Differentiation of Strains of *Xylella fastidiosa* by a Variable Number of Tandem Repeat Analysis

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Short sequence repeats (SSRs) with a potential variable number of tandem repeat (VNTR) loci were identified in the genome of the citrus pathogen *Xylella fastidiosa* and used for typing studies. Although mono- and dinucleotide repeats were absent, we found several intermediate-length 7-, 8-, and 9-nucleotide repeats, which we examined for allelic polymorphisms using PCR. Five genuine VNTR loci were highly polymorphic within a set of 27 *X. fastidiosa* strains from different hosts. The highest average Nei's measure of genetic diversity (*H*) estimated for VNTR loci was 0.51, compared to 0.17 derived from randomly amplified polymorphic DNA (RAPD) analysis. For citrus *X. fastidiosa* strains, some specific VNTR loci had a *H* value of 0.83, while the maximum value given by specific RAPD loci was 0.12. Our approach using VNTR markers provides a high-resolution tool for epidemiological, genetic, and ecological analysis of citrus-specific *X. fastidiosa* strains.

Xylella fastidiosa has been associated with diseases in economically important crops such as grapevine, plum, almond, peach, citrus, and more recently, coffee (13, 17) as well as some diseases in ornamental plants (3). Reciprocal transmission tests including *X. fastidiosa* from several hosts evidenced the occurrence of host infection groups (for details, see reference 10). In Brazil, *X. fastidiosa* has been responsible for causing diseases in important crops such as citrus and coffee (5, 13), although it has also been observed in plum (7) and *Hibiscus schizopetalus* (E. W. Kitajima, H. D. Coletta-Filho, M. A. Machado, and Q. S. Novaes, 33th Congr. Brazil Phytopathol. Soc., abstr. 323, 2000). The major economic damage is in the sweet orange crop (*Citrus sinensis* Osb.) which has suffered an annual loss of about \$100 million. However, the total cost to Brazilian agriculture is probably higher because the economic damage caused to the coffee crop by *X. fastidiosa* has not been estimated.

Methods for distinguishing between bacterial strains are important for detecting disease outbreaks for epidemiological analysis, and for understanding the genetic structure of microbial populations. Molecular techniques of DNA profiling based on PCR of randomly amplified polymorphic DNA (RAPD) and repetitive element polymorphism PCR (rep-PCR) have been used with great success for studies of genetic variation and the relationships between *X. fastidiosa* strains (2, 6, 8, 16). However, the data produced by these methods are biallelic, which limits the amount of genetic information per locus and thus the use of these methods in estimating genetic diversity. Interlaboratory reproducibility is also a weakness of RAPD and rep-PCR analysis (20). Short sequence repeats (SSRs) with a potential variable number of repeats (VNTR) within prokaryotic DNA have been used as markers for discrimination between strains (1, 9, 12, 23). Some of these repetitive

regions are located within or near DNA coding regions and so could have the potential to affect gene expression (22).

The complete sequencing of the genome of the pathogenic *X. fastidiosa* strain 9a5c (19) has allowed the identification of repetitive DNA motifs. In the present study, we conducted a search for SSR size variation in different strains of *X. fastidiosa*, comparing the results with the frequently used RAPD method.

MATERIALS AND METHODS

Strains and DNA isolation. Table 1 shows the *X. fastidiosa* strains used, all of which were cultured on BCYE agar medium (24) for 7 to 10 days at 28°C. The hexadecyltrimethylammonium bromide (CTAB) miniprep method (25) was used for DNA preparation from each strain. Briefly, cells were harvested, washed twice in washing buffer (pH 8.0) containing 20 mM Tris HCl and 10 mM EDTA, and treated with proteinase K and sodium dodecyl sulfate. Proteins and other cellular components were removed by using CTAB and by two chloroform extractions. The DNA present in the aqueous phase was precipitated with absolute ethanol followed by washing with 70% ethanol. The pellet obtained was dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA) (pH 8.0) containing 20 mM RNase. The DNA was subjected to electrophoresis in a 0.8% agarose gel. After staining with ethidium bromide, the samples were visualized under ultraviolet light and the DNA concentration was estimated. The DNA solution was diluted to 5 ng/μl and stored at –20°C.

