Differentiation of *Xanthomonas* species by PCR-RFLP of *rpf*B and *atp*D genes

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Abstract

The genetic characterization of *Xanthomonas* species remains a challenge. Several DNA-based techniques have been previously employed, including the analysis of the 16S rRNA and 16S–23S rRNA genes in order to differentiate and classify the *Xanthomonas* species. However, several species could not be distinguished in these studies, due to the high degree of conservation of these molecular markers. In order to obtain more efficient markers, and to better understand the phylogenetic relationships between the *Xanthomonas* species, two genes commonly found in the different species have been analyzed. The genes *rpf*B and *atp*D involved in the regulation of pathogenicity factors and in the synthesis of ATP, respectively, were amplified in *Xanthomonas* species and further analyzed by PCR-restriction fragment length polymorphism. Dendrograms with the data sets of the *rpf*B and *atp*D analyzed separately and combined were constructed. The results obtained revealed that several *Xanthomonas* species, previously grouped together, could be successfully distinguished using these markers. The results obtained herein provide an alternative method for the distinction of the *Xanthomonas* species and contribute to a better understanding of the genetic and phylogenetic relationships of *Xanthomonas*.

Introduction

The genus *Xanthomonas* is formed by a complex group of bacterial species with diverse physiological and phytopathological traits. This group of phytopathogens infects many economically important plants such as cotton, beans, rice, crucifers, among others and therefore, these bacteria represent a threat to agriculture around the world. Some of the most important diseases caused by *Xanthomonas* species include citrus canker, caused by *Xanthomonas axonopodis* pv. *citri* and black rot, caused by *Xanthomonas campestris* pv. *campestris*. Additionally, *Xanthomonas* is also important in biotechnology due to the production of a polysaccharide known as xanthan gum, which is used as a stabilizer and emulsifier in the food and cosmetic industry, among other applications (Bradbury, 1984; Backer *et al*., 1998).

The genetic characterization of *Xanthomonas* species remains a challenge. This genus was first proposed by Dowson (1939), who described 60 species. At that time, bacteria were classified according to phenotypic, biochemical, morphological and pathogenicity characteristics. However, with the advances in molecular biology, it was necessary to re-evaluate the taxonomic positions of *Xanthomonas* species. Several efforts were made by different research groups to reclassify X*anthomonas* (De Vos & De Ley, 1983; Swings *et al*., 1983; Van den Mooter & Swings, 1990; Yang *et al*., 1993). In 1995, Vauterin *et al*. (1995), based on DNA:DNA homology and the utilization of carbon sources, suggested the classification of the genus *Xanthomonas* into 20 species and this has been the most accepted grouping at the species level. These results were further confirmed by rep-PCR and amplified fragment length polymorphism (AFLP) analyses (Rademaker, 1999; Rademaker *et al*., 2000).

Several DNA-based techniques have been used for the characterization and determination of phylogenetic relationships of different bacterial species. One of these methods is DNA–DNA reassociation, which is the main determinant to define a bacterial species. The study of the 16S rRNA and 16S–23S rRNA genes spacer region has also been extensively used. However, in some cases, a high level of similarity in these regions is found for different species and therefore new approaches need to be developed in order to assess the phylogenetic relationships within the group correctly.

The phylogenetic relationships of the 20 *Xanthomonas* species were evaluated based on the 16S rRNA gene (Hauben

et al., 1997), and only three groups could be distinguished due to the high level of similarity found in this gene (*c.* 98% similarity among the species). In 2002, Gonçalves & Rosato (2002) distinguished six phylogenetic groups of *Xanthomonas*, based on the analysis of the 16S–23S rRNA gene spacer region, which presented a diversity approximately ninefold higher than the 16S rRNA gene. However, these authors report that the largest cluster, formed by most species, could not be differentiated (Gonçalves & Rosato, 2002).

