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Amplification polymorphism among *Xanthomonas albilineans* strains, using a single oligonucleotide primer

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Abstract

A genomic library was produced by a subtractive hybridisation method using DNA from *Xanthomonas albilineans* serovar I and *X. albilineans* serovar II, originating from Mauritius. The cloned fragments were amplified and used as probes for Southern hybridisation. Probe F20 was selected on the basis of the RFLP pattern. Upon hybridisation of *X. albilineans* DNA with probe F20, strains of serovars I and II were differentiated by their banding profiles. This probe was sequenced and oligonucleotide primers were designed. Fragment number and length polymorphisms were obtained after PCR amplification of *X. albilineans* DNA using F20A as a single primer. The number and size of bands obtained with this primer was correlated to the serotypes of the strains and to the DNA grouping reported by Alvarez et al. (1996). The two serotypes I and II which exist in Mauritius were differentiated by the PCR using primer F20A. The probe and primer developed provide rapid and precise tools for the differentiation of genetic variants within the species *X. albilineans*.

Introduction

Leaf scald, caused by *Xanthomonas albilineans*, is a major disease of sugarcane. Several sudden outbreaks in different parts of the world have emphasised the importance of vigilance in the control of the disease. It was first described by two investigators working independently, Wilbrink in Java and North in Australia and Fiji (Wilbrink, 1920). It is thought to have originated from New Guinea and has been disseminated with the spread of the crop for cultivation in various parts of the world. Major epidemics of leaf scald have occurred after prolonged periods of absence. This underlines the unpredictable nature of the disease whose severity is a result of environmental factors as well as bacterial strains and varietal susceptibility. Sugarcane plantations in Mauritius and in Florida were severely affected by this pathogen in 1989. The strain isolated during the epidemic was shown to be genetically different from those which were present before (Comstock and Shine, 1992; Davis, 1992).

Xanthomonas albilineans is a Gram-negative, xylem-invading organism which spreads throughout the plant via the vascular bundles. This pathogen produces an anti-microbial compound, albicidin (Birch and Patil, 1985) which inhibits prokaryotic DNA replication in bacteria and chloroplasts, and is toxic to a large number of Gram-negative and Gram-positive bacteria. It is responsible for inducing chlorosis and for the appearance of white-pencil line stripes during infection (Birch and Patil, 1987). Albicidin plays an important role in the establishment of the leaf scald disease.

The pathogen is disseminated among plants in the field through the use of cutting knives, infected planting material, and through guttation droplets on leaf surfaces (Autrey et al., 1991; Klett and Rott, 1994). Several authors have classified the pathogen on the

basis of different typing methods. Yang et al. (1993) reported the identification of two protein clusters (1 and 2) among 65 strains of *X. albilineans*. The main differences were in the range of 36–66 kDa protein bands. The strains in cluster 2 were from tropical Africa and Fiji. Pillay et al. (1995) have analysed five strains of *X. albilineans* from Australia, Mauritius, South Africa and Fiji. The two strains from Mauritius and one from Australia had the same LPS bands, which were different from the strains from Fiji and South Africa. Rott et al. (1986, 1994) identified three serological groups: serovars I, II and III. Isolates from Mauritius are in serovars I and II.

In order to follow the evolution of microbes and to evaluate their genetic stability, it is essential to use assays that have the required sensitivity to detect small differences in DNA sequences. Although analysis of bacterial proteins, lipopolysaccharide composition and antigenic properties can reveal polymorphism, minor differences such as those occurring within the genome, are not depicted by these methods. Intraspecific variation in *X. albilineans* has been revealed by analysis of restriction fragments of the bacterial genomic DNA on conventional polyacrylamide gel electrophoresis (Alvarez et al., 1996) and by high-resolution pulsed-field gel electrophoresis (Davis et al., 1997). A report about the use of RFLP by hybridisation with specific probes has revealed the polymorphic nature of the pathogen (Jaufeerally-Fakim et al., 2000).

This report describes the development and the use of a probe and a single oligonucleotide primer for the differentiation of *X. albilineans* by hybridisation and PCR. The genetic diversity within the species was demonstrated by different DNA hybridisation patterns and by amplification, using a single primer, of a DNA region probably containing inverse repeats. In this study, Mauritius isolates as well as those from different geographical origins were used.

Materials and methods

Bacterial isolates

Bacterial isolates are listed in Tables 1 and 2. All *Xanthomonas* species were grown in Wilbrink agar or broth at 27 °C. Long-term storage of isolates was in 15% glycerol Wilbrink broth at −80 °C. Isolates of *X. albilineans* were identified as either serovar I or serovar II based on immunodiffusion using polyclonal antisera specific for each serotype. The antisera were produced in rabbits. All of the bacterial strains indicated in Table 1 were used as negative controls for hybridisation with the probe. *Escherichia coli* DH5α, used in cloning experiments, was grown at 37 °C on Luria Bertani (LB) medium and stored in LB containing 15% glycerol at −80 °C (Sambrook et al., 1989).

