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 *mangiferaeindicae* (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. *mangiferaeindicae* (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. *mangiferaeindicae* (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).

Abbreviations: AFLP, amplified fragment length polymorphism; CBS, cashew bacterial spot; DDH, DNA–DNA hybridization; EGD, evolutionary genome divergence; MBC, mango bacterial canker; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NJ, neighbour-joining; nsps, nucleotide substitutions per site; rep-PCR, repetitive extragenic palindromic PCR.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences of *X. citri* pv. *mangiferaeindicae* CFBP 1716, *X. citri* pv. *anacardii* CFBP 2913 and *X. axonopodis* pv. *spondiae* CFBP 2547 are respectively EF989732, EF989733 and EF989734. Those of the partial sequences used in the MLSA study are EU015124–EU015156, EU015158–EU015215 and EU333904–EU333906 (*atpD*), EU015216–EU015248, EU015250–EU015307 and EU333907–EU333909 (*dnaK*) and EU015308–EU015340, EU015342–EU015399 and EU333910–EU333912 (*gyrB*).

Details of strains and primers and ML trees derived from partial *atpD*, *dnaK* and *gyrB* sequences are available as supplementary material with the online version of this paper.

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 *et al.*, 1992), but provides for the orderly reporting of bacterial plant pathogens, for scientific and technical reporting, for plant protection regulation and for quarantine (Young *et al.*, 1992).

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 T_m –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; Mougél *et al.*, 2002; On *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 (Mougél *et al.*, 2002). Recently, multilocus sequence analysis (MLSA), based on sequence analysis of several housekeeping genes, has been developed for species delineation (Gevers *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. *anacardii*, 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 (NCPBP, 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^{-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), 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 *SacI*, 2 U *MspI* (New England Biolabs) and $1 \times$ BSA in $1 \times$ reaction buffer NEB 1. 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 *SacI* adaptor (Supplementary Table S2) (Applied Biosystems) and 2 U T4 DNA ligase (New England Biolabs) in $1 \times$ 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

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 fluorochromes) (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 15 000 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 weighted neighbour-joining (NJ) tree (Gascuel, 1997; Saitou & Nei, 1987) using the R software (version 2.3.1; R Development Core Team). The robustness of the tree was assessed by bootstrap (1000 resamplings).

Gene amplification and sequencing. Amplification of 16S rRNA genes was performed by PCR in 50 µl reaction mixtures using the BD Advantage 2 polymerase mix kit (Clontech), as recommended by the manufacturer, for strains CFBP 1716, CFBP 2913 and CFBP 2547 (pathotype strains for pv. *mangiferaeindicae*, *anacardii* and *spondiae*, respectively) (Ah-You *et al.*, 2007). The specific primers were FGPS6 (5'-GGAGAGTTAGATCTTGCTCAG-3') and FGPS1509 (5'-AAGGAGGGGATCCAGCCGCA-3'), described by Nesme *et al.* (1995), which complement bases 6–27 and bases 1522–1541 (*Escherichia coli* 16S rRNA gene sequence numbering), respectively. PCRs were performed in a PE9600 thermocycler (Applied Biosystems). The amplification program included denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension to 72 °C for 2 min and a final extension step at 72 °C for 3 min. Amplified fragments were purified using a QIAquick PCR purification kit (Qiagen) and cloned into the pGEM-T easy plasmid (Promega) as recommended by the manufacturer. Sequence data were obtained by primer-walking double-strand analysis (Sequencia) using primers T7 and SP6, which flank the cloning region in the pGEM-T easy plasmid. Internal

primers were 16SU579 (5'-ACTCCACCGCTTGTGC-3'), 16SU1124 (5'-CGCGGCATGGCTGGAT-3'), 16SL573 (5'-GCGGTGGAGTA-TGTGG-3') and 16SL1169 (5'-ACGGGAGGCAGCAGTG-3'). Sequence data were compared to those of other xanthomonads (Hauben *et al.*, 1997; Roumagnac *et al.*, 2004; Trébaol *et al.*, 2000) by alignment using the CLUSTAL_X software (Thompson *et al.*, 1997). Comparisons were based on a partial sequence of 1475 bp, corresponding to the shortest region published for a *Xanthomonas* type strain (i.e. GenBank accession no. X95922 from *X. populi* LMG 5743^T).

