Genetic Diversity and a PCR-Based Method for *Xanthomonas axonopodis* Detection in Passion Fruit

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ABSTRACT

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Xanthomonas axonopodis pv. *passiflorae* causes bacterial spot in passion fruit. It attacks the purple and yellow passion fruit as well as the sweet passion fruit. The diversity of 87 isolates of pv. *passiflorae* collected from across 22 fruit orchards in Brazil was evaluated using molecular profiles and statistical procedures, including an unweighted pair-group method with arithmetical averages-based dendrogram, analysis of molecular variance (AMOVA), and an assigning test that provides information on genetic structure at the population level. Isolates from another eight pathovars were included in the molecular analyses and all were shown to have a distinct repetitive sequence-based polymerase chain

reaction profile. Amplified fragment length polymorphism technique revealed considerable diversity among isolates of pv. *passiflorae*, and AMOVA showed that most of the variance (49.4%) was due to differences between localities. Cluster analysis revealed that most genotypic clusters were homogeneous and that variance was associated primarily with geographic origin. The disease adversely affects fruit production and may kill infected plants. A method for rapid diagnosis of the pathogen, even before the disease symptoms become evident, has value for producers. Here, a set of primers (Xapas) was designed by exploiting a single-nucleotide polymorphism between the sequences of the intergenic 16S-23S rRNA spacer region of the pathovars. Xapas was shown to effectively detect all pv. *passiflorae* isolates and is recommended for disease diagnosis in passion fruit orchards.

Additional keywords: molecular diagnosis, *Passiflora*.

The bacterium *Xanthomonas axonopodis* belongs to a group of gram negative, yellow-pigmented plant pathogens that are known to cause significant damage to a variety of commercially important crops. For example, *X. axonopodis* pv. *passiflorae*, as proposed by Gonçalves and Rosato (6), formerly *X. campestris* pv. *passiflorae* (Pereira 1969) Dye 1978b (22), is responsible for bacterial leaf spot in passion fruit, a disease that has spread rapidly since it was first observed in Brazil in the early 1970s. It affects leaves and fruit, leading to commercial losses, particularly under high-temperature and high-humidity conditions. The pathogen attacks the purple (*Passiflora edulis*) and the yellow (*P. edulis* f. *flavicarpa*) forms of passion fruit as well as the sweet passion fruit (*P. alata*). Chemical controls have produced inconsistent results. In Brazil, passion fruit grown and harvested on small farms using family labor constitutes a significant portion of all fruit produced for local consumption and commercial sale. Although the demand for aromatic passion fruit juice in the European and American markets is very encouraging, the occurrence of bacterial leaf spot makes production unreliable, thus hampering the consistency of exports (28,32). Controlling the disease through the development of resistant varieties is one promising solution to this problem.

Ongoing research has focused on the construction of *P. edulis* f. *flavicarpa* linkage maps (2,19). Particular attention was given to

bacterial resistance characteristics in the mapping population, leading to the detection of at least one quantitative resistance locus that explained 16% of the total phenotypic variation for the diseased leaf area (15). The assessment of genetic diversity within a pathogen collection obtained from different locations would also be helpful in understanding effective pathogen resistance in host species. Repetitive sequence-based polymerase chain reaction (rep-PCR) and amplified fragment length polymorphism (AFLP) techniques were useful for characterizing *X. axonopodis* population structures (24,27). The large number of loci (bands) revealed by the AFLP technique provide substantial information about the genetic relatedness of isolates as well as the structure from each planting region (14).

Through early diagnosis of *X. axonopodis* pv. *passiflorae* infection, growers may be able to take action to avoid serious losses and to reduce spreading of the disease. PCR-based techniques are fast and sensitive and can be utilized by diagnostic laboratories to detect disease symptoms in commercial fresh fruit, plants, and seedlings even before they are visibly evident. For instance, specific primers for PCR detection of *X. axonopodis* pv. *citri*, which causes the citrus canker disease, were developed by sequencing of the intergenic 16S-23S rRNA internal transcribed spacer (ITS) region (3).

The objectives of this study were to (i) establish a collection of *X. axonopodis* pv. *passiflorae* obtained from different geographic locations in Brazil; (ii) measure the genetic diversity within the collection using the AFLP technique; (iii) infer details of the structure of the bacterial population, assigning every isolate to a subpopulation or cluster; and (iv) construct PCR primers based on the analysis of the ITS region of 16S-23S rRNA for pathovar

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diagnostic purposes. Two methods that are generally applied to eukaryotic studies were successfully used in this study.

