Phytopathologia Mediterranea (2017) 56, 1, 40–49
DOI: 10.14601/Phytopathol_Mediterr-18736

RESEARCH PAPERS

Genetic diversity of *Xanthomonas citri* subsp. *citri* in citrus orchards in northwest Paraná state, Brazil

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Summary. *Xanthomonas citri* subsp. *citri*, which causes Asiatic citrus canker (ACC), is an important pathogen of citrus in Brazil and elsewhere. The genetic diversity of *X. citri* subsp. *citri* pathotype 'A' has not been studied in Brazil at a local scale (up to 300 km). Forty isolates were sampled from lesions of ACC on citrus in three orchards in Paraná state, Brazil. Twelve minisatellite markers were used to characterize the genetic diversity of the isolates. An Unweighted Paired Group Method of Arithmetic Averages tree was used for identifying unique multilocus haplotypes but there was no association between haplotypes and source locations. An analysis of molecular variance among populations showed that 98% of the variance was accounted for within the populations, and only 2% was accounted for among populations. Differences among populations was not significant (Φ=0.018, P=0.2). The relatively high, yet uniform, genetic diversity among isolates and low degree of spatial differentiation between populations of *X. citri* subsp. *citri* suggests that the populations in Paraná state have a common origin and strong historical epidemiological links.

Key words: Citrus canker, haplotype, minisatellite marker.

Introduction

The bacterium *Xanthomonas citri* subsp. *citri* (Schaad *et al*., 2006) causes Asiatic citrus canker (ACC), and is a pathogen of major concern to the citrus industry in many tropical and sub-tropical citrus growing regions of the world (Schubert *et al*., 2001). The pathogen has a wide host range among species of *Citrus* and its relatives (Gottwald *et al*., 2002; de Carvalho *et al*., 2014). ACC causes direct yield losses, and also damages fruit reducing their market value. (Behlau *et al*., 2010; Gottwald *et al*., 2009).

There are several pathotypes of *X. citri* subsp. *citri* (Gabriel, 2001; Brunings and Gabriel, 2003; Schaad *et al*., 2006), making the pathogen a potentially useful model for host-pathogen interaction studies. Pathotypes are classified into three pathogenic variants based on differences in host range, but not on symptomatology (Vernière *et al*., 1998). Pathotype 'A' is present in all citrus growing regions of the world where ACC causes disease, and is the only pathotype reported in Brazil. This pathotype has the widest host range (almost all citrus species and related genera). *Citrus* species and cultivars exhibit differences in susceptibility to this pathotype. In contrast, pathotypes 'A^*' and 'A^w' have narrow host ranges, mainly restricted to key lime (*Citrus aurantifolia*) and 'Alemow' (*C. macrophylla*) (Vernière *et al*., 1998; Cubero and Graham, 2002; Sun *et al*., 2004). The 'A^w' pathotype is distinguished from 'A^*' based on the hypersensitive response to this pathotype in grapefruit (*C. paradisi*), sweet orange (*C. sinensis*), sour orange (*C. aurantium*), lemon (*C. medica*), tangelo (*C. reticulata* × *C. paradisi*) and trifoliate orange (*Poncirus trifoliata*) (Sun *et al*., 2004).

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ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm

Much research has been aimed to identify and group different pathotypes of *X. citri* subsp. *citri* using molecular tools and understanding of genetic diversity of the 'A' strain, at global, national and regional scales (Hartung and Civerolo, 1989; Pruvost *et al*., 1992; Cubero *et al*., 2001; Cubero and Graham, 2002; Graham *et al*., 2004; Li *et al*., 2007; Golmohammadi *et al*., 2007; Bui Thi Ngoc *et al*., 2010; Scuderi *et al*., 2010; Jaciani *et al*., 2012). However, relatively little research has considered population genetic diversity of individual pathotypes of at small, local scales, in and among fields at the scale of tens of kilometers. A previous study of genetic diversity was based on PCR of the enterobacterial repetitive intergenic consensus and BOX elements (Graham *et al*., 2004). This showed pathotype subgroups and geographic associations that allowed inference of the origins of exotic populations. Subsequently, Li *et al*. (2007) used insertion event scanning (IES) to demonstrate relatedness of historical specimens through genetic matching. Genetic diversity of pathotype A from Brazil at a national scale was first reported in 2012 by Jaciani *et al.* (2012), based on a range of effector genes combined with RFLPs, and demonstrating that the pathogen was relatively diverse. Using other molecular markers, Bui Thi Ngoc *et al.* (2010) demonstrated genetic diversity in different pathotypes of the pathogen. Bui Thi Ngoc *et al.* (2009) developed 14 minisatellite markers based on the *in silico* information of the pathogen's genome, which are suitable for population genetic studies of individual pathotypes, including pathotype A. These minisatellites are a useful tool to study genetic diversity of pathotype 'A' of *X. citri* subsp. *citri* in Brazil, and will provide further information on the characteristics of populations among orchards and their relatedness at a local scale in Brazil.

Knowledge of pathogen diversity can also assist in developing effective disease management strategies, including host resistance (Adhikari *et al*., 1999; Ochiai *et al*., 2000; Restrepo *et al*., 2000; Jaciani *et al*., 2012). The present study evaluated the genetic diversity of *X. citri* susbp. *citri* at a local spatial scale in selected commercial and experimental orchards in the state of Paraná, Brazil.

