr-25 (5′-GGACT-GATGCCTACCAAGAAACAAT-3′) yielded the most reproducible results in the SNuPE protocol, whereas migrations by the other three primers were not reproducible among different runs for the same isolate and the efficiency of the amplification was lower (data not shown). The results obtained with the SNuPE protocol were in complete agreement with those derived from the sequencing of the *gyrB* gene. The results for a given isolate were also identical when the SNuPE reactions were performed with the same PCR products in different runs, or with PCR products resulting from independent amplifications (data not shown). An example of an electropherogram of two SNuPE reactions for bacterial isolates of haplotypes A and G is shown in Fig. 1. For each genotype, a single peak was observed for each of the isolates: green (adenine) for isolates belonging to the A haplotype (e.g., isolate E2 from coffee, A haplotype), and blue (guanine) for isolates belonging to the G haplotype (e.g., isolate 6750 from citrus, G haplotype).

The SNuPE technique was originally developed to detect the Phe508 mutation in the human *CFTR* gene [20]. However, it has been recently applied to bacteria, in particular, to identify bacterial species and variants relevant to animal or human health as well as in the identification and differentiation of various probiotic bacteria at the species or sub-species level [9,12,16,17]. However, ours is the first application of the technique to differentiate among strains or haplotypes of plant pathogenic bacteria.
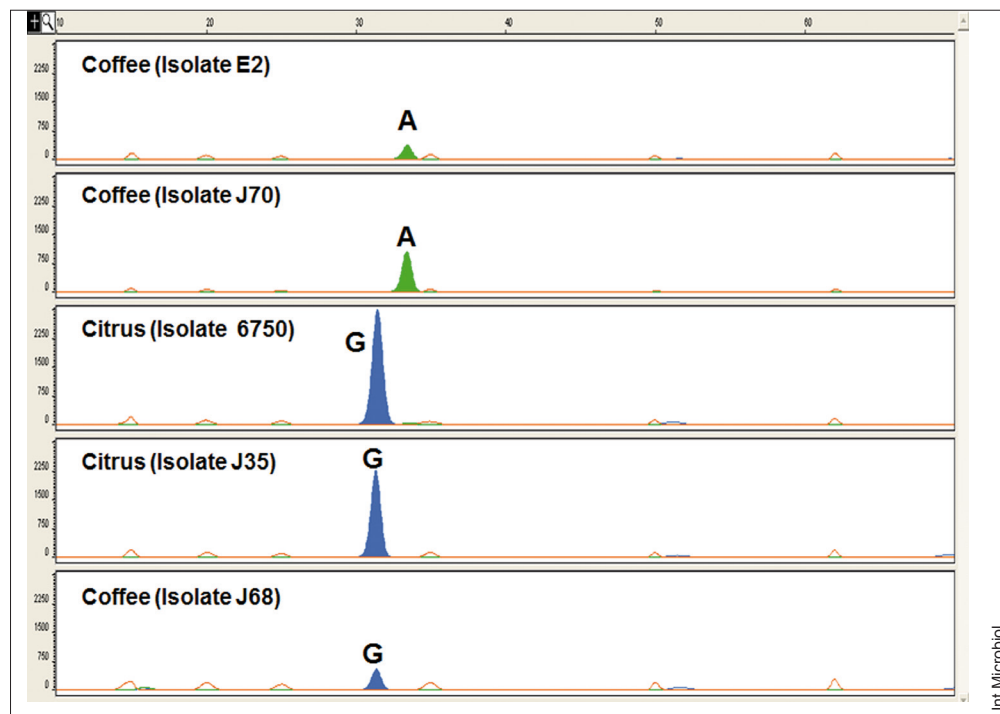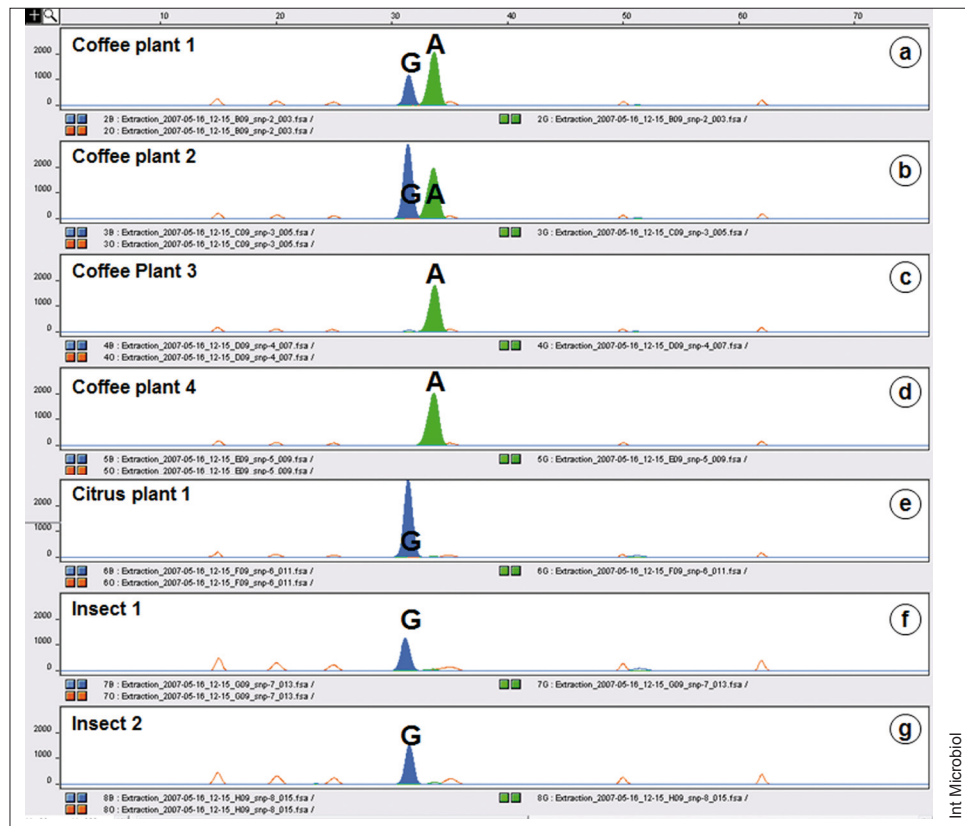
Fig. 1. SNaPshot analysis migration profile of *Xylella fastidiosa* subsp. *pauca* (*Xfp*) isolates E2 and J70 from coffee (A genotype; green peak, adenine), and isolates 6750 and J35N from citrus and J68 from coffee (G genotype; blue peak, guanine). The migrations correspond to the primer length. Note the slightly different migration for the adenine to guanine incorporation after the SNaPshot reaction.

