The Journal of Microbiology, October 2008, p. 482-490 Vol. 46, No. 5
DOI 10.1007/s12275-008-0072-8

Isolation and Molecular Characterization of *Xylella fastidiosa* from Coffee Plants in Costa Rica

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(Received March 18, 2008 / Accepted August 14, 2008)

Coffee plants exhibiting a range of symptoms including mild to severe curling of leaf margins, chlorosis and deformation of leaves, stunting of plants, shortening of internodes, and dieback of branches have been reported since 1995 in several regions of Costa Rica's Central Valley. The symptoms are referred to by coffee producers in Costa Rica as "crespera" disease and have been associated with the presence of the bacterium *Xylella fastidiosa*. Coffee plants determined to be infected by the bacterium by enzyme linked immunosorbent assay (ELISA), were used for both transmission electron microscopy (TEM) and for isolation of the bacterium in PW broth or agar. Petioles examined by TEM contained rod-shaped bacteria inside the xylem vessels. The bacteria measured 0.3 to 0.5 μm in width and 1.5 to 3.0 μm in length, and had rippled cell walls 10 to 40 nm in thickness, typical of *X. fastidiosa*. Small, circular, dome-shaped colonies were observed 7 to 26 days after plating of plant extracts on PW agar. The colonies were comprised of Gram-negative rods of variable length and a characteristic slight longitudinal bending. TEM of the isolated bacteria showed characteristic rippled cell walls, similar to those observed in plant tissue. ELISA and PCR with specific primer pairs 272-1-int/272-2-int and RST31/RST33 confirmed the identity of the isolated bacteria as *X. fastidiosa*. RFLP analysis of the amplification products revealed diversity within *X. fastidiosa* strains from Costa Rica and suggest closer genetic proximity to strains from the United States of America than to other coffee or citrus strains from Brazil.

***Keywords*:** citrus variegated chlorosis, coffee leaf scorch, crespera, Pierce's disease

Xylella fastidiosa Wells is a Gram-negative, xylem limited, plant pathogenic bacterium (Wells *et al*., 1987) responsible for important diseases such as Pierce's disease (PD) of grapevine and almond leaf scorch (ALS) in California, and citrus variegated chlorosis (CVC) of sweet orange in Brazil. It is also the causal agent of several other leaf scorch diseases in crops, ornamental plants and forest and urban trees (Hopkins and Purcell, 2002). It has been reported mainly in the Americas, but there are also reports from Taiwan (Leu and Su, 1993) and Europe (Berisha *et al*., 1998; Güldür *et al*., 2005).

In Brazil, *X. fastidiosa* is the causal agent of coffee leaf scorch (CLS) (Beretta *et al*., 1996; De Lima *et al*., 1998). The disease was first observed in São Paulo State in 1995 and is distributed throughout the State, as well as in other regions of Brazil. The disease is characterized by short internodes with reduced branch growth, chlorosis, deformation, and scorch of leaves, defoliation, reduction in fruit size and quantity, and death of lateral branches and shoots (De Lima *et al*., 1998).

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Pierce's disease of grapevine was reported in Costa Rica in 1979 (Goheen *et al*., 1979). Recently, *X. fastidiosa* was also detected in coffee and citrus plants in Costa Rica (Rodriguez *et al*., 2001; Aguilar *et al*., 2005b). *X. fastidiosa* has been associated with a coffee disease known as "crespera" by the growers, due to irregular growth of leaves with an atypical curling of the margins of leaf blades. Others symptoms of "crespera"-affected Costa Rican coffee plants include reduced leaf size and malformation, shortening of internodes and severe chlorotic mosaic of leaves (Rodriguez *et al*., 2001), as seen in coffee leaf scorch in Brazil. The symptoms have been reported by coffee producers since 1995. The disease may have important economic effects because preliminary results showed a significant difference in the number of coffee berries produced by "crespera" compared to healthy looking plants (Solórzano *et al*., 2001). Thus, the presence of *X. fastidiosa* in coffee in Costa Rica may represent a threat to the industry in Costa Rica and possibly to other Latin American coffee producing countries.