Materials and methods

Pathogen isolation and culture

A total of 40 isolates of *X. citri* susbp. *citri* were sampled from trees of various cultivars in three orchards, all in the State of Paraná [Congonhinhas (23°29'S, 50°29'W and 757 m elevation, four isolates), Paranavaí (23° 1'S, 50° 41'W and 467 m elevation, eight isolates) and Iguatemi (23º21'23''S, 51º04'18''W and 550 m elevation, 28 isolates)] (Table 1). All samples were collected and processed between November 2012 and June 2013. One additional sample of the isolate Xcc 306 (GenBank accession number NC_003919) was included in the study, which was obtained from the collection of pathogen cultures at the Fundo de Defesa da Citricultura (Fundecitrus, Araraquara, São Paulo, Brazil). This is a confirmed *X. citri* susbp. *citri* 'A' pathotype, and has previously been sequenced by the Genoma Project/FAPESP (Da Silva *et al*., 2002).

The diseased leaves, with typical lesions of canker were, disinfected with 1% sodium hypochlorite and washed in distilled water, and the areas with lesions cut out from the healthy tissue. Samples of infected tissue were each transferred to a tube containing 1 mL phosphate buffered saline (PBS, 137 mM NaCl, 76 mM Na_2HPO_4, 25 mM $NaH_2PO_4.H_2O$). A mortar and pestle was used to macerate the plant material. A dilution series was performed (10^{-1} to 10^{-5} bacteria mL^{-1}) to obtain colony forming units for individual isolation. The colonies were grown aseptically in Petri plates on Nutrient Agar (NA) (5 g peptone, 3 g of meat extract, 1 g NaCl, 15 g agar, 1,000 mL H_2O), prepared by autoclaving at 120°C for 30 min. Two replicate plates were prepared. The plates were inverted and incubated at 28°C, and subculturing was to fresh plates. Samples were then transferred to NA in test tubes which served as the stock cultures, which were stored under refrigeration (8°C). When needed, individual isolates were cultured on NA for approx. 48 h at 28°C.

DNA extraction and minisatellite amplification

Total DNA was extracted from the culture of each isolate on NA. After suspending and washing the bacteria with NaCl (5 M), the suspension was centrifuged (8,000 rpm) for 10 min, and the pellet was suspended in 800 µL of extraction buffer (100 mM Tris-HCl pH 8.0, 0.5 M NaCl, 50 mM EDTA, SDS 10%). The samples were incubated at 65°C for 30 min, then 400 µL of potassium acetate (5 M) was added to the samples. The mixture was then incubated on ice for 20 min. Samples were centrifuged (12,000 rpm for 15 min), after which a phenol/chloroform/isoamyl

Table 1. The isolates of *Xanthomonas citri* subsp. *citri* collected from commercial and experimental orchards from three locations and different *Citrus* host species and cultivars in Paraná state, Brazil.

Isolate code	Isolate	Municipality	Citrus host and cultivar
11	Xcc 213	Congonhinhas	*C. sinensis* 'Pera'
17	Xcc 213-2	Congonhinhas	*C. sinensis* 'Pera'
30	Xcc 215	Congonhinhas	*C. sinensis* 'Pera'
41	Xcc 215-2	Congonhinhas	*C. sinensis* 'Pera'
8	Xcc 212	Paranavaí	*C. sinensis* 'Pera'
15	Xcc 220	Paranavaí	*C. sinensis* 'Pera'
16	Xcc ZWP	Paranavaí	*C. sinensis* 'Pera'
24	Xcc 220-2	Paranavaí	*C. sinensis* 'Pera'
25	Xcc 213	Paranavaí	*C. sinensis* 'Pera'
27	Xcc 208	Paranavaí	*C. sinensis* 'Pera'
40	Xcc 212-2	Paranavaí	*C. sinensis* 'Pera'
48	Xcc 208-2	Paranavaí	*C. sinensis* 'Pera'
1	Xcc 338	Iguatemi	*C. sinensis* 'Valencia Olinda'
2	Xcc 87M	Iguatemi	*C. sinensis* 'Pera mutante'
3	Xcc 78	Iguatemi	*C. sinensis* 'Orange clamor'
4	Xcc 21M	Iguatemi	*C. sinensis* 'Pera mutante'
5	Xcc 114	Iguatemi	*C. sinensis* 'Wetumpka'
6	Xcc 85	Iguatemi	*C. sinensis* 'Valencia Frost'
7	Xcc 362	Iguatemi	*C. sinensis* (unknown)
9	Xcc 347	Iguatemi	*C. sinensis* 'Olivelands'
10	Xcc 214	Iguatemi	*C. sinensis* 'Natal Af. Sul'
12	Xcc 103	Iguatemi	*C. sinensis* 'Moro 27'
13	Xcc 84	Iguatemi	*C. sinensis* 'Valencia'
18	Xcc 74	Iguatemi	*C. sinensis* 'Orange Navelina'
19	Xcc 85-2	Iguatemi	*C. sinensis* 'Valencia Frost'
20	Xcc 103-2	Iguatemi	*C. sinensis* 'Moro 27'
22	Xcc 87M-2	Iguatemi	*C. sinensis* 'Pera mutante'
26	Xcc 214-2	Iguatemi	*C. sinensis* 'Natal Af. Sul'
28	Xcc 362-2	Iguatemi	*C. sinensis* (unknown)
31	Xcc 74-2	Iguatemi	*C. sinensis* 'Orange Navelina'
32	Xcc 78-2	Iguatemi	*C. sinensis* 'Orange clamor'
34	Xcc 327	Iguatemi	*C. sinensis* 'Ovale Mut.'
35	Xcc 216-2	Iguatemi	*C. sinensis* 'Westin'
36	Xcc 21M-2	Iguatemi	*C. sinensis* 'Pera mutante'
37	Xcc 370-2	Iguatemi	*C. tangerina* 'Empress'
38	Xcc 327-2	Iguatemi	*C. sinensis* 'Ovale Mut.'
43	Xcc 318	Iguatemi	*C. sinensis* 'Natal'
44	Xcc 148-2	Iguatemi	*C. sinensis* 'Espanole'
45	Xcc 345-2	Iguatemi	*C. limettioides* 'Monica'
46	Xcc 304	Iguatemi	*C. reticulata* × *C. sinensis* 'Tangor Murcote'
23	Xcc 306	Control isolate	-

