The definition of host range is a central parameter for the understanding and, ultimately, the control of infectious diseases in general and bacterial plant diseases in particular. In phytobacteriology, host range is an important aspect of pathogenicity. Control of diseases can be achieved with resistance genes which reduce host range (50, 64). Epidemiological characteristics are highly dependent on host range, and the emergence of new diseases is sometimes correlated with broadened host ranges (74). Xanthomonads have the particularity of an extremely narrow host range (sometimes reduced to a single plant genus), although a very large number of plant families can be hosts when all members of the genus are considered (33), which led plant pathologists to create the concept of pathovar at an infrasubspecific level. Pathovars were defined as groups of strains sharing logical characteristics highly dependent on host range, indicating that strains of a pathovar have a common ancestral origin (3, 56). Xanthomonas citri pv. citri is the causal agent of Asiatic citrus canker, a severe disease infecting most commercial citrus cultivars and some genera in the Rutaceae family in many citrus-producing areas worldwide (6, 60, 61). This pathovar has two types of strains, which differ in their host ranges: pathotype A has a wide host range and a worldwide distribution and is a permanent threat for citriculture (29); in contrast, the more recently characterized pathotype A* causes citrus canker on Mexican lime (Citrus aurantifolia) and has a much less severe impact on citriculture (72). Strains of this pathovar were considered to belong to the pathovar citri because of their phenotypic and genetic relatedness to pathotype A. Their distribution was initially reported to include Saudi Arabia, Oman, Iran, and India and was recently found to extend to southeast Asia, with reports of these strains in Thailand (10) and Cambodia (11). Finally, strains genetically related to pathotypes A and A* but able to infect Mexican lime and Citrus macrophylla naturally were recently detected in Florida and classified as a pathotype designated A" (68). The molecular basis of the specific interaction of X. citri pv. citri pathotypes A* and A" with a restricted range of citrus hosts is not known (5). An interaction between a host resistance gene and an avr gene product from the pathogen inducing host-pathogen incom-

From Local Surveys to Global Surveillance: Three High-Throughput Genotyping Methods for Epidemiological Monitoring of *Xanthomonas citri* pv. citri Pathotypes†‡

Lan Bui Thi Ngoc,1 Christian Vernière,1 Philippe Jarne,2 Sylvain Brisse,3 Fabien Guérin,1 Sébastien Boutry,1 Lionel Gagnevin,1 and Olivier Pruvost1* CIRAD, UMR Peuplements Végétaux et Bioagresseurs en Milieu Tropical CIRAD-Université de la Réunion, Pôle de Protection des Plantes, 7 chemin de l’Irat, 97410 Saint Pierre, La Réunion, France1; Centre d’Ecologie Fonctionnelle et Evolutive, UMR 5175, campus CNRS, 1919 route de Mende, 34293 Montpellier Cedex 5, France2; and Unité Biodiversité des Bactéries Pathogènes Émergentes, Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France3

Received 30 September 2008/Accepted 10 December 2008

Asiatic citrus canker is a major disease worldwide, and its causal agent, *Xanthomonas citri* pv. citri, is listed as a quarantine organism in many countries. Analysis of the molecular epidemiology of this bacterium is hindered by a lack of molecular typing techniques suitable for surveillance and outbreak investigation. We report a comparative evaluation of three typing techniques, amplified fragment length polymorphism (AFLP) analysis, insertion sequence ligation-mediated PCR (IS-LM-PCR) typing, and multilocus variable-number tandem-repeat analysis (MLVA), with 234 strains originating from Asia, the likely center of origin of the pathogen, and reference strains of pathotypes A, A*, and A", which differ in host range. The typing techniques were congruent in describing the diversity of this strain collection, suggesting that the evolution pattern of the bacterium may be clonal. Based on a hierarchical analysis of molecular variance, the AFLP method best described the genetic variation found among pathotypes whereas MLVA best described the variation found among individual strains from the same countries or groups of neighboring countries. IS-LM-PCR data suggested that the transposition of insertion sequences in the genome of *X. citri* pv. citri occurs rarely enough not to disturb the phylogenetic signal. This technique may be useful for the global surveillance of non-epidemiologically related strains. Although pathological characteristics of strains could be most often predicted from genotyping data, we report the occurrence in the Indian peninsula of strains genetically related to pathotype A* strains but with a host range similar to that of pathotype A, which makes the classification of this bacterium even more complicated.
patibility has not yet been demonstrated for the *X. citri* pv. citri-citrus pathosystem, as it has been previously for other plant pathogenic bacteria (43).

No assumption can be made about whether the apparent contemporary emergence of pathotype A* strains is due to a change in virulence or to environmental or human factors. Host range shifts have sometimes been related to modifications in the repertoire of virulence genes by horizontal gene transfer or intragenomic recombinations or mutations (20, 32, 75). A clear understanding of the evolutionary relationships among pathotypes A, A*, and A** and of the diversity among strains of each pathotype would be helpful for assessing these issues.

Due to the extreme difficulty and cost of the complete eradication of Asiatc citrus canker, several canker-threatened citrus-producing regions rely on integrated pest management strategies for control (30). Data derived from the huge effort put into the molecular typing of human bacterial pathogens (46, 63, 67, 69) suggest that an extensive knowledge of populations of plant pathogenic bacteria may improve our understanding of epidemic situations.