Several DNA analyses have shown that Brazilian sweet orange and coffee strains of *X. fastidiosa* are closely related (Rosato *et al*., 1998; Mehta and Rosato, 2001; Qin *et al*., 2001). Researchers have hypothesized that *X. fastidiosa* populations were first selected from native flora and adapted

to and developed in coffee plants and only later moved into sweet orange in Brazil (Mehta *et al*., 2001; Qin *et al*., 2001; Chen *et al*., 2002). Limited variability has been detected among the sweet orange strains (Rosato *et al*., 1998; Lacava *et al*., 2001; Mehta *et al*., 2001; Qin *et al*., 2001) and relatively few coffee strains have been included in these studies. The Brazilian sweet orange-coffee group is generally distinct from *X. fastidiosa* strains from North America (Pooler and Hartung, 1995a; Qin *et al*., 2001) and a proposal has been made to give the sweet orange strains subspecies status different from all North American strains to reflect this (Schaad *et al*., 2004).

The detection and isolation of *X. fastidiosa* from Costa Rica makes it possible to study distribution and variation among isolates from North, Central, and South America. Moreover, as more strains of the bacterium and more hosts are recognized, it is important to understand the diversity and the relationships among and within strains regarding host species (Chen *et al*., 2005; Hernandez-Martinez *et al*., 2006; Shapland *et al*., 2006; Montero-Astúa *et al*., 2007).

In this work, we report the first isolation of *X. fastidiosa* strains from "crespera" symptomatic coffee plants in Costa Rica. The diversity of these coffee strains and their relationship with other strains of the bacterium were assayed by restriction fragment length polymorphism (RFLP) analysis of *X. fastidiosa* specific amplification products (Minsavage *et al*., 1994; Qin *et al*., 2001). RFLP based techniques may allow the identification of particular restriction sites useful for rapid differentiation of strains and for the design of host or locality specific primers.

Materials and Methods

Field survey

Coffee plantations in the Central Valley of Costa Rica were visited, and plants showing "crespera" symptoms as well as asymptomatic plants were sampled. Leaves from different sections of the same coffee plant were collected and tested for the presence of *X. fastidiosa* as described below by double antibody sandwich enzyme linked immunoabsorbent assay (DAS-ELISA) with commercial antibodies (Agdia Inc., USA).

Bacterial cultures

Coffee tissue used for isolation was rinsed in tap water or superficially cleaned with 70% ethanol. Leaf petioles and midribs were then excised and disinfested in 70% ethanol for 5 min, followed by 1% sodium hypochlorite for 5 min and three rinses, 5 min each, in sterile water (He *et al*., 2000). Afterwards, the tissue was ground in sterile phosphate-buffered saline (PBS) buffer or 'periwinkle wilt' (PW) broth (Davis *et al*., 1981) and allowed to stand for 15~30 min. Aliquots (20 μl) of undiluted and 10^{-1} and 10^{-2} dilutions of the plant extract were used to inoculate PW broth or PW agar plates. Agar plates and broth were incubated at 28°C and plates were periodically evaluated for the presence of *Xylella*-like colonies (Wells *et al*., 1987; De Lima *et al*., 1998). PW broth was used directly for DAS-ELISA as described; meanwhile, suspicious colonies on plates were evaluated by resuspending in PBS buffer and either used directly for ELISA or to inoculate PW broth which was tested 15 days later by ELISA. All ELISA-positive PW broths were plated 15 days after inoculation and the resulting *X. fastidiosa*-like colonies were tested again by ELISA and/or polymerase chain reaction (PCR) with specific primers (Pooler and Hartung, 1995b). Colonies were restreaked three times to assure the purity of the strains. Bacterial cells were Gram stained and observed at 1,000× by phase contrast microscopy.

Enzyme linked immunoabsorbent assay (ELISA)

Petiole and midrib tissue from five leaves from each plant were ground in general extraction buffer (Agdia Inc., USA) supplemented with 3% sodium sulfite. All samples were tested in duplicate wells and plates included positive and negative controls. Samples with absorbance values above the average absorbance values of the known negative samples plus three times the standard deviation were considered to be positive (Sutula *et al*., 1986).

