Polyphasic characterization of xanthomonads pathogenic to members of the Anacardiaceae and their relatedness to species of *Xanthomonas*

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We have used amplified fragment length polymorphism (AFLP), multilocus sequence analysis (MLSA) and DNA–DNA hybridization for genotypic classification of *Xanthomonas* pathovars associated with the plant family Anacardiaceae. AFLP and MLSA results showed congruent phylogenetic relationships of the pathovar *mangiferae-indicae* (responsible for mango bacterial canker) with strains of *Xanthomonas axonopodis* subgroup 9.5. This subgroup includes *X. axonopodis* pv. *citri* (synonym *Xanthomonas citri*). Similarly, the pathovar *anacardii*, which causes cashew bacterial spot in Brazil, was included in *X. axonopodis* subgroup 9.6 (synonym *Xanthomonas fuscans*). Based on the thermal stability of DNA reassociation, consistent with the AFLP and MLSA data, the two pathovars share a level of similarity consistent with their being members of the same species. The recent proposal to elevate *X. axonopodis* pv. *citri* to species level as *X. citri* is supported by our data. Therefore, the causal agents of mango bacterial canker and cashew bacterial spot should be classified as pathovars of *X. citri*, namely *X. citri* pv. *mangiferae-indicae* (pathotype strain CFBP 1716) and *X. citri* pv. *anacardii* (pathotype strain CFBP 2913), respectively. *Xanthomonas fuscans* should be considered to be a later heterotypic synonym of *Xanthomonas citri*.

**INTRODUCTION**

Mango bacterial canker (MBC) (also called mango bacterial black spot) is one of the most important bacterial diseases for mango (*Mangifera indica* L.) industries worldwide (Gagnevin & Pruvost, 2001). MBC was first described in 1915 in South Africa (Doidge, 1915) but may have originated in India, as the disease was observed in herbarium specimens collected in Bihar in 1881 (Patel et al., 1948a, b). The causal agent of MBC was first reported as ‘Bacillus mangiferae’ (Doidge, 1915) and, in 1948, was designated ‘*Pseudomonas mangiferae-indicae*’ (Patel et al., 1948a, b). In the 1970s, the pathogen was named *Xanthomonas campestris* pv. *mangiferae-indicae* (Robbs et al., 1974), in compliance with the international standards for naming pathovars of phytopathogenic bacteria of the International Society for Plant Pathology (Dye et al., 1980).
Some species of the plant-pathogenic genus *Xanthomonas* are subdivided into pathovars. The pathovar classification established by Dye *et al.* (1980) and reviewed by Young *et al.* (1992) is an infrasubspecific classification applied to bacterial plant pathogens by reference to their host range or to their capacity to cause distinctive symptoms. Pathogenicity tests are an essential part of this classification. Pathovar nomenclature is not covered by the International Code of Nomenclature of Prokaryotes (hitherto the International Code of Bacteria; Lapage, 1992). Pathovar names are usually derived from the host plant, but they are not accompanied by a species name. In 1995, reclassification of the genus *Xanthomonas* by a polyphasic approach including DNA–DNA hybridization (DDH) assigned strains into 20 genomospecies (Vauterin *et al.* 1995). More recent results have increased the number of genomospecies to 27 (Jones *et al.*, 2004, 2006; Schaad *et al.*, 2006; Trébaol *et al.*, 2000), but many pathovars, including pathogens of major economic importance such as pv. *mangiferaeindicae*, have not been investigated. Studies based on 16S rRNA gene sequences showed that several genomospecies, as determined by DDH, shared more than 99% sequence identity, making this technique inadequate for species differentiation in the genus *Xanthomonas* (Hauben *et al.*, 1997; Moore *et al.*, 1997). Rademaker *et al.* (2000) showed that amplified fragment length polymorphism (AFLP) analysis and repetitive extragenic palindromic PCR (rep-PCR) data positively correlated with DDH. These two genotyping techniques can be used for routine species identification and can be included in a polyphasic scheme for describing novel species or combinations (Rademaker *et al.*, 2005; Roumagnac *et al.*, 2004; Stackebrandt *et al.*, 2002).

One of the *Xanthomonas* genomospecies, *Xanthomonas axonopodis*, displayed a higher intraspecific heterogeneity based on DDH as well as AFLP and rep-PCR data, and six genetic clusters were described within this species (Rademaker *et al.*, 2000, 2005). Some members of these genetic clusters have been elevated to species rank (*Xanthomonas euvesicatoria, X. perforans, X. alfalfae, X. citri* and *X. fuscans*) (Jones *et al.*, 2004, 2006; Schaad *et al.*, 2005, 2006, 2007). These assignments were based on a polyphasic approach including DDH experiments using the nuclease S1 procedure (Crosa *et al.*, 1973) performed at Tm=15 °C.