The tools most often used for the molecular epidemiology of citrus canker have been repetitive-element-based PCR (rep-PCR) and pulsed-field gel electrophoresis (PFGE) (13, 16, 19, 28, 68). The lack of discriminatory power of rep-PCR and the high labor requirement for PFGE make it difficult to use these techniques extensively for outbreak investigations or regional or global surveillance (67). Therefore, alternative high-resolution and high-throughput molecular typing systems for *X. citri* pv. citri should be developed. Amplified fragment length polymorphism (AFLP) analysis of an Iranian collection of strains causing Asiatc citrus canker suggested previously that this technique has better discriminatory power than the rep-PCR method (39). AFLP has the advantage of generating a large number of randomly located markers over the whole genome. The detected polymorphism may arise from point mutations at the targeted restriction sites or from insertions and/or deletions in the amplified region (73). The determination of the complete sequence of *X. citri* pv. citri strain 306 (17) should facilitate the development of molecular typing tools well-suited for deciphering taxonomy, evolution, and/or epidemiology. For instance, it gave access to specific primers associated with transposable elements present in this bacterium (45), which were used for typing DNA from herbarium specimens showing canker-like symptoms and originating from different geographical origins. This technique revealed an unexpectedly high degree of genetic diversity. However, this typing scheme requires more than 50 PCRs for the full analysis of unknown DNA. A new insertion sequence ligation-mediated PCR (IS-LM-PCR) scheme (9) also revealed considerable diversity and is less labor-intensive. This technique amplifies DNA fragments between an insertion sequence element and a selected restriction site (9). We also recently developed a multilocus variable-number tandem-repeat analysis (MLVA) approach for this bacterium, a promising technique targeting tandem repeats (minisatellite-like loci) for fine-scale epidemiology with distinctive advantages, such as high discriminatory power, maximal reproducibility of results, and portability of equipment (12). The characteristics of these newly developed techniques need to be subjected to a comparative evaluation in order to determine which methods would be most useful for global surveillance and molecular epidemiology on small spatial scales. In this study, we compared the AFLP, MLVA, and IS-LM-PCR techniques to explore the genetic diversity of a collection of pathotype A, A*, and A** strains originating from Asia. Furthermore, we sought to determine the genetic diversity and structure of *X. citri* pv. citri strains, including a large collection of pathotype A* strains for which no extensive characterization study is available at the moment, from the area of origin of the pathogen.

**MATERIALS AND METHODS**

**Bacterial strains and DNA extraction.** A total of 234 bacterial strains isolated from citrus canker lesions and collected from 26 countries in Asia were used in this study, together with reference pathotype A, A*, and A** strains. All strains were assigned to a pathogenicity group on the basis of results from detached-leaf inoculation assays performed on Mexican lime (C. aurantifolia), *C. macrophylla*, and grapefruit (*C. paradisi*) (72). Strains producing canker-like lesions on the three host species were classified as pathotype A strains. Both pathotype A* (n = 59) and A** (n = 6) strains produced canker-like lesions on *C. aurantifolia* and *C. macrophylla* but not on *C. paradisi*. Thus, the two types were pathogenically indistinguishable (see Table S1 in the supplemental material for information on each strain, including the geographical origin, host species, date of collection, and pathogenicity group). Single colonies were subcultured on plates containing YPGA (yeast extract, 10 g liter⁻¹; peptone, 7 g liter⁻¹; glucose, 7 g liter⁻¹; agar, 18 g liter⁻¹; and propionic acid, 20 mg liter⁻¹) for 24 h at 28°C. These subcultures were used to inoculate tubes containing 4 ml of YP broth (yeast extract, 7 g liter⁻¹; peptone, 7 g liter⁻¹; pH 7.2), and the tubes were incubated at 28°C on an orbital shaker for 16 to 18 h. These suspensions were used for DNA extraction with the DNeasy tissue kit according to the instructions of the manufacturer (Qiagen, Courtaboeuf, France). DNA concentrations were estimated by fluorometry with a Tko 100 fluorometer (Hoefer, San Francisco, CA).

**AFLP analysis.** AFLP fingerprinting was performed mainly according to the original protocol by Vos et al. (73) as described previously (2). In brief, 25 ng of DNA was digested with MspI and SacI restriction enzymes as recommended by the manufacturer (New England Biolabs/Ozyme, Saint Quentin en Yvelines, France). Then 2.5-μl aliquots of the digested products were added to 22.5-μl ligation mixtures containing 2 μM MspI adaptor (see Table S2 in the supplemental material), 0.2 μM SacI adaptor (Applied Biosystems, Courtaboeuf, France) (see Table S2 in the supplemental material), and 2 μl of T4 DNA ligase (New England Biolabs/Ozyme, Saint Quentin en Yvelines, France) in 1× T4 DNA ligation buffer. Ligation reactions were performed for 3 h at 37°C before enzyme inactivation at 65°C for 10 min. For preselective PCR, 10-fold-diluted ligation products were used as a template in a mix containing 5 mM MgCl₂, 0.23 μM (each) MspI and SacI primers (see Table S2 in the supplemental material), 0.45 mM (each) dNTPs, 50 units of Taq DNA polymerase (New England Biolabs/Ozyme, Saint Quentin en Yvelines, France), and 0.5 U of Taq DNA polymerase (Goldstar red; Eurogenet, Seraing, Belgium) in 1× Goldstar buffer. The following PCR conditions were used: initial extension to ligate the second strand of the adaptors at 72°C for 2 min; a denaturation step at 94°C for 25 min; cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min; and a final extension step at 72°C for 10 min. Tenfold-dilutions of PCR products were used as templates for selective amplifications. The selective amplifications using the unlabeled MspI+ A, MspI+ C, MspI+ T, or MspI+ G primer and the SacI+ C primer labeled with one of four different fluorochromes (Applied Biosystems, Courtaboeuf, France) (see Table S2 in the supplemental material) were performed under the same conditions as the preselective PCR, except that the SacI+ C primer concentration was 0.12 μM. The following PCR conditions were used: initial denaturation at 94°C for 2 min; 37 cycles of 94°C for 30 s, annealing for 30 s at 65°C in the first cycle, at temperatures decreasing by 0.7°C per cycle for the next 12 cycles, and then at 56°C for the last 24 cycles, and extension at 72°C for 2 min; and a final extension step at 72°C for 10 min. Samples were then prepared for capillary electrophoresis by adding 1 μl of the final PCR product to a mixture of 18.7 μl of formamide and 0.3 μl of a GeneScan 500 LIZ DNA ladder (Applied Biosystems, Courtaboeuf, France) as an internal standard. The samples were then denatured for 5 min at 95°C and placed on ice for at least 5 min. Electrophoresis was performed in an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Courtaboeuf, France) using a performance-optimized polymer, POP-4, at 15,000 V for about 20 min at 60°C, with an initial injection of 66 s. The AFLP fingerprints were analyzed visually using the software GeneScan 3.7 (Applied Biosystems, Courtaboeuf, France). To
test the reproducibility of the results from the AFLP technique, independent DNA extractions were used for all strains and strain 306 of X. citri pv. citri (17) was used as a control in each AFLP experiment.