MATERIALS AND METHODS

Establishing the bacterial collection. A collection of 87 *X. axonopodis* pv. *passiflorae* isolates was established, with most specimens originating from different commercial orchards of yellow passion fruit. This is the largest collection of *Xanthomonas* isolates causing leaf spot in passion fruit. Some of the organisms were obtained from the Culture Collection of Phytopathogenic Bacteria of the Biological Institute (IBSBF, Campinas, Brazil), as were seven isolates belonging to seven pathovars of *X. axonopodis*. Two additional isolates of the pathovar *X. phaseoli* were obtained from the Instituto Agrônômico (IAC, Campinas, Brazil) (Table 1). To isolate bacteria from the infected leaves, an injured area was surface sterilized in 70% ethanol and 2% NaClO solutions for 2 min each, followed by a 2-min wash in autoclaved, distilled water. Each sample was sliced with a surgical blade on a plastic plate containing 0.5 ml of water. After 30 min, ≈0.2 ml of the liquid solution from the plate, containing any exuded bacteria, was plated onto general medium nutrient agar (NA) (8 g of peptone, 3 g of meat extract, 15 g of agar, water-quantum sufficit 1,000 ml, pH 7.0). Bacteria were grown at 28°C for 48 h.

Yellow-colored colonies of convex shape, shiny, and highly mucoid were analyzed for the presence of xanthomonadins, which are membrane-bound pigments unique to the genus *Xanthomonas*. Following the protocol of Starr and Stephens (26), the extraction was performed in methanol (90°C for 10 min), a very efficient

way of extracting these pigments. The presence of xanthomonadins in aliquots of the resulting yellow and translucent solutions was detected by monitoring absorbance at 401 to 500 nm at regular intervals of every 3 nm. The absorption spectrum of xanthomonadins shows a characteristic structure, with a peak at ≈445 nm and shoulders at 425 and 470 nm.

Pathogenicity test. The pathogenicity of the isolates was confirmed by artificial leaf inoculations of a susceptible passion fruit genotype. Isolates were grown at 28°C for 48 h as described above. The plates were flooded with a 0.85% NaCl solution and a bacterial suspension was obtained by scraping the surface of the plates. The suspension was adjusted to an optical density of 0.3 at 600 nm, which corresponds to an approximate bacterial concentration of 10⁸ CFU/ml. Then, cotton swabs were dipped in the bacterial suspensions and spread across the surfaces of a hole made in the leaves with a cork borer. Two leaves were inoculated with each isolate and control plants received only the 0.85% NaCl solution. Plants were kept in a greenhouse and disease assessment was done 15 days after inoculation.

DNA extraction. Colonies for DNA extraction were cultivated in liquid nutrient medium. A simple extraction procedure using sodium dodecyl sulfate (SDS) and proteinase K was adopted (1). Aliquots (1 ml) from the liquid cultures were used to preserve bacterial isolates in 60% glycerol at –80°C. DNA samples were stained with ethidium bromide (5 µg/ml), visualized under UV, quantified in comparison with the fluorescent yield of the standards (lambda DNA) after 0.8% gel electrophoresis (3 V cm⁻¹), and photographed using a MultiDoc-It-UVP (UVP LLC, Upland, CA).

TABLE 1. List of *Xanthomonas axonopodis* according to the pathovar, isolate code, geographic origin, and plant host