Table 1. Bacterial isolates used as controls for testing specificity of probe F20

Bacterium	Strain	Host	Source
X. axonopodis pv. *vasculorum* (sugar cane)	2938	Sugarcane	MSIRI
X. axonopodis pv. *phaseoli*	3001	Bean	MSIRI
X. axonopodis pv. *malvacearum*	633	Cotton	TSL
X. vasicola pv. *holcicola*	1060	Sorghum	TSL
X. translucens pv. *translucens*	1943	wheat	TSL
X. vesicatoria	2593	Tomato	TSL
X. translucens pv. *graminis*	—	wheat	TSL
X. axonopodis pv. *vasculorum* (Broom bamboo)	3301	Broom bamboo	MSIRI
Herbaspirillum rubisubalbicans	3024	Sugar cane	MSIRI
Pectobacterium chrysanthemi	2811	Potato	MSIRI

TSL: The Sainsbury Laboratory, Norwich Research Park, Colney, Norwich, UK NR4 7UH. MSIRI: Mauritius Sugar Industry Research Institute, Reduit, Mauritius.

Subtractive hybridisation

DNA was extracted from the bacterial strains, starting with liquid cultures (50 ml) which had been innoculated with individual colonies. It was done using the CTAB method of Ausubel et al. (1994) with slight modifications as follows: the cells were washed twice with NE buffer (50 mM EDTA, 0.15 M NaCl). After addition of phenol and chloroform, the tubes were centrifuged at 10,000 rpm for 15–20 min. The supernatant was removed and a 0.6 volume of isopropanol was added to precipitate the nucleic acids. DNA was resuspended in 500 µl sterile distilled water and stored at −20 °C.

The subtraction was carried out according to the method of Strauss and Ausubel (1990). Two-hundred micrograms of the driver DNA (Mauritius strain 3509, serovar II) was sonicated to give fragments of ca. 3 kb,

Table 2. Comparison of bands amplified using primer F20A with known serotypes and DNA groups

Strains[a]	Country of origin	Serotype (Alvarez et al., 1996) monoclonal antibodies)	Serotype (Rott et al., 1994.) polyclonal antibodies)	Size of band(s) amplified with F20A (this study)	DNA group (Alvarez et al., 1996)
3506, 3508, 3514, 3516, 3223, 3255	Mauritius	ND	I	1.1 kb; 700 bp	ND
3509, 3510, 3511, 3513, 3518, 3226	Mauritius	ND	II	1.1 kb; 370 bp	ND
3268 (SA-PI)	South Africa	I	ND	1.1 kb; 700 bp	2C
3298 (Xa28)	Reunion	I	ND	1.1 kb; 700 bp	1C
3264 (IBSBF 326)	Brazil	I	I	1.1 kb	1B
3266 (IBSBF 654)	Brazil	I	I	1.2 kb	1B
Xa115 (BRA 115)	Brazil	ND	III	1.1 kb	ND
3196	St Kitts	III	III	1.1 kb	ND
3303, 3304	Guadeloupe	I	ND	1.2 kb	1B
3296 (ROC1-890)	Taiwan	I	ND	1.1 kb; 700 bp	1B
3969, 3981, 3987	Florida – 1991	I	ND	1.1 kb; 700 bp	1B
3360 (XACP-003) 3361 (XACP-005) 3362 (XACP-006)	Florida – 1985, 1986	I	I	370 bp	1A
4158, 4086	Hawaii	I	ND	370 bp	1A
3359 (R5161)	Australia	I	ND	370 bp	1A
3184	Hawaii	I	ND	370 bp	1A
Xa97 (DOM 97)	Dominican Republic	ND	I	370 bp	ND
3993 (82-706-3)	Hawaii	I	ND	1.1 kb; 370 bp	1A
3193 (HV5)	Burkina Faso	II	II	1.1 kb; 370 bp	2B
Xa6 (KEN 6)	Kenya	ND	II	1.1 kb; 700 bp	ND
CIV 40	Ivory Coast	ND	II	1.1 kb; 370 bp	ND
3297	Reunion	ND	ND	1.1 kb; 370 bp	ND
3194 (CI35)	Ivory Coast	II	II	500 bp; 700 bp	2D

ND: Not determined. Additional strains used for hybridisation: 3192 (Guadeloupe) Serotype I, DNA group 1B; 3269 (New Guinea) Serotype 2, DNA group 2B. [a]Original numbers are indicated in brackets.

and labelled with photoreactive biotin according to manufacturer's instructions (Clontech; Palo Alto, CA.). Five micrograms of tester DNA (Mauritius strain 3508, serovar I) was digested with *Sau*3A and mixed with 50 µg of driver DNA. After reassociation, the labelled driver DNA was removed from the mixture by incubating in avidin-coated tubes (Boehringer Mannheim) for 1 h. Two cycles of subtraction were performed and at the end of each, 2 µl of the DNA mix was used for cloning into pBR322. Only ampicillin sensitive and tetracycline resistant clones were kept.