**IS-LM-PCR analysis.** IS-LM-PCR fingerprinting was performed as described previously (9). In brief, aliquots of bacterial genomic DNA were subjected to restriction enzyme and ligated to the adaptor by incubation for 3 h at 37°C in total volumes of 20 μl containing 2 ng of DNA, 9 μl of MspI (New England Biolabs/Ozyme, Saint Quentin en Yvelines, France), 50 μl of T4 DNA ligase (New England Biolabs/Ozyme, Saint Quentin en Yvelines, France), 50 mM NaCl, 1 μM MspI adaptor (Applied Biosystems, Courtaboeuf, France) (see Table S3 in the supplemental material), and 1X bovine serum albumin in 1X T4 DNA ligation buffer, followed by enzyme inactivation at 65°C for 10 min. Tenfold dilutions were used as template DNA in 20 μl of a PCR mix which contained 1 mM (each) deoxynucleoside triphosphates, 5 mM MgCl2, 0.25 μM unlabeled MspI primer (Applied Biosystems, Courtaboeuf, France) (see Table S3 in the supplemental material), 0.25 μM 5′-end-labeled insertion sequence-specific primer (Applied Biosystems, Courtaboeuf, France) (see Table S3 in the supplemental material), and 0.5 μl of Taq DNA polymerase (Goldstar red; Eurogentec, Seraing, Belgium) in 1X Taq Goldstar buffer. The following PCR conditions were used: initial extension to ligate the second strand of the adaptors at 72°C for 2 min; a denaturation step at 94°C for 2 min; 35 cycles at 94°C for 45 s, 60°C for 30 s, 72°C for 45 s followed by a final extension step at 72°C for 10 min. Samples were prepared and subjected to capillary electrophoresis as explained above. To test the reproducibility of the results from the IS-LM-PCR technique, two independent DNA extractions were used for all strains and strain 306 of X. citri pv. citri (17) was used as a control in each experiment.

**MLVA scheme.** Fourteen primer pairs targeting single-locus alleles designed from X. citri pv. citri strain 306 (17) were used in a multiplex PCR format with a PCR kit from Qiagen (Courtaboeuf, France) (12). Briefly, 2 to 5 ng of genomic DNA was used as a template in mixes containing 0.2 μM (each) primers (one of which was marked with one of the fluorescent dyes 6-carboxyfluorescein, NED, PET, and VIC [Applied Biosystems]), 1X QIAGEN multiplex mastermix (containing a hot-start Taq DNA polymerase), 0.5 μl Q-solution (Qiagen, Courtaboeuf, France), and 1XQF-free water to yield a volume of 15 μl. The PCR conditions were performed on a MJ Research PTC-225 thermocycler (Applied Biosystems) under the following conditions: 15 min at 95°C for hot-start activation; 25 cycles of 94°C for 30 s, annealing at temperatures of 60°C to 72°C for 30 s, and a final extension step at 72°C for 10 min. Samples were prepared and subjected to capillary electrophoresis as explained above.

To test the reproducibility of the results from the IS-LM-PCR technique, two independent DNA extractions were used for all strains and strain 306 of X. citri pv. citri (17) was used as a control in each experiment.

**Data scoring and exploratory analysis.** For the ISLMPCR and MLVA techniques, the presence and absence of fragments were scored as a binary matrix and analyzed with the following software: (i) BioNumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium); (ii) R Development Core Team (Vienna, Austria). The size of each fragment in the range of 50 to 500 bp was determined. Fragments with fluorescence above a threshold set to 500 relative fluorescence units were scored. This threshold was found to be suitable for minimizing scoring discrepancies among DNA replicates in earlier studies (2, 53). Only fragments detected for both DNA replicates were scored as positive in the data matrix. Dice dissimilarities were used as distances to construct a weighted neighbor-joining tree for a specific sample set (19). To test the reproducibility of results from the MLVA technique, two independent DNA extractions were used for all strains and strain 306 of X. citri pv. citri (17) was used as a control in each experiment.

**Data scoring and exploratory analysis.** For the AFLP and IS-LM-PCR techniques, the presence and absence of fragments were scored as a binary matrix and analyzed with the following software: (i) BioNumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium); (ii) R Development Core Team (Vienna, Austria). The size of each fragment in the range of 50 to 500 bp was determined. Fragments with fluorescence above a threshold set to 500 relative fluorescence units were scored. This threshold was found to be suitable for minimizing scoring discrepancies among DNA replicates in earlier studies (2, 53). Only fragments detected for both DNA replicates were scored as positive in the data matrix. Dice dissimilarities were used as distances to construct a weighted neighbor-joining tree for a specific sample set (19). To test the reproducibility of results from the MLVA technique, two independent DNA extractions were used for all strains and strain 306 of X. citri pv. citri (17) was used as a control in each experiment.

**Data scoring and exploratory analysis.** For the AFLP and IS-LM-PCR techniques, the presence and absence of fragments were scored as a binary matrix and analyzed with the following software: (i) BioNumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium); (ii) R Development Core Team (Vienna, Austria). The size of each fragment in the range of 50 to 500 bp was determined. Fragments with fluorescence above a threshold set to 500 relative fluorescence units were scored. This threshold was found to be suitable for minimizing scoring discrepancies among DNA replicates in earlier studies (2, 53). Only fragments detected for both DNA replicates were scored as positive in the data matrix. Dice dissimilarities were used as distances to construct a weighted neighbor-joining tree for a specific sample set (19). To test the reproducibility of results from the MLVA technique, two independent DNA extractions were used for all strains and strain 306 of X. citri pv. citri (17) was used as a control in each experiment.
RESULTS

Predictive in silico AFLP, IS-LM-PCR, and MLVA typing. In the AFLP analysis, 111 of 147 predicted fragments (76%) for the sequenced X. citri pv. citri strain 306 were always recorded and 8 predicted fragments were amplified in an irreproducible way. AFLP markers were scattered throughout the X. citri pv. citri genome. In the IS-LM-PCR analysis, 34 of 39 predicted fragments (87%) were always recorded, 3 predicted fragments were occasionally amplified, and 4 unpredicted fragments were reproducibly recorded. In MLVA, all predicted fragments were observed.