Computer analysis of repetitive DNA. The genomic DNA sequence of *X. fastidiosa* strain 9a5c (available on the UNICAMP website at <http://onsona.lbi.ic.unicamp.br/xf>) was screened for repetitive DNA with the Tandem Repeat Finder version 2.02 software (4), freely available at <http://c3.biomath.mssm.edu/trf/>. This software scores all DNA motif categories, although in our study, only perfect repeats were selected. BLASTN searches of the regions upstream and downstream of the repeat motif were conducted on the complete *X. fastidiosa* 9a5c genome to localize the repeats and related genes.

Repeat analysis. For some 7-, 8-, and 9-nucleotide repeats, sets of primers (Table 2) with the potential for locus-specific amplification were designed using Lasergene 99 software (DNASTAR, Inc). Most of the primers were deduced from sequences bordering the repeat, 5 nucleotides upstream and downstream of the locus. The other primers (SSR26, SSR30, and SSR32) were designed up to 30 nucleotides distant from the repeat locus. Amplifications were conducted in a volume of 25 μl containing 10% of 10× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2.5 mM MgCl₂, 0.25 mM (each) deoxynucleoside triphosphate (GIBCO BRL), 50 ng (each) primer, 50 ng of template DNA, and 1.5 U of *Taq* DNA polymerase (GIBCO BRL). An initial denaturation step at 94°C for 3 min was followed by a touchdown amplification program. DNA was denatured at 94°C for 1 min, and primers were annealed for 30 s and extended at 72°C for 1 min. The initial annealing temperature was 64°C for 1 cycle. The temperature

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TABLE 1. *X. fastidiosa* strains used in this study

Strain	Host	Geographic origin	Source
9a5c	<i>Citrus sinensis</i>	São Paulo, Brazil	INRA ^a
B14	<i>Citrus sinensis</i>	São Paulo, Brazil	CCSM ^b
11779	<i>Citrus sinensis</i>	Santa Catarina, Brazil	IAPAR ^c
M2-1	<i>Citrus sinensis</i>	São Paulo, Brazil	CCSM
ITAa	<i>Citrus sinensis</i>	São Paulo, Brazil	CCSM
11834	<i>Citrus sinensis</i>	Paraná, Brazil	IAPAR
NP3.2	<i>Citrus sinensis</i>	São Paulo, Brazil	CCSM
11775	<i>Citrus sinensis</i>	Rio Grande do Sul, Brazil	IAPAR
11066	<i>Citrus sinensis</i>	Paraná, Brazil	IAPAR
11067	<i>Citrus sinensis</i>	Paraná, Brazil	IAPAR
NP2.3	<i>Citrus sinensis</i>	São Paulo, Brazil	CCSM
10438	<i>Citrus sinensis</i>	Paraná, Brazil	IAPAR
11038	<i>Citrus sinensis</i>	São Paulo, Brazil	IAPAR
11347	<i>Citrus sinensis</i>	Paraná, Brazil	IAPAR
SR9.2	<i>Citrus sinensis</i>	São Paulo, Brazil	CCSM
11037	<i>Citrus sinensis</i>	São Paulo, Brazil	IAPAR
11399	<i>Citrus sinensis</i>	Sergipe, Brazil	IAPAR
11380	<i>Citrus sinensis</i>	Santa Catarina, Brazil	IAPAR
SL4	<i>Citrus sinensis</i>	Minas Gerais, Brazil	CCSM
C3	<i>Coffea arabica</i>	São Paulo, Brazil	CCSM
C6	<i>Coffea arabica</i>	São Paulo, Brazil	CCSM
C13	<i>Coffea arabica</i>	São Paulo, Brazil	CCSM
12288	<i>Coffea arabica</i>	Paraná, Brazil	IAPAR
8935	<i>Vitis vinifera</i>	United States	ATCC 35879 ^d
Hib. 5	<i>Hibiscus schizopetalus</i>	Brasília, Brazil	CCSM
9746	<i>Prunus salicina</i>	Paraná, Brazil	IAPAR
12319	<i>Catharantus</i> sp.	São Paulo, Brazil	IAPAR

^a INRA, Institut National de la Recherche Agronomique et Université Victor Ségalé, Bordeaux, France.