DDH may be considered the gold standard method for genotypic delineation of bacterial species (Wayne *et al.*, 1987). AFLP, a technique also recommended for bacterial taxonomy (Stackebrandt *et al.*, 2002), has the advantage of generating a large number of randomly located markers over the whole genome. AFLP was useful for evaluating the species status of several genera (Aabenhus *et al.*, 2005; Hong *et al.*, 2005; Huys *et al.*, 2000; Leal-Klevezas *et al.*, 2005; Mougel *et al.*, 2002; Oñ *et al.*, 2003; Thompson *et al.*, 2003), including xanthomonads (Janssen *et al.*, 1996; Rademaker *et al.*, 2000; Roumagnac *et al.*, 2004; Boudon *et al.*, 2005; Schaad *et al.*, 2005). Based on AFLP data, evolutionary genome divergences (EGD) or current genome mispairing (CGM) provide a measurement of genetic divergences between genomes (Mougel *et al.*, 2002). Recently, multilocus sequence analysis (MLSA), based on sequence analysis of several housekeeping genes, has been developed for species delineation (Geyers *et al.*, 2005; Hanage *et al.*, 2005a, b; Richter *et al.*, 2006; Chelo *et al.*, 2007; Martens *et al.*, 2007). MLSA has the advantage of analysing phylogenetic relationships of large sets of strains with a better portability than genotyping techniques such as AFLP.

The purpose of this study was to perform a detailed genetic characterization, based on DDH, AFLP and MLSA, of the pathovar *mangiferaeindicae sensu* Dye *et al.* (1980) and to evaluate its relatedness to different *Xanthomonas* species. We show that the causal agent of MBC is genetically related to *X. citri* (syn. *X. axonopodis* group 9.5). Our data support the elevation of *X. axonopodis* pv. *citri* as *X. citri* and show that the causal agents of MBC and cashew bacterial spot (CBS) should be classified as pathovars of this genomospecies, namely *X. citri* pv. *mangiferaeindicae* and *X. citri* pv. *anacardi*., respectively.

**METHODS**

**Bacterial strains and media.** *Xanthomonas* strains isolated from members of several plant genera within the family Anacardiaceae, the type strains of 27 *Xanthomonas* species (Jones *et al.*, 2004; Schaad *et al.*, 2006; Trébaol *et al.*, 2000; Vauterin *et al.*, 1995) and some additional *Xanthomonas axonopodis* pathovars were used in this study (Supplementary Table S1, available in IJSEM Online). Some strains included in this study were deposited in the Collection Française de Bactéries Phytopathogènes (CFBP, INRA Angers, France), the BCCM/LMG (Belgian Coordinated Collections, University of Ghent, Belgium) and the National Collection of Plant Pathogenic Bacteria (NCPBB, CSL, York, UK). Cultures were stored after lyophilization and/or in a –80 °C freezer. They were checked for purity and routinely cultivated on YPGA (1 %, 7 g yeast extract, 7 g peptone, 7 g glucose, 18 g agar, 20 mg propiconazole; pH 7.2) at 28 °C, except for *Xanthomonas populi* strains, which were grown at 19 °C. Strains that grew poorly on YPGA were cultivated on modified Wilbrink medium (Rott *et al.*, 1988).

**AFLP analysis.** Genomic DNA was extracted from bacteria using the DNeasy tissue kit (Qiagen) following the manufacturer’s instructions and DNA concentrations were estimated by fluorimetry (TKO 100 fluorometer; Hoefer). AFLP experiments were performed in 96-well plates in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) and DNA concentrations were estimated by fluorimetry (TKO 100 fluorometer; Hoefer). AFLP experiments were performed in 96-well plates in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems), as described previously (Ah-You *et al.*, 2007). Digestions were carried out in a 25 µl volume for 1 h at 37 °C and contained 100 ng bacterial genomic DNA, 10 U Sau3A, 2 U MspI (New England Biolabs) and 1 x BSA in 1 x reaction buffer NEB I. Next, 2.5 µl of the digested products was added to 22.5 µl of a ligation mixture containing 2 µM MspI adaptor (Supplementary Table S2), 0.2 µM SclI adaptor (Supplementary Table S2) (Applied Biosystems) and 2 U T4 DNA ligase (New England Biolabs) in 1 x T4 DNA ligation buffer. Ligations were performed for 3 h at 37 °C before enzyme inactivation at 65 °C for 10 min. Ligation products were diluted 10-fold with

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HPLC-grade water before preselective PCR. The reactions were done in 15 μl and contained 5 μl diluted ligation product, 2.5 mM MgCl₂, 0.23 μM each of the MspI and SacI primers (Supplementary Table S2), 0.45 mM of each dNTP (New England Biolabs) and 0.5 U Taq DNA polymerase (Goldstar Red; Eurogentec) 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 2 min, 25 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. PCR products were diluted 10-fold with HPLC-grade water before selective amplification.