Gene portions of *dnaK* (encoding the 70 kDa heat-shock protein; Hsp70), *atpD* (F₁–F₀ ATPase subunit) and *gyrB* (DNA gyrase beta subunit) (Table 1) were amplified by PCR in 50 µl reaction mixtures using the BD Advantage 2 polymerase mix kit (Clontech), as recommended by the manufacturer. PCRs were performed in a PE9600 thermocycler as follows: heating to 95 °C for 3 min and 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 66 °C and extension to 68 °C for 1 min, followed by a final extension step at 68 °C for 7 min. For a small number of strain–gene combinations, the annealing temperature was modified (63–68 °C) for optimal PCR quality or yield. The PCR products were sequenced by Macrogen. The *dnaK*, *atpD* and *gyrB* sequences from *Stenotrophomonas maltophilia* K279a (http://www.sanger.ac.uk/Projects/S_maltophilia/) were used as outgroups.

Phylogenetic analyses of housekeeping genes. DNA sequences for each gene (*dnaK*, *atpD* and *gyrB*) were aligned using the CLUSTAL w-based subalignment tool (Thompson *et al.*, 1997) available in MEGA 3.1 program (Kumar *et al.*, 2004). Tajima's D (Tajima, 1989), which tests the hypothesis that observed mutations are selectively neutral by measuring differences between the population mutation rate and the mean number of nucleotide differences, was performed for each gene portion using DnaSP, version 4.0 (Rozas *et al.*, 2003). The method of Nei & Gojobori (1986) was used to evaluate non-synonymous/synonymous substitution ratios (K_a/K_s) using Saaap software version 1.0.2 (<http://www.bacteriamuseum.org/SWAAP/SwaapPage.htm>). Nucleotide and amino acid sequence identities and transition/transversion ratios were also evaluated using Saaap software. Detection of potential recombinant sequences, identification of likely parental sequences and localization of possible recombination breakpoints were carried out on a concatenated sequence alignment using the rdp, geneconv, bootscan, maximum chi-squared, chimera and sister scan recombination detection methods as implemented in the RDP3 software (Martin *et al.*, 2005). The analysis was performed with default settings for the different detection methods and a Bonferroni-corrected *P*-value cut-off 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 concatenated genes were conducted both in PAUP* and in PHYL (Guindon & Gascuel, 2003). The model of substitution was chosen using the R software and the APE package implemented in R (Paradis, 2006) and PHYL. 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 7.0×10^6 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 + Γ + I, based on concatenated sequences (Paradis, 2006). PT, Pathotype strain.

Strain	Pathovar	<i>X. axonopodis</i> LMG 982 ^T		<i>X. citri</i> LMG 9322 ^T		<i>X. fuscans</i> LMG 826 ^T	
		AFLP	MLSA	AFLP	MLSA	AFLP	MLSA
CFBP 2913 ^{PT}	<i>anacardii</i>	0.1285	0.0422	0.0710	0.0210	0.0455	0.0032
LA100	<i>anacardii</i>	0.1340	0.0422	0.0667	0.0223	0.0422	0.0041
CFBP 1716 ^{PT}	<i>mangiferaeindicae</i>	0.1530	0.0411	0.0463	0.0027	0.0770	0.0202
JN570	<i>mangiferaeindicae</i>	0.1516	0.0411	0.0495	0.0027	0.0787	0.0202
CFBP 2935	<i>mangiferaeindicae</i>	0.1472	0.0411	0.0527	0.0027	0.0767	0.0202
JV1121	<i>mangiferaeindicae</i>	0.1601	0.0411	0.0503	0.0027	0.0767	0.0202
CFBP 2547 ^{PT}	<i>spondiae</i>	0.1194	0.0432	0.1088	0.0423	0.1056	0.0462
CFBP 2623	<i>spondiae</i>	0.1175	0.0432	0.1050	0.0423	0.0996	0.0462

neously and sampled every 100 generations. Variations in the ML scores in these samples were examined graphically using the Tracer software (<http://evolve.zoo.ox.ac.uk/software.html?id=tracer>). Trees generated prior to stabilization of ML scores were discarded (burn-in of 10 %).