Table 2. Minisatellite primers used to screen 40 isolates of *Xanthomonas citri* subsp. *citri* (and one control isolate of pathotype 'A') sampled from orchards in Paraná state, Brazil.

Name	Primer	Annealing temp. (°C)
XL1[1]	5¢- TTGCCACGGGCCAGGGTCGC-3¢	64
	5′-GCCCAGATGCTCGATCAAGG-3′	
XL10	5′- GCGAGGCGATTGCCTGGCTCA-3′	70
	5′-GCGGATGCATTTGCCGGGTGAGT-3′	
XL2	5′ -TGGGAAGCGGTCAGTAGGCA-3′	68
	5′-CCACATAAGCACGGCAACAA-3′	
XL3	5′ -TTCAAGGAATTGCCCGAGAG-3′	64
	5′-CAGACAGCGGCGGATTGATG-3′	
XL4	5′ -TTGCCCATCGTTGCTGAAGCAGC-3′	64
	5′-ATGCCGCTGTTGTGTGAGCGC-3′	
XL13	5′--GCGCTCCCTCGCTGCGAGGTA-3′	64
	5′-CAGCAGATACAGGGCGAACGCGAT-3′	
XL5	5′ -TCCGCTCGATCATCGTCAAGG-3′	64
	5′-ACGCTGATGCGCCCAGCGCTTGC-3′	
XL14	5′-CCAAAGGCTATGCGGATCTG-3′	64
	5′-CGTTCGACCCGGAATAAG-3′	
XL6	5′-TGACAAGCAGGAGCAGGCGCATGG-3′	70
	5′-ATCGCACAGCAGCAACGAAGG-3′	
XL7	5′ PET-TGCGCAAGCTGGTCAAGTGG-3′	68
	5′-TCCTGCGATGGCGAGTGG-3′	
XL8	5′ FAM-GGCGGTGGCGGCGTAGTCAC-3′	68
	5′-TCGGCTGCTGGCGTTGATTG-3′	
XL9	5′ VIC-TCAGTCAGCCATCTCTACAAGC-3′	64
	5′-CGACAAGGTCGACGAATACC-3′	

[1] Minisatellite markers, drawn from the complete genome sequence of *X. citri* subsp. *citri* 306 isolate (Bui Thi Ngoc *et al*., 2009).

alcohol solution (25:1:24) was added and the supernatant collected. Subsequently, 150 µL of RNase was added to the samples, which were maintained at 37°C for one further precipitation with isopropanol. After a final centrifugation (12,000 rpm for 10 min), the precipitate was suspended in Tris-EDTA (TE) (adapted from Nunes *et al*., 2008). DNA quantification and quality were assessed on 1% agarose gels stained with ethidium bromide.

The amplification of the DNA followed the protocols of Bui Thi Ngoc *et al.* (2009). Twelve minisatellite primer pairs, drawn from the complete genome sequence of *X. citri* subsp. *citri* isolate 306 (Da Silva *et al*., 2002), were used to amplify the sample DNA (Table 2). The PCR reactions were each conducted in a total volume of 25 µL, consisting of 2.5 µL of buffer (10×, 200 mM Tris-HCl, pH 8.4, 500 mM KCl), Milli-Q water, 2.5 mM $MgCl_2$, 10 mM DNTP, 15 ng of

each primer, 40 ng of sample DNA and 1 U of Taq-DNA-polymerase (Invitrogen®). The reactions were performed individually for each isolate × primer combination, and were not multiplexed as described by Bui Thi Ngoc *et al.* (2009).

PCR amplifications conditions were: 15 min at 95°C for the initial DNA denaturation, followed by 25 cycles at 94°C for 30 s, annealing temperatures ranging from 64 to 70°C depending on primer, for 90 s and an extension step of 72°C for 90 s, followed by a final extension for a further 30 min at 72°C (Bui Thi Ngoc *et al*., 2009).