Pathovar	Isolate ^a	Locality and state of origin ^b	Plant host
<i>passiflorae</i>	Abj5-3 (1); Abj5-4 (2); Abj14-2 (3); Abj15-3 (4); Abj20-4 (5); Abj24-2 (6); Ac1-3 (7); Ac2-2 (8); Ac6-4 (9)	Avaí, SP (A)	Yellow passion fruit
	FDsa1-1 (10); FDsa6-1 (11); FDsa14-2 (12); FDsa16-2 (13); FDns6 (14); FDns20 (15); FDe2-4 (16); FDe5-2 (17); FDe10-1 (18)	Fernão, SP (B)	...
	LP1-1a (19); LP2-3 (20); LP12-4 (21); LP14-2b (22); LP16-3 (23); LP32-2 (24); LP35-1 (25)	Lençóis Paulista, SP (C)	...
	SMA1a (26); SMA3a (27); SMA4a (28); SMA5a (29); SMA1b (30); SMA2b (31); SMA1c (32)	São Miguel Arcanjo, SP (D)	...
	AP302 (33)	Adamantina, SP (E)	...
	B1 (34); B2 (35); B4 (36); B6 (37); B8 (38); B10 (39); B11 (40); B15 (41)	Bauru, SP (F)	...
	PA1-2 (42); PA6-1 (43); PA8-2 (44); PA8-4 (45); PA11-3 (46); PA16-1 (47); PA20-1 (48); PA20-2 (49)	Piratinga, SP (G)	...
	LM2a (50); LM4a (51); LM6a (52); LM9a (53); LM12a (54)	Limeira, SP (H)	...
	LM2d (55)	Limeira, SP	Sweet passion fruit
	IBSBF941 (56)	Vera Cruz, SP (I)	Yellow passion fruit
	IBSBF996 (57)	Tupã, SP (J)	...
	M110 (58); M111 (59); M129 (60)	Araguari, MG (K)	...
	M89 (61); M116 (62)	Araxá, MG (L)	...
	M113 (63)	Patrocínio, MG (M)	...
	M144 (64)	Pedrinópolis, MG (N)	...
	M92 (65); M94 (66); M117 (67); M145 (68)	Perdizes, MG (O)	...
	M81 (69)	Sacramento, MG (P)	...
	M83 (70)	Uberaba, MG (Q)	...
	Mudas (71)	Uberlândia, MG (R)	...
	M115 (72); AP1 (73); AP2 (74); AP3 (75); AP4 (76); AP5 (77); AP6 (78)	Carmo do Paranaíba, MG (S)	...
	IBSBF1257 (79)	S. Sebastião da Amoreira, PR (T)	...
	Br5d (80); Br6d (81); Br8d (82); Br10d (83); Br11d (84)	Planaltina, DF (U)	Sweet passion fruit
	Br12a (85); Br13a (86)	Planaltina, DF	Yellow passion fruit
	IBSBF1325 (87)	Vicente Pires, DF (V)	Sweet passion fruit
	IBSBF1387 (88)	Brasilândia, MG	Pepper
<i>vesicatoria</i>	IBSBF1478 (89)	Arujá, SP	<i>Anthurium</i>
<i>dieffenbachiae</i>	IBSBF1553 (90)	Biritiba Mirim, SP	Lettuce
<i>vitians</i>	IBSBF1770 (91)	Sao Paulo State	Onion
<i>allii</i>	IBSBF1954 (92)	Primavera do Leste, MT	Cotton
<i>malvacearum</i>	IBSBF1994 (93)	Valinhos, SP	Cassava
<i>manihotis</i>	IBSBF2008 (94)	Jaú, SP	Sugarcane
<i>vasculorum</i>	IAC11280 (95)	Cosmópolis, SP	Common bean
<i>phaseoli</i>	IAC13755 (96)	Mococa, SP	Common bean

^a Numbers within parentheses refer to bacterial isolates or pathovars.

^b Letters within parentheses refer to localities of origin.

Rep-PCR. Repetitive DNA sequences known as BOX, ERIC, and REP are currently used for bacterial fingerprinting, including the distinction of closely related plant pathovars. Isolates of *X. axonopodis* pv. *passiflorae* as well as all different pathovars of *X. axonopodis* were submitted for PCR analysis with the following primer sets (17): BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3'); ERIC1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGAAGTGGGTGAGCG-3'); and REP1R-I (5'-IIICGICGICATCIGGC-3') and REP2-I (5'-ICGI CTTATCIGGCCTAC-3'). A 20- μ l mixture used to amplify REP and ERIC elements contained 1 $\times$ reaction buffer, 1.2 μ M each primer, 0.4 mM each dNTP, 4 mM MgCl₂, 1.5 U of Go *Taq* Flexi Promega Corp. (Madison, WI), and 50 ng of template DNA. For the BOX element, the same mixture was used with the following modifications: 2.0 μ M primer, 0.5 mM each dNTP, and 2 U of enzyme. PCR conditions were as follows: 95°C for 7 min; 30 cycles at 94°C for 1 min, 44, 52, or 53°C for the REP, ERIC, and BOX primers, respectively, for 1 min, and 72°C for 1.5 min; and a final extension at 72°C for 15 min, in a Gen Amp PCR System 9700 thermocycler (Applied Biosystems, Carlsbad, CA). Amplicons were electrophoresed in agarose gel (1.4%) at 3 V cm⁻¹, visualized under UV after DNA stained with ethidium bromide (5 μ g/ml), and photographed in a MultiDoc-It-UVP.