To further demonstrate the usefulness of the newly developed SNuPE technique, the robustness of the protocol was tested in blind tests using total DNA extracted from bacterial isolates or from infected plants. All bacterial isolates were assigned correctly to their respective host of origin by the SNuPE protocol; e.g., isolate J35 (citrus, G haplotype) and isolate J70 (coffee, A haplotype) (Fig. 1). As determined from the sequence analysis of *gyrB,* a small proportion (13.3%) of the bacterial isolates from coffee in the orchard located in São Gotardo, MG, showed the G haplotype (e.g., isolate J68). In addition, amplification of the *gyrB* gene showed that all symptomatic coffee and citrus plants were infected by *Xfp*. The SNuPE protocol indicated that all citrus-infected plants showed the G haplotype, as expected (Fig. 2E), whereas the analysis of coffee plants indicated that seven were infected by bacteria of the A haplotype (Fig. 2C,D), and three were infected jointly by bacteria of the two haplotypes (Fig. 2A,B). These plants came from the CLS-affected orchard in São Gotardo, MG, from which isolates of *Xfp* belonging to haplotypes A and G were isolated. This is the first reported demonstration that two *Xfp* haplotypes can co-infect trees in a single orchard, and more importantly a single coffee plant within an orchard. This observation may explain the results reported by Almeida et al. [2] and Nunney et al. [28], in which CVC and CLS were shown to be caused by genetically distinct, frequently recombining groups of *X. fastidiosa*. Recombination is thought to occur widely in this bacterium*,* allowing both its adaptation to new host plants and speciation. In addition, horizontal gene transfer of pathogenicity factors may drive the emergence of new diseases caused by *X. fastidiosa* [2].

