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Short Communication

Genetic diversity of *Xanthomonas axonopodis* pv. citri based on plasmid profile and pulsed field gel electrophoresis

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Abstract

Xanthomonas axonopodis pv. citri strains that cause disease in citrus were investigated by pulsed field and plasmid profile analysis. For the first method, genomic DNA was digested by the rare-cutting enzymes *Xba* I and *Vsp* I. The strains evaluated were collected in seven different States of Brazil and in Argentina, Bolivia, Paraguay and Uruguay. Genetic variability was found among strains of *X. axonopodis* pv. citri from different geographical areas Argentina, Bolivia and Uruguay, with similarities varying from 0.62 to 0.83. However, the strains collected in Brazil, despite being from different States, have shown a genetic similarity ranging from 0.83 to 1.00. Cluster analysis showed a relationship between genomic similarity and geographical origin of the strains. Plasmids were observed in all strains, with a total of five different plasmids, with sizes between 57.7 and 83.0 kilobases. The 72.6 kb plasmid was the most frequent, present in 15 out of 22 strains, while the 68.1 kb plasmid was observed in two strains only. Although the plasmid diversity detected in the present study was not very great, the *X. axonopodis* pv. citri strains evaluated showed a considerable degree of diversity with regard to this extrachromosomal genetic element.

Key words: genetic variability, bacterium, citrus, PFGE.

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Species and pathovars of bacteria belonging to the genus *Xanthomonas* cause important diseases to several plant species of major economical value, such as citrus canker (*Xanthomonas axonopodis.* pv. citri), passion fruit angular leaf spot disease (*X. axonopodis* pv. passiflorae), bacterial blight of common beans (*X. axonopodis* pv. phaseoli), cassava bacteriosis (*X. axonopodis* pv. manihotis) and bacterial spot (X. *axonopodis* pv. vesicatoria). Citrus canker is an important disease for the Brazilian citrus industry (Leite *et al.*, 1987; Negri, 1996), due to the susceptibility of the host and the poor efficiency of the available control methods (Namekata, 1991). Since citrus canker was detected in Brazil for the first time (Bitancourt, 1957), a rigorous program has been implemented for the eradication of the disease. However, difficulties to enforce the eradication campaign did not allow its complete elimination in the Brazil. As a consequence, the disease has spread to several new regions of the country (Leite, 1990).

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The genome of *X. axonopodis* pv. citri (strain 306) has been completely sequenced (Da Silva *et al.*, 2002). This pathogen has one circular chromosome comprising 5,175,554 base pairs (bp) and two plasmids: pXAC33 (33,699 bp) and pXAC64 (64,920 bp) (Da Silva *et al.*, 2002). Comparisons between the genomes of *X. campestris* pv. campestris (pathogens of crucifers) and *X. axonopodis* pv. citri revealed a high degree of similarity, approximately 80% (Da Silva *et al.*, 2002). Nevertheless, both genomes have a large number of genes that are specific for each one of the sequenced strains. These genes may explain the host specificity and the differences in the pathogenesis processes (Da Silva *et al.*, 2002).

Several molecular techniques have been used to study the population structure of different plant pathogens, including RAPD, Rep and Eric-PCR, RFLP, plasmid profile analysis, PCR amplification, SDS-PAGE, sequencing of the 16S rDNA, and pulsed field gel electrophoresis (Hartung and Civerolo, 1987; Lazo *et al*., 1987; Cooksey and Graham, 1989; Graham *et al*., 1990; Leach *et al*., 1990; Egel *et al*., 1991; Pruvost *et al*., 1992; Pooler *et al*., 1996; Hauben *et al*., 1997).

The objective of this research was to study the diversity of *X. axonopodis* pv. citri strains collected from different citrus species and geographic regions of Brazil and other South American countries. The study was based on comparison of plasmid analysis and profile of the entire bacterium genome obtained by pulsed field gel electrophoresis.