AFLP. The protocol described by Lopes et al. (15) and modified from Vos et al. (29) was used to obtain the AFLP markers. Briefly, 250 ng of genomic DNA from each isolate was double digested with *Eco*RI (NE Biolabs, Ipswich, MA) and *Mse*I (Promega Corp.) enzymes (6 U of each) in a solution with 1 $\times$ One Phor-All buffer (Amersham Biosciences, Buckinghamshire, United Kingdom), 1 $\times$ bovine serum albumen (NE Biolabs), and ultrapure water q.s.p. 20 μ l. Reactions occurred during 4 h at 37°C and then were terminated by heat inactivation (65°C for 20 min). All resulting fragments were ligated to adapter sequences by adding 1 $\times$ T4 DNA ligase reaction buffer, 0.25 μ M *Eco*RI adapter (denoted E), 2.5 μ M *Mse*I adapter (denoted M), 67 U of T4 DNA ligase (NE Biolabs), and ultrapure water q.s.p. 20 μ l. Ligation reactions were incubated at 16°C for 14 h and terminated as described above. Primers, complementary to the adapters, with zero, one, or two selective nucleotides at the 3' end were used for selective amplification. The 20- μ l PCR mix contained 1 $\times$ reaction buffer, 0.25 μ M each primer, 0.25 mM each dNTP, 1.5 mM MgCl₂, 1 U of Go *Taq* Flexi Promega Corp., and 5 μ l of the adapter-ligated DNA (diluted fourfold). PCR conditions were as follows: 94°C for 2 min; 26 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min in a Gen Amp PCR System 9700 thermocycler. Samples (2 μ l) of amplified products were denatured with 1 $\times$ loading buffer (0.2% each of bromophenol blue and xylene cyanol, 10 mM EDTA, and 95% formamide at pH 8.0) at 95°C for 5 min before they were loaded onto the gels. AFLP bands

were resolved in 5% (wt/vol) denaturing polyacrylamide gels (19:1; acrylamide:bis-acrylamide) using the electrophoresis apparatus Sequi-Gen GT (Bio-Rad, Hercules, CA) for 4 h at 80 W, and visualized by silver staining.

Statistical treatment of AFLP data. Data were scored for the presence (=1) or absence (=0) of amplification products by means of indicator variables. This type of scoring was done for each polymorphic locus across all 87 isolates of *X. axonopodis* pv. *passiflorae*. Pairwise comparisons of isolates based on shared and unshared amplicons were used to generate similarity coefficients (12). Dendrograms were generated based on the unweighted pair-group method with arithmetical averages (UPGMA), employing the sequential, agglomerative, hierarchic, and nonoverlapping (SAHN) clustering methods. The statistical package NTSYS-PC software (version 2.1; Setauket, NY) (25) was used for computations. Bootstrap analysis with 1,000 resamples was completed with WINBOOT software (International Rice Research Institute, Manila, Philippines) to determine the confidence limits of the UPGMA dendrogram. "Goodness" of the dendrogram was verified by direct comparison of the original similarity matrix and the cophenetic value matrix derived from the dendrogram, as described by Rohlf (25).

Analysis of molecular variance (AMOVA) was used to estimate the components of variation directly from AFLP data, using ARLEQUIN software (version 2.000; Genetic and Biometry Laboratory, University of Geneva, Switzerland). Total variation was subdivided, reflecting variation between the geographical Brazilian states, between the localities within each of the states, and within the localities.

Each isolate was probabilistically assigned to a genetically distinct cluster based on Bayesian models using STRUCTURE software (version 2.2.3; Department of Human Genetics, University of Chicago) (23), available online from Cornell University at <http://cbsuapps.tc.cornell.edu/structure.aspx>. This package allowed us to assess the genetic structure of the *X. axonopodis* pv. *passiflorae* collection assuming that all sampled isolates belong to an unknown number of clusters (*K*) that are defined by the means of the frequency of AFLP bands. The *K* statistic, which is based on the rate of chance in the log probability of data between successive *K* values (5), was calculated. Ten runs were performed for each number of clusters (*K* of 1 to 22, a burn-in of 200,000 and 300,000 Markov chain-Monte Carlo steps after the burn-in period). This analysis indicated the number of clusters that best fit the data.