A nested-PCR assay for the *gyrB* gene [35] using total DNA extracted from insects previously fed for 2 days on a citrus plant infected with the G haplotype of a *Xfp* isolate yielded positive results. A subsequent assay using the SNuPE protocol confirmed the identity of the isolate as the G haplotype (Fig. 2F,G). No PCR amplification occurred when DNA extracted from healthy laboratory-reared insects allowed to feed on non-infected citrus plants was used as the template (data not shown). Thus, the SNuPE technique also has applications in genotyping for host association of *X. fastidiosa* using total DNA extracted directly from plant and insect tissues. The need for a nested-PCR protocol for the analysis of insect DNA samples in our study was probably due to the low number of *X. fastidiosa* cells in the insect vector, as previously

Fig. 2. SNaPshot analysis migration profile of plant samples. DNA samples extracted from four different coffee (**a**, **b**, **c,** and **d**) or citrus (**e**) representative plants showing CLS or CVC symptoms, respectively, and from insect vectors allowed to feed on *Xfp*-infected citrus tree (**f**). Note the presence of both genotypes (A genotype; green peak, adenine; and G genotype; blue peak, guanine) in representative coffee plants 1 and 2.

observed in culture and by the quantification of colony-forming units [14]. Nevertheless, the combined use of the nested-PCR and the SNuPE assays in a sequence would be very useful for epidemiological studies of CVC and CLS diseases, as well as for finding new potential vectors in *Xfp*-susceptible crops. Also, the identification of new SNPs in *gyrB* genes and other loci will facilitate the implementation of the SNuPE protocol in a multiplex approach to analyze and differentiate *Xfp* variants, such as the one associated with OQDS in Italy, as well as the remaining *X. fastidiosa* subspecies. Indeed, the combination of only four SNPs was shown to be sufficient to differentiate all *X. fastidiosa* subspecies, including the one associated with OQDS in Italy (M. Montes-Borrego, M. Saponari, B.B. Landa, unpublished results).

Characteristics of SSR loci, genotypes and genetic diversity. SSR amplification was successful with the three primer pairs, which amplified products ranging from 224 to 367 bp, as scored with the multicapillary sequencer

and GenScan software. A double dendrogram (one for the isolates and another for the SSR loci) was generated with the three sets of SSR loci to visually summarize the variability within the collection of *Xfp* isolates (Fig. 3). Overall, 41 SSR products were scored with the three primer pairs and the 90 *Xfp* isolates analyzed (Fig. 3). All loci were polymorphic, with the number of alleles ranging from a minimum of 11 (OSSR17) to a maximum of 16 (CSSR20). Among the 56 genotypes (i.e., combined SSR profiles) that were identified, 34 were from coffee and 22 from citrus; 37 were unique (i.e., a single isolate). Although some of the loci were common to coffee and citrus isolates (i.e., 2/11, 5/14 and 7/16 for OSSR17, CSSR16, and CSSR20 respectively), no common combined SSR profiles were detected among coffee and citrus isolates. The frequencies of some alleles varied considerably with respect to the host source and geographic origin (states of Brazil and counties within each state) of the bacterial isolates (Fig. 3). Among the products amplified by the SSR primer pairs CSSR6, CSSR20, and OSSR17, those of sizes 253, 290, and

Similarity (%)