The S–H test was also used to compare tree topologies (NJ, ML and Bayesian methods) based on concatenated data.

DNA–DNA hybridization. DNA was extracted and purified according to Brenner *et al.* (1982), except for *X. axonopodis* LMG 982^T, for which DNA was extracted using the CTAB method (Ausubel *et al.*, 1991). Native DNAs of *X. axonopodis* LMG 982^T and of pathotype strains of pv. *mangiferaeindicae* (CFBP 1716) and pv. *citri* (CFBP 2525) were labelled *in vitro* with tritium-labelled nucleotides by random priming using the Megaprime DNA labelling system 1604 (GE Healthcare). The S1 nuclease/trichloroacetic acid (TCA) method was used for DDH experiments as described by Grimont *et al.* (1980), except that hybridizations were performed for 24 h instead of 16 h. The reassociation temperatures used were 70 and 75 °C. DDH experiments were performed twice.

Thermal stability of DNA reassociation. For strains sharing reassociation values between 50 and 70 %, the thermal stability of DNA was determined (Crosa *et al.*, 1973; Grimont, 1988). This technique determines by interpolation the DNA T_m (thermal denaturation midpoint), which is the temperature at which half of the reassociation of the DNA is obtained. The T_m value depends on both DNA base composition and ionic strength: the T_m of a DNA can be lowered by decreasing the sodium molarity (Grimont, 1988). This property was used to reduce artificially the theoretical T_m of our strains (102 °C after calculations). After 24 h hybridizations at 75 or 70 °C in 0.42 M NaCl, the hybridization mixture was diluted 2-fold, in order to have a sodium molarity of 0.2 M, which decreased the T_m by 5 °C. Then, as described by Grimont (1988), the temperature was raised in 5 °C increments (from 80 to 100 °C). At each step, a sample was taken, and nuclease S1 buffer was added that contained 0.67 M of NaCl in order to obtain the 0.42 M of sodium required for nuclease S1 digestion. Further steps of T_m determination were similar to the original method (Crosa *et al.*, 1973; Grimont, 1988). ΔT_m or divergence corresponds to the difference between the T_m of a homologous reaction and the T_m of a heterologous reaction (Grimont, 1988).

RESULTS

AFLP analysis

Cluster analysis, based on 1308 AFLP fragments, placed most *Xanthomonas* genomospecies in distinct lineages, supported by high bootstraps 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, 2917, 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 982^T) 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 *bauhiniae*, *cajani*, *citri*, *clitoriae*, *desmodiilaxiflori*, *glycines*, *malvacearum* and *mangiferaeindicae*. Pathovar *mangiferaeindicae*

Nucleotide transitions exceeded transversions, with ratios ranging from 1.47 (SD=1.50) (*atpD*) to 3.51 (SD=2.77) (*gyrB*). The K_a/K_s ratios of the three genes ($\ll 1$) 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

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 (K_s) and non-synonymous (K_a) 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 ($P>0.10$).

Gene	<i>atpD</i>	<i>gyrB</i>	<i>dnaK</i>
Length (bp)	747	773	762
Sequence identity (%)			
Nucleotide	96.96	96.57	96.81
Amino acid	99.26	99.55	99.67
Ts/Tv	1.4667 (1.5185)	3.5412 (2.7858)	3.1145 (1.8021)
K_a	0.0084 (0.0072)	0.0020 (0.0030)	0.0015 (0.0014)
K_s	0.1235 (0.0740)	0.1666 (0.1053)	0.1509 (0.0806)
K_a/K_s	0.0678 (0.0478)	0.0119 (0.0118)	0.0102 (0.0239)
Tajima's D	-0.529	-0.618	-0.124

single gene sequences (*atpD*, *dnaK* and *gyrB*) showed that xanthomonads originating from members of the Anacardiaceae [i.e. *X. campestris* pv. *mangiferaeindicae* sensu Dye *et al.* (1980)] are distributed into three different lineages (not shown). Each tree showed that strains of pathovars *mangiferaeindicae* and *anacardii* grouped with strains of subgroup 9.5 and 9.6, respectively, regardless of which gene was analysed. The pathovar composition of subgroups 9.5 and 9.6 was identical to that described for AFLP analysis. Subgroups 9.5 and 9.6 were always closely related, an association supported by high bootstrap values ($\geq 80\%$) for *atpD* and *gyrB* (Supplementary Figs S1–S3). Strains identified as pv. *spondiae* grouped with subgroup 9.1 (*gyrB*) or subgroup 9.4 (*atpD* and *dnaK*) (Supplementary Figs S1–S3).