PCR amplification of inserts from the subtracted libraries

Inserts were amplified from the recombinant plasmids. PCR was carried out using primers 16 (5′-ATG CGT CCG GCG TAG A-3′) and 20 (5′-CAC TAT CGA CTA CGC GAT CA-3′) corresponding to sequences on each side of the *Bam*H1 site of the vector pBR322 (Sigma, St Louis, MO). Reactions were carried out in 25 µl volumes containing 0.2 mM each dNTP, 0.2 U *Taq* polymerase and 1 × PCR buffer (Boehringer Mannheim) and 25 pmol of each primer. The reaction mixture was heated to 95 °C for 2 min, cycled through 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, 30 times before a final extension at 72 °C for 10 min. Inserts larger than 100 bp were selected for labelling. Inserts cloned in pBR322 were amplified using primers 16 and 20, and labelled with digoxigenin for use as probes. DNA from *X. albilineans* and several other bacteria (Table 1) were tested by Southern blotting. From this subtraction, 10 inserts were screened.

Screening and sequencing of selected probes

A preliminary screening of the probes was carried out using dot blots with DNA from *X. albilineans* and *X. axonopodis* pv. *vasculorum*. Dot blots and Southern hybridisations were performed at 65 °C in a hybridising solution containing 5 × SSC, 0.1%

N-laurylsarcosine, 0.02% SDS and 1% blocking reagent (Boehringer Mannheim). Probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by random priming or by PCR. For the latter, the final concentrations of DIG-dUTP and dTTP in the reaction mix were 66 and 134 μM, respectively. Detection, requiring anti-DIG Fab fragment conjugated with alkaline phosphatase and the substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1.^{3,7}]decan}-4-yl)phenyl phosphate (CSPD)® was carried out according to the manufacturer's instructions (Boehringer Mannheim). Only the probes that gave positive signals with *X. albilineans* and not with *X. axonopodiss* pv. *vasculorum* were kept for use. Southern hybridisations were performed using bacterial DNA cut with either *Bam*H1 or with *Eco*R1 and *Hind*III. DNA from several other organisms (Table 1) were used as controls.

Inserts which gave polymorphic bands with *X. albilineans* DNA and no band with the control bacteria, were re-amplified, purified on Qiagen columns (Qiagen Inc., Valencia, CA.) and used in cycle sequencing reactions. The latter were performed with a Silver Sequence kit (Promega, Madison, WI) using DIG-labelled primers 16 and 20 (Boehringer Mannheim) both according to the manufacturer's instructions. DIG labelling of the primers was essential, as clear bands could not be obtained upon silver staining of the gel. Cycling was as follows: 95 °C for 2 min, 95 °C for 30 s, 55 °C for 30 s, 70 °C for 1 min for 45 cycles. Samples were electrophoresed through a 0.6% polyacrylamide gel at 60 W for 2–5 h before transferring the DNA onto nylon membrane (Boehringer Mannheim). Detection was performed as described above for Southern blots.

PCR with primers derived from probe sequences

Upon hybridisation of probe F20 to *X. albilineans* DNA, polymorphic bands were obtained and no band was obtained with any of the control bacteria. It was then sequenced as described above and, based on the sequence, oligonucleotide primers were designed. Several primers were tested for their ability to give amplification polymorphisms. However, in a control PCR, designed to contain only one of each primer, polymorphic fragments were amplified with primer F20A : (5′-TTC GCC CTT AAC ACC GCC C-3′; T_m = 62 °C).

The PCR mix contained 0.66 μM of primer F20A, 0.2 mM each dNTP, 0.6 U of *Taq* polymerase and 1 × PCR buffer (Boehringer Mannheim) per 30 μl reaction volume. The following cycling programme was used: 95 °C for 2 min; 94 °C for 1 min, 58 °C (for primer F20A) for 1 min, 72 °C for 1 min for 35 cycles and, finally 72 °C for 10 min.

Results

Identification of X. albilineans*-specific inserts*

Ten cloned fragments were tested on Southern blots of digested bacterial DNA. One of them, termed probe F20, was a DNA fragment of 380 bp. It produced banding patterns with strains of serovars I, which were different from those obtained with serovar II. On a DNA blot of eight Mauritius and five Louisiana strains of *X. albilineans* cut with *Eco*R1 + *Hind*III, hybridisation with probe F20 revealed three RFLP groups (Figure 1) corresponding to the Mauritius strains of serovars I and II and the five Louisiana strains. Strains of serovar I produced a 15 kb band while all serovar II isolates gave a 3.5 kb band. Neither of these two bands was observed with the five strains from Louisiana (Figure 1).