The analyses were carried as follows:

a) Bacterial strains and culture medium: Twenty-two strains of *X. axonopodis* pv. citri were obtained from the bacterial collection of Instituto Agronômico do Paraná (IAPAR), Londrina, PR, Brazil. These strains were recovered from cultures maintained at -80 °C (Schaad, 1988) and were originated from different States of Brazil and from Argentina, Bolivia, Paraguay and Uruguay (Table 1).

b) Pulsed Field Gel Electrophoresis (PFGE): Total genomic DNA of *X. axonopodis* pv. citri strains was extracted by using the protocol described by Cooksey and Graham (1989) and Egel *et al*. (1991), with few modifications. The *X. axonopodis* pv. citri strains were grown on NA (Nutrient Agar) medium for 48 h at 28 °C. Pure colonies were transferred to test tubes containing NB (Nutrient Broth) and kept on a rotatory shaker (150 rpm) for 24 h at 28 °C. Cells (5 x 10^9 CFU/mL) were washed in 1 mL of SE buffer (75 mM NaCl, 25 mM EDTA, pH 8.0) and resuspended in 0.5 mL of the same buffer. The cell suspension was mixed with 0.5 mL of melted, cooled, low-melting-point agarose solution (10 mM Tris [pH 8.0], 10 mM $MgCl_2$, 0.1 mM EDTA [pH 8.0], 2% [wt/vol] low-melting-point agarose in sterile distilled water) and pipetted into a plastic mold (Pharmacia, Uppsala, Sweden). The mold was kept at 4 °C for 10 to 15 min, and the inserts were then removed and transferred to a lysing solution (0.5 mg of proteinase K per mL, 1% [wt/vol] N-lauryl Sarkosyl, 0.5 mM EDTA, pH 9.5) in sterile tubes. The tubes were placed into a water bath at 50 °C, and the cells were lysed overnight for at least 15 h. After lysis, the inserts were removed from the solution and placed in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). After 15 min at room temperature, the TE solution was changed and the inserts were incubated for an additional 6 to 8 h. Slices of 1-2 mm were cut from the insert and placed in a microcentrifuge tube with 200 μL of restriction buffer provided by the manufacturer (Boehringer Mannheim, Indianapolis, IN, USA); the rest of the insert was saved in 250 mM EDTA, pH 8.0, at 4 °C. After 15 min of incubation at room temperature, the restriction buffer was changed, and 30 U of either *Xba*I or *Vsp*I restriction enzymes (Boehringer Mannheim, Indianapolis, IN, USA) were added. The tubes were incubated for at least 8 h at 37 °C in a horizontal position. After incubation, the restriction buffer was removed, and 500 μL of lysing solution (without proteinase k) were added. Samples were incubated in a water bath at 50 °C for 2 h, then the lysing solution was changed and the samples were incubated for an additional 2 h at room temperature.

Table 1 - List of *Xanthomonas axonopodis* pv. citri strains included in this study.

Strain	Origin	Year of isolation
8946	Paraguay (Par)	1989
8947	Paraguay (Par)	1989
12413	São Paulo, Brazil (SP)	1999
12427	São Paulo, Brazil (SP)	1999
12712	São Paulo, Brazil (SP)	1999
12714	São Paulo, Brazil (SP)	1999
12758	Bolivia (Bol)	1999
12789	Bolivia (Bol)	2000
12842	Santa Catarina, Brazil (SC)	1996
12843	Santa Catarina, Brazil (SC)	1996
12849	Paraná, Brazil (PR)	1996
12917	Paraná, Brazil (PR)	1997
12919	Paraná, Brazil (PR)	1997
12955	Paraná, Brazil (PR)	1997
12970	Uruguay (Uru)	1993
12971	Argentina (Arg)	1993
12972	Goiás, Brazil (GO)	1996
12973	Rio Grande do Sul, Brazil (RS)	1999
12974	Mato Grosso, Brazil (MT)	2000
12975	Minas Gerais, Brazil (MG)	2000
12976	Rio Grande do Sul, Brazil (RS)	1993
12977	Rio Grande do Sul, Brazil (RS)	1994

The agarose slices prepared for electrophoresis were placed in wells of a 1% gel made with 0.5X TBE (0.45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). The wells were sealed with cooled 2% agarose. The gel was placed in a Gene-Navigator (Pharmacia) containing approximately 0.5X TBE and run at 185 V (14.8 V/cm of gel) at 10 °C. Pulse times for DNA restricted with both *Xba*l and *Vsp*l were 1 h at 4 s and 22 h at 15 s. Phage λ concatamers from Bio-Rad (Bio-Rad, Hercules, CA, EUA) were used as molecular markers. Gels were stained with 0.5 mg ethidium bromide per liter and photographed with type 667 Polaroid film (Polaroid Corporation, Cambridge, MA, EUA).