AFLP analysis. Although fingerprints obtained for DNA replicates sometimes differed in fragment intensity, the scoring system allowed overall good reproducibility of AFLP results, with >95% of the corresponding fragments in replicates being identically assigned. A total of 182 fragments, 100 (55%) of which were polymorphic, were scored. Ninety-four haplotypes were identified within the strain collection (n = 234) (Table 1). Based on the NJ tree (see Fig. S1A in the supplemental material) and the MDS plot (Fig. 1A), a clear-cut separation between pathotype A and A*/A" strains was most often observed. A noticeable exception concerned a set of pathotype A strains from Bangladesh and India that were genetically close to pathotype A" strains but produced canker-like lesions on all assayed Citrus species (data not shown). The two graphical representations also suggested greater polymorphism within the A*/A" group, an indication supported by the calculation of Nei's unbiased total gene diversity index (Ht) for pathotype A*/A" strains, which is a good indicator of the real K (21). We examined the clustering of strains of X. citri pv. citri for the inferred number of groups. We used different ancestry models in STRUCTURE according to the data sets. The linkage model (24) was used for MLVA data. For the biallelic markers (AFLP and IS-LM-PCR data), we used the admixture model and performed clustering without using prior population information under the F model, which assumes that the allele frequencies in the populations are correlated (23).

IS-LM-PCR analysis. The overall reproducibility of IS-LM-PCR results was similar to that described for AFLP results. The global diversity (Hr) ranged from 0.06 (for insertion element ISXac2) to 0.13 (for ISXac1) (Table 1). Based on Mantel test results, all distance matrices derived from a single primer pair were significantly correlated (P < 0.001). Pooling data ensured that the NJ tree had a robust structure, as indicated by bootstrap values (data not shown). When data derived from the four insertion sequence primer pairs were pooled, a total of 336 markers, 329 (98%) of which were polymorphic, were scored. The relationships among the 146 haplotypes that were identified within the strain collection (n = 234) are shown by an NJ tree (see Fig. S1B in the supplemental material) and the MDS plot in Fig. 1B. The two first MDS axes described 85.8% of the total variation. A clear-cut separation between pathotype A and A*/A" strains was observed, which is consistent with AFLP results (Fig. 1A and B). Pathotype A" strains formed a subclade supported by a bootstrap value of 87% but were closely related to pathotype A* strains from India, forming a cluster with a maximal bootstrap value. The A*/A" group formed a total of 10 robust clusters supported by maximal bootstrap values (data Fig. S1B in the supplemental material). This high level of diversity was confirmed by calculating the genotypic diversity. The gene diversity (Nei's unbiased gene diversity index [Hi]) calculated for pathotype A*/A" strains (Ht = 0.20) was greater than that calculated for pathotype A strains (Ht = 0.05). The strain compositions of A*/A" clusters derived from AFLP and IS-LM-PCR techniques were highly consistent. Pathotype A strains did not generally form robust clusters (see Fig. S1B in the supplemental material), which is consistent with AFLP data. Due to the greater discriminatory power of IS-LM-PCR, haplotypes that included a large number of strains were rare. Nevertheless, as in the AFLP analysis, it was possible to identify a few haplotypes of pathotype A strains that were found in several countries. For example, haplotype 10 (seven strains) was detected in China, Japan, and Taiwan. Pathotype A strains that showed the greatest genetic relatedness to pathotype A* strains originated from western Asia (Bangladesh, India, and the Maldives), which is consistent with AFLP data.

MLVA scheme. A total of 209 haplotypes among the 234 strains were detected when data from the 14 loci were pooled, with allele numbers per locus ranging from 6 (for locus XL1) to 29 (for locus XL2). Five pathotype A" strains and two pathotype A* strains from India did not produce amplicons for the XL5 locus. The mean numbers of
observed alleles for types A and A* were 12.3 and 9.8, respectively, revealing a slightly higher degree of allelic richness in pathotype A strains (Table 1). Strains with the same allelic profile were primarily those isolated from the same site during the same year. In a few cases, strains sharing the same allelic profile were isolated from sites several miles apart in the same year or from the same site over two years. Similarly, each MLVA type originated from a single country. The relationships among the haplotypes that were identified within the strain collection are shown by an NJ tree (see Fig. S1C in the supplemental material) and the MDS plot in Fig. 1C. An eBURST analysis of the 209 MLVA types yielded 32 groups and 108 singletons, which is consistent with AFLP and IS-LM-PCR data. In order to determine whether tandem repeats evolve by following an SMM, we computed all the differences in the number of repeats along the evolutionary path deduced by the eBURST analysis within each eBURST group. Generally, the distribution of occurrences showed a single mode centered around zero. For most loci where at least four changes occurred in total (e.g., loci XL1, XL3, XL7, and XL11 to XL14), the most frequent change was either +1 or –1 repeat unit and the symmetric change was generally the second most frequent. Two noticeable exceptions were locus XL10, with more...

### Table 1. Genetic diversity parameters for *X. citri* pv. *citri* strains estimated by using MLVA (14 VNTR loci), AFLP (4 conditions), and IS-LM-PCR (4 conditions)