In order to obtain a better understanding of the phylogenetic relationships of this group, other more informative genes and DNA sequences common to all species need to be analyzed and may represent more efficient markers for the genetic relatedness among the *Xanthomonas* species. The objective of the present work was to search for these markers and validate the use of these genes to verify the variability found in the different species of the *Xanthomonas* genus.

Materials and methods

Bacterial strains and culture conditions

All *Xanthomonas* strains [reference or type (^{T}) strains] were obtained from the Microbiology Laboratory of the University of Gent (Belgium). The following strains were used in this study: *Xanthomonas axonopodis* pv. *axonopodis* LMG538^T, *Xanthomonas bromi* LMG947^T, *X. campestris* pv. *campestris* LMG568^T, *Xanthomonas cassavae* LMG 673^T, *Xanthomonas codiaei* LMG 8678^T, *Xanthomonas cucurbitae* LMG690^T, *Xanthomonas hortorum* pv. *hederae* LMG 733, *Xanthomonas hyacinthi* LMG739^T, *Xanthomonas melonis* LMG8670^T, *Xanthomonas pisi* LMG 847^T, *Xanthomonas sacchari* LMG 471^T, *Xanthomonas theicola* LMG 8684^T, *Xanthomonas translucens* pv. *translucens* LMG 876^T, *Xanthomonas vasicola* pv. *holcicola* LMG 736^T, *Xanthomonas vesicatoria* LMG 911^T, *Xanthomonas oryzae* LMG 5047^T, and *Xanthomonas arboricola* LMG 443^T. *Xanthomonas albilineans* ICMP 196 was obtained from the International Collection of Plant Microorganisms, New Zealand, and *Xanthomonas fragariae* – IBSBF 89 was provided by Instituto Biológico, Bacteriologia Fitopatológica (IBSBF), Campinas, SP, Brazil. All strains were grown on NYG medium (Turner *et al.*, 1984) at 30 °C.

Sequence analysis and primer design

Sequences of the genes *rpf*B, associated with the regulation of pathogenicity factors and *atp*D involved in the synthesis of ATP, were searched and retrieved from public databases. Alignment of the sequences from different *Xanthomonas* species was performed using the CLUSTALW program (Thompson *et al.*, 1994) and primers were designed using the GENE RUNNER program, version 3.0 (Hastings Software Inc.), based on conserved regions.

DNA Extraction and PCR conditions

DNA extraction was conducted as described by Gonçalves & Rosato (2002). Amplifications were performed in a total volume of 25 μL containing 20 ng DNA, 1.25 mM $MgCl_2$, 100 μM dNTP, 0.5 mM of each primer and 0.5 U of *Taq* DNA polymerase (Amersham). The primer sequences for the *atp*D gene were ATPDF: 5′-GGC AAG ATC GTT AGA TC-3′ and ATPDR: 5′-CTG CCG ACC ATG TAG AAC-3′, and the primers used for *rpf*B were RPFBF: 5′-GTC CTT GGT TGC AAA GTT ATC C-3′ and RPFBR: 5′-AGG ATC TTG CCG ACG TTG G-3′. Control reactions without DNA template were also included. At least two repetitions were performed in order to confirm the results obtained. The PCR reactions for the amplification of *rpf*B were performed with an initial denaturation at 94 °C for 1 min, followed by 40 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min. A final extension at 72 °C for 1 min was performed. The amplification of *atp*D was performed as described above, with an annealing temperature of 57 °C.

PCR-restriction fragment length polymorphism (RFLP)

All *Xanthmonas* species used in this study produced amplification products using the primers designed for the genes *rpf*B and *atp*D, and the PCR products were used for PCR-RFLP analyses. The selection of the restriction enzymes to be used was performed using DNASIS 6.0 (Hitachi Software Engineering Co. Ltd) based on the predicted restriction sites of the sequences used for the primer design. Enzymes that produced a smaller number and low-molecular-weight fragments were preferred. Using these criteria, the enzymes AluI (New England), BstxI (Biolabs) and HaeIII (New England) were selected to digest the amplified *rpf*B gene and AluI (New England), HincII (New England) and HinfI (New England) to analyze the amplified *atp*D gene. The digestion conditions were performed in a final volume of 10 μL, as described by the manufacturer, and repeated at least twice.