F20 was also used to probe a blot of *X. albilineans* DNA cut with *Bam*H1. Again the two serovars I and II were differentiated. The DNA groups (Alvarez et al., 1996) 1A and 1B (Figure 2; lanes 12–15 and 8–11) gave two different banding patterns. Serovar II strains from Mauritius and Burkina Faso (Figure 2, lanes 1–3 and 17) gave a 12 kb band, which was not seen with any of the other isolates.

PCR amplification of X. albilineans *DNA using primer F20A*

Hybridisation with probe F20 revealed a high degree of polymorphism among the strains of *X. albilineans* (Figures 1 and 2). Several oligonucleotide primers derived from the sequence of this probe (Figure 3) were tested. A single primer, F20A, produced amplification products of 370, 700 and 1.1 kb (Figures 4–7) with strains from diverse geographical locations. While the serovar I and II strains from Mauritius were clearly distinguishable (Figure 4), similar banding patterns were produced from other *X. albilineans* strains outside Mauritius (Figures 5–7). The 370 bp band is typical of serovar II strains from Mauritius, Burkina Faso and Ivory Coast. Eight strains (4158, 4086, 3184, 3993, 3359, 3360, 3361, 3362) from Hawaii, Australia and Florida-pre1991, gave the 370 bp band and had all been

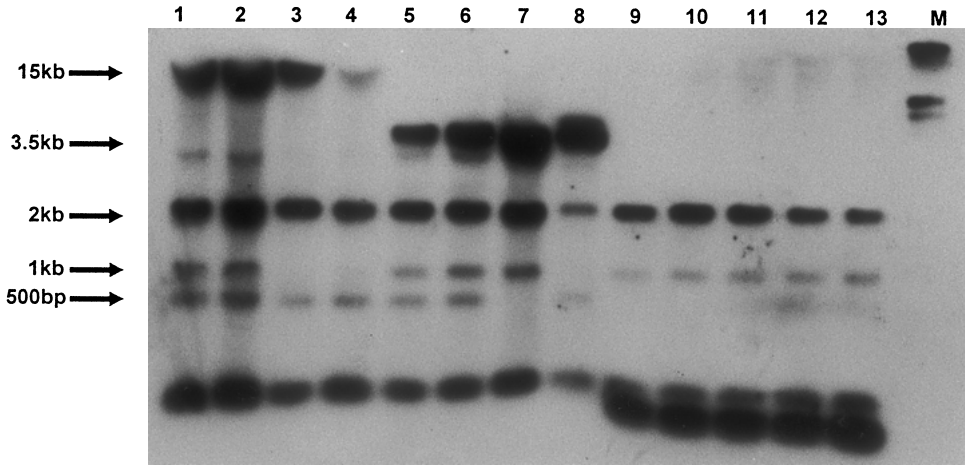

Figure 1. Hybridisation of probe F20 with *Eco*R1/*Hind*III digests of *X. albilineans* DNA. Lanes 1–4: 3508, 3514, 3516, 3536 (Serovar I); lanes 5–8: 3509, 3510, 3511, 3512 (Serovar II); lanes 9–13: SCB11, SCB12, SCB40, SCB41, SCB42 (Louisiana); lane 14: DNA molecular weight marker III (Boehringer Mannheim).

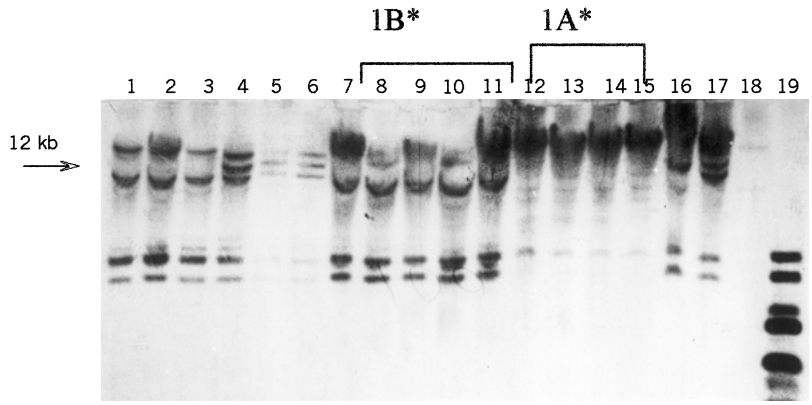

Figure 2. Hybridisation of probe F20 with *Bam*H1 digests of *X. albilineans*. Lanes 1–3: 3506, 3514, 3516 (Serovar I); lanes 4–6: 3510, 3512, 3513 (Serovar II); lane 7: 3298 (Reunion); lane 8: 3264 (Brazil); lane 9: 3969 (Florida, 1991); lane 10: 3192 (Guadeloupe); lane 11: 3296 (Taiwan); lane 12: 3360 (Florida, 1985); lane 13: 3359 (Australia); lanes 14 and 15: 4086, 4158 (Hawaii); lane 16: 3269: (P. New Guinea); lane 17: 3193 (B. Faso); lanes 18 and 19: DNA markers III and VI (Boehringer Mannheim). *Alvarez groups (1996).