The resulting PCR products were visualized using electrophoresis in 2% agarose gels, stained with ethidium bromide. The gels were photographed under ultraviolet light with photo-documentation equipment to record the banding pattern (UVP GDS-8000 system, UVP Inc.). The size of the amplicon on the gel was recorded (bp) relative to the size standard used in each gel (1 kb Plus DNA ladder, Invitrogen®).

Data analyses

To assess relationships among all 41 isolates of *X. citri* susbp. *citri* (including the control isolate, Xcc 306), an Unweighted Paired Group Method of Arithmetic Averages (UPGMA) cluster analysis (PROC CLUSTER, method=average; SAS Institute) was applied to the matrix of minisatellite marker and isolate data. A Mantel test was performed to assess the association between population pairwise physical and genetic distance. Relationships among isolates from different populations were investigated. The control isolate Xcc 306 was not included in the population genetic anlaysis for the three locations sampled. The data were analyzed as codominant markers using the genetic analysis software GenAlex (Peakall and Smouse, 2006; 2012), with the genetics assumed to be based on a haploid organism. The percent polymorphic loci and the number of private alleles were calculated. Shannon's information index (${}^{s}H_A$) and genetic diversity (H_T) were calculated for each population based on the minisatellite allele frequency data, and used to evaluate the level of genetic diversity in populations (Lewontin, 1972). Nei's genetic identity and Shannon's mutual information index (${}^{s}H_{uA}$) were used to further characterise each population and to measure population differentiation as defined by Peakall and Smouse (2006, 2012). Missing values, where they occurred, were coded according to the program instructions.

Results

The 12 pairs of minisatellite primers used in this study amplified genomic regions of *X. citri* subsp. *citri* in all of the tested isolates. The minisatellite markers were highly discriminating and were effective for detecting differences between all individual isolates from the state of Paraná, Brazil. The UPGMA analysis separately identified each isolate as a unique multi locus haplotype (MLHT) (Figure 1). However, there was no tendency for differentiation of the isolates by source location based on this analysis, with isolates from the three populations being equally closely related to one another. The control isolate Xcc 306 was genetically similar to the other isolates sampled, confirming the identities as *X. citri* subsp. *citri* pathotype 'A'.

The numbers of alleles for each locus and in each population varied (Table 3). In total, there were 56 alleles among the twelve primers. The greatest number of alleles (seven) was found with primers XL5, XL6 and XL10, while the fewest (two) were found with primer XL7. Among the populations sampled, the greatest number of alleles were found in the FEI-Iguatemi population (48 alleles, or 86% of total alleles identified). Most samples were collected from the FEI-Iguatemi population, although the Paranavaí population had 73% of all alleles identified, and the Congonhinhas population had 55% of all alleles identified, despite smaller sample sizes. There was a high degree of polymorphism in each of the populations from which isolates were collected (Table 4), ranging from 92 to 100% of loci. The mean number of different alleles per locus was 3.33 over all populations, with a range of 2 to 7 alleles per locus among all isolates depending on the minisatellite marker. Shannon's information index (${}^{s}H_A$) and genetic diversity (H_T) (which are both measures of genetic diversity) were similar for all three populations sampled, despite differences in sample size; the number of private alleles was low in all the populations sampled. The analysis of molecular variance among populations showed that 98% of the variance was accounted for within the populations, and only 2% was accounted for among populations (Table 5). The differences among populations were not statistically significant (Φ=0.018, P=0.2).