<table>
<thead>
<tr>
<th>Method</th>
<th>Locus(i) or primer combination(s)</th>
<th>No. of alleles</th>
<th>Total no. of fragments</th>
<th>No. of haplotypes</th>
<th>$H_T$</th>
<th>$H_w$</th>
<th>$F_{ST}^b$</th>
<th>$R_{ST}^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLVA</td>
<td>XL1</td>
<td>19</td>
<td>0.92</td>
<td>0.84</td>
<td>0.11***</td>
<td>0.23NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL2</td>
<td>29</td>
<td>0.93</td>
<td>0.58</td>
<td>0.38***</td>
<td>0.84***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL3</td>
<td>11</td>
<td>0.87</td>
<td>0.70</td>
<td>0.25***</td>
<td>0.37 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL4</td>
<td>22</td>
<td>0.93</td>
<td>0.77</td>
<td>0.21***</td>
<td>0.78***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL5</td>
<td>15</td>
<td>0.89</td>
<td>0.73</td>
<td>0.19***</td>
<td>0.49***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL6</td>
<td>26</td>
<td>0.93</td>
<td>0.83</td>
<td>0.15***</td>
<td>0.56***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL7</td>
<td>18</td>
<td>0.89</td>
<td>0.60</td>
<td>0.27***</td>
<td>0.17 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL8</td>
<td>10</td>
<td>0.73</td>
<td>0.44</td>
<td>0.33***</td>
<td>0.22 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL9</td>
<td>14</td>
<td>0.74</td>
<td>0.49</td>
<td>0.33***</td>
<td>0.60 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL10</td>
<td>10</td>
<td>0.83</td>
<td>0.47</td>
<td>0.43***</td>
<td>0.50 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL11</td>
<td>6</td>
<td>0.33</td>
<td>0.23</td>
<td>0.49***</td>
<td>0.53 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL13</td>
<td>11</td>
<td>0.47</td>
<td>0.31</td>
<td>0.39***</td>
<td>0.80*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL14</td>
<td>23</td>
<td>0.94</td>
<td>0.62</td>
<td>0.35***</td>
<td>0.86***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XL15</td>
<td>18</td>
<td>0.86</td>
<td>0.70</td>
<td>0.19***</td>
<td>0.51 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All <em>X. citri</em> loci</td>
<td>232</td>
<td>0.80</td>
<td>0.59</td>
<td>0.27***</td>
<td>0.62 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Loci from pathotype A strains</td>
<td>172.6</td>
<td>0.77</td>
<td>0.64</td>
<td>0.13***</td>
<td>0.38***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Loci from pathotype A* strains</td>
<td>137.6</td>
<td>0.79</td>
<td>0.48</td>
<td>0.54***</td>
<td>0.92***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method</th>
<th>Locus(i) or primer combination(s)</th>
<th>No. of alleles</th>
<th>Total no. of fragments</th>
<th>No. of haplotypes</th>
<th>$H_T$</th>
<th>$H_w$</th>
<th>$F_{ST}^b$</th>
<th>$R_{ST}^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS-LM-PCR</td>
<td>pMsp/ISXac1-D3</td>
<td>80</td>
<td>0.13</td>
<td>0.03</td>
<td>0.78**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pMsp/ISXac1-GF</td>
<td>95</td>
<td>0.12</td>
<td>0.03</td>
<td>0.76**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pMsp/ISXac2-D3</td>
<td>96</td>
<td>0.12</td>
<td>0.03</td>
<td>0.63**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pMsp/ISXac3-IRF</td>
<td>65</td>
<td>0.08</td>
<td>0.02</td>
<td>0.83**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All combinations</td>
<td>336</td>
<td>0.10</td>
<td>0.03</td>
<td>0.78**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Loci from pathotype A strains</td>
<td>216</td>
<td>0.10</td>
<td>0.03</td>
<td>0.39***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Loci from pathotype A* strains</td>
<td>249</td>
<td>0.20</td>
<td>0.03</td>
<td>0.87**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Notes

- $H_T$, Nei’s mean index of gene diversity within populations.
- $F_{ST}$ estimates were calculated for eight and three geographical groups (with 10 or more members) composed of pathotype A and A* strains, respectively.
- $R_{ST}$ estimates from VNTR data were calculated for eight and three geographical groups (with 10 or more members) composed of pathotype A and A* strains, respectively.
- Parameters were calculated for 225 strains, as 7 pathotype A*/A strains did not produce any amplicon for the XL5 locus.
- Loci originally described as pathotype A* (68) were included in the pathotype A* group for data derived from all three genotyping techniques.
changes \((n = 6)\) than \(-1 (n = 3)\) and \(+1 (n = 3)\) changes, and locus XL9, with more \(+2 (n = 4)\) than \(-1 (n = 3)\) or \(+1 (n = 1)\) changes. However, the numbers were small in these two cases.

The \(\lambda\) calculated for the whole strain collection or for each pathotype separately showed that there was significant linkage disequilibrium \((P < 0.001)\) between loci.

**Genetic diversity and population structure.** The discriminatory abilities of the AFLP, IS-LM-PCR, and MLVA techniques was determined and compared by calculating \(D\) for 234 strains typed by all three methods. MLVA differentiated 209 strains and showed the best level of discrimination, with a \(D\) value of 0.998. IS-LM-PCR and AFLP markers distinguished 146 strains \((D = 0.991)\) and 94 strains \((D = 0.970)\), respectively (Table 2). The combination of MLVA and IS-LM-PCR data improved the discrimination among the strains to a \(D\) value of 0.999 \(\) of 218 of 234 strains were discriminated. The three methods were more discriminative than rep-PCR based on the data from a collection of 34 strains (Table 2).

Mantel test results suggested that data derived from the three typing techniques were congruent \((P < 0.001)\) (Table 3). The highest values of correlation between individual genetic distances were observed for AFLP and IS-LM-PCR data \((r = 0.866; P < 0.001)\). Lower but highly significant values of correlation between MLVA and IS-LM-PCR data \((r = 0.618, P < 0.001)\) and MLVA and AFLP data \((r = 0.599; P < 0.001)\) were found. Correlations for individual pairs of genetic distances were also highly significant when determined by considering pathotype A and A*/Aw strains separately (Table 3).

Hierarchical AMOVA revealed highly significant genetic variation between pathotypes \((P < 0.01)\), as well as between populations or within populations, whatever the molecular markers used (Table 4). The patterns of partitioning of the

### Table 2. Discriminatory powers of AFLP, IS-LM-PCR, and MLVA methods based on results for a collection of 234 strains of *X. citri* pv. citri and a subset of 34 strains typed by rep-PCR

<table>
<thead>
<tr>
<th>Genotyping method</th>
<th>No. of haplotypes identified ((n = 234))</th>
<th>(D)</th>
<th>No. of haplotypes identified ((n = 34))</th>
<th>(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLP</td>
<td>94</td>
<td>0.970</td>
<td>19</td>
<td>0.932</td>
</tr>
<tr>
<td>IS-LM-PCR</td>
<td>146</td>
<td>0.991</td>
<td>31</td>
<td>0.994</td>
</tr>
<tr>
<td>MLVA</td>
<td>209</td>
<td>0.998</td>
<td>33</td>
<td>0.998</td>
</tr>
<tr>
<td>Rep-PCR</td>
<td>ND(^b)</td>
<td>ND</td>
<td>ND</td>
<td>0.854</td>
</tr>
</tbody>
</table>

\(^a\) Rep-PCR data are from Cubero and Graham (16).
\(^b\) ND, not determined.