Transmission electron microscopy (TEM)

Midrib samples (2 mm×1 mm) were taken from older leaves of field-grown, ELISA-positive coffee plants showing severe symptoms of "crespera" and from healthy, ELISA-negative coffee plants grown in a greenhouse. Samples were fixed with Karnovsky's fixative for 48 h at room temperature. Samples were rinsed five times in 0.05 M cacodylate buffer, pH 7.2 and post-fixed in 1% osmium tetraoxide buffered in 0.05 M cacodylate buffer pH 7.2 at room temperature for 2 h. Samples were rinsed two times with 0.05 M cacodylate buffer, pH 7.2 followed by two rinses with distilled water. Then the post-fixed tissue was dehydrated in a graded ethanol series (50, 75, 95, and 100%; 1 h each). Exposure to 100% ethanol was repeated two times and followed by propylene oxide:ethanol (1:3, 2:2, 3:1; 1 h each). Tissue was then embedded in Spurr's medium. Semi-thin sections (~125 nm) were cut with glass knives, stained with toluidine blue (1%) and observed at 1,000× with a light microscope. The blocks with xylem vessels that took up the stain were selected for ultra-thin sectioning. Ultra-thin sections (70~90 nm) were cut with a diamond knife on an ultra microtome (Ultracut, Leica) and stained for 5 min with 2% uranyl acetate in 50% ethanol and for 2 min with lead citrate. Sections on grids were analyzed and photographed with a transmission electron microscope (Hitachi H 7100) at 100 Kv.

Isolated bacterial cells from one of the cultured strains (described previously) were prepared for TEM following a procedure similar to that of (Leu and Su, 1993). One milliliter of a bacterial culture was mixed with 1 ml of Karnovsky's fixative for 1 h at room temperature. The suspension was centrifuged at 2,000 rpm for 5 min and the pellet gently mixed with 0.5 ml of 2% melted Bacto agar at 50°C. Agar blocks with embedded bacterial cells were cut into 1 mm^3 pieces, and then were fixed with 1% osmium tetraoxide for 1 h at room temperature. Afterwards, the agar blocks were washed three times with 0.05 M cacodylate buffer pH 7.2, dehydrated, resin infiltrated, sectioned, and stained as described above for TEM of plant tissue.

PCR amplification

PCR amplification was carried out using *X. fastidiosa* colo-

nies, broth cultures, or DNA extracts of cultured bacteria as template. DNA was extracted with a CTAB miniprep method for bacterial genomic DNA (Ausubel, 1992). PCR was performed with specific primer pairs 272-1-int/272-2-int as described (Pooler and Hartung, 1995b) and RST31/RST33 (Minsavage *et al*., 1994) in a final volume of 50 μl; each reaction contained 2 μl of template (DNA, resuspended bacteria or PW broth), 3 mM $MgCl_2$, 0.1 mM dNTPs, 3 μM each primer, 1× PCR buffer, and 1.25 units *Taq* DNA Polymerase (Fermentas, Germany). The amplification program was followed as previously reported (Minsavage *et al*., 1994) on a MJ Research Thermal Cycler (Model PTC-100 or PTC-200). Electrophoresis of products was performed in 1.5% agarose gels at 80 Volts for 25 min. Amplification products were stained in a 0.5 μg/ml ethidium bromide solution and visualized with UV light. All sets of PCR reactions included *X. fastidiosa* positive and PCR mix negative controls. All gels included a 100-bp DNA Ladder (Fermentas, Germany).

Restriction fragment length polymorphism (RFLP) analysis

Seven Costa Rican coffee strains (Table 1) were selected for RFLP analysis of PCR-amplification products. The molecular comparisons included two grapevine (VvIc1 and VvIIIc1) and two citrus (CspIc1 and CspIIIc1) strains from Costa Rica (Aguilar *et al*., 2005a, 2005b); five strains from Brazil and three from North America (DNA extracted at the Fruit Laboratory, ARS-USDA). The Costa Rican isolates are kept at the Centro de Investigación en Biología Celular y Molecular (CIBCM), Universidad de Costa Rica, Costa Rica. Replicates of strains CaIc2, CaIIc2, CaIIIc3, CaVc1, CspIc1, and VvIc2; together with the coffee and orange strains from Brazil: Café20/11, Fund2, Fund4, Lar20/11, and Taq30 are kept at the USDA-ARS Molecular Plant Pathology Laboratory, Beltsville, MD. Strains with an ATCC prefix are from the American Type Culture Collection, Manassas, VA. Strain ATCC 35783 was isolated from elm (Kostka *et al*., 1981; Wells *et al*., 1987) and strains ATCC 35868 and ATCC 35869 were isolated from mulberry (Kostka *et al*., 1986; Wells *et al*., 1987). The restriction pattern for strains 9a5c (isolated from sweet orange, Brazil) (Simpson *et al*., 2000) and Temecula1 (isolated from Grapevine, USA) was predicted from the corresponding sequences in GenBank (Table 2).