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* grouped with strains belonging to subgroups 9.5 and 9.6, respectively (Fig. 2).

Distance matrices calculated with the model GTR+ Γ +I, based on concatenated sequences, allowed us to assess relationships between strains. Distances within subgroup 9.5 were very short (≤ 0.004 nsps). Subgroups 9.5 and 9.6 were closely related to each other, with a mean sequence distance of 0.022 nsps ($SD=0.002$). The distance between the type strains of *X. citri* and *X. fuscans* was 0.021 nsps. The distance between the pathotype 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). Mean distance values between subgroup 9.5 and other *X. axonopodis* subgroups (9.1–9.4) ranged from 0.036 to 0.047 nsps. Strains of pv. *spondiae* were equidistantly related to subgroups 9.1 (0.020 nsps) and 9.4 (0.019 to 0.023 nsps). The latter two subgroups were closely related, with a mean sequence distance of 0.023 nsps ($SD=0.002$).

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 0.033 to 0.078 nsps.

DDH and ΔT_m 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%. ΔT_m values obtained between CFBP 1716 and the type strains of *Xanthomonas oryzae*, *X. melonis* and *X. axonopodis* were >5.0 °C (Table 3). Other ΔT_m 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*)

Table 3. Levels of DNA–DNA reassociation between the pathotype strain of pathovar *mangiferaeindicae* 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.

Strain	Current nomenclature	RBR (%) with labelled DNA from CFBP 1716 ^{PT}	ΔT_m (°C) with labelled DNA from:		
			CFBP 1716 ^{PT}	CFBP 2525 ^T	LMG 982 ^{PT}
LMG 8678 ^T	<i>X. codiae</i>	23	ND	ND	ND
LMG 911 ^T	<i>X. vesicatoria</i>	22	ND	ND	ND
LMG 568 ^T	<i>X. campestris</i>	39	ND	ND	ND
LMG 8670 ^T	<i>X. melonis</i>	56	11.0	ND	ND
LMG 876 ^T	<i>X. translucens</i>	9	ND	ND	ND
LMG 471 ^T	<i>X. sacchari</i>	11	ND	ND	ND
CFBP 4188 ^T	<i>X. cynarae</i>	29	ND	ND	ND
LMG 673 ^T	<i>X. cassavae</i>	31	ND	ND	ND
LMG 5047 ^T	<i>X. oryzae</i> pv. <i>oryzae</i>	58	7.5	ND	ND
LMG 982 ^T	<i>X. axonopodis</i> pv. <i>axonopodis</i>	ND	7.5	7.0	–
CFBP 1716 ^{PT}	<i>X. axonopodis</i> pv. <i>mangiferaeindicae</i>	ND	–	1.5	4.5
CFBP 2940	<i>X. axonopodis</i> pv. <i>mangiferaeindicae</i>	ND	0.5	ND	ND
CFBP 2525 ^{PT}	<i>X. citri</i> pv. <i>citri</i>	ND	1.0	ND	6.0
CFBP 2913 ^{PT}	<i>X. axonopodis</i> pv. <i>anacardii</i>	ND	2.0	ND	7.0
LA98	<i>X. axonopodis</i> pv. <i>anacardii</i>	ND	2.5	ND	ND
LMG 7387	<i>X. axonopodis</i> pv. <i>cajani</i>	ND	3.5	ND	ND

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 vasicola* LMG 736^T (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* XV938^T (GenBank accession no. AF123091) or *X. euvesicatoria* XV153 (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. *mangiferaeindicae* 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. *mangiferaeindicae* supported its partition into three pathovars, namely pv.

