Molecular and Pathogenic Diversity Among Brazilian Isolates of *Xanthomonas albilineans* Assessed with SSR Marker Loci

Ana C. Tardiani, Centro de Cana, Instituto Agronômico, Campinas–IAC, Rod. Antonio Duarte Nogueira Km 321, CP 206, Ribeirão Preto, SP, Brazil, and Universidade Estadual Paulista–UNESP, Av. Prof. Paulo Donato Castellane, 14884-900, Jaboticabal, SP, Brazil; **Dilermando Perecin**, Universidade Estadual Paulista–UNESP; **Rafael F. Peixoto-Junior**, **Alvaro Sanguino**, and **Marcos M. G. Landell**, Centro de Cana, Instituto Agronômico, Campinas–IAC, Rod. Antonio Duarte Nogueira Km 321, CP 206, Ribeirão Preto, SP, Brazil; **Luis O. Beriam**, Laboratório de Bacteriologia Vegetal–CEIB, Instituto Biológico, Campinas, 13001-970, SP, Brazil; **Daniel S. Nunes**, Centro de Cana, Instituto Agronômico, Campinas–IAC, Rod. Antonio Duarte Nogueira Km 321, CP 206, Ribeirão Preto, SP, Brazil; **Luis E. A. Camargo**, Departamento de Fitopatologia e Nematologia, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Av. Pádua Dias, 11, 13418-900, Piracicaba, SP, Brazil; and **Silvana Creste**, Centro de Cana, Instituto Agronômico, Campinas–IAC, Rod. Antonio Duarte Nogueira Km 321, CP 206, Ribeirão Preto, SP, Brazil

Abstract

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Leaf scald is one of the most important diseases of sugarcane in Brazil. Despite its importance, little is known about the genetic and pathogenic variability of its causal agent, *Xanthomonas albilineans*. The genetic diversity of 44 *X. albilineans* isolates from diverse geographic regions of Brazil was assessed using 15 newly developed short sequence repeat (SSR) loci designed from the genome sequence of *X. albilineans* strain GPE PC73. In addition, the aggressiveness of each isolate was evaluated by inoculating on a susceptible sugarcane cultivar and scoring the disease severity. Of the 15 SSR loci, 12 were polymorphic and produced 54 polymorphic alleles. The average number of

polymorphic alleles per locus was 4.5, and ranged from 2 to 12 alleles. Phenetic analysis based on Nei's unbiased genetic distance, clustered the isolates into two major groups. Group I included 32 isolates from all four geographic regions studied, whereas group II included 9 isolates from two regions. Three isolates did not cluster within these groups. Analysis of disease severity data also revealed variability in aggressiveness among isolates but no correlation could be established with either SSR haplotypes or phenetic groups. Isolates with identical haplotypes differed in aggressiveness and vice versa. However, single marker-trait analysis revealed two markers associated with this trait.

Xanthomonas albilineans (Ashby 1929) Dowson 1943 is a systemic, xylem-invading bacterium that causes leaf scald disease in sugarcane. The disease affects yields by reducing the germination of seed canes, affecting the development of infected plants, and reducing sucrose content. The major symptom in the chronic form of the disease is a white stripe that develops longitudinally in the leaf and may vary in intensity whereas, in the acute form, complete wilting and necrosis of the leaves result in plant death in susceptible cultivars (9). Additionally, a common symptom in mature cane is the early sprouting of buds from basal setts. The frequent occurrence of latent infections with no visible symptoms represents a major obstacle in the control of the disease in nurseries and commercial crops because it may result in the undesirable spread of contaminated planting material. Although *X. albilineans* is thought to be mainly transmitted by using contaminated cutting instruments, plant-to-plant aerial transmission has been reported in certain locations together with severe outbreaks of the disease (2,11,15).

Control of leaf scald is achieved by using resistant cultivars and by adopting sanitary measures, which include using disease-free seed cane and disinfecting cutting instruments during planting and harvest (37). Other factors such as drought stress might contribute to increase the occurrence of leaf scald in some areas. Selection of resistant genotypes, although feasible, is hampered by the latent infections, which restrict efficient selection (A. Sanguino, unpublished).

It is well known that the pathogenicity of *X. albilineans* varies according to the strain of the pathogen, indicating the existence of different pathotypes within the species (9,15,29). The albicidin toxin plays a key role in pathogenicity (6,7); however, other pathogenicity factors exist, because albicidin-deficient mutant strains are still able to efficiently colonize sugarcane (37).