Electrophoresis and data analysis

The amplified and digested fragments were analyzed by electrophoresis in agarose gel 0.8% and 1.4%, respectively. The fragments were visualized after ethidium bromide staining. Dendrograms were constructed using the data sets obtained after digestion of *rpf*B and *atp*D amplified fragments with the restriction enzymes, using the Jaccard coefficient, the UPGMA algorithm and the NTSYS-PC program (Rohlf, 1989).

Results and discussion

In this study, molecular markers to be used in the characterization of the genetic and phylogenetic relationships

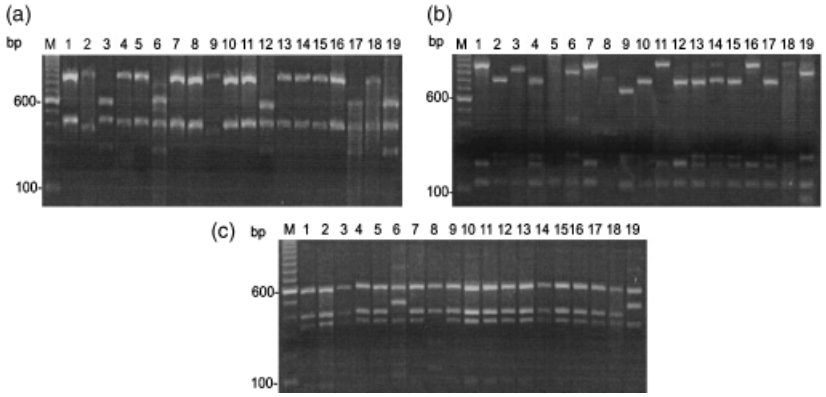

Fig. 1. PCR-RFLP profiles of the *atp*D gene of the *Xanthomonas* species obtained after digestion with the restriction enzymes (a) Hinc II, (b) Alu I and (c) HinfI. M, molecular marker 100 bp (Invitrogen). 1 *Xanthomonas campestris*, 2 *Xanthomonas axonopodis*, 3 *Xanthomonas hyacinthi*, 4 *Xanthomonas translucens*, 5 *Xanthomonas vesicatoria*, 6 *Xanthomonas sacchari*, 7 *Xanthomonas vasicola*, 8 *Xanthomonas cucurbitae* 9 *Xanthomonas melonis*, 10 *Xanthomonas cassavae*, 11 *Xanthomonas hortorum*, 12 *Xanthomonas theicola*, 13 *Xanthomonas bromi*, 14 *Xanthomonas pisi*, 15 *Xanthomonas codiaei*, 16 *Xanthomonas arboricola*, 17 *Xanthomonas fragariae*, 18 *Xanthomonas oryzae*, 19 *Xanthomonas albilineans*.

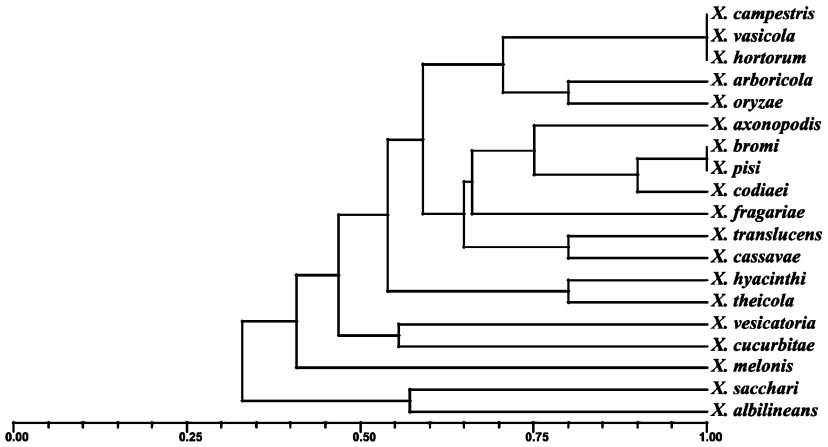

Fig. 2. Dendrogram obtained for the different species of *Xanthomonas* based on the PCR-RFLP profile of the *atp*D gene.