^b CCSM, Centro de Citricultura Sylvio Moreira, Instituto Agrônomo, Cordeiropolis, São Paulo, Brazil.

^c IAPAR, Instituto Agrônomo do Paraná, Paraná, Brazil.

^d ATCC, American Type Culture Collection, Manassas, Va.

was subsequently dropped 1°C every cycle until a final annealing temperature of 55°C was reached. For the remaining 22 cycles, the annealing temperature was 55°C. A final extension step of 4 min at 72°C was followed by 10°C soak. PCR products were separated by gel electrophoresis in 3% agarose gels and stained

with ethidium bromide, with the fragment size estimated based on migration relative to that of a 100-bp size marker (GIBCO BRL) loaded together with the samples in the gel.

RAPD analysis. RAPD analysis was performed in a volume of 13 µl containing 10% 10× PCR buffer, 2.0 mM MgCl₂, 0.25 mM (each) deoxynucleoside triphosphate (GIBCO BRL), 15 ng of template DNA, 1.5 U of *Taq* DNA polymerase (GIBCO BRL), and 15 ng of primer (Operon kit of 10-mer primers; OPG10, OPG17, OPG19, OPH03, OPH07, OPH12, OPN04, OPQ05, and OPW07). Amplification was performed using a temperature profile, with initial denaturation at 94°C for 3 min followed by 36 cycles of denaturation (1 min at 94°C), annealing (1 min at 36°C), and extension (2 min at 72°C) with a final 10-min extension step at 72°C. The RAPD reaction products were separated by electrophoresis in 1.3% agarose gels and stained with ethidium bromide, with the RAPD band size estimated by comparison with a 1-kb DNA ladder marker (GIBCO BRL).

Data analysis. All genotypes used for SSR and RAPD were included in the analysis. For the RAPD methodology, only high-intensity markers of unambiguous interpretation and good reproducibility were scored. Nei's measure of genetic diversity was calculated as $H = [1 - \sum p_i^2]$, where p_i is the frequency of allele i at the locus (15).

RESULTS

Database searches for SSRs. There were no 1- or 2-base SSRs, but there were 67 perfect SSRs with 3 to 33 nucleotides per unit (Table 3). Most common were 8-nucleotide SSRs, which were present at twelve loci at copy numbers varying from 3 to 37 copies per repeat. In one of these loci, we encountered mixed repeats harboring the (TTGGGTAG)₂₂/(TTGGGTAA)₃₅ motif.

SSR amplification by PCR. For DNA polymorphism analysis of the SSR regions, we selected two 7-nucleotide SSRs, six 8-nucleotide SSRs, and one 9-nucleotide SSR (Table 2). These SSRs were selected as potential VNTRs because they were of intermediate size, uncommon for prokaryotic SSRs (22), and presented a large number of copies per repeat unit. Amplifications using all these potential VNTR primers were performed in *X. fastidiosa* strains 9a5c and B14 (Fig. 1), although for two SSR primers (SSR32 and SSR34), the amplification step failed even though variations in the amplification program and reaction mixtures were tried. Although the SSR26 and SSR36 primers generated the same product for both strains

TABLE 2. SSR regions monitored for polymorphism in 27 strains of *X. fastidiosa* isolated from different hosts^a

Potential VNTR name	Repeat motif	Repeat position in genome	Primer sequence for VNTR (5' → 3' amplification)
SSR20	(ATTGCTG) ₁₃	18527–18619	ATGAAGAAGCCAGGATACAT GCTACACGTGCAACAAC
SSR21	(TGTTATC) ₂₁	52902–53049	AACACGGATCAAGCTCATG GGAACACGCAATAGTAAGAC
SSR26	(GTGTGTGA) ₃₇	49439–49733	CTGTGATCGGTGAATTGA TCAAGCACACTTCTACG
SSR28	(GTGTGCCT) ₁₁	1046415–1046503	GCAACGCTGTTATCTCAAT ATTACGCTTCTTATCGCTGT
SSR30	(TGATCCTG) ₁₅	1512303–1512422	TACGCTGCACCTGTCTG CTGTGAACTTCCATCAATCC
SSR32	(CTGATGTG) ₉	2050614–2050688	AGATGAACCTCGCCAC GTACTCATCTGCGATGG
SSR34	(TTGGGTAG) ₂₂ /(TTGGGTAA) ₃₅	2289266–2289727	TGATAGAACTGTTTGACGCATTG TCGGGAAGTTTGGGGTGAC
SSR36	(TGTTGGGG) ₁₀	2639987–2640070	ATGTCACCTCAGGTCAGG CAGAACCACCGACTG
SSR40	(GAAGGCGTA) ₂₇	1220096–1220340	ACCTTGACGACGGATG TAGGAAGTGTGCTACTGAT