The first studies on the genetic variability of *X. albilineans* reported the existence of three serovars and six lysovars (35,36). These were followed by several reports using diverse techniques, including artificial inoculation (32), serological methods (1,36), and molecular techniques such as restriction fragment length polymorphism (RFLP; 17), gene sequencing (42), repetitive extragenic palindrome (rep) polymerase chain reaction (PCR) (27), and amplified fragment length polymorphism (AFLP; 40). An analysis of 218 isolates from 31 geographic locations by RFLP with pulse-field gel electrophoresis revealed the existence of at least eight genetic groups (A through H), and most isolates associated with outbreaks in sugarcane crops belonged to group B (17). However, the correlation between genetic diversity and variation in aggressiveness has not been intensively investigated (1,22,23, 27,40). Brazil is the largest producer of sugarcane in the world, and the crop currently occupies 9.2 mi ha (43). The expansion of sugarcane cultivation to drought-prone marginal growing areas, such as central Brazil or the northeastern region of the State of São Paulo, together with the increasing use of mechanical harvesting (without burning), have contributed to increasing the incidence of the disease in these areas, turning this pathogen into one of the most important. Despite its importance, little is known regarding the diversity of *X. albilineans* in the major producing regions of Brazil.

Tandem repeat sequences (TRS), which are common in eukaryotic genomes, have been identified in prokaryotes as well and represent a major source of genetic diversity for many pathogenic bacteria (3). Among the various classes of TRS, short sequence

Corresponding author: S. Creste, E-mail: screste@iac.sp.gov.br

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repeats (SSRs) are highly polymorphic and, therefore, constitute a very useful marker class for the analysis of genetic relationships among isolates in prokaryotic organisms (12,25). In this study, the complete genome sequence of *X. albilineans* strain GPE PC73 (33) was mined for TRS, and SSR primers were designed to characterize *X. albilineans* isolates from different Brazilian sugarcane-growing regions. Additionally, the aggressiveness of each isolate was assessed by inoculating in a susceptible cultivar.

Materials and Methods

***X. albilineans* isolates.** In total, 44 isolates of *X. albilineans* were used, which included 43 isolated from symptomatic plants collected from different sugarcane-growing regions in Brazil and strain IBSBF 1374 (Table 1) from the Mauritius Island, which was used as an outgroup. All isolates were established from single colonies grown on *X. albilineans*-selective (XAS) medium (16) after 5 to 7 days of growth at 28°C. The identity of each isolate was confirmed by PCR using the primer pair NPRS-2 (34).

Development of SSR markers. The complete genome sequence of the *X. albilineans* strain GPE PC73 (GenBank accession FP565176.1) was mined using the Tandem Repeat Finder program (version 4.03; <http://tandem.bu.edu/trf/trf.html>) to identify tandem repeat sequences. Primers flanking SSR loci were designed using the PRIMER3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and their quality was checked using the NETPRIMER software (www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html).

DNA extraction and PCR conditions. Bacterial isolates were grown on XAS medium (16) and used for total genomic DNA extraction (21). Each PCR reaction was performed with 30 ng of genomic DNA, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM each primer, and 1 U of Taq DNA polymerase in an appropriate buffer (Invitrogen) with a final reaction volume of 15 µl. Amplifications were performed in a *MyCycler* thermocycler (Bio-Rad) programmed with a touchdown cycle having an initial denaturation step at 95°C for 5 min followed by 10 cycles of 40 s at 95°C, 30 s starting at 68°C and decreasing 1°C every cycle to 59°C, and 30 s at 72°C. The amplification was followed by 30 identical cycles of 40 s at 95°C, 30 s at 58°C, and 30 s at 72°C, with a final extension cycle of 72°C for 5 min. Electrophoresis was performed in denaturing 6% polyacrylamide gels (7 M urea), and the products were visualized by silver staining (13). Amplicon sizes were estimated using a 10-bp DNA ladder (Life technologies, Invitrogen).

SSR data analysis. The amplified SSR fragments were scored in terms of loci and alleles. Relationships among the isolates were evaluated using phenetic cluster analysis with unweighted pair-grouping and arithmetic average clustering, using Nei's unbiased genetic distance (31), and the results plotted as a dendrogram using NTSYS-pc 2.1 (Exeter Software). Bootstrap *P* values were calculated with the Winboot program (44) with 1,000 samplings.