Sequencing of the intergenic 16S-23S rRNA ITS region. The intergenic 16S-23S rRNA spacer region of four isolates of pv. *passiflorae* (Abj20-4, LP1-1a, PA6-1, and Br5d; GenBank accession nos. FJ828864, FJ828866, FJ828867, and FJ828865, respectively) and eight pathovars of *X. axonopodis* (IBSBF1387, IBSBF1478, IBSBF1553, IBSBF1770, IBSBF1954, IBSBF1994,

Fig. 1. Repetitive sequence-based polymerase chain reaction profiles generated with the primers **A**, REP; **B**, ERIC; and **C**, BOX. After the DNA molecular size marker (100-bp, Invitrogen), the first three lanes of all three gels correspond to *Xanthomonas axonopodis* pv. *passiflorae* while the following nine lanes correspond to other pathovars of *X. axonopodis* (Table 1, 88 to 96).

IBSBF2008, and IAC13755; GenBank accession nos. FJ828868, FJ828869, FJ828870, FJ828871, FJ828872, FJ828873, FJ828874, and FJ828875, respectively) was amplified using the primer pair pHr 5'-TGCGGCTGGATCACCTCCTT-3' (18) and p23Suni322anti 5'-GGTTCTTTTCACCTTTCCTC-3' (10). PCR was conducted using a thermocycler PTC-100 (MJ Research, Inc., Waltham, MA) as described previously (20), except that the annealing temperature was 58°C. PCR products were precipitated with polyethylene glycol and ethanol, and sequenced using the DYEnamic ET dye terminator Cycle Sequencing kit (Amersham Biosciences) with the abovementioned primers. Reactions were carried out in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) and electrophoresed in a MegaBace 1000 Molecular Dynamics sequencer (Amersham Biosciences). Sequences were assembled using the Phred/Phrap/Consed package (University of Washington, Seattle) applying a quality threshold value ≥ 20 . If the Phred score of a base exceeded this threshold, the base was considered high quality.

Sequence alignment and primer designing. The ITS sequences were initially aligned using Clustal X Software (version 1.81; Rockville Pike, Bethesda, MD). Nucleotide differences among the aligned sequences were visually searched and a single-nucleotide polymorphism (SNP) of interest was detected. A three-primer set was designed and included a forward (Xapas-F), an outer reverse (Xapas-Ro), and an inner reverse (Xapas-Ri) primer. To enhance pathovar specificity, a second deliberate mismatch (an A instead of a G nucleotide) at position -2 from the 3' end of the inner primer was also incorporated, as suggested by Ye et al. (31). The primers were mixed to amplify two bands. Xapas-F and Xapas-Ro produce a larger and common band because both anneal to conserved sequences in all pathovars, whereas Xapas-F and Xapas-Ri produce a smaller band specific to *pv. passiflorae* allele because the Xapas-Ri 3'-terminal base was positioned at the SNP. The GeneRunner program (<http://www.generunner.com>) was used to calculate the primer melting temperatures as the percentage of GC, and also determine the formation of secondary structures like hairpins, dimers, and loops within primer sequences.

For primer validation, all 87 isolates from *pv. passiflorae*, nine from other pathovars of *X. axonopodis*, and one isolate of *X. campestris pv. campestris* were used. The 20- μ l PCR mix contained 1 $\times$ reaction buffer (Promega Corp.), 1 μ M inner primer, 0.1 μ M outer reverse primer, 0.1 μ M forward primer, 0.2 mM each dNTP, 2.0 mM MgCl₂, 1 U of Go Taq Flexi (Promega Corp.), and 40 ng of template DNA. PCR conditions were as follows: 94°C for 3 min; 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 5 min in a Gen Amp PCR System 9700 thermocycler. Amplicons were electrophoresed at 3 V cm⁻¹ and visualized under UV after DNA SYBR Safe (Invitrogen, Carlsbad, CA) (0.5 $\times$) staining; photographs were taken to document results.

A bioassay for detection and quantification of *X. axonopodis pv. passiflorae* in plant tissues. Inoculums were produced by growing the Abj5-3 isolate on NA as previously described. The plates were flooded with a 0.85% NaCl solution and a bacterial suspension was obtained by scraping the surface of the plates. The suspension was adjusted to an optical density of 0.3 at 600 nm, which corresponds to an approximate bacterial concentration of 10⁸ CFU/ml.