Data was analyzed based on the occurrence of specific prominent bands, which was scored as 1 and their absence as 0. Similarity coefficients were determined by pairs, using the Dice coefficient. To calculate genetic similarity between strains, the following equation proposed by Nei and Li (1979) was used: $F = 2nxy / (nx + ny)$, where nxy is the number of fragments shared by two strains, and nx and ny are the total number of fragments of each strain. Relationships among strains were evaluated by phylogenetic analysis made with the computer program NTSYS and the UPGMA method (Rohlf, 1993).

c) Plasmid extraction: The *X. axonopodis* pv. citri strains were cultivated in NB (Schaad, 1988) and incubated

at 28 °C for 12 to 16 h under agitation. The bacterial cell suspension was adjusted to 10^8 UFC/mL, and DNA from plasmids was extracted by using the Mini-prep method (Birnboim and Doly, 1979). The plasmidial DNA was analyzed by agarose gel (0.7%) electrophoresis (3 V/cm). Gels were stained with ethidium bromide (0.5 mg/L) and photographed with type 667 Polaroid film.

d) Determination of plasmid size: Plasmids of *Erwinia stewartii* strains SW2 were used as molecular markers. Thirteen plasmids were present in the *E. stewartii* isolate, with bands ranging from 4.1 kb to 318 kb (Coplin *et al*., 1981) which were extracted by the Mini-prep method (Birnboim and Doly, 1979), with minor modifications. Molecular weights were determined by regression of $\log_{10}$ of the molecular weight versus $\log_{10}$ of the relative mobility of the plasmids.

The following results were obtained:

a) Genomic analysis: Genetic diversity of *X. axonopodis* pv. citri was determined through fragment profiles of the genomic DNA generated by restriction endonuclease of infrequent recognition sites. The *X. axonopodis* pv. citri strains restricted with the endonucleases *Xba*I and *Vsp*I produced distinct genetic profiles among the 22 isolates, resulting in 13 different haplotypes (Figures 1 and 2). The number of fragments produced when the bacterial DNA was restricted with the endonucleases was variable, ranging from 16 to 20 fragments in each profile. The sizes of the fragments ranged from 30 kb to 200 kb for all strains (Figures 1 and 2).

Based on the proportion of common fragments, comparing fragments larger than 40 kb, the similarity coefficients among the strains ranged from 0.62 to 1.0. In comparison with the other strains included in the study, strain 12970 from Uruguay and strains 12789 and 12758 from Bolivia showed the lowest similarity coefficients, ranging from 0.62 to 0.87. Nevertheless, strains 12789 and 12758 from Bolivia showed 98% of similarity. Strain 12843 (SC, Brazil) showed 90% of similarity with strains 12919 and 12955 from the State of Paraná, Brazil. Among the four strains from São Paulo, three showed the same haplotypes (12427, 1271 and 12712), and two out of four isolates from Paraná, 12919 and 12955, produced unique profiles.

The highest genetic variability was observed among strains 12970 from Uruguay, 12971 from Argentina, and 12758 and 12789 from Bolivia (Figures 1, 2 and 3).

Three major branches were observed in the similarity dendrogram. One branch grouped the two strains from Bolivia, another branch included strain 12970 from Uruguay, and the third branch comprised all the other strains (Figure 3). The genomic profile cluster analyses of *X. axonopodis* pv. citri strains revealed genetic differences among the Brazilian strains and the strains from Bolivia and Uruguay, suggesting that the distance among them could be related to their geographic origin (Figure 3).