Selective amplifications using the unlabelled MspI+A, C, T or G primer and the labelled SacI+C primer (with four different fluorochrome groups) (Supplementary Table S2) 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 followed by 37 cycles at 94 °C for 30 s, annealing for 30 s at 65 °C for the first cycle, decreased by 0.7 °C per cycle for the next 12 cycles and then 56 °C for the last 24 cycles and extension at 72 °C for 2 min, with 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 18.7 μl formamide and 0.3 μl LIZ500 DNA ladder (Applied Biosystems) as an internal standard. The mixture was 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) using performance-optimized polymer POP-4 at 15000 V for about 20 min at 60 °C, with an initial injection of 66 s. AFLP fingerprints were analysed visually using GENESCAN software 3.7 (Applied Biosystems). To test the reproducibility of the AFLP technique, two independent DNA extractions were used for all strains, and strain 306 of X. citri pv. citri (Da Silva et al., 2002) was used as a control in each AFLP experiment.

The presence and absence of fragments were scored as a binary matrix. The threshold for assigning a peak was set to 200 relative fluorescence units. EGD were calculated from Dice similarity indices and corrected to account for unobserved substitutions by using the standard Jukes–Cantor model, which assumes equal rates of substitution between all pairs of bases (Mougel et al., 2002; Portier et al., 2006). EGD values were used as distances to construct a concatenated sequence alignment using the dpd, geneconv, bootscans, maximum chi-squared, chimaera and sister scan recombination detection methods as implemented in the RDP3 software (Martin et al., 2005). The analysis was performed with default settings for each different detection methods and a Bonferroni-corrected P-value cutoff of 0.05. Recombination events were accepted when detected with three detection methods or more. The breakpoint positions and recombinant sequence(s) inferred for every detected potential recombination event were checked visually and adjusted where necessary by using the extensive phylogenetic and recombination signal analysis features available in RDP3.

Maximum-likelihood (ML) trees were calculated for each of the three genes by using PAUP⁺ (version 4.0b10). The Shimodaira–Hasegawa (S–H) method (Shimodaira & Hasegawa, 1999), as implemented in PAUP⁺, was used to test whether the tree topologies based on each locus fall within the same confidence limits.

Phylogenetic analyses including NJ and ML trees of the alignment of the concatenated gene sequences were conducted both in PAUP⁺ and in PHYML (Guindon & Gascuel, 2003). The model of substitution was chosen using the R software and the AIC package implemented in R (Paradis, 2006) and PHYML. Bootstrap analyses were done with 1000 replicates for NJ and ML. For the Bayesian approach, MrBayes software (version 3.1.2) (Huelsenbeck & Ronquist, 2001) was used. Two runs with four Markov chains (using default heating values) consisting of 7.0 × 10⁶ generations starting from a random initial tree were run simulta-
Table 1. Genetic relatedness between xanthomonads pathogenic to members of the Anacardiaceae and selected type strains

Data marked AFLP are EGD values (Mougel et al., 2002) based on four combined AFLP conditions. The mean EGD value (derived from AFLP) among 15 strains of pathovar mangiferaeindicae was 0.010 nsps (sd=0.0029). The mean EGD value among four strains of pathovar anacardii was 0.016 nsps (sd=0.0093). Data marked MLSA are genetic distances based on the evolution model GTR+G+I, based on concatenated sequences (Paradis, 2006). PT, Pathotype strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pathovar</th>
<th>X. axonopodis LMG 982T</th>
<th>X. citri LMG 9322T</th>
<th>X. fuscans LMG 826T</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFBP 2913PT</td>
<td>anacardii</td>
<td>0.1285</td>
<td>0.0710</td>
<td>0.0455</td>
</tr>
<tr>
<td>LA100</td>
<td>anacardii</td>
<td>0.1340</td>
<td>0.0667</td>
<td>0.0422</td>
</tr>
<tr>
<td>CFBP 1716PT</td>
<td>mangiferaeindicae</td>
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<td>0.0770</td>
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<tr>
<td>JN570</td>
<td>mangiferaeindicae</td>
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<td>JV1121</td>
<td>mangiferaeindicae</td>
<td>0.1600</td>
<td>0.0503</td>
<td>0.0767</td>
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<td>CFBP 2547PT</td>
<td>spondiae</td>
<td>0.1194</td>
<td>0.1088</td>
<td>0.1056</td>
</tr>
<tr>
<td>CFBP 2623</td>
<td>spondiae</td>
<td>0.1175</td>
<td>0.1050</td>
<td>0.0996</td>
</tr>
</tbody>
</table>