Inoculations were performed by cutting off a 2-cm strip from the tip of fully expanded leaves with a pair of scissors that had been immersed in the bacterial suspension. Three leaves of each of three 120 day-old plants of *P. alata* were inoculated (a total of nine inoculations). A control plant received only the 0.85% NaCl solution. Plants were kept in a greenhouse under controlled conditions of temperature (minimum temperature of 13 $\pm$ 2°C and maximum temperature of 30 $\pm$ 2°C) and humidity (minimum of 14 to 18% and maximum of 88 to 99%). One leaf was collected

on days 5, 12, and 19 post inoculation, and each leaf was separated into two halves. One half was used for quantifying *X. axonopodis pv. passiflorae* by reisolating and direct counting of CFU using a single dilution series; the other half was further subdivided into two more segments, proximal and distal to the inoculation point, to determine possible bacterial migration. These segments were used for DNA extraction (4), with 60 ng of DNA

Fig. 2. Amplified fragment length polymorphism profiles generated with the primers M+T/E+G of a collection of *Xanthomonas axonopodis*: the number assigned to each isolate of *pv. passiflorae* (1 to 87) and to other pathovars of *X. axonopodis* (88 to 96) is indicated at the left of each lane. M is a mixture of 100- and 25-bp DNA molecular size markers (Invitrogen).

used as a template following the same PCR conditions as described for molecular diagnosis. Positive controls received bacterial colonies as PCR templates.

RESULTS

Bacterial identification and pathogenicity. Following the protocol of Starr and Stephens (26), the yellow extracts obtained from all the isolates showed a maximum absorbance at 443 nm

and shoulders at 470 and 425 nm, which are typical of xanthomonadins, confirming that all isolates belong to the genus *Xanthomonas*. The leaf lesions (minimum of 1.6 cm²) provoked by the artificial inoculations confirmed that all isolates were pathogenic.

Molecular diversity. All ERIC, BOX, and REP primer sets, as well as several combinations of AFLP selective primers, generated consistent molecular patterns for all 87 of the *X. axonopodis* pv. *passiflorae* isolates, and very distinct profiles for each

Fig. 3. Dendrogram generated by cluster analysis using unweighted pair-group method with arithmetical averages based on amplified fragment length polymorphism profiles of *Xanthomonas axonopodis* pv. *passiflorae* isolates (Table 1, 1 to 87). Bootstrap values are indicated at the branches and clusters were delineated at an arbitrary similarity level of 0.70. The scale bar indicates the genetic similarities measured by the Jaccard's coefficient.

of the pathovars. Although there was an agreement between rep-PCR and AFLP results, the rep-PCR fingerprints revealed a limited amount of polymorphic loci (31) within the *pv. passiflorae* isolates. Therefore, the rep-PCR technique was not useful to identify a significant clustering pattern (data not shown), because a high number of isolates need to be compared. However, this method was important to reveal that *X. axonopodis* pathovars were molecularly very distinct (Fig. 1).

Out of 408 AFLP loci obtained from nine selective primer combinations, 89 were polymorphic. The primer combinations M+0/E+AA and M+CA/E+CG revealed 15 polymorphic loci each, M+T/E+G revealed 13, and M+G/E+A and M+G/E+T revealed 10 and 9, respectively. Nine loci were indicated by M+AG/E+T, eight from M+A/E+G, six from M+T/E+C, and four from M+C/E+A. Fragments sizes varied from 90 to 880 bp (Fig. 2).

Estimates of overall pair-wise genetic similarities between isolates generated by the Jaccard coefficient were 0.16 to 1.0. With 0.7 as the minimum threshold for designating genetic simi-

larity, 12 groups were formed as shown in the dendrogram generated from the UPGMA cluster analysis (Fig. 3). The cophenetic correlation—the correlation between the distances at which a pair of observations are joined in a dendrogram and the dissimilarity values for that pair—was 0.9255, meaning that clustering was very successful in partitioning the data. The dendrogram shows the exclusive occurrence of isolates from some localities. For example, all isolates from localities U and D were clustered into group II, and isolates from F, H, and G were clustered into groups III, IV, and XII, respectively. In contrast to the location-specific clustering of some isolates, those collected from A were clustered into two groups, I and VII.

The similarity analyses and resulting dendrograms supported the results of the AMOVA, which indicated considerable between-locality variation and less within-locality variation. In all, $\approx 50\%$ of the total molecular variance was due to differences between localities, 36% was due to differences within the localities, and 14% of the variance was partitioned between the geographical states.