classified by Alvarez et al. (1996) in DNA group 1A. Eight others (3264, 3266, 3303, 3304, 3296, 3969, 3981, 3987) from Brazil, Guadeloupe, Taiwan and Florida-1991 did not produce this 370 bp band and had been classified in Alvarez group 1B.

Using primer F20A, three strains from Florida, collected in 1985 and 1986 (3360, 3361, 3362) were compared with three others collected in 1991 (3969, 3981, 3987). Strains 3360, 3361 and 3362 produced bands of 370 and 1 kb, while strains 3969, 3981 and 3987 from 1991 produced the 1 kb band only (Figure 7). PCR with F20A produced reproducible results with all the strains tested.

Discussion

Many DNA probes have been isolated for the study of plant pathogens (Rasmussen and Reeves, 1992). The DNA profiles obtained upon hybridisation can distinguish between pathovars of the same organism as well as among strains of the same pathovar. Two pathovars of forage grasses *X. translucens* pv. *phlei* and *X. translucens* pv. *graminis* have been differentiated using a 1.6 kb DNA probe (Wang et al., 1995). The distribution of five genetic groups of *X. oryzae* pv. *oryzae,* which causes bacterial blight of rice, has been determined in South-East Asia using two DNA probes

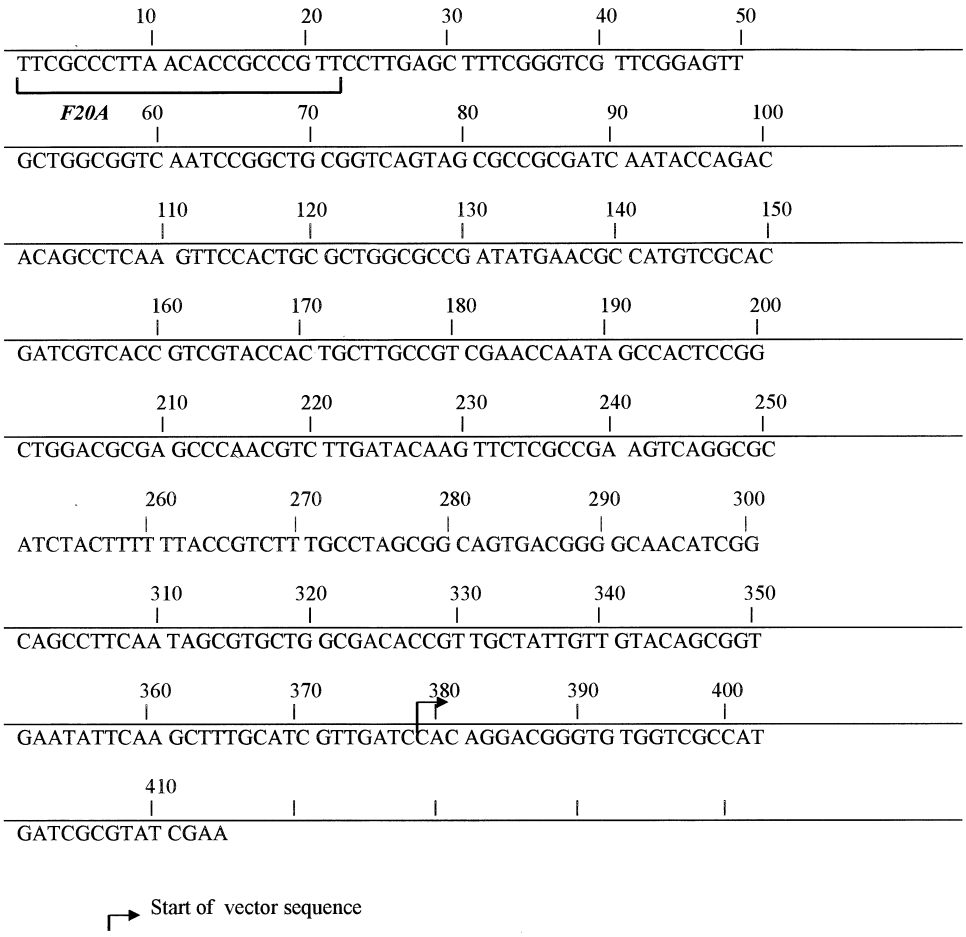

Figure 3. Nucleotide sequence of probe F20 cloned in vector pBR322.