between *Xanthomonas* species have been searched. One of the genes analyzed was *atp*D. Sequences were searched in the database and three relevant *Xanthomonas* sequences were identified: *X. campestris* gi|21229478, *X. oryzae* gi|58424217 and *X. campestris* gi|21110025. The alignment of the sequences revealed a high similarity of *c.* 94%. Primers were constructed based on conserved regions and used for amplification. A fragment of *c.* 1.3 kb was obtained for all strains and this amplification product was digested with the restriction enzymes AluI, HinfI and HincII separately, revealing different PCR-RFLP profiles (Fig. 1). The fragment sizes ranged between 120 and 1000 bp.

The PCR-RFLP results obtained were used to construct a dendrogram (Fig. 2), which demonstrated the distinction between some species of *Xanthomonas*. The genetic similarity ranged from 34% to 100%, and at 55% similarity, the dendrogram revealed five clusters (Fig. 2). The most distinct cluster was formed by the species *X. albilineans* and *X. sacchari*, which showed a similarity of 34% with the other species. The dendrogram obtained also showed that most *Xanthomonas* species were grouped in Cluster I. Two groups, one formed by *X campestris*, *X. vasicola* and *X. hortorum*, and the other by *X. bromi* and *X. pisi*, shared 100% similarity within the group (Fig. 2). These results show that *atp*D was a useful marker to distinguish some species and that other markers need to be tested in order to obtain a higher level of diversity.

In the *rpf*B analysis, sequences were searched in the database and two relevant *Xanthomonas* sequences were identified: *X. campestris* gi|21112960 and *X. oryzae* gi|15723357. The alignment of both sequences revealed a similarity of 89%. These results indicate that this gene is conserved in the *Xanthomonas* genus, however, with a degree of variation that may be useful for phylogenetic analysis. Primers were constructed based on conserved regions and used for amplification. The *rpf*B gene was amplified in all 19 species analyzed and a fragment of *c.* 1.5 kb was obtained. This amplification product was

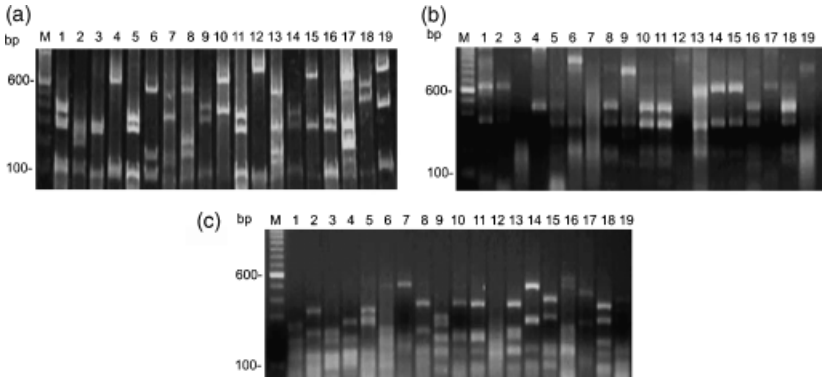

Fig. 3. PCR-RFLP profiles of the *rpf*B gene of the *Xanthomonas* species obtained after digestion with the restriction enzymes (a) AluI, (b) BstxI and (c) HaeIII. M, molecular marker 100 bp (Invitrogen). 1 *Xanthomonas campestris*, 2 *Xanthomonas axonopodis*, 3 *Xanthomonas hyacinthi*, 4 *Xanthomonas translucens*, 5 *Xanthomonas vesicatoria*, 6 *Xanthomonas sacchari*, 7 *Xanthomonas vasicola*, 8 *Xanthomonas cucurbitae* 9 *Xanthomonas melonis*, 10 *Xanthomonas cassavae*, 11 *Xanthomonas hortorum*, 12 *Xanthomonas theicola*, 13 *Xanthomonas bromi*, 14 *Xanthomonas pisi*, 15 *Xanthomonas codiaei*, 16 *Xanthomonas arboricola*, 17 *Xanthomonas fragariae*, 18 *Xanthomonas oryzae*, 19 *Xanthomonas albilineans*.