Fig. 4. Geographic map of the Brazilian localities (A–V) where the isolates of *Xanthomonas axonopodis* *pv. passiflorae* were collected (1 to 87). A, DF, Distrito Federal; MG, Minas Gerais; SP, São Paulo; and PR, Paraná State. B, Result of the assignment test: each vertical bar corresponds to a bacterial isolate and each color corresponds to a genotypic cluster. Y-coordinate values correspond to membership coefficients (Q) that reveal how each sample (isolate) fits into a specific cluster.

Molecular data were analyzed by the computer program Structure using Bayesian models to probabilistically assign each genotype to a genetically distinct cluster. Data were arranged using the number of localities (i.e., 22) (Fig. 4A, A to V) to complete the assigning task. The best K value found according to the model described by Evano et al. (5) was 13, meaning that all 87 isolates were assigned to 13 clusters, which are represented by a color-coded bar graph shown in Figure 4B. These findings were very similar to the UPGMA results, because the number of groups in the dendrogram was 12 (Fig. 3).

In essence, the assignment test determined whether a cluster had a molecular genotype that was typical of the locality where samples originated (as in F) or whether it was divided among one or more different localities (as in the blue-colored cluster). The Y-coordinate values correspond to membership coefficients (Q) that reveal how each sample (isolate) fits into a specific cluster (Fig. 4B).

The assignment test revealed that A, B, and C populations were genetically diverse. The populations of D, F, G, H, and U, in contrast, were uniform (Fig. 4B). These results reflect those of the dendrogram obtained using the UPGMA algorithm. There was no genetic variation within 10 localities because just one isolate

occurred in each. However, the assignment test showed that eight of those isolates were contained in different clusters ($Q \leq 66\%$). The remaining isolates (64 and 87) were contained in one single cluster with membership coefficients of $Q = 98.3$ and 96.2% , respectively (Fig. 4B).

Molecular diagnosis of *X. axonopodis* pv. *passiflorae*. The alignment of the fragment (1,000 bp) of the 16S-23S rRNA intergenic spacer region showed the presence of an SNP within the pv. *passiflorae* sequence. Comparing the 12 ITS sequences, a G nucleotide was detected within the pv. *passiflorae* sequence instead of a T nucleotide that was detected in the other pathovars. The inner primer Xapas-Ri was designed to be specific to pv. *passiflorae*, with its 3'-terminal base positioned at the SNP, and a second deliberate mismatch introduced to enhance specificity (Fig. 5A). As expected, a larger amplicon of 641 bp was produced in all pathovars because the primers Xapas-F and Xapas-Ro were designed to anneal to a conserved region of the ITS sequence; a second and smaller fragment of 520 bp was amplified exclusively from all 87 isolates of the pv. *passiflorae* (Fig. 5B). In samples of our studies, the PCR-based method for the detection of pv. *passiflorae* described before (7) failed to detect isolates from different locations.

Fig. 5. A, Part of the sequence alignment of the 16S-23S rRNA intergenic spacer region in which a single nucleotide polymorphism (in bold) that distinguishes the four isolates of pv. *passiflorae* from the other pathovars of *Xanthomonas axonopodis* was detected. Sequences of the primer set Xapas are shown on the top as the deliberate mismatch introduced at position -2 (*). **B**, Agarose gel showing a larger amplicon (641 bp) produced in all pathovars of *Xanthomonas axonopodis* (lanes 1 to 96) as the primers Xapas-F and Xapas-Ro were designed to anneal to a conserved region of the internal transcribed spacer sequence, and a smaller amplicon (520 bp, indicated by arrow) produced exclusively from all isolates of the pv. *passiflorae* (lanes 1 to 87); lanes 88 to 96 correspond to other pathovars of *X. axonopodis*; lane 97, *Xanthomonas campestris* pv. *campestris*; lane 98, polymerase chain reaction assay without DNA template; M, 100-bp ladder, DNA molecular size marker.

Passion fruit plants started to show typical symptoms of infection 8 days after artificial bacterial inoculation, though on day five we were able to detect amplicons with the primer set (Xapas) in DNA extracted from a leaf segment proximal to the inoculation point. These amplicons were weak in relation to those produced in samples collected on days 12 and 19 from locations proximal or distal to the inoculation point. Over time, bacteria colonized the leaf and became increasingly abundant as PCR repeatedly revealed conspicuous bands from all plants (Fig. 6). The bacterial densities in infected plants were measured during the evaluation period. Average concentrations were 0.60×10^7 CFU/g on day 5, 5.86×10^7 CFU/g on day 12, and 14.31×10^7 CFU/g on day 19. Neither symptoms nor colonies were detected in the control plant.