Aggressiveness assay. Two trials were conducted simultaneously in a greenhouse to evaluate the aggressiveness of the isolates on healthy plants of the susceptible 'SP78-4467' (14) produced in vitro from meristem tips. Individual plants were cultivated in 5-liter pots containing a 3:1:1 mixture of soil, sand, and pine and coconut bark substrate (Tropstrato). Four-month-old plants were inoculated by the decapitation method (38), using 100 µl of each bacterial suspensions adjusted to 10⁸ CFU/ml. The first trial consisted of 45 treatments (44 isolates plus a control) distributed in four randomized complete blocks with one replicate of one plant each. In the second experiment, the same isolates were used, except that it consisted of five randomized complete blocks. The difference concerning the number of blocks among the experiments was due the number of disease-free plants available. The control treatment consisted of plants mock inoculated with sterile distilled water.

Disease severity on leaves was evaluated 30 days after inoculation using a symptom rating scale developed in this study. The scale ranged from 1 to 8, where 1 indicated no symptoms, 2 indi-

cated initial chlorosis with small chlorotic lines, 3 indicated leaf chlorosis with one or two white pencil-line streaks, 4 indicated three to five white pencil-line streaks, 5 indicated five to six white pencil-lines streaks with one large line, 6 indicated two or three larger white lines and the beginning of necrosis, 7 indicated two or three larger white lines with more than six pencil-line streaks and necrosis in the lateral leaf blade extension, and 8 indicated necrosis and death of the shoots.

Disease severity was transformed using the Ln(*x* + 1) transformation and analyzed using the SAS computer software, version 9.2 (2008). Means were compared by the *t* test (least significant difference [LSD], *P* < 0.05).

Association of aggressiveness with marker genotypes. The association between SSR markers and aggressiveness was investigated for each marker allele by simple linear regression using disease severity values of each isolate as the dependent variable and their genotypes for a given allele, scored as presence or absence of fragment, as the independent one. Aggressiveness data were based on the combined analysis of two trials. The regression significance was tested by the *F* test (*P* < 0.05) and the effect of marker on aggressiveness was estimated by the *R*² value. In addition, marker associations were also investigated using the stepwise multiple

Table 1. Sugarcane hosts, origin, and year of isolation of *Xanthomonas albilineans* isolates used in this study

Isolate	Hosts ^y	Origin	Year of isolation
Xa11	SP90-7027	Conchal, SP	2010
Xa4	IBSBF2256 ^z	Santa Adélia, SP	2010
Xa37	IACSP08-1592	Piracicaba, SP	2011
Xa40	nr	Maceió, AL	2011
Xa41	nr	Maceió, AL	2011
Xa42	nr	Maceió, AL	2011
Xa8	CTC 17	Goianésia, GO	2010
Xa20	IACSP04-656	Pindorama, SP	2010
Xa36	Sh106	Valparaíso, SP	2011
Xa1	Brava de Pericó	Ribeirão Preto, SP	2010
Xa12	nr	Conchal, SP	2010
Xa25	IACSP04-656	Mococa, SP	2010
Xa6	IACSP95-5000	Goianésia, GO	2010
Xa16	nr	Maceió, AL	2010
Xa18	nr	Maceió, AL	2010
Xa19	nr	Maceió, AL	2010
Xa24	IACSP04-656	Vargem Grande Sul, SP	2010
Xa29	SP81-1763	Ribeirão Preto, SP	2010
Xa32	IBSBF654 ^z	Araras, SP	2011
Xa13	nr	Conchal, SP	2011
Xa2	IBSBF2254 ^z	Santa Adélia, SP	2010
Xa38	nr	Conceição Alagoas, MG	2011
Xa39	SP79-1011	Conceição Alagoas, MG	2011
Xa15	nr	Maceió, AL	2010
Xa17	nr	Maceió, AL	2010
Xa7	CTC 11	Goianésia, GO	2010
Xa44	nr	Maceió, AL	2011
Xa34	IACSP04-704	Ribeirão Preto, SP	2011
Xa33	IBSBF2113 ^z	Piracicaba, SP	2011
Xa10	IACSP93-3046	Indiara, GO	2010
Xa14	nr	Conchal, SP	2010
Xa35	Sh185	Lins, SP	2011
Xa31	326-01	Piracicaba, SP	2011
Xa9	IACSP04-6165	Ribeirão Preto, SP	2010
Xa26	IACSP07-4616	Mococa, SP	2010
Xa3	IBSBF2255 ^z	Santa Adélia, SP	2010
Xa23	RB86-7515	Araçatuba, SP	2010
Xa28	POJ 2878	Ribeirão Preto, SP	2010
Xa5	RB72-454	Conchal, SP	2010
Xa27	CTC 14	Ribeirão Preto, SP	2010
Xa22	CV5007 1899	Araçatuba, SP	2010
Xa30	IBSBF1374 ^z	Mauricius Island	2011
Xa43	nr	Maceió, AL	2011
Xa21	nr	Araçatuba, SP	2010

^y Abbreviation: nr = information not recorded.