Isolate	Host	County, State	Year	Tree
J4	Coffee	Cravinhos, SP	2003	
J79	Coffee	Sao Gotardo, MG	2006	
J58	Coffee	Planaltina, DF	2005	3
J61	Coffee	Planaltina, DF	2005	5
J82	Coffee	Sao Gotardo, MG	2006	
J83	Coffee	Sao Gotardo, MG	2006	
J73	Coffee	Sao Gotardo, MG	2006	
J32	Coffee	Muritinga do Sul, SP	2003	
J71	Coffee	Sao Gotardo, MG	2006	
J59	Coffee	Planaltina, DF	2005	4
J63	Coffee	Planaltina, DF	2005	8
J51	Coffee	Lavras, MG	2005	E1.5
J60	Coffee	Planaltina, DF	2005	6
J50	Coffee	Lavras, MG	2005	E3.2
J62	Coffee	Planaltina, DF	2005	7
J56	Coffee	Planaltina, DF	2005	1
J72	Coffee	Sao Gotardo, MG	2006	
J80	Coffee	Sao Gotardo, MG	2006	
J29	Coffee	Garsa, SP	2003	
J81	Coffee	Sao Gotardo, MG	2006	
J70	Coffee	Sao Gotardo, MG	2006	
J74	Coffee	Sao Gotardo, MG	2006	
J75	Coffee	Sao Gotardo, MG	2006	
J76	Coffee	Sao Gotardo, MG	2006	
J77	Coffee	Sao Gotardo, MG	2006	
J47	Citrus	Gaviao Peixoto, SP	2005	3
E19	Coffee	Ervália, MG	2003	B
E26N	Coffee	Sao Gotardo, MG	2005	K
E15	Coffee	Ervália, MG	2003	A
E17	Coffee	Ervália, MG	2003	A
E18	Coffee	Ervália, MG	2003	A
E1N	Coffee	Ervália, MG	2005	A
E2N	Coffee	Ervália, MG	2005	A
J57	Coffee	Planaltina, DF	2005	2
E10N	Coffee	Ervália, MG	2005	B
J17	Coffee	Neves Paulista, SP	2003	
E3	Coffee	Ervália, MG	2003	B
6756	Coffee	Matão, SP	1999	
E1	Coffee	Ervália, MG	2003	A
E2	Coffee	Ervália, MG	2003	A
E11	Coffee	Ervália, MG	2003	C
E22	Coffee	Ervália, MG	2003	C
E3N	Coffee	Ervália, MG	2005	A
E15N	Coffee	Ervália, MG	2005	D
E22N	Coffee	Sao Gotardo, MG	2005	G
E23N	Coffee	Sao Gotardo, MG	2005	H
E25N	Coffee	Sao Gotardo, MG	2005	J
E4	Coffee	Ervália, MG	2003	C
E5	Coffee	Ervália, MG	2003	C
E8	Coffee	Ervália, MG	2003	C
E10	Coffee	Sao Gotardo, MG	2003	E
J33	Coffee	Varginha, MG	2002	
J41	Citrus	Taquaritinga, SP	2005	6
E13	Coffee	Sao Gotardo, MG	2003	E
9a5c	Citrus	Macaubal, SP	1993	
E14	Coffee	Sao Gotardo, MG	2003	E
E24N	Coffee	Sao Gotardo, MG	2005	I
J48	Coffee	Lavras, MG	2005	E1.3
J49	Coffee	Lavras, MG	2005	E2.1
E13N	Coffee	Ervália, MG	2005	C
E21	Coffee	Sao Gotardo, MG	2003	E
E27N	Coffee	Sao Gotardo, MG	2005	L
J68	Coffee	Sao Gotardo, MG	2006	
J69	Coffee	Sao Gotardo, MG	2006	
J78	Coffee	Sao Gotardo, MG	2006	
J12	Citrus	Sao Carlos, SP	2003	
J19	Citrus	Sao Carlos, SP	2003	
J52	Citrus	Comendador Gomes, MG	2005	1
J53	Citrus	Comendador Gomes, MG	2005	2
J55	Citrus	Frutal, MG	2005	5
J65	Citrus	Itapirucu, BA	2005	
J1	Citrus	Bebedouro, SP	2003	
J45	Citrus	Gaviao Peixoto, SP	2005	1
J54	Citrus	Frutal, MG	2005	3
J42	Citrus	Taquaritinga, SP	2005	7
J46	Citrus	Gaviao Peixoto, SP	2005	2
J18	Citrus	Sao Carlos, SP	2003	
J44	Citrus	Ubirajara, SP	2005	1
J15	Citrus	Sao Carlos, SP	2003	
J35	Citrus	Comendador Gomes, MG	2002	
6750	Citrus	Bebedouro, SP	1997	
J20	Citrus	Sao Carlos, SP	2003	
J11	Citrus	Sao Carlos, SP	2003	
J6	Citrus	Bebedouro, SP	2003	
J43	Citrus	Taquaritinga, SP	2005	8
J2	Citrus	Bebedouro, SP	2003	
J5	Citrus	Bebedouro, SP	2003	
J16	Citrus	Sao Carlos, SP	2003	
J7	Citrus	Bebedouro, SP	2003	
J8	Citrus	Bebedouro, SP	2003	