DISCUSSION

Genomic-based fingerprints have been used to identify pathogens and strains that were previously not distinguishable by other classification methods (11,16,17). In the present study, we observed different DNA patterns for all nine pathovars of *X. axonopodis* revealed by rep-PCR and AFLP profiling (Figs. 1 and 2), reflecting the adaptation of bacteria to specific hosts.

We found a close association between the geographical origin of the 87 isolates of *X. axonopodis* pv. *passiflorae* and AFLP-based polymorphism; Restrepo et al. (24) reported a similar relationship for *X. axonopodis* pv. *manihotis*. There was a predominance of only a few clusters in most of the sample or production locations (Fig. 3). The assignment test provided important information on the genetic structure of the bacterial population and its relationship to pathogen migration. The blue-colored cluster, for example, was predominant in the localities B, C, D, H, U, and V, the latter two being geographically distant from the others. It suggests that the pathogen was disseminated through infected cuttings from a production area to the country's center. The test also indicated that some genotypes are more successful in their ability to colonize the orchards, particularly the ones colored in brown (predominant in F) and green (predominant in G), though the respective locations are very close to genetically diverse areas (A and B). All of these data have important implications for disease control.

In contrast, 10% of the isolates were admixed (13, 16, 18, 23, 33, 56, 57, 61, and 71), sharing their molecular profiles with several clusters. Each of these isolates was given a membership coefficient for each cluster where $Q < 60\%$, the sum of membership coefficients being 1. For example, the localities A and B were very diversified, with admixed isolates that probably migrated from neighboring areas and colonized the vines.

The high level of genomic diversity found among the bacterial isolates is possibly associated with the outcrossing behavior of

cultivated passion fruit. In Brazil, however, passion fruit is a recent crop and propagation of cultivated varieties is often through vegetative means. Therefore, pathogens at some localities are under less selection pressure, resulting in less diversification. Similar results were reported for *Xylella fastidiosa* when comparing SNP-based molecular profiles of two strains from the same host collected from distinct geographical areas, as well as from different hosts (30). A significant effect of geographic location on genetic diversity of *Xanthomonas arboricola* pv. *juglandis*, the cause of walnut blight, and *X. translucens* pv. *graminis*, which causes bacterial wilt in forage grasses, was detected by cluster analyses using AFLP markers (13,16).

Our research investigated diversity in *X. axonopodis* pv. *passiflorae* using an UPGMA-based dendrogram and an assigning test, as well as AMOVA. Similar conclusions can be drawn from the data obtained from different methods; for example, there was greater variability between different sample locations than within most of them. Similar results were reported for *X. axonopodis* pv. *manihotis* (24). Population studies such as this expand our current knowledge about pathogen dissemination and have important implications for disease control. Our data suggest that the dispersal of isolates is reduced and that dissemination occurs primarily through infected cuttings.

Molecular diagnostics of plant pathogens for disease management is gaining importance. Park et al. (21) designed a primer pair to amplify a sequence of the *hrpF* gene to detect *X. campestris* pv. *campestris* but the ITS sequence was preferentially used to design primers for distinguishing *Xanthomonas* spp. and pathovars (8,9) and detecting the pathogen in cultivated plants (3). In the present work, a set of primers (Xapas) was shown to efficiently detect all isolates of *X. axonopodis* pv. *passiflorae* as well as the presence of the pathogen in plant tissues even before outward symptoms were apparent. As far as we know, this is the first report on the use of an assigning test to provide information on the genetic structure of a bacterial population, and to utilize a primer set specially designed for bacterial diagnosis based on a SNP-anchored approach (31).

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Fig. 6. Agarose gel showing the amplicons generated by polymerase chain reaction (PCR) with the primer set Xapas and DNA extracted from passion fruit plants (1, 2, and 3) after 5, 12, and 19 days of inoculation with *X. axonopodis* pv. *passiflorae*; M, 100-bp ladder, DNA molecular size marker; T, the control plant; A and B correspond to DNA extracted from leaf segments proximal and distal to the inoculation point, respectively; C⁺, positive control received bacterial colonies as the PCR template; C⁻, PCR assay without DNA template as a negative control.

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