^z Genotype from phyto bacteria culture collection of Instituto Biológico, Campinas, Brazil.

linear regression ($P < 0.05$). All analyses were performed with Statistical Analysis System, version 9.2 (2008).

Results

TRS analysis. In total, 429 TRS were identified in the complete genome sequence of *X. albilineans* GPE PC73. The motif sizes ranged from 1 to 210 nucleotides, with copy numbers varying from 2 to 27. The most common motif was 6 nucleotides in length (33%), with copy numbers varying from 5 to 11, followed by 3 nucleotides (26%), with copy numbers varying from 8 to 14. Furthermore, sequences with 9 (6%), 12 (6%), 18 (13%), and 36 (6%) nucleotides long, with copy number ranging from 7 to 23, were selected. Two sequences had overlapping repeats. Based on the size of the motif and on the number of copies, 16 sequences were selected for primer design, of which 12 comprised perfect (noninterrupted sequences) and 4 were imperfect (interrupted sequences) repeats. Of these 16 sequences, 15 SSR primers were constructed, because 2 sequences had overlapping repeats. All primers produced well-defined discrete amplicons, of which 12 were polymorphic among isolates and three were monomorphic (Table 2). The number of alleles per locus ranged from 2 to 12, with a total of 54 polymorphic alleles and an average of 4.5 alleles per locus.

Genetic diversity of *Xanthomonas albilineans*. Molecular characterization of the isolates with the 12 polymorphic SSRs revealed extensive genetic variability, because 36 haplotypes were identified. The average genetic distance was 0.33. The highest distance among Brazilian isolates (0.67) occurred between isolates Xa4 and Xa36 from Santa Adélia and Valparaíso, respectively, both of which are in the state of São Paulo. The minimum distance (0) was found among 10 isolates.

Cluster analysis (Fig. 1) grouped the isolates into two main clusters. Group I consisted of 32 isolates collected from the four regions, whereas group II comprised 11 isolates, all of which were collected in the states of Alagoas and São Paulo. Isolates Xa35 (Ribeirão Preto), Xa4 (Santa Adélia), and IBSBF1374 (Mauritius Island) did not cluster into any group and were considered outgroups.

The dendrogram showed that, within group I, the SSR markers were not able to detect polymorphisms among some isolates. These

included isolates Xa1, Xa27, Xa28, Xa29 (Ribeirão Preto/SP), Xa12 (Conchal/SP), and Xa22 (Araçatuba/SP); isolates Xa11 (Conchal/SP), Xa40, and Xa44 (Maceió/AL); and isolates Xa38 and Xa39 from Conceição das Alagoas/MG. Also, isolates did not cluster according to their state or region of origin.

Aggressiveness of *X. albilineans* isolates. Statistical analyses (analysis of variance) conducted with the individual experiments (Table 3) revealed high homogeneity of the variances, as shown by the ratio the of mean square errors: (error trial I/error trial II) = 1.14. The combined analysis revealed no statistically significant difference between trials. Also, no trial-isolate interaction was detected (Table 3). For this reason, the means were pooled over trials and used to compare the aggressiveness of isolates using the LSD test (Table 3). Isolates Xa11, Xa4, and Xa37 (and 17 others with mean > 1.81; Table 3) were the most aggressive whereas Xa3, Xa23, Xa5, Xa27, Xa22, Xa30, Xa43, and Xa21 were the least. Isolate IBSBF1374 from the Mauritius Island was ranked among the three least aggressive isolates.

When comparing the dendrogram with the aggressiveness values, group I included a larger proportion (15 isolates) of the more aggressive isolates than group II (5 isolates). However, no difference in aggressiveness was detected between these groups based on a *t* test ($P < 0.05$; data not shown). Furthermore, aggressive isolates were present in all four regions sampled, indicating no relationship between their origin and their level of aggressiveness.

Association of aggressiveness and molecular markers. Mean disease severity of *X. albilineans* isolates based on the rating scale and genotyping of 54 alleles are shown in Table 4.