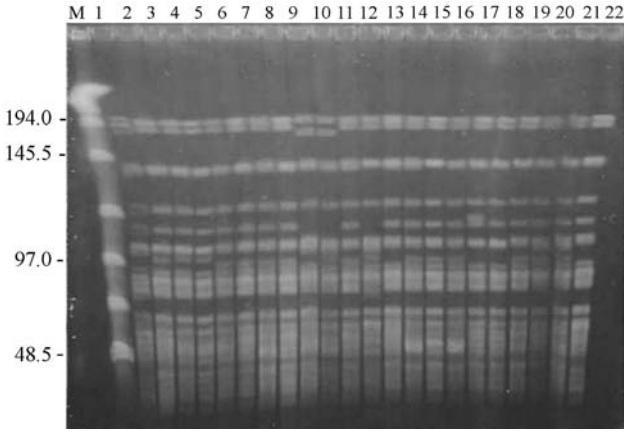

Figure 1 - Restriction endonuclease profiles of *Xanthomonas axonopodis* pv. citri strains obtained with endonuclease *Xba* I. Pulsed field gel electrophoresis for 1 h at 4 s and 22 h at 15 s. Line M, phage λ; 12970 (1), 12971 (2); 12972 (3); 12973 (4); 12974 (5); 12975 (6); 12976 (7); 12977 (8); 12789 (9); 12758 (10); 12842 (11); 12843 (12); 12413 (13); 12427 (14); 12714 (15); 12712 (16); 8946 (17); 8947 (18); 12917 (19); 12919 (20); 12955 (21); 12849 (22). The number adjacent to the bands indicates the size in kilobases (kb).

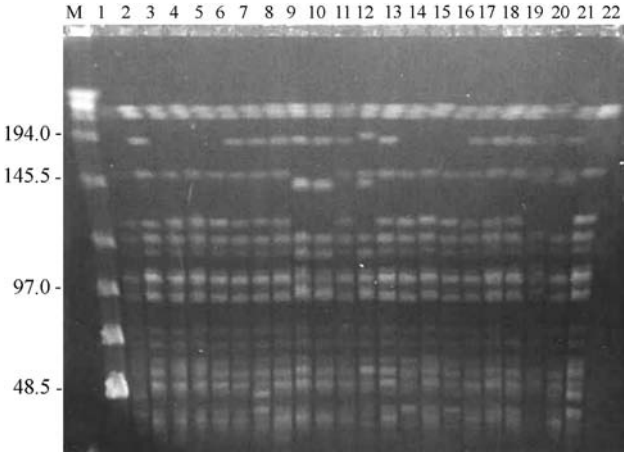

Figure 2 - Restriction endonuclease profiles of *Xanthomonas axonopodis* pv. citri strains obtained with endonuclease *Vsp* I. Pulsed field gel electrophoresis for 1 h at 4 s and 22 h at 15 s. Lanes: M, phage λ; 12970 (1), 12971 (2); 12972 (3); 12973 (4); 12974 (5); 12975 (6); 12976 (7); 12977 (8); 12789 (9); 12758 (10); 12842 (11); 12843 (12); 12413 (13); 12427 (14); 12714 (15); 12712 (16); 8946 (17); 8947 (18); 12917 (19); 12919 (20); 12955 (21); 12849 (22). The number adjacent to the bands indicates the size in kilobases (kb).

The genetic similarity between the strains from Paraguay and Bolivia was 98%, and the 16 strains from Brazil revealed a genetic similarity ranging from 83 to 100%.

Some of the 22 strains presented an identical profile. Strains 12972 and 12973 (from GO and RS, Brazil) belonged to the same haplotype; strains 12975 (MG), 12976 (RS), 12977 (RS), 12842 (SC), 12413 (SP) and 8947 (Paraguay) represented another haplotype; strains 12427, 12714 and 12712 from the State of São Paulo presented a third haplotype, and strains 12919 and 1955 from the State of Paraná repre-

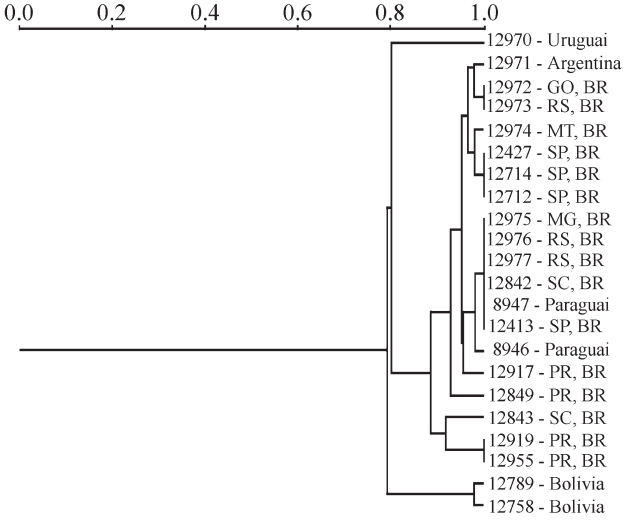

Figure 3 - Similarity dendrogram of *Xanthomonas axonopodis* pv. citri inferred from genomic profiles obtained by pulsed field gel electrophoresis and constructed with the software package NTSYS, using the UPGMA method (Rohlf, 1993).