^a All data are based on the genome sequence of *X. fastidiosa* strain 9a5c.

TABLE 3. Perfect SSR regions encountered in the genome of *X. fastidiosa* strain 9a5c by computerized screening

Repeat unit length	No. of copies ^a
3.....8, 10	
4.....7, 8, 13	
5.....11, 12, 21, 22	
6.....(5) ^b 4, 7, 8, 10, 15, 16	
7.....4, 5, (2) 7, 13, 21	
8.....(3) 3, 4, 5, (2) 9, 10, 11, 15, 22, 35, 37	
9.....(2) 3, 27	
10.....3, 4	
11.....2	
12.....(7) 2, 3	
13.....(2) 2	
14.....2	
15.....(6) 2	
17.....2	
18.....4	
21.....2	
23.....2	
29.....2	
33.....2	

^a The number of copies in different positions in the genome is shown.

^b Number of times that the number of copies of repeat were encountered through the genome.

9a5c and B14, they were still used for typing studies together with primers SSR20, -21, -28, -30, and -40.

VNTR analysis. Table 4 shows the SSR and RAPD primers used and the genetic diversity values obtained. For the SSR26 and SSR36 primers, no variation in the number of repeats was observed in the strains. Five pairs of primers (SSR20, -21, -28, -30, and -40) showed definite length polymorphism in their respective SSR regions and thus appear to represent genuine VNTRs. Even though the reactions were repeated, doublet bands were sometimes observed when the SSR20 and SSR40 primers were used with strain 12319 and when the SSR30 primer was used in some other strains. This indicates the presence of two alleles for the same SSR locus in some strains, although no doublet bands were observed in strain 9a5c.

A minimum of six and a maximum of eight alleles per locus were observed with the VNTR primers, generating a high level of polymorphism. The discriminatory power of each VNTR locus and RAPD analysis was estimated from the genetic diversity (*H*) values based on the number of alleles and their frequency. In the analysis of citrus and coffee *X. fastidiosa* strains, the average *H* value was higher for VNTR markers

TABLE 4. Mean *H* values for the SSR and RAPD loci in 27 strains of *X. fastidiosa* isolated from different hosts

Locus ^a	<i>H</i> value for <i>X. fastidiosa</i> strain isolated from:			
	All hosts	Citrus	Coffee	Other hosts ^b
SSR				
SSR20	0.77	0.70	0.50	0.72
SSR21	0.44	0.28	0.63	0.38
SSR26	0.00	0.00	0.00	0.00
SSR28	0.81	0.80	0.75	0.63
SSR30	0.69	0.64	0.63	0.48
SSR36	0.00	0.00	0.00	0.00
SSR40	0.83	0.83	0.63	0.56
Avg	0.51	0.46	0.45	0.39
RAPD				
OPG10	0.23	0.12	0.09	0.36
OPG17	0.16	0.02	0.15	0.33
OPG19	0.22	0.16	0.05	0.27
OPH03	0.14	0.07	0.06	0.39
OPH07	0.16	0.04	0.08	0.43
OPH12	0.13	0.09	0.06	0.21
OPN04	0.19	0.11	0.06	0.35
OPQ05	0.07	0.00	0.00	0.28
OPW07	0.20	0.00	0.13	0.48
Avg	0.17	0.07	0.07	0.34

^a SSR and RAPD primers.