Restriction endonucleases for RFLPs were selected based on previous reports (Minsavage *et al*., 1994; Qin *et al*., 2001) or by an *in silico* analysis in BioEdit Sequence Alignment Editor (version 7.0.5) software (Hall, 1999) with sequences

Table 1. Digestion patterns[a] generated with 12 restriction endonucleases and specific PCR amplification products from *Xylella fastidiosa* strains from Brazil (Br), Costa Rica (CR), and United States (US)

Strain	Origin	Primer set 272-1-int/272-2-int product						Primer set RST31/RST33 product						Reference
		*Hpy*F10VI	*Nco*I	*Mnl*I	*Taq*I	*Cfo*I	*Bsp*143I	*Bsp*68I	*Psu*I	*Hin*1II	*Bsp*PI	*Rsa*I	*Alu*I	
Café20/11	Coffee, Br	A	C	E	I	K	M	O	Q	S	V	X	Z	Qin *et al*. (2001)
Fund2	Coffee, Br	B	C	E	J	L	M	O	Q	S	V	X	Z	
Fund4	Coffee, Br	A	C	E	I	K	M	O	Q	S	V	X	Z	
Lar20/11	Citrus, Br	B	C	E	J	L	M	O	Q	T	V	X	Z	
Taq30	Citrus, Br	B	C	E	J	L	M	O	Q	T	V	X	Z	
9a5c[b]	Citrus, Br	B	C	E	J	L	M	O	Q	T	V	X	Z	Simpson *et al*. (2000)
CaIc1	Coffee, CR	A	C	F	J	K	M	P	R	U	W	X	α	This work,
CaIIc2	Coffee, CR	A	C	F	J	K	M	P	R	U	W	X	α	Chacón-Díaz, C.
CaIIIc3	Coffee, CR	A	C	G	J	K	N	P	R	U	W	X	α	
CaVc1	Coffee, CR	A	C	G	J	K	N	P	R	U	W	X	α	
CaVIIc3	Coffee, CR	A	C	F	J	K	M	P	R	U	W	X	α	
CaVIIIc3	Coffee, CR	A	C	G	J	K	N	P	R	U	W	X	α	
CaXc1	Coffee, CR	A	C	H	J	K	N	P	R	U	W	Y	α	
CspIc1	Citrus, CR	A	C	H	J	K	N	P	R	U	W	Y	α	Aguilar *et al*. (2005b)
CspIIIc1	Citrus, CR	A	C	E	I	K	M	O	Q	T	V	X	β	
VvIc2	Grapevine, CR	A	C	F	J	K	M	P	R	U	W	X	α	Aguilar *et al*. (2005a)
VvIIIc1	Grapevine, CR	A	C	F	J	K	M	P	R	U	W	X	α	
ATCC 35873	Elm, USA	A	D	E	J	K	M	P	R	U	W	Y	α	Kostka *et al*. (1981)
ATCC 35868	Mulberry, US	A	C	G	J	K	N	P	R	U	W	Y	α	Kostka *et al*. (1986)
ATCC 35869	Mulberry, US	A	C	G	J	K	N	P	R	U	W	Y	α	
Temecula-1[b] (ATCC 700964)	Grapevine, US	A	C	G	J	K	N	P	R	U	W	X	α	ATCC, Depositor H. Feil

[a] Each letter represents a different restriction pattern.
[b] Restriction patterns for strains 9a5c and Temecula-1 were predicted from the corresponding sequences analyzed with BioEdit Sequence Alignment Editor (v. 7.0.5).