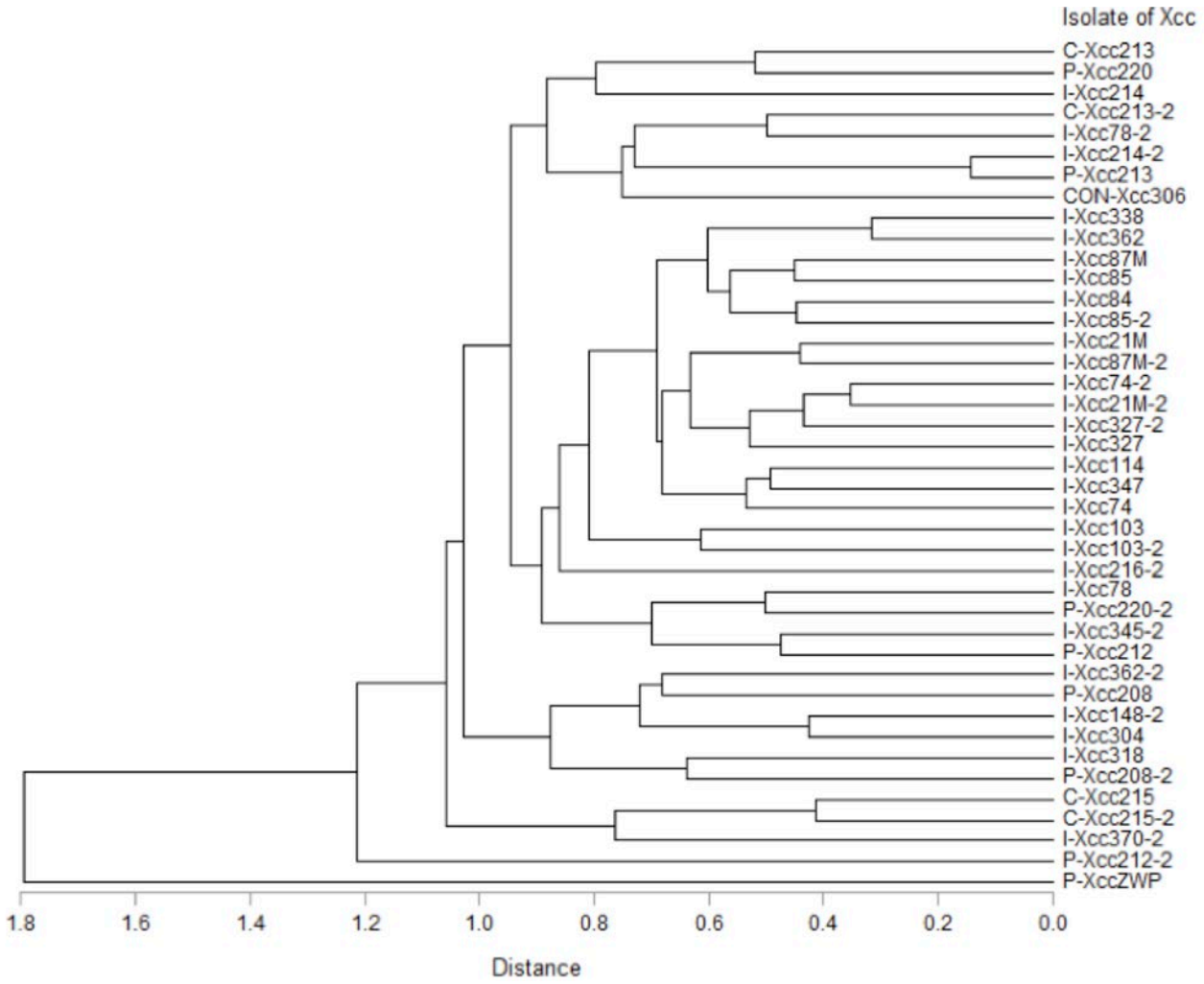

Figure 1. Unweighted paired group method of arithmetic averages cluster analysis showing the genetic diversity of 41 isolates of *Xanthomonas citri* subsp. *citri*, characterized by minisatellite markers from different locations in Paraná state, Brazil. Scale shows Euclidean distance. The letter prefix for each isolate indicates source population: C = Congonhinhas, P = Paranavaí, I = FEI-Iguatemi and CON = control (type sample = Xcc 306).

The pairwise population comparison of Nei's genetic identity and Shannon's information index were used to evaluate the level of population subdivision and genetic diversity (Table 6). Despite differences in population sample sizes, genetic identity was comparable (Nei's genetic identity = 0.657–0.828), with only limited population differentiation ($^{s}H_{uA}$ = 0.061–0.204). This further confirmed the similarity among isolates from the different locations. There was no association between physical distance (log km) and genetic distance ($^{s}H_{u}$) (r=0.369, *P*=0.8), although the number of locations was very small.

Discussion

This study has demonstrated that there was high genetic diversity in *X. citri* subsp. *citri* samples from Paraná, with polymorphisms existing at each locus, and each isolate being identified as a unique MLHT. These results are in general agreement with previous observations (Jaciani *et al*., 2012), that demonstrated that pathotype 'A' of the pathogen in Brazil had high genetic diversity. However, the isolates in the previous study were from diverse locations in Brazil, while our study focused specifically on a local scale,

Table 3. Number of alleles per locus (primer) for the minisatellites used to screen 40 isolates of *Xanthomonas citri* subsp. *citri* sampled from orchards in Paraná state, Brazil. Numbers in parentheses are the percentage of the total number of alleles observed.

Locus[1] (primer name)	Population			Total
	Congonhinhas	FEI-Iguatemi	Paranavaí	
XL1	3 (100)	3 (100)	3 (100)	3
XL2	2 (50)	2 (50)	3 (75)	4
XL3	2 (67)	2 (67)	3 (100)	3
XL4	3 (60)	4 (80)	4 (80)	5
XL5	2 (29)	7 (100)	6 (86)	7
XL6	3 (43)	6 (86)	4 (57)	7
XL7	1 (50)	1 (50)	2 (100)	2
XL8	3 (50)	6 (100)	3 (50)	6
XL9	2 (50)	4 (100)	4 (100)	4
XL10	4 (57)	7 (100)	4 (57)	7
XL13	4 (100)	3 (75)	2 (50)	4
XL14	2 (50)	3 (75)	3 (75)	4
Total	31 (55)	48 (86)	41 (73)	56

[1] Minisatellite markers, drawn from the complete genome sequence of *X. citri* subsp. *citri* 306 isolate (Bui Thi Ngoc *et al*., 2009).

Table 4. Measures of polymorphism and genetic diversity of three populations of *Xanthomonas citri* subsp. *citri* sampled from different orchards in Paraná state, Brazil (based on minisatellite markers).