mangiferaeindicae, 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*, *mangiferaeindicae* and *spondiae* suggested that their assignment to *X. axonopodis* would be incorrect. Data from both techniques indicated that pathovars *mangiferaeindicae* 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 ($T_m - 25^\circ\text{C}$) (Johnson, 1984), and strengthened our results, when appropriate, by evaluating the thermal stability of DNA reassociation (ΔT_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. codiae*, *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%. ΔT_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, ΔT_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 ΔT_m of 4.5°C . ΔT_m values in the range $6\text{--}7^\circ\text{C}$ (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 ΔT_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 ΔT_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 ΔT_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). ΔT_m values for the latter strains were only $2.0\text{--}2.5^\circ\text{C}$. 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* (*citri* B, C and D groups), *X. citri* pv. *bauhiniae*, *X. citri* pv. *cajani*, *X. citri* pv. *citri* (*citri* A group), *X. citri* pv. *clitoriae*, *X. citri* pv. *desmodiilaxiflori*, *X. citri* pv. *dieffenbachiae* (strains not pathogenic to anthurium), *X. citri* pv. *glycines*, *X. citri* pv. *malvacearum*, *X. citri* pv. *mangiferaeindicae*, *X. citri* pv. *phaseoli* var. *fuscans*, *X. citri* pv. *rhynchosiae*, *X. citri* pv. *sesbaniae*, *X. citri* pv. *vignaeradiatae* 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_m values of 2.0 and 2.5 °C, respectively. PT, Pathotype strain.

Strain	Pathovar	pv. <i>anacardii</i>				pv. <i>phaseoli</i> var. <i>fuscans</i>							
		CFBP 2913 ^{PT}		LA100		LMG 826 ^T		LMG 837		LMG 7511		LMG 8036	
		AFLP	MLSA	AFLP	MLSA	AFLP	MLSA	AFLP	MLSA	AFLP	MLSA	AFLP	MLSA
LMG 558 ^{PT}	<i>cajani</i>	0.0673	0.0197	0.0617	0.0210	0.0719	0.0202	0.0737	0.0206	0.0789	0.0202	0.0761	0.0206
LMG 9322 ^T	<i>citri</i>	0.0710	0.0210	0.0667	0.0223	0.0798	0.0214	0.0787	0.0219	0.0847	0.0214	0.0831	0.0219
CFBP 2525 ^{PT}	<i>citri</i>	0.0724	0.0210	0.0662	0.0223	0.0818	0.0214	0.0816	0.0219	0.0876	0.0214	0.0862	0.0219
306*	<i>citri</i>	0.0715	0.0210	0.0654	0.0223	0.0788	0.0214	0.0787	0.0219	0.0846	0.0214	0.0832	0.0219
LMG 9045 ^{PT}	<i>clitoriae</i>	0.0761	0.0202	0.0718	0.0214	0.0784	0.0206	0.0783	0.0211	0.0855	0.0206	0.0826	0.0211
LMG 9046 ^{PT}	<i>desmodiilaxiflori</i>	0.0805	0.0210	0.0777	0.0223	0.0795	0.0228	0.0811	0.0232	0.0852	0.0227	0.0821	0.0232
LMG 712 ^{PT}	<i>glycines</i>	0.0818	0.0208	0.0791	0.0221	0.0809	0.0213	0.0825	0.0217	0.0883	0.0213	0.0889	0.0217
LMG 8026	<i>glycines</i>	0.0880	0.0208	0.0896	0.0221	0.0852	0.0213	0.0887	0.0217	0.0913	0.0213	0.0919	0.0217
LMG 761 ^{PT}	<i>malvacearum</i>	0.0804	0.0210	0.0736	0.0223	0.0754	0.0228	0.0792	0.0232	0.0817	0.0228	0.0822	0.0232
LMG 7429	<i>malvacearum</i>	0.0821	0.0210	0.0774	0.0223	0.0793	0.0228	0.0809	0.0232	0.0834	0.0228	0.0838	0.0232
CFBP 1716 ^{PT}	<i>mangiferaeindicae</i>	0.0730	0.0197	0.0636	0.0210	0.0770	0.0202	0.0788	0.0206	0.0829	0.0202	0.0816	0.0206
JN570	<i>mangiferaeindicae</i>	0.0748	0.0197	0.0660	0.0210	0.0787	0.0202	0.0803	0.0206	0.0842	0.0202	0.0829	0.0206

*Complete genome sequence reported by Da Silva *et al.* (2002).

The type strain is strain 3213^T = ATCC 49118^T = ICMP 15804^T = ICPB 10518^T = LMG 9322^T.

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