Table 2. Sequences obtained from GenBank and used for in silico restriction fragment length analysis in BioEdit Sequence Alignment Editor (v. 7.0.5)

Strain	Accession number	Origin	Primer set
Temecula-1	AE009442	Grapevine, US	
	position 479091-478620		272-1-int/272-2-int
	position 726358-7277078		RST31/RST33
9a5c	AE003849	Citrus, Brazil	
	position 1051239-1051710		272-1-int/272-2-int
	position 1298144-1298877		RST31/RST33
Found-4	AF344190	Coffee, Brazil	272-1-int/272-2-int
Found-5	AF344191	Citrus, Brazil	272-1-int/272-2-int
JB-USNA	AY196792	*Fagus crenata*	272-1-int/272-2-int

available in GenBank (www.ncbi.nlm.nih.gov) corresponding to amplification products with primers 272-1-int/272-2-int or RST31/RST33 (Table 2). Sequences were downloaded, aligned and restriction maps were generated for each one. Restriction endonucleases capable of distinguishing the strains were identified.

Enzymes *Hpy*F10VI, *Nco*I, *Mnl*I, *Taq*I, *Sau*3AI (Fermentas, Germany), and *Cfo*I (Promega) were used to digest the 272 primer set amplification product; the RST primer set amplification product was digested with the enzymes *Bsp*68I, *Psu*I, *Hin*1II, *Bsp*PI, *Rsa*I, and *Alu*I (Fermentas, Germany). Digestions were carried out in 15 µl final volume containing 2.5 U of restriction endonuclease and 10 µl of the amplification product and incubated for 3 h. Products were separated by electrophoresis through 3% agarose gels and visualized. Contrast adjustment in AlphaEaseFC™ (version 3.2.1) software and negative effect and balance adjustment in Microsoft Photo Editor (version 3.0.2.3) software was applied to the gel pictures to improve the visualization of DNA bands. Fragment sizes were estimated based on migration relative to a 50-bp molecular marker (Fermentas, Germany). Fragments longer than 100 bp were scored for analysis.

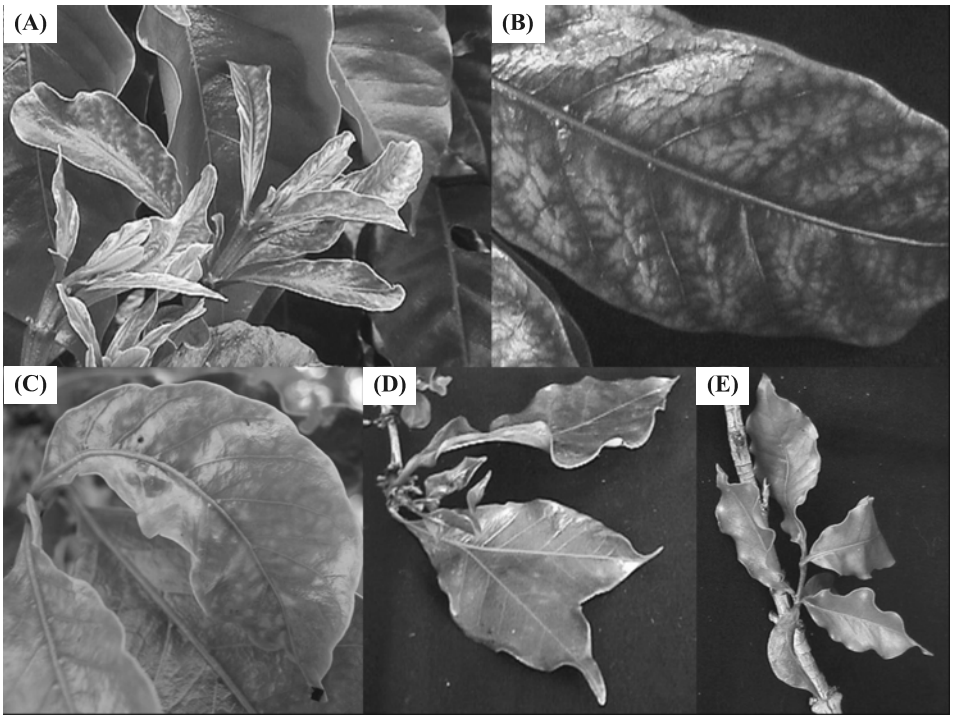

Fig. 1. Symptoms associated with "crespera" disease of coffee plants in Costa Rica. Stunting, leaf chlorosis, and deformation in new shoots (A); interveinal leaf chlorosis (B); curling of leaf margins and leaf deformation by arching of the midrib (C) or by splitting of the midrib (D); and defoliation of lateral branches with the appearance of new shoots with curling leaves (E).