(Adhikari et al., 1995). Such molecular tools are particularly attractive for studying non-culturable organism. A thorough characterisation of the pathogenic organism, which causes the important greening disease of citrus, was only possible when DNA probes became available. These probes could differentiate the Asian from the African strains of the organism and in addition their DNA sequences allowed its classification as a Liberobacter, (Villechanoux et al., 1992). The DNA probe and primer described here were used for the differentiation of *X. albilineans* strains. They can differentiate the two groups that are found in Mauritius, serovars I and II, and can also identify DNA groups 1A and 1B described by Alvarez et al. (1996).

Probe F20 was obtained from a subtracted genomic library of *X. albilineans*. Genomic subtraction is performed to enrich tester sequences. The denaturation and renaturation steps allow fragments of different origins to anneal and form hybrids on the basis of their homology. The approach used here was partly based on the method of Strauss and Ausubel (1990) for the isolation of DNA sequences corresponding to deletions in a yeast strain. In this study, avidin-coated tubes were used instead of avidin-coated beads. Since only 10 inserts were screened, no DNA fragment specific for the serovar II strains was obtained. However, probe F20 and the oligonucleotide primer derived from it, F20A, could distinguish between the two serovars.

The hybridisation profiles obtained with probe F20 and the amplification products of primer F20A, allow the differentiation between serovars I and II strains of Mauritius and suggest that these strains were of different origins. In addition, there was a high degree of polymorphism among the *X. albilineans*

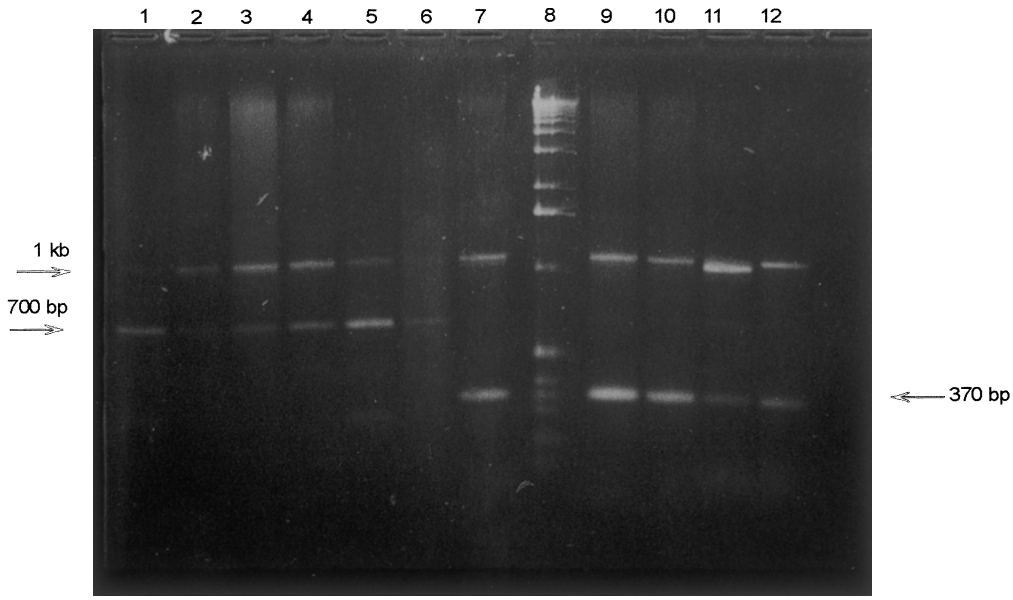

Figure 4. PCR amplification of *X. albilineans* DNA with primer F20A. Lanes 1–6: 3506, 3508, 3514, 3516, 3223, 3255 (Mauritius, serovar I); lane 7: 3509 (Mauritius, serovar II); lane 8: DNA molecular weight marker X (Boehringer Mannheim); lanes 9–12: 3510, 3511, 3513, 3226 (Mauritius, serovar II).