Location	Percent polymorphic loci	Number of different alleles (standard error)	Shannon's information index[2] (standard error)	Genetic diversity[3] (standard error)	Number of private alleles
Congonhinhas	91.7	2.58 (0.26)	0.851 (0.113)	0.525 (0.059)	0.083
Iguatemi	91.7	4.00 (0.59)	1.046 (0.140)	0.568 (0.062)	0.583
Paranavaí	100.0	3.42 (0.31)	1.018 (0.102)	0.569 (0.045)	0.583
All populations	94.4 (2.78)	3.33 (0.25)	0.972 (0.069)	0.554 (0.032)	

[1] Variables caluculated using Genalex (Peakall and Smouse, 2006, 2012).
[2] Shannon's information index was calculated as , where where h is the total number of alleles, and P_i is the frequency of allele i (Peakall and Smouse, 2006, 2012).
[3] Genetic diversity was calculated as , where where h is the total number of alleles, and P_{Ti} is the frequency of allele i over the whole population (Peakall and Smouse, 2006, 2012).

with isolates from trees in three orchards in Paraná. Our results demonstrate that although genetic diversity exists, the populations of *X. citri* subsp. *citri* at this local scale could not be differentiated.

Unlike the study of Jaciani *et al*. (2012), we were not able to demonstrate the existence of specific MLHTs that were more common than others, as each isolate was identified as a unique genotype

Table 5. Molecular variance[1] of three populations of *Xanthomonas citri* subsp. *citri* from Paraná state, Brazil.

Source	D.f.	SS	MS	Estimated variance	% of variance	Φ (P-value)
Among populations	2	8.68	4.34	0.069	2	0.018 (0.2)
Within populations	37	137.19	3.71	3.708	98	
Total	39	145.87		3.777	100	

[1] Analysis of molecular variance for the populations of *X. citri* subsp *citri* was based on 1000 permutations using Genalex (Peakall and Smouse, 2006, 2012). D.f.: degrees of freedom, SS: sum of squares, MS: mean square error.

Table 6. Pairwise population matrix of Nei's measure of genetic identity and the mean Shannon mutual information index ($^{s}H_{uA}$) over all loci, for *Xanthomonas citri* subsp. *citri* isolates from citrus orchards in Brazil.

Location	Congonhinhas	Iguatemi	Paranavaí
Congonhinhas		0.772	0.657
Iguatemi	0.061		0.828
Paranavaí	0.204	0.099	

Geogrpaphic distance between the Congonhinhas and FEI-Iguatemi locations = 217 km, between Congonhinhas and Paranavaí locations = 261 km, and between FEI-Iguatemi and Paranavaí locations = 59 km. A mantel test was used to explore association between physical and genetic distance.

using these minisatellites. The populations in Paraná state are all likely to have the same source (Jaciani *et al*., 2012), and our results using minisatellites confirm this conclusion - no isolates tested were strikingly different to the group as a whole. A region-wide study in Asia that included pathotype 'A' of the pathogen also identified each isolate as a unique genotype (Bui Thi Ngoc *et al*., 2009). As suggested by these authors, these minisatellites are thus very useful for epidemiological typing and for population genetic studies at a relatively small spatial scale. Furthermore, Pruvost *et al.* (2011) used these primers developed for *X. citri* subsp. *citri* to study the diversity of the related pathogen *X. citri* subsp. *mangiferaeindicae*, and readily demonstrated differentiation of pathotypes. Our results did not show consistent differences between populations that would allow grouping at a sub-pathotype level. Recent introduction to Brazil, and local spread of the pathogen may preclude differences, at the scale of individual orchards, becoming established in contiguous populations over distances of 100 to 200 km. All the isolates were very closely related genetically, and location of origin was not obvious from their genetic profiles.

Despite isolates coming from different *Citrus* species or hybrids, there was no evidence that the genetic diversity of haplotypes was related to source host. Furthermore, Bui Thi Nogoc *et al.* (2010) demonstrated that although there is differentiation among pathotypes of *X. citri* subsp. *citri* (for example 'A*' and 'Aw' pathotypes), they are all closely related. Our results confirm the relatedness of isolates from different hosts and locations for pathotype 'A' at a local scale in Brazil. Also, an evaluation of 42 pathotypes of collected from different citrus species in Argentina from orchards in the Tucuman region showed that each isolate was genetically unique based on the molecular markers used, and most showed similar pathogenicity to the reference isolate of *X. citri* subsp. *citri* used in the study (Chiesa *et al*., 2013). Lin *et al.* (2012) evaluated 21 isolates of the pathogen in Taiwan (pathogenic on *C. aurantifolia*, *C. paradisi* and *C. sinensis*), and confirmed all as *X. citri* subsp. *citri* pathotype 'A' by genetic characterization. The results we present support these previous observations.

More extensive and comprehensive studies using minisatellites and other molecular markers (Jaciani *et al.*, 2012) can be used to provide valuable information on the population genetic diversity, relatedness and gene flow of pathotype 'A' in Brazil. This information is not only important for understanding pathogen distribution and movement, but potentially for regulatory purposes, and identifying novel pathotypes of the pathogen at early stages in their progress. The genetic characterization of populations of these bacteria will provide useful information for monitoring the disease epidemics they cause, in addition to evaluating origins, genetic and evolutionary aspects of the pathogen. Further research on the biology and epidemiology of *X. citri* subsp. *citri* is necessary, as several important questions regarding the species, their population biology and epidemiology remain unresolved.