RESULTS

AFLP analysis

Cluster analysis, based on 1308 AFLP fragments, placed most Xanthomonas genomospecies in distinct lineages, supported by high bootstrap values (>80 %) (Fig. 1). Strains identified as pathovars of X. axonopodis were distributed in six clusters, corresponding to subgroups 9.1 to 9.6 sensu Rademaker et al. (2005). Each subgroup was supported by bootstrap values above 80 %. Strains associated with members of the Anacardiaceae (indicated in bold in Fig. 1) were heterogeneous and grouped in three of the six subgroups in X. axonopodis. Among strains pathogenic to members of the Anacardiaceae, pathovar mangiferaeindicae from mango (CFBP 1716, 2916, 2927, 2932, 2933 and 2935, A11-1, JF30-1, JK147-1, JN570 and JV1121) and Brazilian pepper (CFBP 2938 and 2940 and JP758) was most closely related to subgroup 9.5 (synonym X. citri) (Table 1), with EGD ≥ 0.042 nucleotide substitutions per site (nsps), and constituted a novel clade within this subgroup (bootstrap 100 %) (Fig. 1). Pathovar anacardii from cashew (LA98 and LA100) and mango (CFBP 2913 and 2914) grouped with strains identified as subgroup 9.6 (synonym X. fuscans) (Table 1), with EGD ≥ 0.025 nsps, and pathovar spondiae from ambarella was most closely related to X. axonopodis subgroup 9.4 (with EGD ≥ 0.048 nsps).

Comparisons between the type strain of X. axonopodis (LMG 982T) and the pathotype strains of pathovars anacardii (CFBP 2913), mangiferaeindicae (CFBP 1716) and spondiae (CFBP 2547) suggested that strains pathogenic to members of the Anacardiaceae should not be classified as X. axonopodis (EGD ≥ 0.118 nsps) (Table 1). The type strains of X. citri and X. fuscans diverged by 0.080 nsps.

Subgroup 9.5 contained strains of pathovars baumhiniace, cajani, citri, citoriae, desmodialiisflori, glycines, malvaceaearum and mangiferaeindicae. Pathovar mangiferaeindicae
was most closely related to pathovar *citri*. Subgroup 9.6 contained strains of pathovars *anacardii*, *aurantifolii*, *cajani*, *dieffenbachiae* and *phaseoli* var. *fuscans*. Strains of pathovar *anacardii* were most closely related to *X. axonopodis* pv. *aurantifolii*. Strains of pathovar *spondiae* were most closely related to the pathotype strain of *X. axonopodis* pv. *dieffenbachiae* (subgroup 9.4).

**Multilocus sequence analysis**

The nucleotide identity of the three gene portions ranged from 96.53% (*gyrB*) to 96.94% (*atpD*) (Table 2).

Nucleotide transitions exceeded transversions, with ratios ranging from 1.47 (SD=1.50) (*atpD*) to 3.51 (SD=2.77) (*gyrB*). The $K_T/K_S$ ratios of the three genes ($<1$) indicated that these genes are under purifying selection (i.e. a type of selection in which genetic diversity decreases as the population stabilizes on a particular trait value). No recombination event was detected within *X. citri*.

The S–H test performed on ML trees from each gene showed that the topologies of the *atpD*, *dnaK* and *gyrB* ML trees fell within the same confidence interval as that of the concatenated dataset (1000 bootstraps). ML trees based on
Characterization of xanthomonads from the Anacardiaceae

Table 2. Sequence variation for three housekeeping genes within Xanthomonas strains used in this study

Transition/transversion ratios (Ts/Tv) were determined using Kimura’s two-parameter method (Kimura, 1980). Synonymous (Ks) and non-synonymous (Kn) substitution rates were determined using the method of Nei & Gojobori (1986). Values in parentheses are standard deviations. The values of Tajima’s D are not significant (P>0.10).