The single-marker analysis revealed no significant association between markers and aggressiveness as inferred from the severity ratings for the majority of the SSR loci, with the exception of allele 4 of IACXa3 and of allele 5 of IACXa13 (Table 4; $P < 0.10$). In the first case, the allele corresponded to a 160-bp fragment present in eight isolates. Seven of these isolates were evaluated as being aggressive (Xa6, Xa8, Xa16, Xa18, Xa19, Xa24, and Xa25). In the second case, the allele corresponded to a 230-bp fragment present only in one isolate (Xa4), which presented the second highest aggressiveness value (6.77). The mean aggressiveness of isolate pre-

Table 2. Characteristics of 15 short sequence repeat primers developed from the complete sequence of *Xanthomonas albilineans* GPE PC73

Primer	Sequence	Motif	Allele size (bp)	Number of alleles
IACXa1-F	TCATCGGCATTACGACAAAG	...	...	...
IACXa1-R	CAACATCAACGCTGCAAGAT	(CCG) ₁₁	109	1
IACXa2-F	GGTCAGTCAAAAGCAAAAC	...	...	...
IACXa2-R	TTTCAACGTGGCTGAAAG	(AGCAACAGCACA) ₁₉	312	12
IACXa3-F	AACTGTTGCAAGCGTCTGTG	...	...	...
IACXa3-R	ATATTTCGGCTACCCCGTCAC	(ACACCG) ₁₁	149	8
IACXa4-F	GTCGCTATTGACGGTGTACT	...	...	...
IACXa4-R	TTCCACTAGCGGTGGTAGC	(GTGGAGCCGCCCCACCT) ₂₃	493	1
IACXa5-F	GAACATTAACGCGCAGAG	...	...	...
IACXa5-R	GATATGTTTTCGCAATAGCC	(GGCGGCTCGACGGGCGGTGGCGGTTCCTACTGGTGGC) ₁₀	483	2
IACXa6-F	GATGACCTTGGTGCTGAA	...	...	...
IACXa6-R	GTTGAGATCGGTGCGAAA	(GGAGCCACCACCGCT) ₈	174	4
IACXa7-F	TTTCAGATCGGCCTGGATT	...	...	...
IACXa7-R	GCCACTCTAAGCGACACCAT	(CGGAGA) ₅	156	3
IACXa8-F	CTTCGCATATCACCTCAAC	...	...	...
IACXa8-R	GTGTCTTGAGCACCGTCA	(GGTGGTGAA) ₇	148	3
IACXa9-F	GAATTTGGAGGAGGAGGA	...	...	...
IACXa9-R	GTTGCGTTGACTTCTTGTG	(GGT) ₁₁	143	1
IACXa10-F	ACACAGGCCAGTTCAAGAGGTG	...	...	...
IACXa10-R	ACCACGACCATGATA	(GTCGTG) ₉	129	4
IACXa11-F	GAGAAAATGCGAAGAAAGC	...	...	...
IACXa11-R	TCAGGTCGGGTAGGTCT	(GAGAAG) ₅	128	4
IACXa12-F	GATGTTGCATAAGCTGTGG	...	...	...
IACXa12-R	GTCGACGATGACATGAGTG	(GGT) ₁₄ + (TGGCAA) ₆	130	6
IACXa13-F	TTAACCTGTTGCATAAGCTG	...	...	...
IACXa13-R	TATCGACGATGACATGAGTG	(GGTGGTGGTGGCTCCACC) ₁₅	346	5
IACXa14-F	GCGTCGTAGTAGTTGTGGT	...	...	...
IACXa14-R	GGCATGTTCTTGTTCGAG	(CCA) ₈	134	2
IACXa15-F	CTGCCGTATGTGTTCTCTG	...	...	...
IACXa15-R	ACACGTCTCTCTTCATC	(GGATGA) ₅	149	2

senting both alleles was 5.55, whereas that of isolates without these alleles was 4.70.

The stepwise multiple regression analysis using all markers simultaneously confirmed the association of these two alleles with aggressiveness ($P < 0.05$). The resulting double linear regression equation, which accounted for 15.96% of the total variation in aggressiveness among isolates, was expressed as $\text{severity} = 1.74 + 0.11y (\text{allele 4 marker}) + 0.31y (\text{allele 5 marker})$ ($R^2 = 0.16$).

Interestingly, although isolates Xa1, Xa27, Xa28, and Xa29 and isolate Xa22 presented the same haplotype, they differed in aggressiveness.