Acknowledgements

The authors thank 'Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior' (CAPES) for financial support. A.M.O. Gonçalves-Zuliani is a recipient of a PhD grant from CAPES, Ministerio de Educacao de Brazil. W.M.C. Nunes was supported by a grant from Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq).

Literature cited

Adhikari T.B., T.W. Mew and J.E. Leach, 1999. Genotypic and pathotypic diversity in *Xanthomonas oryzae* pv. *oryzae* in Nepal. *Phytopathology* 89, 687–694.

Behlau F., J. Belasque Jr, J.H. Graham and R.P. Leite Jr, 2010. Effect of frequency of copper application on control of citrus canker and the yield of young bearing sweet orange trees. *Crop Protection* 29, 300–305.

Brunings A.M. and D.W. Gabriel, 2003. *Xanthomonas citri*: Breaking the surface. *Molecular Plant Pathology* 4, 141–157.

Bui Thi Ngoc L., C. Vernière, K. Vital, F. Guerin, L. Gagnevin, S. Brisse, N. Ah-You and O. Pruvost, 2009. Development of 14 minissatellite markers for the citrus canker bacterium, *Xanthomonas citri* pv. *citri*. *Molecular Ecology Resources* 9, 125–127.

Bui Thi Ngoc L., C. Vernière, E. Jouen, N. Ah-You, P. Lefeuvre, F. Chiroleu, L. Gagnevin and O. Pruvost, 2010. Amplified fragment length polymorphism and multilocus sequence analysis-based genotypic relatedness among pathogenic variants of *Xanthomonas citri* pv. *citri* and *Xanthomonas campestris* pv. *bilvae*. *International Journal of Systematic and Evolutionary Microbiology* 60, 515–525.

Carvalho F.M.S., P.C.L. Caramori and R.P. Leite Jr, 2005. Genetic diversity of *Xanthomonas axonopodis* pv. *citri* based on plasmid profile and pulsed field gel electrophoresis. *Genetics and Molecular Biology* 28, 446–451.

Chiesa M.A., M.F. Siciliano, L. Omella, R.A. Roeschlin, M.A. Favaro, N.P. Delgado, L.N. Sendin, I.G. Orce, L.D. Ploper, A.A. Vojnov, J.G. Vacas, M. Filippone, A.P. Castagnaro and M.R. Marano, 2013. Characterization of a variant of *Xanthomonas citri* subsp. *citri* which triggers a host-specific defense response. *Phytopathology* 103, 555–564.

Cubero J. and J.H. Graham, 2002. Genetic relationship among worldwide strains of *Xanthomonas* causig canker in citrus species and design of new primers for their identification by PCR. *Applied and Environmental Microbiology* 68, 1256–1264.

Cubero J., J.H. Graham and T.R. Gottwald, 2001. Quantitative PCR method for diagnosis of citrus bacterial canker. *Applied and Environmental Microbiology* 67, 2849–2852.

Da Silva A.C., J.A. Ferro, F.C. Reinach, C.S. Farah, L.R. Furlan, Quaggio R.B., C.B. Monteiro-Vitorello, M.A. Van Sluys, N.F. Almeida, L.M. Alves, A.M. do Amaral, M.C. Bertolini, L.E.A. Camargo, G. Camarotte, F. Cannavan, J. Cardozo, F. Chambergo, L.P. Ciapina, R.M. Cicarelli, L.L. Coutinho, J.R. Cursino-Santos, H. El-Dorry, J.B. Faria, A.J. Ferreira, R.C. Ferreira, M.I. Ferro, E.F. Formighieri, M.C. Franco, C.C. Greggio, A. Gruber, A.M. Katsuyama, L.T. Kishi, R.P Leite Jr, E.G. Lemos, M.V. Lemos, E.C. Locali, M.A. Machado, A.M. Madeira, N.M. Martinez-Rossi, E.C. Martins, J. Meidanis, C.F. Menck, C.Y. Miyaki, D.H. Moon, L.M. Moreira, M.T. Novo, V.K. Okura, M.C. Oliveira, V.R. Oliveira, H.A. Pereira, A. Rossi, J.A. Sena, C. Silva, R.F. de Souza, L.A. Spinola, M.A. Takita, R.E. Tamura, E.C. Teixeira, R.I. Tezza, M. Trindade dos Santos, D. Truffi, S.M. Tsai, F.F. White, J.C. Setubal and J.P. Kitajima, 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* 417, 459–463. doi:10.1038/417459.

Gabriel D.W., 2001. Citrus canker. In: *Encyclopedia of Plant Pathology*, O C Maloy and T D Murray, eds. John Wiley and Sons (p 215-217), 1368 pp. ,

Golmohammadi M., J. Cubero, J. Peñalver, J.M. Quesada, M.M. López and P. Llop, 2007. Diagnosis of *Xanthomonas axonopodis* pv. citri, causal agent of citrus canker, in commercial fruits by isolation and PCR-based methods. *Journal of Applied Microbiology* 103, 2309–2315.

Gottwald T.R., X. Sun, T. Riley, J.H. Graham, F. Ferrandino and E.L. Taylor, 2002. Geo-referenced spatiotemporal analysis of the urban citrus canker epidemic in Florida. *Phytopathology* 92, 361–377.