Groups: Ia, Ib, Ic, IIa, IIb, IIc

Int Microbiol

Table 3. Descriptive statistics and genetic diversity of *Xylella fastidiosa* subsp. *pauca* isolates across three microsatellite loci, as determined in Brazilian populations of the bacterium collected in coffee and citrus plants showing leaf scorch and variegated chlorosis symptoms, respectively

Population	Locus	Number of isolates	Number of alleles	Number of effective alleles	Haploid genetic diversity	Frequency of occurrence of alleles
Coffee	CSSR6	62	10	1.99	0.498	0.016–0.694
	CSSR20	62	13	10.01	0.900	0.016–0.161
	OSSR17	62	8	5.29	0.811	0.016–0.226
Citrus	CSSR6	28	9.00	5.44	0.816	0.036–0.286
	CSSR20	28	10.00	5.85	0.829	0.036–0.321
	OSSR17	28	5.00	1.86	0.462	0.036–0.714
Total	CSSR6	90	14.00	3.84	0.739	0.011–0.478
	CSSR20	90	16.00	11.16	0.910	0.011–0.178
	OSSR17	90	11.00	6.72	0.851	0.011–0.233

234 bp were the most frequent (frequencies of 0.478, 0.178, and 0.233, respectively) and the most effective in discriminating *Xfp* groups (Fig. 3; dendrogram for SSR loci). Locus-based haploid diversity ranged from 0.498 to 0.900 for coffee isolates and from 0.462 to 0.829 for citrus isolates. The overall haploid genetic diversity was very similar for coffee (0.736) and citrus isolates (0.702) (Table 3). Almeida et al. [2] also analyzed the genetic diversity among citrus and coffee strains from Brazil using microsatellite analysis, but they did not provide any information concerning the number of alleles, genotypes, or haploid genetic diversity of each population.

We also wondered whether *Xfp* isolates collected from symptomatic citrus and coffee plants in different Brazilian states could be grouped genetically based on the host plant. The AMOVA results showed a statistically significant difference between the two population groups ($Fst = \Phi PT = 0.664$; $P = 0.001$), supporting the hypothesis that the genetic structures of the groups of isolates were driven by the plants from which they were obtained. Indeed, most of the variation observed occurred among populations (66%). In contrast, Almeida et al. [2] did not find significant genetic differences associated with the host source of 46 of *X. fastidiosa* isolates. Most of the variation occurred among individuals within populations, although populations within groups showed statistical differences when grouped by state, indicative of genetic differ-

ences between coffee and citrus isolates from a same region. The discrepancies between our results and those of Almeida et al. [2] might be due to differences in the sizes of the datasets (90 vs. 46 isolates, respectively) and in the SSR loci selected for our study. Thus, we used three SSR loci showing the highest number of different alleles among those described by Lin et al. [22], whereas Almeida et al. [2] used six SRR loci from those described by Coletta-Filho et al. [9], which in general showed lower haploid genetic diversity than was the case in our study (0–0.85 for citrus isolates and 0–0.75 for coffee isolates).

Genetic relationship and structure. Two main clusters (I and II) and six subclusters (Ia, Ib, Ic, and Id, and IIa, IIb, and IIc) were identified in the cluster analysis of the 90 bacterial isolates that were defined based on a 35.5% and 60.0% similarity coefficient, respectively. This clustering of the isolates correlated well with their host of origin and bacterial haplotype, with a few exceptions (Fig. 3). For instance, all isolates in cluster I were isolated from coffee plants and had the A haplotype, with the exception of one isolate from citrus (J47) that was of the G haplotype (subcluster Ia). Within cluster II, subclusters IIb and IIc were formed only by citrus strains, whereas subcluster IIa was the most diverse and included all coffee isolates that were of the G haplotype and some citrus isolates (Fig. 3).