<table>
<thead>
<tr>
<th>Gene</th>
<th>atpD</th>
<th>gyrB</th>
<th>dnaK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (bp)</td>
<td>747</td>
<td>773</td>
<td>762</td>
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<tr>
<td>Sequence identity (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleotide</td>
<td>96.96</td>
<td>96.57</td>
<td>96.81</td>
</tr>
<tr>
<td>Amino acid</td>
<td>99.26</td>
<td>99.55</td>
<td>99.67</td>
</tr>
<tr>
<td>Ts/Tv</td>
<td>1.4667 (1.5185)</td>
<td>3.5412 (2.7858)</td>
<td>3.1145 (1.8021)</td>
</tr>
<tr>
<td>Ks</td>
<td>0.0084 (0.0072)</td>
<td>0.0020 (0.0030)</td>
<td>0.0015 (0.0014)</td>
</tr>
<tr>
<td>Kn</td>
<td>0.1235 (0.0740)</td>
<td>0.1666 (0.1053)</td>
<td>0.1509 (0.0806)</td>
</tr>
<tr>
<td>Ks/Kn</td>
<td>0.0678 (0.0478)</td>
<td>0.0119 (0.0118)</td>
<td>0.0102 (0.0239)</td>
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<tr>
<td>Tajima’s D</td>
<td>−0.529</td>
<td>−0.618</td>
<td>−0.124</td>
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</tbody>
</table>

When using concatenated sequences, the general time reversible (GTR) model with gamma (Γ) variations and a proportion of invariable sites (I) was the most suitable model, based on the Akaike information criterion. This model was used for building NJ, ML and Bayesian trees. The three methods gave congruent results in tree topologies, supported by high bootstrap and probability values. Based on the S–H test, the best tree likelihood was obtained with the ML method. All strains of X. axonopodis sensu Vauterin et al. (1995), together with X. euvesicatoria and X. perforans (Jones et al., 2004, 2006), clustered in a very robust but heterogeneous group. Consistent with data from single gene sequences, strains of pathovars mangiferaeindicae and anacardii, both pathogenic to members of the Anacardiaceae, was 0.020 nsps. X. axonopodis subgroup 9.5 and 9.6 constituted robust clades, as did the clade composed of the two subgroups (Fig. 2).

Distances between the pathotype strains of pathovars mangiferaeindicae, anacardii and spondiae (pathogenic to members of the Anacardiaceae) and the type strain of X. axonopodis sensu Vauterin et al. (1995) were 0.041, 0.048 and 0.045 nsps, respectively. These strains from members of the Anacardiaceae were also distantly related to the type strain of the recently described Xanthomonas species X. perforans, X. euvesicatoria and X. gardneri, with distances ranging from 0.053 to 0.078 nsps.

DDH and ΔTm values

The level of DNA reassociation between pv. mangiferaeindicae pathotype strain CFBP 1716 (labelled) and the type strains of most selected species of the genus Xanthomonas was below 40 %. ΔTm values obtained between CFBP 1716 and the type strains of Xanthomonas oryzae, X. melonis and X. axonopodis were >5.0 °C (Table 3). Other ΔTm results (Table 3) indicated that the pathotype strains of X. axonopodis pathovars citri (CFBP 2525), anacardii (CFBP 2913) and mangiferaeindicae (CFBP 1716) should not be classified as members of X. axonopodis and should be classified within a single species.

Sequencing of the 16S rRNA gene

A fragment of 1545 bp was amplified from strains CFBP 1716 (pv. mangiferaeindicae), CFBP 2547 (pv. spondiae)
Fig. 2. ML tree derived from the GTR + Γ + I model, based on concatenated partial atpD, dnaK and gyrB sequences, showing the relationships between X. axonopodis subgroups (Rademaker et al., 2000, 2005) and xanthomonads pathogenic to members of the Anacardiaceae. Branches with bootstrap values lower than 80% are represented by dotted lines. Isolation sources of strains pathogenic to members of the Anacardiaceae (in bold) are indicated by * (mango), † (Brazilian pepper), ‡ (cashew) and § (ambarella).
Table 3. Levels of DNA–DNA reassociation between the pathotype strain of pathovar manguiferaeindicae and selected Xanthomonas type strains and pathotype strains