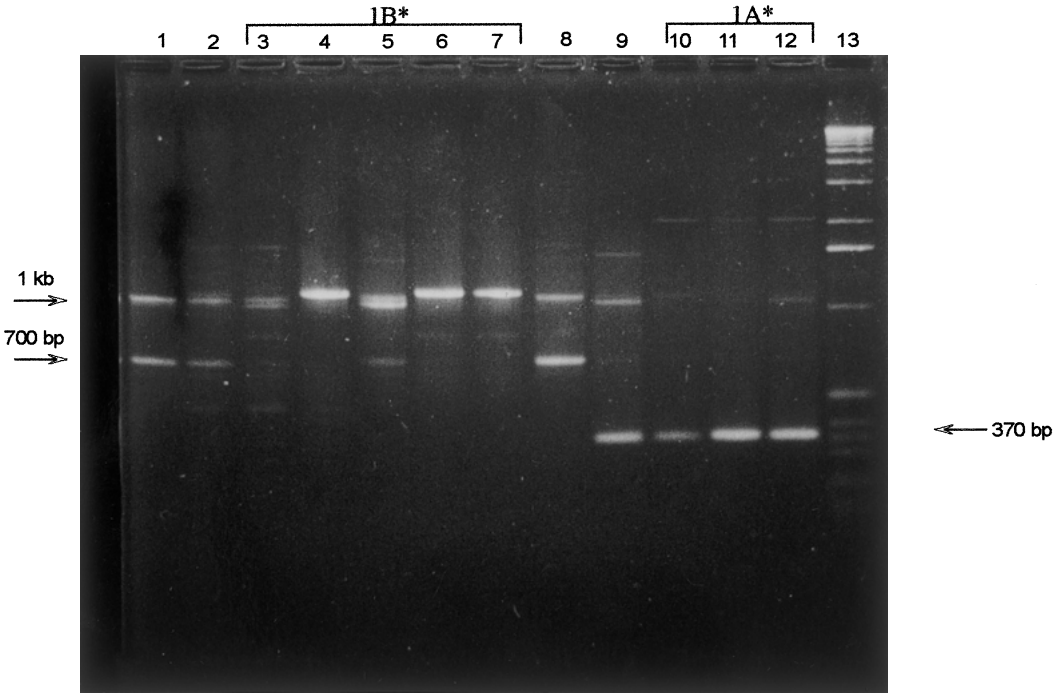

Figure 5. PCR amplification of *X. albilineans* DNA with primer F20A. Lane 1: 3268 (South Africa); lane 2: 3298 (Reunion); lanes 3 and 4: 3264, 3266 (Brazil); lane 5: 3296 (Taiwan); lanes 6 and 7: 3303, 3304 (Guadeloupe); lane 8: Xa 6 (Kenya); lane 9: 3193 (Burkina Faso); lane 10: 3359 (Australia); lanes 11 and 12: 4158, 4086 (Hawaii); lane 13: DNA marker X. (Boehringer Mannheim); *Alvarez groups (1996).

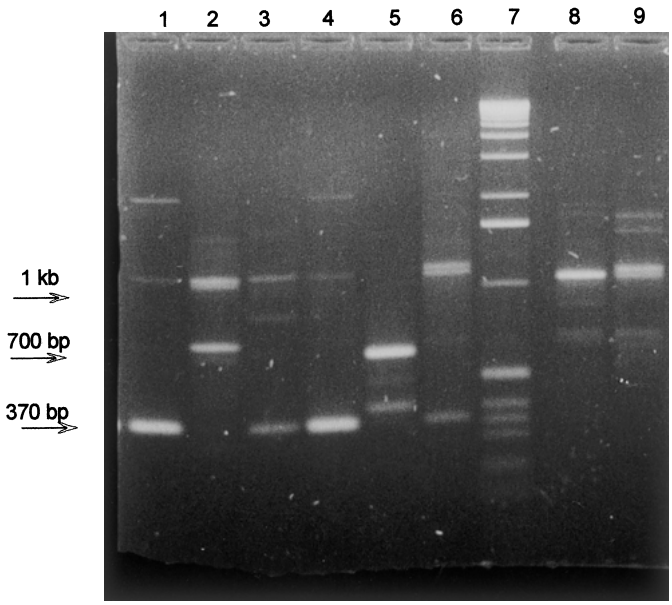

Figure 6. PCR Amplification of *X. albilineans* DNA with primer F20A. Lane 1: Xa 97 (Dominican Republic); lane 2: Xa6 (Kenya); lane 3: CIV 40 (Ivory Coast); lane 4: 3993 (Hawaii); lane 5: 3194 (Ivory Coast); lane 6: 3297 (Reunion); lane 7: DNA marker X (Boehringer Mannheim); lane 8: 3196 (St Kitts); lane 9: Xa 115 (Brazil).