← **Fig. 3.** Dendrogram based on similarity data (simple matching coefficient) from short sequence repeats (SSR) fragment analysis with the Ward algorithm using Bionumerics 6.6 software for the 90 *Xfp* isolates used in the study. BA = Bahia, MG = Minas Gerais, SP = Sao Paulo, DF = Distrito Federal. Cophenetic correlation values are indicated in each node. *Cluster and subcluster groups were defined based on a cluster cutoff value of 35.5% and 60.0%, respectively.
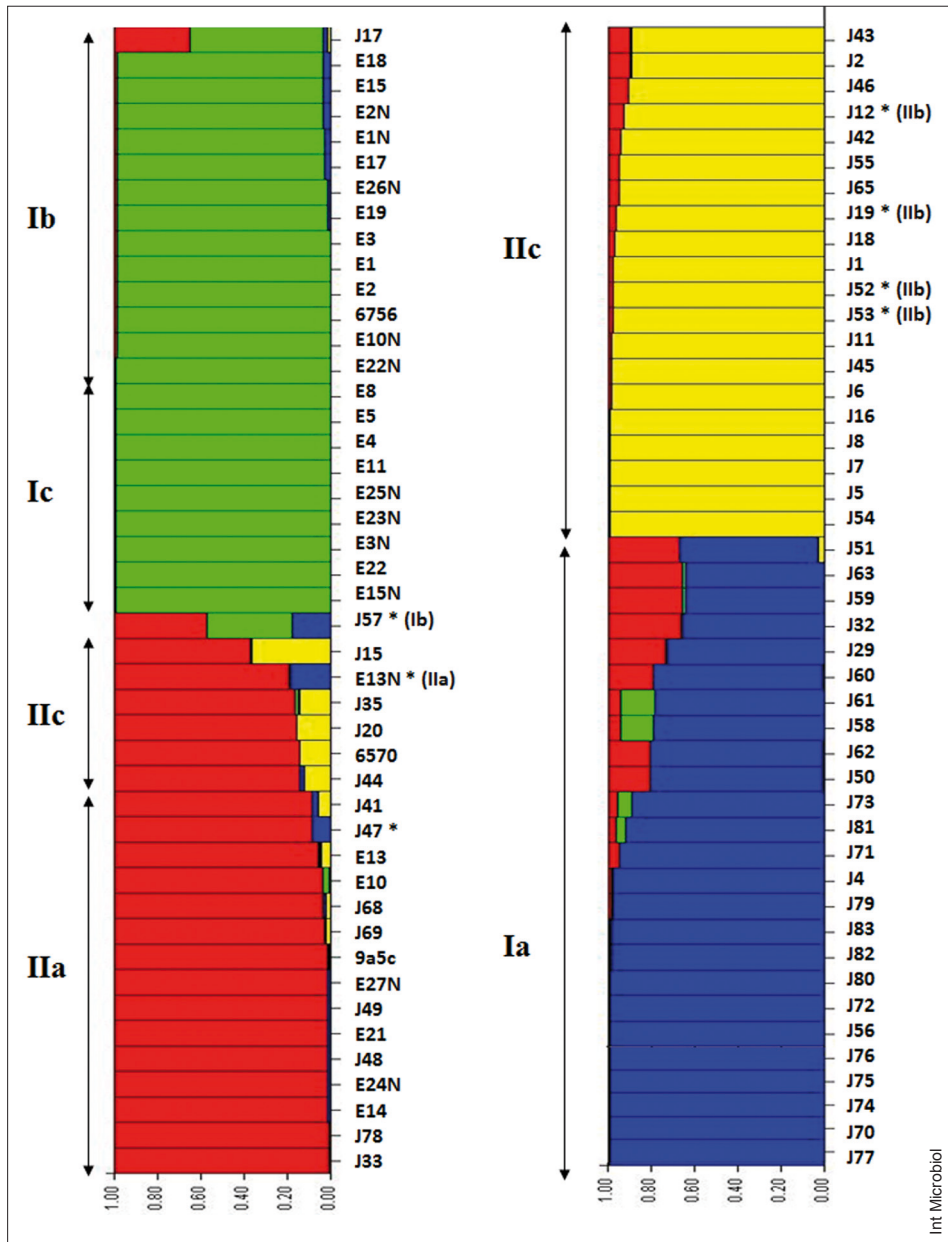
Fig. 4. Individual assignments of '*X. fastidiosa* subsp. *pauca*' isolates to four genetic clusters obtained by STRUCTURE analysis and indicated in different colors. Disagreement with respect to the assignment of isolates with the clustering obtained in Fig. 3 is indicated by an asterisk (*).