Values in italics indicate that the considered bacteria should be classified within a single species, according to the defined threshold for bacterial species (Wayne et al., 1987). Values in bold indicate that the considered bacteria should be classified within separate species. PT, Pathotype strain; RBR, relative binding ratio; ND, not determined.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Current nomenclature</th>
<th>RBR (%) with labelled DNA from CFBP 1716&lt;sup&gt;PT&lt;/sup&gt;</th>
<th>ΔT&lt;sub&gt;m&lt;/sub&gt;(°C) with labelled DNA from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>CFBP 1716&lt;sup&gt;PT&lt;/sup&gt;</td>
</tr>
<tr>
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<td>ND</td>
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<tr>
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<td>X. axonopodis pv. anacardii</td>
<td>ND</td>
<td>2.5</td>
</tr>
<tr>
<td>LMG 7387</td>
<td>X. axonopodis pv. cajani</td>
<td>ND</td>
<td>3.5</td>
</tr>
</tbody>
</table>

and CFBP 2913 (pv. anacardii). Their respective DNA sequences shared more than 99% similarity with members of the X. campestris rRNA gene core and were more distantly related to the Xanthomonas sacchari and Xanthomonas translucens core. The sequence obtained for CFBP 1716 differed from those of strains CFBP 2913 and CFBP 2547 by six and five nucleotides, respectively. It was most closely related to those of X. axonopodis pv. citri strain 306 (GenBank accession no. NC_003919) and Xanthomonas vascula LMG 736<sup>T</sup> (Y10755), with a sequence difference of one nucleotide. In contrast, the sequences of strains CFBP 2913 and CFBP 2547 were most closely related to those of X. perforans XY938<sup>T</sup> (GenBank accession no. AF123091) or X. euvesicatoria XY153 (AF123089) (the latter two sequences were 100% identical), from which they differed by three and four nucleotides, respectively.

**DISCUSSION**

Our study aimed to determine the taxonomic position of X. campestris pv. manguiferaeindicae sensu Dye et al. (1980). We used AFLP and MLSA on a broad collection of strains to define a relevant subset of strains to be used in DDH experiments in order to refine relationships of these strains in relation to Xanthomonas species and the infraspecific genetic clusters of X. axonopodis reported by Rademaker et al. (2000) and Roumagnac et al. (2004). Variations in pathogenicity within X. campestris pv. manguiferaeindicae supported its partition into three pathovars, namely pv. manguiferaeindicae, pv. anacardii and pv. spondiae (Ah-You et al., 2007). The AFLP and MLSA data supported placement of these three pathovars separately in three of the six subgroups defined within X. axonopodis (Rademaker et al., 2005). However, divergence between the type strain of X. axonopodis sensu Vauterin et al. (1995) and strains of pathovars anacardii, manguiferaeindicae and spondiae suggested that their assignment to X. axonopodis would be incorrect. Data from both techniques indicated that pathovars manguiferaeindicae and anacardii were most closely related to X. citri (syn. X. axonopodis subgroup 9.5) and X. fuscans (syn. X. axonopodis subgroup 9.6), respectively. The strains of pathovar spondiae were most closely related to subgroup 9.4 of X. axonopodis by AFLP, but equidistantly related to subgroups 9.1 and 9.4 by MLSA. This difference complicates the classification of these strains and illustrates the relatively close relationships within group 9 (Vauterin et al., 1995). Additional analysis, including an extended MLSA scheme, may clarify the classification of the strains of pathovar spondiae. With the exception of this example, pathovar assignment to X. axonopodis subgroups was identical by AFLP and MLSA, and our data support conclusions based on previously published rep-PCR data (Rademaker et al., 2005).

Sequence-based analyses of the structure of the genus Xanthomonas have been published, but they have targeted either the ribosomal operon (Goncalves & Rosato, 2002; Hauben et al., 1997; Schaad et al., 2005) or a single housekeeping gene (Cubero & Graham, 2004). To our knowledge, our study is the first step towards an MLSA
scheme for the genus *Xanthomonas*. The three studied housekeeping genes (*dnaK*, *gyrB* and *atpD*) were under purifying selection, and no recombination event concerned members of *X. citri*. All three single-sequence analyses yielded ML tree topologies non-significantly different from that derived from the concatenated dataset based on the S–H test, indicating that the observed groups were congruent. When using concatenated gene datasets, NJ, ML and Bayesian trees were of similar structures, with ML having the highest likelihood (S–H test). MLSA data (Fig. 2) were consistent with AFLP results (Fig. 1), and both techniques appeared to be powerful tools for studying the taxonomy of *Xanthomonas*.