sented a fourth haplotype. The nine remaining strains represented nine different haplotypes (Figures 1 and 2).

b) Characterization of plasmidial profiles: The presence of plasmids was observed in all 22 bacterium strains included in this study (Figure 4, data for strain 12973 not shown). Strains 12971, 12972, 12974, 12975, 12977, 12789, 12758, 12842, 12413, 12919 and 12955 presented two plasmids with sizes ranging from 57.7 to 83.0 kb. Strains 12970, 12976, 12843 and 12917 had a single plasmid of 83.0 kb. The two strains from Paraguay (8446 and 8947) showed three plasmids with sizes of 57.7, 72.6 and 83.0 kb, respectively. Strains 12427 and 12712 revealed the presence of two plasmids with sizes of 60.8 and 68.1 kb, respectively, and strains 12973 (data not shown in Figure 4) and 12849 presented a single plasmid of 72.6 kb (Figure 4 and Table 2).

The results obtained for all strains in the PFGE analysis with the endonucleases *Xba*I and *Vsp*I indicated that more than 50% of the bacterium genome was represented

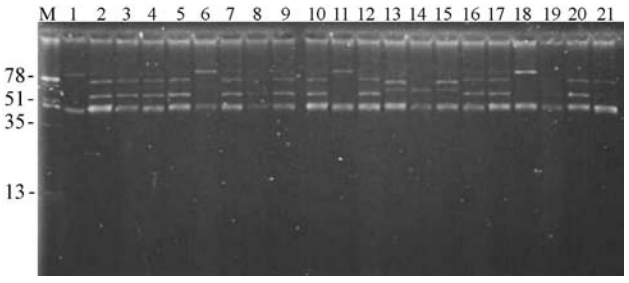

Figure 4 - Plasmid profiles of *Xanthomonas axonopodis* pv. citri strains. Lanes: M are plasmids from *Erwinia stewartii* (SW2) used as molecular size markers. Lanes: 12970 (1), 12971 (2); 12972 (3); 12974 (4); 12975 (5); 12976 (6); 12977 (7); 12789 (8); 12758 (9); 12842 (10); 12843 (11); 12413 (12); 12427 (13); 12714 (14); 12712 (15); 8946 (16); 8947 (17); 12917 (18); 12919 (19); 12955 (20); 12849 (21).

Table 2 - Plasmid profiles of *Xanthomonas axonopodis* pv. citri strains.

Strains	Plasmid profile	Number of plasmids	Plasmid size (kb)				
			57.7	60.8	68.1	72.6	83.0
12970 (Uru)	1	1					+
12971 (Arg)	2	2	+			+	
12972 (GO)	2	2	+			+	
12973 (RS)	6	1				+	
12974 (MT)	2	2	+			+	
12975 (MG)	2	2	+			+	
12976 (RS)	1	1					+
12977 (RS)	2	2	+			+	
12789 (Bol)	2	2	+			+	
12758 (Bol)	2	2	+			+	
12842 (SC)	2	2	+			+	
12843 (SC)	1	1					+
12413 (SP)	2	2	+			+	
12427 (SP)	4	2		+	+		
12714 (SP)	5	1		+			
12712 (SP)	4	2		+	+		
8946 (Par)	3	3	+			+	+
8947 (Par)	3	3	+			+	+
12917 (PR)	1	1					+
12919 (PR)	2	2	+			+	
12955 (PR)	2	2	+			+	
12849 (PR)	6	1				+	

by the restriction patterns. The dissimilarities found among the strains from Bolivia and Uruguay suggest that the genetic distance may be related to the geographical origin of the strains. Cooksey and Graham (1989) also demonstrated a correlation between genetic distance and origin of the strains in *X. campestris* pv. vesicatoria. Likewise, unique profiles were previously found for strains of citrus canker groups A and B from Florida and Argentina, respectively (Egel *et al*., 1991). A genetic distance matrix revealed higher similarity when strains from the same region were compared, such as two strains from Bolivia (12789 and 12758) which had 98% of similarity and two strains from Paraguay (8946 and 8947) which had also 98% of similarity. The strains from Brazil showed a similarity ranging from 83 to 100%. These results indicate a correlation between genetic distance and geographic origin of the strains. The present study produced data which help understanding the fragment variability analyses obtained from the restriction endonuclease analysis of infrequently occurring recognition sites in genomic DNA fragments separated by PFGE. Although it was not able to clearly assess the real distance between strains that belong to the genus *Xanthomonas*, this tool can be of great value compared to other techniques.