^b Data from strains isolated from plum, grapevine, hibiscus, and periwinkle.

than for RAPD markers (Table 4), with VNTR primers SSR28 and SSR40 showing the highest *H* values. For citrus strains, these values reached 0.80 (SSR28) and 0.83 (SSR40). It has previously been shown that VNTR typing of *Bacillus anthracis* and *Yersinia pestis* has produced *H* values of 0.80 and 0.82, respectively (1, 11). Table 4 also shows that in the analysis of *X. fastidiosa* strains isolated from other hosts (plum, grapevine, hibiscus, and periwinkle), the average VNTR marker *H* value (0.39) was only slightly higher than the average RAPD marker *H* value (0.34).

DISCUSSION

The presence of SSRs and their usefulness for typing have previously been shown for both human and animal pathogens (22), but this is the first time that these molecular markers have been used to type phytopathogenic bacteria.

The characteristics of the SSR regions found in the *X. fastidiosa* genome were quite different from the SSRs already known in other bacteria. No mono- or dinucleotide SSRs were detected in *X. fastidiosa*, but there was a large number of 7- and 8-nucleotide repeats (Table 3), a fact which led us to use intermediate-sized repeats in our typing studies. Shorter, 1- or 2-nucleotide repeats are abundant in other species of pathogenic bacteria such as *Helicobacter*, *Neisseria*, *Mycobacterium*, and *Escherichia coli*, while intermediate-sized repeats have rarely been found (22). This phenomenon appears to be independent of genome size, because in the 4.2-Mb chromosome of *Bacillus subtilis*, there are only repeats with short sequences, while in the 0.58-Mb chromosome of *Mycoplasma genitalium*, there are other classes of repeats (21).

FIG. 1. Gel electrophoresis of seven SSR primers for two *X. fastidiosa* strains. *X. fastidiosa* strains 9a5c (lanes 1) and B14 (lanes 2) and SSR primers SSR36 (A lanes), SSR20 (B lanes), SSR26 (C lanes), SSR21 (D lanes), SSR28 (E lanes), SSR40 (F lanes), and SSR30 (G lanes) were used. The intensely stained band represents a 600-bp fragment (arrow). Lanes M, 100-bp ladder marker (GIBCO BRL).

FIG. 2. Gel electrophoresis of SSR20, SSR21, SSR26, SSR28, SSR30, and SSR40 primers from 12 *X. fastidiosa* strains. *X. fastidiosa* strains 9a5c, B14, 11779, M2.1, ITAa, 11834, 9713, C13, C3, Hib 5, 9746, and NP3.2 are shown in lanes 1 to 12, respectively (see Table 1 for host identification). The intensely stained bands represent a 600-bp fragment (arrows). Lanes M, 100-bp ladder marker (GIBCO BRL).

The VNTR typing method presented in this paper is a powerful method for the genetic characterization of *X. fastidiosa* isolates. The potential VNTR regions selected for typing were conserved in all *X. fastidiosa* strains tested, irrespective of host (Table 4 and Fig. 2). The genetic diversity (H) values based on VNTR primers were extremely high, especially for citrus- and coffee-specific *X. fastidiosa* strains. This contrasts with the RAPD technique, which although it has allowed the construction of well-defined *X. fastidiosa* host groups (5, 16) has shown limited discriminatory capacity between *X. fastidiosa* strains isolated from citrus species (18). Although the RAPD technique has the potential for genome-wide analysis, SSRs have the advantage that they can link to the genomic hypervariable regions produced as a result of variation in sequence composition and DNA polymerase activity. It seems that the high discriminatory power of VNTR locus analysis is a result of the hypervariability of this molecular marker rather than the occurrence of artifacts due to serial subculture. The VNTR assay is also fairly reproducible (1, 14), and VNTR mutation rates, as seen in *Bacillus anthracis*, are less than 10^{-5} (11).

The growing numbers of prokaryotic DNA sequences (including those from plant pathogens) in databases and computer programs available for the detection of SSR loci have facilitated the evaluation of SSR within DNA sequences. Not only are the hypervariability and reproducibility of SSRs markers useful, but if the primers are *X. fastidiosa* specific, there is also the possibility of in situ analysis of a known gene without isolating the bacteria. This approach would greatly facilitate epidemiological, genetic, and ecological studies of fastidious bacteria, such as *X. fastidiosa*, which are normally difficult to isolate and grow.

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