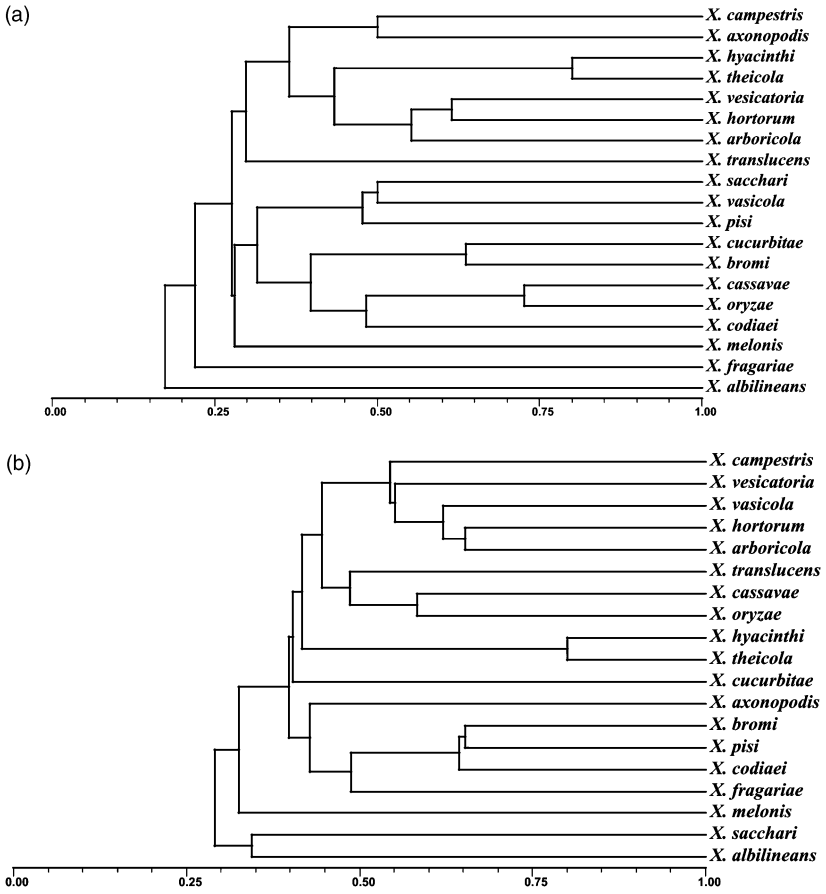

Fig. 4. (a) Dendrogram obtained from different *Xanthomonas* species based on PCR-RFLP profile of the gene *rpf*B. (b) Dendrogram of the combined data sets of PCR-RFLP of the *rpf*B and *atp*D genes.

digested with the restriction enzymes AluI, BstxI and HaeIII and revealed variable PCR-RFLP profiles for the different *Xanthomonas* species (Fig. 3). The fragments sizes ranged between lower than 100 and 900 bp.

The PCR-RFLP results obtained were used to construct a dendrogram (Fig. 4a), which demonstrated the distinction between most species of *Xanthomonas*. The genetic similarity ranged from 18% to 80%, and at 55% similarity, the dendrogram revealed 14 clusters (Fig. 4a). The most distinct cluster was formed by the species *X. albilineans*, which showed a similarity of only 18% with the other species.