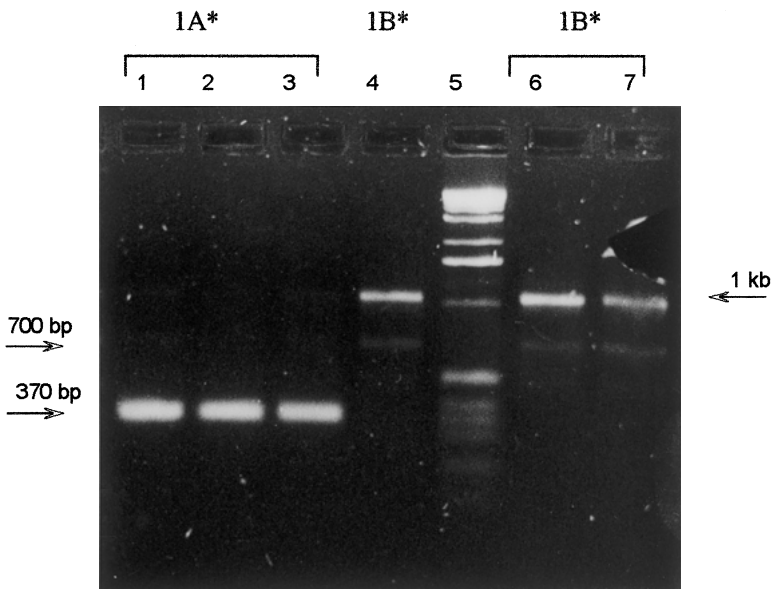

Figure 7. PCR Amplification of *X. albilineans* DNA with primer F20A. Lanes 1–3: 3360, 3361, 3362 (Florida 1985, 1986); lane 4: 3969 (Florida 1991); lane 5: DNA marker X (Boehringer Mannheim); lanes 6 and 7: 3981, 3987 (Florida 1991). *Alvarez groups (1996).

strains studied. With probe F20, polymorphic bands revealed five hybridisation profiles when eight strains from Mauritius and six from Louisiana were compared. Probe F20 also detected differences within the serovars I and II, indicating that these groups are not homogeneous at the DNA level.

PCR with the single primer F20A produced bands of 370, 700 and 1.1 kb. The 370 bp band was obtained with

all strains of serovar II strains of Mauritius. Four strains from Hawaii, one each from Australia, Burkina Faso, Dominican Republic and Ivory Coast also produced the 370 band (results for strain 3184 is not shown in the figures provided here). None of the Mauritius strains of serovar I, or the strains from Brazil, South Africa, Guadeloupe and Taiwan produced the 370 bp band. Table 2 shows the serotype of the strains used here, typed using either monoclonal or polyclonal antibodies. Since the two classifications are comparable, they can be used to relate to the DNA groups. With the Mauritius strains, the two serovars can be differentiated by PCR using primer F20A. All serovar II strains gave the 1.1 kb and the 370 bp bands except 3194 (I. Coast) and Xa6 (Kenya). Strain 3194 was classified in a group of its own by Alvarez et al. (1996).

Two subgroups may exist in the serovar I strains. Subgroup 1a (to differentiate from Alvarez grouping) gave the 370 bp band with primer F20A and this includes strains from Florida (1985–1986), Hawaii, Australia and Dominican Republic. These samples had already been classified together in DNA group 1A by Alvarez et al. (1996). The other subgroup, 1b, did not amplify the 370 bp band and included strains from Brazil, Guadeloupe, Taiwan and Florida (1991), all of which were in Alvarez DNA group 1B. Two strains of serovar III, Xa115 and 3196, were tested by PCR and they did not produce the 370 bp band typical of serovar II and subgroup 1a. More strains of this serotype need to be tested in order to define their genetic relatedness.

Primer F20A could be targeting a region of inverse repeats in the genome of *X. albilineans*. The repeat feature is illustrated by the multiplicity of the band size; the 700 bp band is nearly twice the 370 while 1.1 kb is about three times 370. However, this needs to be confirmed by sequencing the amplified fragments. The PCR reaction was done at the annealing temperature of 58 °C, which is only three degrees below the T_m (melting temperature) of primer F20A. This reaction is therefore not a RAPD type reaction. In conclusion, probe F20 and primer F20A, described here, provide rapid and simple tools for the typing of *X. albilineans* strains. We have confirmed and defined the genetic diversity of *X. albilineans* strains from Mauritius and compared them with strains of other geographical origins. The data presented here indicate that at least two DNA groups, with possible subgroups, of *X. albilineans* are present in Mauritius.

Of the six strains of *X. albilineans* from Florida, the first three were collected before the epidemic of 1989 and the other three were obtained afterwards. Interestingly, the two sets of strains were distinguishable with primer F20A. The amplification polymorphism described here with primer F20A is a simpler approach to study genetic diversity, than for example the identification of restriction fragments on polyacrilamide gels (Alvarez et al., 1996) or the PFGE (pulsed-field gel electrophoresis) technique (Davis et al., 1997). A large number of samples can be handled at the same time. The combined use of this probe and this primer can improve our understanding of the intraspecific variation of *X. albilineans* strains at the molecular level, and can assist in epidemiological studies and possibly for relating strain genotypes to their virulence on particular sugarcane varieties.

Acknowledgements

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