We performed DDH, using the nuclease S1/TCA method under internationally recommended stringency conditions (*T_m*–25 °C) (Johnson, 1984), and strengthened our results, when appropriate, by evaluating the thermal stability of DNA reassociation (Δ*T_m*), which is recommended for species delineation, especially when DDH values are in the range 50–75% (Crosa et al., 1973; Grimont et al., 1980; Grimont, 1988). Based on AFLP data, 11 genomospecies were selected for DDH experiments (*X. axonopodis*, *X. citri*, *X. codiaeae*, *X. vesicatoria*, *X. campestris*, *X. melonis*, *X. translucens*, *X. sacchari*, *X. cynarae*, *X. cassavae* and *X. oryzae*). When the pathotype strain of pathovar *mangiferaeindicae* was labelled, most species had DDH values lower than 40%. Δ*T_m* values with the three species that were most closely related to pathovar *mangiferaeindicae* (*X. oryzae*, *X. melonis* and *X. axonopodis*) were greater than the widely accepted threshold of 5 °C for species delineation (Wayne et al., 1987). The relationships between the pathotype strains of pathovars *mangiferaeindicae* and *citri* and the type strain of *X. axonopodis sensu* Vauterin et al. (1995) were further examined. Whatever the labelled strain, Δ*T_m* values between the type strain of *X. axonopodis* and these two members of subgroup 9.5 were greater than 5 °C; the only exception was hybridization of *X. axonopodis* (labelled) to pv. *mangiferaeindicae*, which indicated a Δ*T_m* of 4.5 °C. Δ*T_m* values in the range 6–7 °C (obtained between *X. citri* and *X. axonopodis*) corresponded to EGD values (derived from AFLP) of about 0.12 and to genetic distances (derived from MLSA) of about 0.04 nsps. Our data fully support the elevation of *X. axonopodis* pv. *citri* to species rank, as *X. citri* (ex Hasse 1915) Gabriel et al. 1989 emend Schaad et al. 2006.

Reciprocal DDH and thermal stability of DNA reassociation showed a close relationship between pathovars *mangiferaeindicae* and *citri*, indicating that these pathovars should both be part of the *X. citri* genomospecies. The distances between these strains by AFLP and MLSA were fully consistent with Δ*T_m* results (Table 1). Based on AFLP and MLSA data, all pathovars of *X. axonopodis* subgroup 9.5 formed a homogeneous group, with AFLP distances ranging from 0.042 to 0.065 nsps and MLSA distances lower than 0.005 nsps. We therefore propose that all pathovars presently identified as *X. axonopodis* subgroup 9.5 sensu Rademaker et al. (2005) should be reclassified as pathovars of *X. citri*.

Values of Δ*T_m* between the pathotype strain of pathovar *mangiferaeindicae* (subgroup 9.5) and strains CFBP 2913 and LA98 of pathovar *anacardii* (subgroup 9.6) were 2.0 and 2.5 °C, respectively. These values are below the 5 °C threshold for species delineation (Wayne et al., 1987), suggesting that they are members of the same species. These Δ*T_m* values corresponded to distances of 0.062 and 0.073 nsps (AFLP) and 0.020 and 0.021 nsps (MLSA), respectively.

Our AFLP and MLSA results confirmed the genetic relatedness of *X. citri* and *X. fuscans* (*X. axonopodis* subgroups 9.5 and 9.6, respectively), already outlined using different AFLP conditions (Rademaker et al., 2000; Roumagnac et al., 2004) and rep-PCR (Rademaker et al., 2005). Recently, Schaad et al. (2005) proposed the elevation of two pathovars of *X. axonopodis* subgroup 9.6 to species level as *X. fuscans*. Distances derived from AFLP and MLSA between the type strains of *X. citri* and *X. fuscans* were slightly greater but similar to distances between the pathotype strains of pathovars *anacardii* and *mangiferaeindicae* (Table 4). Δ*T_m* values for the latter strains were only 2.0–2.5 °C. Globally, our data do not support the classification of *X. axonopodis* subgroup 9.6 as *X. fuscans*. We therefore propose that this name be considered to be a later synonym of *X. citri*, for which an emended description is provided below.

**Emended description of Xanthomonas citri** (ex Hasse 1915) Gabriel et al. 1989

*Xanthomonas citri* (ci’tri. L. gen. n. citri of citrus).

Later heterotypic synonym: *Xanthomonas fuscans* Schaad et al. 2007.