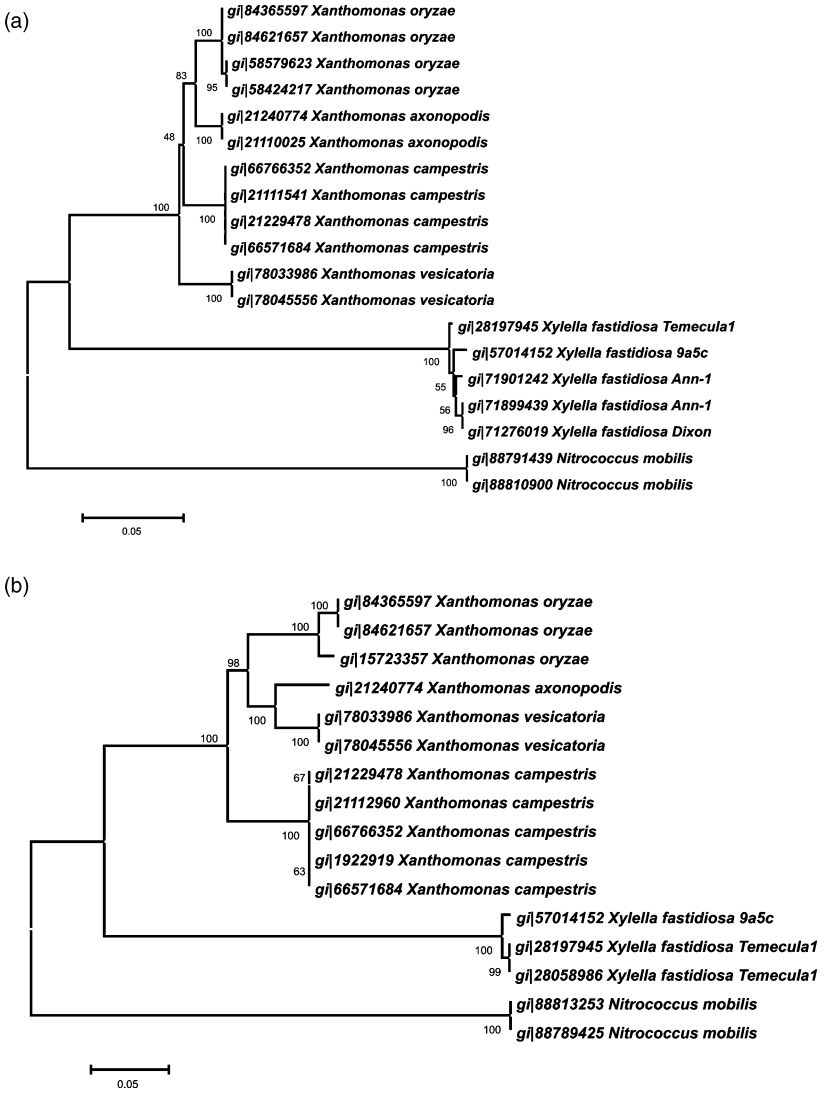

Fig. 5. (a) Phylogenetic tree using sequences of the *atp*D gene obtained from GenBank. (b) Phylogenetic tree using sequences of the *rpf*B gene obtained from GenBank. The trees were constructed using the Neighbor-Joining method with the MEGA program version 3.0 (Kumar *et al.*, 2004). The accession numbers of the sequences used as well as the bootstrap values are indicated.

The combined data sets of the *atp*D and *rpf*B genes were also used to construct a dendrogram (Fig. 4b), and at 55% similarity, 11 Clusters could be identified. Cluster I was formed by *X. campestris*, *X. vesicatoria*, *X. vasicola*, *X. hortorum* and *X. arboricola*; Cluster II was formed by *X. translucens*; and Cluster III by *X. cassavae* and *X. oryzae*. As expected, *X. hyacinthi* and *X. theicola* were grouped together and formed Cluster IV. Similar results were obtained by Gonçalves & Rosato (2002) while analyzing the 16S–23S rRNA gene. *Xanthomonas cucurbitae* formed Cluster V and *X. axonopodis* formed Cluster VI. *Xanthomonas bromi*, *X. pisi* and *X. codiaei* were grouped together and constituted Cluster VII. *Xanthomonaas fragariae*, *X. melonis*, *X. sacchari* and *X. albilineans* formed Clusters VIII, IX, X and XI, respectively.