Discussion

SSRs are hypermutable sequences of DNA and it has been proposed that they represent a main source of genetic diversity for many pathogenic bacteria (3). As a result, the interest of these sequences as tools in molecular studies of bacterial pathogens has significantly increased. This type of genetic marker proved to be variable in *X. albilineans* as well, because the majority of the isolates (36 of 44) presented a unique haplotype. This finding also illustrates the remarkable genetic variability of this pathogen in Brazil. The use of such sequences for characterizing plant pathogenic bacteria is uncommon and was first reported in *Xylella fastidiosa* (12). The authors concluded that SSR markers represented a high-resolution tool for epidemiological and genetic analysis of this pathogen of citrus. Subsequent studies have characterized other species of animal pathogenic bacteria, such as *Nisseria meningitidis* (45), *Mycobacterium tuberculosis* (24), and *Salmonella enterica* (26).

The molecular characterization performed in the present study revealed remarkable genetic variability of *X. albilineans* in Brazil. However, there was no correlation between their clustering based on genetic distances with either the state or region where they were collected or with their degree of aggressiveness. This can result from the intense flow of plants with latent infections within the major sugarcane-producing regions of Brazil, which contributes to the spread of the pathogen and favors gene flow between the populations of *Xanthomonas albilineans*.

Several studies reported on the genetic diversity of *X. albilineans* in Brazil and other countries. For instance, the genetic diversity of 50 isolates of *X. albilineans* from commercial areas of the state of São Paulo, Brazil was assessed by rep-PCR (41) and grouped the isolates into three groups. However, unlike the present study, most isolates clustered according to their origin, although this was probably due to the fact that all isolates were from the same state. A study with 215 isolates of *X. albilineans* from 28 different locations around the world identified three serotypes: serotype I was the largest, comprising isolates from various geographic locations; serotype II contained only African isolates; and serotype III consisted of isolates from the Caribbean islands (36). Genomic DNA fingerprinting and specific monoclonal antibodies were used to compare 38 isolates of *X. albilineans* from 13 different countries (1). The clusters of isolates that were genetically and serologically related were not necessarily collected at nearby locations. These data corroborate the results obtained in the present study where it was not possible to cluster the isolates according to their origin. BOX primers were used to characterize *X. albilineans* isolates from four countries, which were distinguished into five groups, the majority clustering according to the country of origin (27). Similarly, the polymorphism revealed by *gyrB* and *rpoD* genes with 21 isolates of *X. albilineans* from Okinawa (Japan) and 10 other isolates from other countries showed that isolates from more geographically distant regions exhibited greater genetic variability (42). These findings corroborate with the results obtained in our study, in which the isolate from Mauritius (IBSBF 1374) remained separated from the Brazilian isolates.

The isolates used in our study differed in aggressiveness, which made it possible to identify isolates of low, intermediate, and high aggressiveness. Differences in aggressiveness of *X. albilineans* based on the ability to colonize sugarcane or cause symptoms varies according to the pathogen isolate (9). These results corroborate those obtained previously by other authors (10), where a wide variation in aggressiveness was observed with 75 isolates of *X. albilineans* from Guadalupe, Mexico, confirming the existence of different pathotypes of *X. albilineans* in this geographical location.

Fig. 1. Estimates of genetic distance among *Xanthomonas albilineans* isolates collected from four Brazilian States based on short sequence repeat loci. Bootstrap values (1,000 replicates) for branch nodes ranged from 10 to 100%.

AFLP and fluorescent AFLP were used in the differentiation of *X. albilineans* from several countries (40). Although the markers successfully revealed genetic diversity among isolates, it was not possible to distinguish *X. albilineans* and non-*X. albilineans* from South Africa. In our study, the SSR markers developed are specific to *X. albilineans*, and proved to be a powerful and reliable method for isolate identification and typing.

In the current study, a 160-bp allele of IACXa03 was exclusively amplified in 8 isolates, of which 7 (Xa6, Xa8, Xa16, Xa18, Xa19, Xa24, and Xa25) were among the 18 most aggressive isolates identified. This locus represents a functional gene that codes for a non-ribosomal peptide synthetase (NRPS), a class of enzymes involved

Table 3. Disease severity on leaves of sugarcane SP70-4467 after inoculation with 44 isolates of *Xanthomonas albilineans*