Gottwald T.R., J.H. Graham, C.H. Bock, G. Bonn, E. Civerolo, M. Irey, R.P. Leite Jr, M. Lopez, G. McCollum, P. Parker, J. Ramallo, T. Riley, T. Schubert, B. Stein and E. Taylor, 2009. The epidemiological significance of post-packinghouse survival of *Xanthomonas citri* ssp *citri* for dissemination of Asiatic citrus canker via infected fruit. *Crop Protection* 28, 508–524.

Graham J.H., T.R. Gottwald and J. Cubero, D.S. Achor, 2004. *Xanthomonas axonopodis* pv *citri*: factors affecting successful eradication of citrus canker. *Molecular Plant Pathology* 5, 1–15.

Hartung, J.S., E.L. Civerolo, 1989. Restriction fragment length polymorphisms distinguish *Xanthomonas campestris*

strains isolated from Florida citrus nurseries from *X. c.* pv. *citri*. *Phytopathology* 79, 793–799.

Jaciani FJ, J.A Ferro, M.I.T. Ferro, C. Vernière, O. Pruvost and J. Belasque Jr, 2012. Genetic diversity of a Brazilian strain collection of *Xanthomonas citri* subsp. *citri* based on the type III effector protein genes. *Plant Disease* 96, 193–203.

Lewontin R.C., 1972. The apportionment of human diversity. *Evolutionary Biology* 6, 381–398.

Li W., J.S. Hartung and L. Levy, 2007. Evaluation of DNA amplification methods for improved detection of "Candidatus Liberibacter species" associated with citrus huanglongbing. *Plant Disease* 91, 51–58.

Lin H.C., C.Y. Feng, Y.A. Chang and H. Chang, 2012. Molecular Typing and Genetic Characterization of pathogenic variants of *Xanthomonas axonopodis* pv. *citri* in Taiwan. *Journal of Phytopathology* 160, 475–483.

Nunes W.M.C., M.J. Corazza, S.A.C.D Souza, S.M. Tsai and E.E. Kuramae, 2008. Characterization of *Xanthomonas axonopodis* pv *phaseoli* isolates. *Summa Phytopathologica* 34, 228–231.

Ochiai H., O. Horino, K. Miyajima and H. Kaku, 2000. Genetic diversity of *Xanthomonas oryzae* pv. *oryzae* strains from Sri Lanka. *Phytopathology* 90, 415–421.

Peakall R. and P.E. Smouse, 2006. GENALEX 6: genetic analysis in Excel Population genetic software for teaching and research. *Molecular Ecology Notes* 6, 288–295.

Peakall R. and P.E. Smouse, 2012. GenAlEx 65: genetic analysis in Excel Population genetic software for teaching and research–an update. *Bioinformatics* 28, 2537–2539.

Pruvost O., A. Couteau and J. Luisetti, 1992. Pepper tree (*Schinus terebenthifolius* Radii), a new host plant for *Xanthomonas campestris* pv. *mangiferaeindicae*. *Journal of Phytopathology* 135, 289–298.

Pruvost O., C. Vernière, K. Vital, F. Guérin, E. Jouen, F. Chiroleu, N. Ah-You and L. Gagnevin, 2011. Insertion sequence and tandem repeat-based genotyping techniques for *Xanthomonas citri* pv *mangiferaeindicae. Phytopathology* 101, 887–893.

Restrepo S., C.M. Vélez and V. Verdier, 2000. Measuring the genetic diversity of *Xanthomonas axonopodis* pv *manihotis* within different fields in Colombia. *Phytopathology* 90, 683–690.

Schaad N.W., E. Postnikova, G. Lacy, A. Sechler, I. Agarkova, V.K. Stromberg and A.K. Vidaver, 2006. Emended classification of xanthomonad pathogens on citrus. *Systematic and Applied Microbiology* 29, 690–695.

Schubert T.S., S.A.Rizvi, *X.* Sun, T.R. Gottwald, J.H. Graham and W.N. Dixon, 2001. Meeting the challenge of eradicating citrus canker in Florida-Again. *Plant Disease* 85, 340–356.

Scuderi G., M. Golmohammadi, J. Cubero, M.M. López, G. Cirvilleri and P. Llop, 2010. Development of a simplified NASBA protocol for detecting viable cells of the citrus pathogen *Xanthomonas citri* subsp. c*iti* under different treatments. *Plant Pathology*, 59, 764–772.

Sun X., R.E. Stall, J.B. Jones, J. Cubero, T.R. Gottwald, J.H. Graham, W.N. Dixon, T.S. Schubert, P.H. Chaloux, V.K. Stromberg, G.H. Lacy and B.D. Sutton, 2004. Detection and characterization of a new strain of citrus canker bacteria from Key / Mexican lime and alemow in South Florida. *Plant Disease* 88, 1179–1188.

Vernière C., J.S. Hartung, O.P. Pruvost, E.L. Civerolo, A.M. Alvarez, P. Maestri and J. Luisetti, 1998. Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv *citri* from Southwest Asia. *European Journal of Plant Pathology* 104, 477–487.

Accepted for publication: December 15, 2016
Published online: May 10, 2017