### Table 3. Mantel test results for pairwise correlations between genetic distances among individual strains for the different typing methods and pairwise population genetic differentiation

<table>
<thead>
<tr>
<th>Population(s)</th>
<th>(r_A)</th>
<th>(r_B)</th>
<th>(r_C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathotype A strains</td>
<td>0.472</td>
<td>0.430</td>
<td>0.673</td>
</tr>
<tr>
<td>Pathotype A* strains</td>
<td>0.818</td>
<td>0.801</td>
<td>0.759</td>
</tr>
<tr>
<td>All populations</td>
<td>0.866</td>
<td>0.599</td>
<td>0.618</td>
</tr>
</tbody>
</table>

\(^a\) \(r_A\), AFLP versus IS-LM-PCR data; \(r_B\), AFLP versus MLVA data; and \(r_C\), IS-LM-PCR versus MLVA data. \(P < 0.001\) for all values.
total variance differed among the different data sets. For AFLP, most of the total genetic variation (66.5%) was found between pathotypes and the remainder was distributed evenly among or within populations. Most of the genetic variation indicated by IS-LM-PCR was also between pathotypes, but to a lesser extent (52.5%), and the second source of variation was found at the population level. In contrast, analysis of the MLVA data set revealed that most of the total genetic variance was found among individual strains within populations (65.5%) and that the genetic variation between pathotypes accounted for only 12.2% of the total variation (Table 4).

For the eight defined *X. citri* pv. citri pathotype A populations, significant correlation was found between the logarithmic geographical distances and $F_{ST}/(1 − F_{ST})$, as estimated from IS-LM-PCR and MLVA data sets ($P < 0.05$) but not from the AFLP data set, with correlation values of 0.536, 0.507, and 0.371, respectively.

Fisher exact tests revealed little differentiation among the eight groups of pathotype A strains. The Indian peninsula group was differentiated from the group defined for Thailand ($P < 0.05$; Fisher exact test) and, with some significance, from those defined for China and the Philippines ($P < 0.10$), whatever the genotyping method (Table 5). Genetic differentiation between pathotype A strains from Thailand and the groups from China and the Philippines ($P < 0.05$) was also observed, but only for the MLVA data set. Unlike pathotype A strains, the three defined groups of pathotype A* strains, i.e., those from Saudi Arabia, Iran, and Thailand, were found to be genetically differentiated, whatever the typing technique ($P ≤ 0.01$; Fisher exact test). Based on $F_{ST}$ analogues, when pathotypes were analyzed separately, larger differentiation estimates were obtained for pathotype A* strains than for pathotype A strains, whatever the typing technique (Table 1). Furthermore, genetic differentiation was observed in pairwise population analyses performed with biallelic markers, with AFLP-based and IS-LM-PCR-based $F_{ST}$ estimates showing 27 of 28 and 28 of 28 pairs, respectively, to be significantly different ($P < 0.01$). $F_{ST}$ estimates, when significant, varied from 0.07 (Taiwan/Thailand) to 0.62 (South Korea/the Maldives) and from 0.059 (Japan/Taiwan) to 0.67 (Japan/the Maldives) for AFLP and IS-LM-PCR, respectively. The level of genetic differentiation revealed by the MLVA-based $F_{ST}$ values and $R_{ST}$ values was lower, with 24 of 28 and 26 of 28 pairs, respectively, being significantly different ($P < 0.01$). Differentiation estimates varied from 0.07 (Philippines/Thailand) to 0.31 (Japan/the Maldives) and from 0.07 (Philippines/Thailand) to 0.81 (China/the Maldives) for MLVA-based $F_{ST}$ and $R_{ST}$ estimates, respectively. All the analyzed pairs of pathotype A* strains ($n = 3$) revealed genetic differentiation ($P < 0.01$) when $F_{ST}$ and $R_{ST}$ were estimated from biallelic markers or MLVA data. $F_{ST}$ estimates were between 0.47 and 0.87, 0.78 and 0.93, and 0.46 and 0.63 for AFLP, IS-LM-PCR, and MLVA data, respectively. MLVA-based $R_{ST}$ estimates for comparisons among pathogen A* groups varied from 0.90 to 0.95.

Bayesian clustering was used to analyze multilocus haplotypes to infer the genetic ancestry of the individual strains from both pathotypes separately. When pathotype A strains ($n = 167$) were considered, the analyses showed a pattern typical of an unstructured population, whatever the typing technique. No plateau in the estimates of the log likelihoods was reached, and no consis-

### Table 4. Hierarchical AMOVA of *X. citri* pv. citri populations (eight pathotype A and three pathotype A* groups) by using AFLP, IS-LM-PCR, and VNTR markers

<table>
<thead>
<tr>
<th>Level at which variation occurred</th>
<th>df</th>
<th>Variance by:</th>
<th>% Total variation by:</th>
<th>Φ statistic&lt;sup&gt;b&lt;/sup&gt; for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AFLP analysis</td>
<td>IS-LM-PCR analysis</td>
<td>MLVA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between pathotypes</td>
<td>1</td>
<td>7.96</td>
<td>14.61</td>
<td>0.77</td>
</tr>
<tr>
<td>Among populations within pathotypes</td>
<td>9</td>
<td>1.75</td>
<td>8.02</td>
<td>1.37</td>
</tr>
<tr>
<td>Within populations</td>
<td>173</td>
<td>2.24</td>
<td>5.19</td>
<td>4.04</td>
</tr>
<tr>
<td>Total</td>
<td>183</td>
<td>11.95</td>
<td>27.83</td>
<td>6.17</td>
</tr>
</tbody>
</table>

<sup>a</sup> df, degrees of freedom.

<sup>b</sup> ***, $P < 0.001$. Populations/geographical groups with 10 or more strains were considered.

### Table 5. Genetic differentiation between *X. citri* pv. citri pathotype A subpopulations as estimated by Fisher exact test<sup>a</sup>