Isolate, ANOVA ^y	Mean disease severity		
	Trial I	Trial II	Pooled trial ^z
Xa11	2.14	2.02	2.07 A
Xa4	2.17	1.97	2.06 A
Xa37	2.07	2.04	2.06 A
Xa40	1.91	2.07	2.00 A–B
Xa41	1.99	1.97	1.98 A–B
Xa42	1.87	2.00	1.94 A–C
Xa8	1.92	1.91	1.91 A–D
Xa36	1.77	2.02	1.91 A–D
Xa20	1.79	1.98	1.89 A–E
Xa12	1.78	1.97	1.89 A–E
Xa25	1.86	1.88	1.87 A–F
Xa6	1.86	1.86	1.86 A–F
Xa16	1.83	1.88	1.86 A–F
Xa18	1.80	1.91	1.86 A–F
Xa19	1.63	2.02	1.84 A–G
Xa24	1.84	1.83	1.83 A–G
Xa29	1.86	1.79	1.82 A–G
Xa32	1.76	1.88	1.83 A–G
Xa1	1.76	1.85	1.81 A–H
Xa13	1.62	1.96	1.81 B–H
Xa2	1.83	1.75	1.79 B–H
Xa38	1.68	1.86	1.78 B–H
Xa39	1.83	1.75	1.79 B–H
Xa15	1.81	1.74	1.77 B–H
Xa17	1.59	1.92	1.77 B–H
Xa7	1.81	1.71	1.76 B–I
Xa44	1.69	1.81	1.76 B–I
Xa34	1.70	1.79	1.75 B–I
Xa33	1.87	1.62	1.73 B–I
Xa10	1.72	1.70	1.70 C–I
Xa14	1.69	1.71	1.70 C–I
Xa35	1.56	1.80	1.70 C–I
Xa31	1.66	1.68	1.67 D–I
Xa9	1.64	1.69	1.67 D–I
Xa26	1.66	1.61	1.63 E–I
Xa28	1.83	1.43	1.61 F–I
Xa3	1.64	1.55	1.59 G–J
Xa23	1.60	1.58	1.59 G–J
Xa5	1.58	1.58	1.58 G–J
Xa27	1.58	1.58	1.58 G–J
Xa22	1.54	1.56	1.55 H–J
Xa30	1.73	1.40	1.54 H–J
Xa43	1.44	1.54	1.49 I–J
Xa21	1.39	1.29	1.34 J
ANOVA			Mean square (P value)
Trial	...	...	0.0660 ($P > 0.05$)
Isolate	...	...	0.2190 ($P < 0.0001$)
Trial × isolate	...	...	0.0570 ($P > 0.05$)
Block (within trial)	...	...	0.1151 ($P > 0.05$)
Error (MS)	0.0885	0.0778	0.0824
Error (df)	129	172	301
CV (%)	16.93	15.64	15.89
LSD ($P > 0.05$)	0.42	0.35	0.27

^y ANOVA = analysis of variance.

^z Means of severity followed by the same letter do not differ significantly by *t* test (least significant difference [LSD], $P > 0.05$).

Table 4. Relative frequencies (RFs) of alleles of 12 polymorphic short sequence repeat (SSR) loci among 44 isolates of *Xanthomonas albilineans* and mean disease severity of isolates with the presence or absence of each allele^y