Dendrogram calculated from RFLP data

A binomial matrix was constructed for the absence/presence of each band in each restriction digest pattern. A genetic distance dendrogram was generated using Treecom (version 1.3) software (Van De Peer and Wachter, 1994) with the unweighted pair group arithmetic mean average (UPGMA) algorithm with 1,000 permutations.

Results and Discussion

Field survey

Coffee plants with crespera-like symptoms (Fig. 1) were found throughout the Central Valley of Costa Rica. Coffee plants showed a range of symptoms from mild curling of leaf borders and chlorosis to severe stunting, leaf deformation, defoliation, shortening of internodes, and branch dieback. No leaf scorch symptoms were observed in field-grown plants in Costa Rica. This symptom was only observed in greenhouse-grown plants with severe water stress.

Bacterial cultures

Twelve strains of *X. fastidiosa* were obtained from coffee plants representing 5 different localities in the Central Valley of Costa Rica. Both the time required for colonies to appear on PW agar after inoculation and colony morphology were variable. Colonies of *X. fastidiosa* from coffee were observed on plates 7 to 26 days after inoculation with plant extracts or PW broth. Colonies were circular, dome- shaped and varied from opalescent white to pale pink or beige. The size of the colonies varied from less than a millimeter to circa 5 ml. Variations in the time required for colonies to appear and in colony morphology have been noted before (Davis *et al*., 1981; Campanharo *et al*., 2003; Chen *et al*., 2005).

The isolated bacteria were identified as *X. fastidiosa* by Gram staining and observations of cell morphology in phase contrast microscopy and constant positive reactions in ELISA tests with commercial antibodies against *X. fastidiosa*. The observations revealed a Gram-negative, rod-shaped, slender bacillus with varying cell length (Lee *et al*., 2001). Many cells presented a characteristic slight longitudinal curve, resembling a comma.

ELISA

X. fastidiosa was detected by ELISA in field-grown coffee plants with and without "crespera" like symptoms. Variability of symptom expression and symptomless but infected coffee and citrus plants have been also reported in Brazil (De Lima *et al*., 1998) (Araújo *et al*., 2002; Lacava *et al*., 2004). Symptom expression may depend on coffee plantation management, weather, and site conditions and interactions with other endophytic bacteria as has been observed in sweet orange plants in Brazil (Araújo *et al*., 2002; Lacava *et al*., 2004). Alternatively, the lack of symptoms in some infected plants may be due to genetic variation in either the host or pathogen (De Lima *et al*., 1998).

TEM

TEM observations revealed the presence of bacteria in the lumen of xylem vessels of midribs from symptomatic, ELISA positive plants (Fig. 2). Bacteria were never observed in midribs of asymptomatic, ELISA negative, greenhouse-grown plants. The bacteria were approximately 0.3 to 0.5 μm wide and from 1.5 to 3.0 μm long, and exhibited a rippled cell envelope, 10 to 40 nm in thickness. Bacteria were found in higher densities and were most easily observed at the junction of the petiole and midrib of mature leaves. Amorphous material was also observed in the xylem vessels colonized