Several virulence and avirulence factors have been associated with genes present in plasmids such as *Agrobacterium tumefaciens, A. rhizogenes and Pseudomonas savastonoi* (Comai and Kosuge, 1980; Nester and Kosuge, 1981). Factors related with ecological adaptability of bacteria are thought also to be present in plasmids (Coplin, 1982). The plasmidial diversity found in the twenty-two *X. axonopodis* pv. citri strains studied was not very great, which is in agreement with the findings of Pruvost *et al.* (1992) and Hartung (1992), who demonstrated that plasmidial DNA in *X. axonopodis* pv. citri is highly conservative.

In the present work, we did not observe a strong relationship between the presence of a certain plasmid and the geographical origin of the strain. However, the presence of a 60.8 kb plasmid was observed in only three strains from the State of São Paulo (12427, 12712 and 12714). These three strains showed the same haplotypes when analyzed by PFGE. The results obtained showed a consistent genetic similarity among strains from São Paulo. The presence of 60.8 and 68.1 kb plasmids in the strains from this State indicates a common geographic origin for this genetic trait. The frequency of 72.6 kb plasmids in 15 (12971, 12972, 12974, 12975, 12977, 12789, 12758, 12842, 12413, 8946, 8947, 12919, 12955, 12973 and 12955) out of the 22 strains, as well as the 72.6 kb plasmid that was present in 13 of the 22 strains (12971, 12972, 12974, 12975, 12977, 12789, 12758, 12842, 12413, 8946, 8947, 12919 and 12955), suggest that this extrachromosomal element may play an important role in *X. axonopodis* pv. citri. All of the 15 strains carrying the 72.6 kb plasmid (with the exception of strains 12789 and 12758 from Bolivia) fall within a large branch when we analyze the similarity dendrogram (Figure 3). The 83.0 kb plasmid was the only one found in strains 12970 from Uruguay, 12976 from Rio Grande do Sul, 12843 from Santa Catarina, and 12917 from Paraná. All these strains were found in the southernmost areas of each region and were obtained in the years of 1996 and 1997; therefore, they may be related to the year of introduction of the plasmid in these regions. Furthermore, it can be observed that all strains from the southeastern and midwestern regions of Brazil (GO, 12972; MT, 12974; MG, 12975; SP 12413, 12712 and 12714) did not present this plasmid, nor did the strains from Bolivia (12789 and 12758) and Argentina (12971), which are located near to the regions in the southeast and midwest of Brazil and further apart from regions where the strains containing the 83.0 kb plasmid were found.

The presence of plasmids indicates their importance in the genome of *Xanthomonas axonopodis* pv. citri, considering that within each of the 22 strains sampled in the present study at least one extrachromosomal element was detected. The bacterium *X. axonopodis* pv. citri was introduced for the first time in South America in 1957, and it is believed that this was the only introduction. However, the genetic diversity observed in the present study between *X. a.* pv. citri strains from different geographic regions of South America points to more than one introduction of the bacterium. Therefore, *X. axonopodis* pv. citri from Bolivia and Uruguay may have originated from different introductions of the bacterium in South America. Similarly, Graham and Cubero (2002) suggested that *X. axonopodis* pv. citri type A had at least three introductions in Florida since 1980, based on PCR analyses that revealed differences in the plasmid sequences of two type A strains with distinct characteristics. There is a need for further studies to better evaluate the relationship of the *X. axonopodis* pv. citri strains present in South America and to compare them with strains that occur in other continents. On the other hand, the bacterium originated from a single introduction may have undergone changes through genetic recombination in its genome. The results obtained in this research support the previous knowledge regarding the diversity of the *X. axonopodis* pv. citri population that occurs in Brazil and neighboring countries.

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