SSR locus, allele	RF	Presence	Absence
IACXa2			
1	1/44	1.90 (5.70)	1.76 (4.81)
2	1/44	1.90 (5.70)	1.77 (4.87)
3	3/44	1.78 (4.93)	1.77 (4.87)
4	3/44	1.84 (5.30)	1.77 (4.87)
5	5/44	1.67 (4.31)	1.78 (4.93)
6	4/44	1.74 (4.70)	1.77 (4.87)
7	4/44	1.74 (4.70)	1.77 (4.87)
8	7/44	1.81 (5.11)	1.76 (4.81)
9	9/44	1.78 (4.93)	1.77 (4.87)
10	2/44	1.76 (4.81)	1.77 (4.87)
11	2/44	1.76 (4.81)	1.77 (4.87)
12	1/44	1.76 (4.81)	1.77 (4.87)
IACXa3			
1	1/44	1.70 (4.47)	1.77 (4.87)
2	2/44	1.69 (4.41)	1.77 (4.87)
3	3/44	1.79 (5.0)	1.77 (4.87)
4	8/44	1.85 ^z (5.36)	1.75 ^z (4.75)
5	8/44	1.73 (4.64)	1.78 (4.93)
6	17/44	1.76 (4.81)	1.77 (4.87)
7	2/44	1.85 (5.35)	1.76 (4.81)
8	3/44	1.66 (4.25)	1.78 (4.93)
IACXa 5			
1	42/44	1.77 (4.87)	1.72 (4.58)
2	2/44	1.72 (4.60)	1.77 (4.87)
IACXa 6			
1	1/44	1.54 (3.66)	1.77 (4.87)
2	2/44	1.63 (4.10)	1.77 (4.87)
3	28/44	1.77 (4.87)	1.76 (4.81)
4	11/44	1.80 (5.04)	1.76 (4.81)
IACXa 7			
1	42/44	1.77 (4.87)	1.79 (4.99)
2	2/44	1.80 (5.04)	1.77 (4.87)
IACXa 8			
1	1/44	1.54 (3.66)	1.77 (4.87)
2	1/44	1.69 (4.42)	1.77 (4.87)
3	42/44	1.75 (4.75)	1.62 (4.05)
IACXa 10			
1	5/44	1.77 (4.87)	1.77 (4.87)
2	33/44	1.76 (4.81)	1.79 (5.99)
3	5/44	1.82 (5.17)	1.76 (4.81)
4	1/44	1.69 (4.42)	1.77 (4.87)
IACXa 11			
1	2/44	1.90 (5.70)	1.76 (4.81)
2	39/44	1.76 (4.81)	1.80 (5.04)
3	2/44	1.82 (5.17)	1.76 (4.81)
4	1/44	1.54 (3.66)	1.77 (4.87)
IACXa 12			
1	1/44	1.54 (3.66)	1.77 (4.87)
2	2/44	1.74 (4.70)	1.77 (4.87)
3	1/44	1.84 (5.30)	1.77 (4.87)
4	2/44	1.80 (5.04)	1.77 (4.87)
5	10/44	1.82 (5.17)	1.75 (4.75)
6	28/44	1.75 (4.75)	1.79 (5.99)
IACXa 13			
1	1/44	1.54 (3.66)	1.77 (4.87)
2	1/44	1.75 (4.75)	1.77 (4.87)
3	10/44	1.78 (4.93)	1.76 (4.81)
4	31/44	1.76 (4.81)	1.78 (4.93)
5	1/44	2.05 ^z (6.77)	1.76 ^z (4.81)
IACXa 14			
1	43/44	1.77 (4.87)	1.54 (3.66)
2	1/44	1.54 (3.66)	1.77 (4.87)
IACXa 15			
1	43/44	1.77 (4.87)	1.54 (3.66)
2	1/44	1.54 (3.66)	1.77 (4.87)
Means	...	1.73 (4.64)	1.76 (4.81)

^y Presence = severity mean when the allele is present and Absence = severity mean when the allele is absent. Values in parentheses = severity mean recorded (not transformed).

^z $P < 0.10$.

in the production of secondary metabolites such as antibiotics, immunosuppressors, and toxins in many bacteria and fungi (28). It has been proposed that these toxins represent bacterial virulence factors (5,20). In *X. albilineans*, it is known that two NRPS genes (*albIV* and *albIX*) are involved in the biosynthesis of albicidin (34), which acts as a bactericide against a wide range of gram-positive and gram-negative bacteria, likely warranting to *X. albilineans* advantages when competing against other bacteria in the same site of infection (8). Another allele at the IACXa13 locus was amplified in only one isolate (Xa4) but was classified as the second most aggressive, causing a disease severity rate of 6.77. This locus corresponds to a precursor of an endoglucanase produced by phytopathogenic bacteria, which plays an important role in disease development (19).

The association of functional SSR markers with virulence factors has been reported for some prokaryotic organisms (18). These markers are responsible for controlling the expression of surface determinants at the population level, resulting in phase variations, a strategy by which bacteria facilitate niche selection and avoid the mechanisms of immune response of the host, causing changes in the phenotype (4). Thus, bacterial pathogens exploit localized hypermutations through the shifting of DNA polymerase from SSRs to generate variation in the phenotype, thus increasing its adaptability to changes in the environmental conditions (30). These SSRs are located in reading frames or in the promoter regions of a set of genes, frequently termed contingency loci, whose functions are usually involved in direct interactions with the host structures (3,30,39).

In conclusion, SSR loci were used to characterize isolates of *X. albilineans*, which proved to be a powerful tool in revealing the existence of molecular and pathogenic variability. The aggressiveness analysis revealed that the isolates differed for this trait, existing isolates with low, intermediate, and high aggressiveness. However no correlation could be established with either SSR haplotypes or phenetic groups. Also, single-marker-trait analysis revealed two markers associated with aggressiveness.

The present study opens new possibilities for studies to improve knowledge about this important sugarcane pathogen.

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