Our results also showed that coffee trees in a single orchard can be infected by different isolates and that a single coffee plant can be infected by different isolates, as determined by different SSR profiles. For instance, isolates E15, E17, and E18 (subcluster Ib) and isolates E1 and E2 (subcluster Ib) were obtained from the same coffee tree from Ervália, MG, in 2003 (Fig. 3, Table 1). This was also the case for isolates E11 and E22 and for isolates E4, E5, and E8 (obtained from the same orchard as those above, but from a different tree), which grouped in subcluster Ic but differed in their SSR profiles, as well as for isolates E10, E13, E14, and E21 (sampled in São

Gotardo, MG, in 2003), which varied in their SSR profiles and were grouped in subcluster IIa (Fig. 3). Note that, although coffee orchards from Ervália and São Gotardo, MG were sampled in different years (Table 1), isolates with the same SSR genotype were recovered from the different coffee plants sampled. Note also that some of the SSR profiles were present in coffee or citrus plants in different states and counties, whereas isolates of other SSR profiles were unique to a single orchard or county (Fig. 3).

To further analyze the possible genetic structure of *Xfp* isolates, a second clustering approach was used to infer the

most likely group of origin of each isolate. An analysis using the STRUCTURE software showed that the probability was the highest with four clusters (*k*), which was confirmed by the larger Δ*k* value with four populations (data not shown). The membership of each isolate obtained from the STRUCTURE analysis, estimated as (*q*), or the ancestry coefficient, is shown in Fig. 4. The *q* values vary between 0 and 1.0; with 1.0 indicating full membership in a population. Individual isolates can be assigned to multiple clusters (with values of *q* summing to 1.0), indicating that they are admixed. If isolates with $q \geq 0.90$ are defined as those of a single lineage, 18 of the 90 *Xfp* isolates can be considered as admixed lineages [18]. The STRUCTURE analysis, without prior information regarding geography or host cultivar, placed all isolates into four major clusters (Fig. 4). The clustering of the isolates was consistent with their lineage assignment, as determined by the cluster analysis (Fig. 3). The exception was a few isolates in cluster IIb, which according to STRUCTURE were grouped with all isolates in cluster IIc. Almeida et al. [2], using the STRUCTURE package, grouped their coffee and citrus *X. fastidiosa* dataset into three genetic clusters, one of which comprised both citrus and coffee isolates, as was the case in the present study for isolates within cluster IIa (Figs. 3 and 4).

The combined use of microsatellite- and SNuPE protocol-based assays in the present study yielded new insights into the genetic relationships among *Xfp* isolates collected from symptomatic citrus and coffee plants in Brazil. Our fingdings support previous results showing that CVC and CLS diseases are caused by genetically distinct, frequently recombining groups of *Xfp* isolates [2]. In addition, we were able to show that some of the isolates obtained from coffee plants shared the G haplotype and clustered with citrus isolates. This result is consistent with previous observations that CVC-associated isolates can multiply to some extent in coffee plants, while CLS-associated isolates do not colonize citrus plants [2,31].

In conclusion, although the use of a single molecular marker for the specific detection of *X. fastidiosa* strains originating from one or another host can have limitations, due to the frequent mutation and recombination events that characterize populations of this bacterium [2,28], the SNuPE protocol developed in this study is a rapid and robust assay that can be used in a first screening to differentiate among *Xfp* haplotypes recovered from citrus or coffee. The combined use of the SNuPE protocol with microsatellite analysis can be very useful for studies on the epidemiology of the diseases caused by *Xfp*, the host specificity of bacterial genotypes, the occurrence of host jumping, insect vector feeding habits, etc., in economically important cultivated host plants and weed host

reservoirs of the bacterium in Brazil. Also, the combined use of microsatellite- and SNuPE protocol-based assays has applications in studies on the new variant of *Xfp* associated with the recent epidemic outbreak of OQDS, devastating olive trees in Italy [5,24,36].

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