<table>
<thead>
<tr>
<th>Population origin</th>
<th>China</th>
<th>Indian peninsula</th>
<th>Japan</th>
<th>South Korea</th>
<th>The Maldives</th>
<th>The Philippines</th>
<th>Taiwan</th>
<th>Thailand</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>0.016</td>
<td>0.295</td>
<td>0.362</td>
<td>0.344</td>
<td>0.062</td>
<td>0.326</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Indian peninsula</td>
<td>0.0001/0.113</td>
<td>0.062</td>
<td>0.080</td>
<td>0.080</td>
<td>0.014</td>
<td>0.065</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>1/1</td>
<td>0.458/1</td>
<td>1.000</td>
<td>1.000</td>
<td>0.265</td>
<td>1.000</td>
<td>0.069</td>
<td></td>
</tr>
<tr>
<td>South Korea</td>
<td>1/1</td>
<td>1/1</td>
<td>1.000</td>
<td>1.000</td>
<td>0.282</td>
<td>1.000</td>
<td>0.097</td>
<td></td>
</tr>
<tr>
<td>Maldives</td>
<td>0.999/1</td>
<td>1/1</td>
<td>0.998/1</td>
<td>1/1</td>
<td>0.288</td>
<td>1.000</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>Philippines</td>
<td>1/1</td>
<td>0.006/0.811</td>
<td>1/1</td>
<td>0.999/1</td>
<td>0.277</td>
<td>0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taiwan</td>
<td>1/1</td>
<td>0.996/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td>1/1</td>
<td>0.024/0.563</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> P values for the AFLP/IS-LM-PCR data sets (below the diagonal) and for the MLVA data set (above the diagonal) are given.
tency among typing techniques for the compositions of putative populations was found. On the contrary, STRUCTURE identified two to four ancestral groups within the collection of pathotype A* strains, depending on the typing technique. Observations of plateaus in the estimates of $\ln(\Pr(X|K))$ and a clear modal value in the distribution of $\bar{H}_{9004}$ indicated values of $K$ of 2, 3, and 4 for the IS-LM-PCR, AFLP, and MLVA data sets, respectively (Fig. 2). The assignments of individual pathotype A* strains to ancestral clusters were consistent among the different data sets and correlated primarily with the strains’ geographical origins. Strains from Florida, together with two strains from the Indian peninsula, were identified as a single population, whatever the typing technique. Two populations of strains from Saudi Arabia were consistently identified. One of the populations identified shared a common ancestor with strains from Iran, based on AFLP and IS-LM-PCR results, but the latter strains were unique based on MLVA data (Fig. 2). The second identified population of strains from Saudi Arabia shared a common ancestor with strains from India, Oman, Thailand, and Cambodia, whatever the genotyping technique (Fig. 2).

**DISCUSSION**

The availability of the complete genome of a Brazilian strain of *X. citri* pv. citri with a wide host range among citrus plants (pathotype A) (17) made it easier to design appropriate markers and predict the DNA fragments produced by different PCR-based techniques (9, 12, 45). This genome is relatively rich in insertion sequence elements and tandem repeats (17, 51). We evaluated the potentials of AFLP, IS-LM-PCR, and MLVA techniques as genotyping methods for molecular epidemiology studies of *X. citri* pv. citri. The analysis of the genetic diversity of large collections or populations requires discriminative, high-throughput techniques able to identify strain types and provide reproducible results. Despite the worldwide distribution and the major economic importance of this quarantine organism, no comparative evaluation of different molecular typing techniques for population structure analyses based on large strain collections has been conducted previously. Earlier studies considered primarily the usefulness of PFGE, AFLP, or rep-PCR markers in terms of their abilities to determine genetic relationships between *Xanthomonas* pathovars that are pathogenic to citrus and *X. citri* pv. citri pathovar variants (16, 19, 39, 44, 68).

All three techniques used in this study typed all strains, except in the case of the XL5 VNTR locus, for which no amplicon was obtained from a limited number of strains within pathotype A*. MLVA revealed the greatest genetic diversity (based on Nei’s and Hunter’s indices), followed by IS-LM-PCR and AFLP analyses. For instance, pathotype A* strains from Saudi Arabia and Iran were not differentiated using AFLP, while IS-LM-PCR analysis and MLVA clearly separated strains from the two origins. Furthermore, MLVA identified each Iranian strain as a haplotype. The discriminatory powers of these methods were markedly greater than that of rep-PCR, based on results for a subset of 34 strains used in our study and a previous study by Cubero and Graham (16). VNTRs are known to exhibit much higher levels of mutation than other parts of the genome (35). The discriminatory power of MLVA was indeed found to be higher than or similar to those of insertion sequence-based typing techniques in popu-

![Figure 2](image-url)
type A* strain was detected in Cambodia in 2007 (11), and this pathotype A* strains, whatever the typing technique. A pathological variants of the pathogen are yet to be discovered. A* strains. It is likely that undescribed genetic and/or pathological effects to pathotype A strains present in southeast Asia or to pathotype A strains that were genetically related either to the presence of two groups of pathotype A* strains and with the greatest genetic diversity revealed to date, with the display the greatest genetic diversity revealed to date, with the presence of two groups of pathotype A* strains and with pathotype A strains that were genetically related either to pathotype A strains present in southeast Asia or to pathotype A* strains. These strains originated from Bangladesh and India. Interestingly, they were all isolated from lime and displayed pathogenicity patterns typical of pathotype A. These observations suggest that variations in host range may have occurred in populations of X. citri pv. citri that originated from the Indian peninsula. Populations of the pathogen from this region display the greatest genetic diversity revealed to date, with the presence of two groups of pathotype A* strains and with pathotype A strains that were genetically related either to pathotype A strains present in southeast Asia or to pathotype A* strains. It is likely that undescribed genetic and/or pathological variants of the pathogen are yet to be discovered.

Clusters identified in relation to the geographical origins of the strains were consistently reproduced among X. citri pv. citri pathotype A* strains, whatever the typing technique. A pathotype A* strain was detected in Cambodia in 2007 (11), and this strain was closely related to pathotype A* strains from Thailand (10). Similarly, pathotype A* strains originating from Florida were closely related to some pathotype A* strains isolated in India: these strains belonged to a single cluster with high bootstrap values, whatever the typing technique, and none of them could be typed using the XL5 VNTR locus. In addition, STRUCTURE suggested a common ancestry for pathotype A* strains and the pathotype A* strains isolated in India. Thus, our molecular data fully support the results of investigations in Florida, which revealed that pathotype A* strains had been isolated from Mexican lime in the yard of a family that had recently arrived from India and did not reveal the source of the diseased plant (62). The distribution area of pathotype A* strains in Asia is larger than previously reported and confirms the epidemiological significance of this narrow-host-range pathotype.

Interestingly, the level of genetic diversity among pathotype A* strains was higher than that among pathotype A strains, whatever the typing technique. This finding suggests that pathotype A* may have a longer evolutionary history. Li et al. (45) analyzed a few lime herbarium specimens collected in western Asia during the first half of the 20th century, and no conclusion could be drawn. Genetic differentiation analyses indicated that pathotype A* strain populations are well-structured. The number of ancestral populations computed by STRUCTURE was genotyping technique dependent, but pathotype A* strains could originate from at least two ancestral populations. Pathotype A* strains from Saudi Arabia were consistently classified into two populations, whatever the typing technique, which may suggest at least two different incidents involving the introduction of the pathogen into this country.