The results presented here revealed the differentiation of *Xanthomonas* species by the analysis of the *atp*D and *rpf*B genes, which showed different levels of diversity. The use of the housekeeping gene *atp*D for the classification of bacterial species has been extensively reported and this marker has successfully established the phylogenetic relationships of several bacterial groups including *Pasteurella*, *Bradyrhizobium*, *Sesbania* and *Mesorhizobium* (Petersen *et al.*, 2001; Weir *et al.*, 2004; Vinuesa *et al.*, 2005a, b). In our analysis, the *atp*D gene showed a higher resolution than that observed in previous analysis with the 16S–23S rRNA (Gonçalves & Rosato, 2002); however, this marker could not differentiate all *Xanthomonas* species. On the other hand, the *rpf*B gene distinguished most species and showed a higher level of diversity. It is possible that the diversity found in the *rpf*B gene may be due to the fact that this gene does not have vital functions in the bacterial cell. It is possible that a high mutation rate in this gene is more tolerated than in genes such as *atp*D.

The analysis of the combined data sets was a useful tool to assess the genetic relationships of *Xanthomonas* species. The

dendrogram obtained by the combined analysis revealed that *X. alibilineans* and *X. sacchari* were the most distant species, showing a similarity of <30% with the other species. The differentiation of *X. sacchari* was previously observed in the analysis of the 16S rRNA gene (Hauben *et al.*, 1997). It was reported that this species alone represented one of the three phylogenetic clusters identified (Hauben *et al.*, 1997). In the present work, only four clusters grouped more than one *Xanthomonas* species (Clusters I, III, IV and VII), and Cluster I, which is the largest group, was formed by five species. These results indicate that the *atp*D and *rpf*B genes can be successfully used as markers to distinguish different *Xanthomonas* groups.

In order to verify whether similar results could be obtained by analyzing sequence data, the complete sequences of the *atp*D and *rpf*B genes of *Xanthomonas* (*X. campestris, X. axonopodis, X. oryzae and X. vesicatoria*), *Xylella fastidiosa* (closely related) and *Nitrococcus mobilis* (distantly related) available in GenBank were used to construct phylogenetic trees (Fig. 5a and b). Both trees constructed showed that the four *Xanthomonas* species analyzed could be separated into different clusters and that strains of the same species grouped together. Interestingly, in the sequence analysis of *atp*D, *X. oryzae* was closer to *X. axonopodis* than to *X. campestris* and when analyzing the *rpf*B sequence, *X. vesicatoria* was closer to *X. axonopodis*, which differed from the results obtained by PCR-RFLP. The comparison of the two data sets (PCR-RFLP and sequencing) must be made with caution, as sequence information of only a few *Xanthomonas* species was available in GenBank. Moreover, in the sequence analysis, the whole gene is being compared, whereas in PCR-RFLP, only a few restriction sites are analyzed. Future studies using the complete sequences of *atp*D and *rpf*B may provide complementary information and may contribute to a more detailed understanding of the relationships between species of this complex genus. Although the sequence analysis is promising, PCR-RFLP was able to distinguish most *Xanthomonas* species and represents a fast and simple alternative to analyze the *Xanthomonas* genus rapidly.

The present work gives a new insight into the genetic relatedness between the different species of *Xanthomonas*. Two genes with variable levels of diversity have been analyzed by the relatively simple technique of PCR-RFLP. It has been reported that PCR-RFLP of selected genes has successfully distinguished other bacterial species such as *Brucella* sp. (Al Dahouk *et al.*, 2005). This technique has been considered a reliable and effective method that shows epidemiological concordance. It has also been reported that PCR-RFLP studies of specific genes can serve as a tool for diagnostic, epidemiological, taxonomic and evolutionary studies (Al Dahouk *et al.*, 2005). Further analysis with other selected genes may be useful to corroborate the results obtained in this study and to further differentiate the *Xanthomonas* species grouped together.

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