The description of the species *X. citri* is encompassed by the description of the genus *Xanthomonas* Dowson 1939 emend. Vauterin et al. (1995) and by the description provided by Gabriel et al. (1989). *X. citri* can be differentiated from all other *Xanthomonas* species by DDH assays (Schaad et al., 2005; this study), rep-PCR profiles (Rademaker et al., 2000, 2005), AFLP (Rademaker et al., 2000; Roumagnac et al., 2004; Schaad et al., 2005; this study) and MLSA (this study). *X. citri* is composed of strains previously identified as *X. axonopodis* clusters 9.5 and 9.6 (Rademaker et al., 2000, 2005). *X. citri* comprises several pathovars, namely *X. citri* pv. *anacardii*, *X. citri* pv. *aurantifolii* (ci’tri B, C and D groups), *X. citri* pv. *buhiniae*, *X. citri* pv. *cajani*, *X. citri* pv. *citri* (ci’tri A group), *X. citri* pv. *clitoriae*, *X. citri* pv. *desmodii*axiliformi, *X. citri* pv. *dieffenbachiae* (strains not pathogenic to anthurium), *X. citri* pv. *glycines*, *X. citri* pv. *malvacearum*, *X. citri* pv. *mangiferaeindicae*, *X. citri* pv. *phasioli* var. *fuscans*, *X. citri* pv. *rhynchosiae*, *X. citri* pv. *sesbaniae*, *X. citri* pv. *vignae*ridatiae and *X. citri* pv. *vignicola*. The DNA G+C content is 64.6–67.5 mol% (Swings & Civerolo, 1993).
Table 4. Genetic relatedness between members of *X. axonopodis* groups 9.5 (syn. *X. citri*) and 9.6 (syn. *X. fuscans*)

Data marked AFLP are EGDs (Mougel *et al.*, 2002) based on four combined AFLP conditions. Data marked MLSA are genetic distances based on the evolution model GTR + Γ + I, based on concatenated sequences (Paradis, 2006). Comparisons between the pathotype strain of pv. *mangiferaeindicae* (CFBP 1716) and two strains of pv. *anacardii* (LA98 and CFBP 2913) indicated that EGD (derived from AFLP) values of 0.062 and 0.073 nsps corresponded to distances (MLSA) of 0.021 and 0.020 nsps and to ΔT$_{in}$ values of 2.0 and 2.5 °C, respectively. PT, Pathotype strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pathovar</th>
<th>pv. <em>anacardii</em></th>
<th>pv. <em>phaseoli var. fuscans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CFBP 2913&lt;sup&gt;PT&lt;/sup&gt;</td>
<td>LA100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AFLP</td>
<td>MLSA</td>
</tr>
<tr>
<td>LMG 558&lt;sup&gt;PT&lt;/sup&gt;</td>
<td><em>cajani</em></td>
<td>0.0673</td>
<td>0.0197</td>
</tr>
<tr>
<td>LMG 9322&lt;sup&gt;T&lt;/sup&gt;</td>
<td><em>citri</em></td>
<td>0.0710</td>
<td>0.0210</td>
</tr>
<tr>
<td>CFBP 2525&lt;sup&gt;PT&lt;/sup&gt;</td>
<td><em>citri</em></td>
<td>0.0724</td>
<td>0.0210</td>
</tr>
<tr>
<td>306*</td>
<td><em>citri</em></td>
<td>0.0715</td>
<td>0.0210</td>
</tr>
<tr>
<td>LMG 9045&lt;sup&gt;PT&lt;/sup&gt;</td>
<td><em>clitoriae</em></td>
<td>0.0761</td>
<td>0.0202</td>
</tr>
<tr>
<td>LMG 9046&lt;sup&gt;PT&lt;/sup&gt;</td>
<td><em>desmodiilaxiflori</em></td>
<td>0.0805</td>
<td>0.0210</td>
</tr>
<tr>
<td>LMG 712&lt;sup&gt;PT&lt;/sup&gt;</td>
<td><em>glycines</em></td>
<td>0.0818</td>
<td>0.0208</td>
</tr>
<tr>
<td>LMG 8026</td>
<td><em>glycines</em></td>
<td>0.0880</td>
<td>0.0208</td>
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<tr>
<td>LMG 761&lt;sup&gt;PT&lt;/sup&gt;</td>
<td><em>malvacearum</em></td>
<td>0.0804</td>
<td>0.0210</td>
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<tr>
<td>LMG 7429</td>
<td><em>malvacearum</em></td>
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<td>0.0210</td>
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<td>CFBP 1716&lt;sup&gt;PT&lt;/sup&gt;</td>
<td><em>mangiferaeindicicae</em></td>
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<td>0.0197</td>
</tr>
<tr>
<td>JN570</td>
<td><em>mangiferaeindicicae</em></td>
<td>0.0748</td>
<td>0.0197</td>
</tr>
</tbody>
</table>

*Complete genome sequence reported by Da Silva *et al.* (2002).
The type strain is strain 3213T = ATCC 49118T = ICMP 15804T = ICPB 10518T = LMG 9322T.

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