Based on NJ trees and MDS, no clear geographically inferred structure among the pathotype A strains was revealed. This result is consistent with those of previous studies targeting diversity by using rep-PCR or transposable elements (16, 45). Significant patterns of isolation by distance were observed at the level of the Asian continent. The low degree of global genetic diversity and the significant isolation by distance suggest a spatially limited gene flow for pathotype A strains and/or a rather short evolutionary history.

The different levels of structuring were also reflected in the different values of differentiation estimates for subpopulations of each pathotype. The elevated \( R_{ST} \) values compared to \( F_{ST} \) estimates calculated from the MLVA data set suggested that the geographical subpopulations of X. citri pv. citri or of each pathotype differed not only in allele frequencies but also significantly in the evolutionary distance between alleles. Different estimates of \( F_{ST} \) from each molecular marker data set revealed lower values for MLVA than for the two biallelic marker systems. MLVA revealed the highest discriminatory power, as shown in this study by the identification of 209 different haplotypes among 234 strains. Estimations of \( F_{ST} \) depend partly on the level of within-population diversity, and estimates derived from highly mutable loci should be considered with caution (14). Gene flow between populations homogenizes allele frequencies and tends to decrease genetic differentiation. Furthermore, the differentiation level is not affected strictly by the mutation potential of the observed loci but also by the ratio between mutation and migration (8). This situation makes \( F_{ST} \) sensitive to the mutation rates for pathogens such as X. citri pv. citri with limited long-distance migration (31). The \( R_{ST} \) analogue values from the MLVA data set are independent of the mutation rate and are more appropriate estimates than \( F_{ST} \) values, given that tandem repeats in our scheme can be considered as evolving by the progressive gain or loss of single repeat units. \( R_{ST} \) values were in accordance with the estimates of genetic differentiation obtained from the two biallelic data sets. It is likely that the significance of the long-distance movement of infected plant material, although globally limited, is pathotype dependent. Pathotype A strains can be transported by most of the citrus species, primarily through propagative material, providing much greater potential for the exchange of pathotype A strains than for that of pathotype A* strains, which are hosted mainly by limes and, to a lesser extent, C. macrophylla. In many Asiatic countries, lime trees are produced
from seed and *C. macrophylla* is used mainly as rootstock, and the pathogen is not seed borne (15). These host characteristics drastically decrease the movement of budwood (a major source of long-distance spread). The low level of migration of pathotype A* strains, together with their narrow host range, minimizes gene flow, which can explain the greater genetic differentiation observed within A* subpopulations. This observation may also be the result of the greater genetic diversity observed within *X. citri* pv. citri pathotype A*.

Significant levels of linkage disequilibrium among the VNTR loci were observed using a multilocus estimate, which revealed the absence of frequent genetic exchanges. Given our strain collection, however, this finding should be considered with caution because specific ecological niche and/or geographical barriers may explain the limited DNA exchange observed (49). Nevertheless, these results, together with the significant congruence among data derived from the three independent genotyping techniques, suggested that both pathotypes of *X. citri* pv. citri are clonal. Testing for nonrandom association of loci on small scales should confirm the level of clonality, which may vary among populations. Extensive sampling of pathotype A* strains in the Indian peninsula, for which admixtures within some individual strains suggest different origins, may reveal a population structure which differs from that in other geographical areas.

The various discriminative powers of the three genotyping techniques made it possible to conduct molecular epidemiology analyses on different spatial scales and for different purposes. Highly variable markers, such as VNTRs, are not adapted for international long-term strain screening (70). Although the concomitant use of several typing techniques strengthens analyses, the increased discriminatory power of the IS-LM-PCR method compared to those of rep-PCR (16) and AFLP (this study) analyses makes this technique most appropriate for the global surveillance of non-epidemiologically related strains of *X. citri* pv. citri. IS-LM-PCR targets transposable elements, similar to the technique developed by Li et al. (45). IS-LM-PCR seems superior because of the smaller number of PCR amplifications required. Furthermore, in contrast with the method used by Li et al. (45), IS-LM-PCR was proved previously to be very useful for the discrimination of anthrax-causing bacterial populations with a low level of genetic diversity (38). We are currently evaluating MLVA for the molecular epidemiology of *X. citri* on different spatial scales and in different epidemiological contexts (such as integrated management and eradication).

ACKNOWLEDGMENTS

We thank E. L. Civerolo for helpful discussion and K. Vital, C. Boyer, and V. Ledoux for their technical expertise. The European Union (FEOGA and FEDER), Conseil Régional de La Réunion, and CIRAD provided financial support. The platform “Genotyping of Pathogens and Public Health” acknowledges financial support from the Institut Pasteur (Paris, France) and the Institut de Veille Sanitaire (Saint-Maurice, France).

REFERENCES

40. Hunter, P. R., and M. A. Gaston. 


66. Slatkin, M. 1995. A measure of population subdivision based on micro-

67. Struelens, M. J., Y. De Gheldre, and A. Deplano. 1998. Comparative and
library epidemiologic typing systems: outbreak investigations versus surveil-

W. N. Dixon, T. S. Schubert, P. H. Chaloux, V. K. Stromberg, G. H. Lacy,
and B. D. Sutton. 2004. Detection and characterization of a new strain of
citrus canker bacteria from key Mexican lime and Alemow in South Florida.
Plant Dis. 88:1179–1188.

69. Van Belkum, A. 2002. Molecular typing of micro-organisms: at the centre of
diagnostics, genomics and pathogenesis of infectious diseases? J. Med. Mi-
crobiol. 51:7–10.

2001. Role of genomic typing in taxonomy, evolutionary genetics, and mi-

from amplified fragment length polymorphism (AFLP) markers show indi-
cation of size homoplasy and of a relationship between degree of homoplasy

Maestri, and J. Luisetti. 1998. Characterization of phenotypically distinct
strains of Xanthomonas axonopodis pv. citri from Southwest Asia. Eur. J.

73. Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Vandelee, M. Hornes, A.

74. Wicker, E., L. Grassart, R. Coranson-Beaudu, D. Mian, C. Guilbaud, M.
Fegan, and P. Prior. 2007. Ralstonia solanacearum strains from Martinique
(French West Indies) exhibiting a new pathogenic potential. Appl. Environ.
Microbiol. 73:6790–6801.

the epidemiology and evolution of species jumps. Trends Ecol. Evol. 20:238–
244.