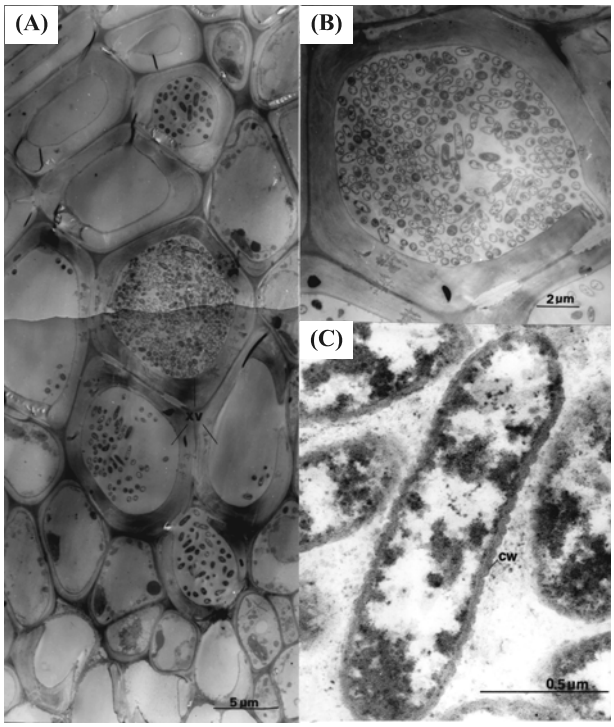

Fig. 2. Xylem vessels from a coffee petiole with "crespera" symptoms; the bacteria may be observed inside of some xylem vessels (xv) while other vessels are not colonized (A). Xylem vessel plugged with bacteria (B). Longitudinal view of a bacterium inside the vessels with the characteristic rippled cell wall (cw) (C).

Fig. 3. Longitudinal view of a rod-shaped bacterium with rippled cell wall representing a strain of *Xylella fastidiosa* isolated from coffee plants with "crespera" symptoms.

by the bacteria. The bacteria observed had characteristics similar to those previously described for grapes with Pierce's disease (Mollenhauer and Hopkins, 1974) as well as in other hosts. TEM observations of cultured bacteria (Fig. 3) documented abundant rod shaped cells with the characteristic rippled cell wall (Lee *et al*., 2001) similar to those observed in xylem vessels of "crespera" coffee plants (Fig. 2).

PCR amplification

PCR with specific primers 272-1int/272-2-int and RST31/33 confirmed the identity of the isolated bacteria as *Xylella fastidiosa*. *In silico* analysis of available sequences in GenBank corresponding to these PCR-amplification products identified several variable positions for RFLP analysis. Six different restriction endonucleases were selected to analyze each fragment, for a total of 12 enzymes.

RFLP analysis

The restriction patterns of the 272 primer set amplicon product were generally not associated with either geography or host origin (Table 1). Enzymes *Bsp*143I and *Mnl*I separated the coffee strains from Costa Rica into two and three different groups, respectively. Moreover, restriction enzyme *Mnl*I generated four different fragment patterns (Fig. 4), two of which were unique to Costa Rican strains. Therefore, the use of this enzyme is recommended as a rapid method to assess variability among Costa Rican strains.

Meanwhile, with the 'RST' product, the enzymes *Alu*I, *Bsp*PI, *Bsp*68I, and *Psu*I differentiated the Brazilian strains from both United States (US) and Costa Rican strains. In addition, when the 'RST' product was treated with endonuclease *Hin*1II three fragment patterns were produced. These patterns differentiated strains from the US and Costa Rica from strains from Brazil, and also distinguished coffee and citrus strains from Brazil (Table 1 and Fig. 4). The only exception obtained with these five endonucleases, was with the RST amplicon from strain CspIIIc1 from sweet orange in Costa Rica, which presented the same pattern as did the Brazilian strains. This result is in contrast to previous work assessing sequence and variability for the 'RST' amplicon, which did not detect any sequence polymorphism among 38 sweet orange strains tested from Brazil, and only one of three coffee strains tested presented a single nucleotide mismatch that distinguished it from the sweet orange isolates (Wendland *et al*., 2003). These patterns differentiated U.S. and Costa Rican strains from Brazilian strains of *X. fastidiosa*.

The results obtained with enzyme *Rsa*I/RST amplicon in this work are consistent with previous results from other strains of *X. fastidiosa* (Minsavage *et al.*, 1994; Hendson *et al*., 2001; Wendland *et al*., 2003). The *Rsa*I/RST amplicon from grapevine from the US and sweet orange strains from Brazil presented the same restriction pattern (two bands, circa 149 and 572 bp) as expected (Minsavage *et al*., 1994; Chen *et al*., 2005). This pattern was also present in strains isolated from coffee, citrus, and grapevine from Costa Rica. Two mulberry strains and one elm strain from the US presented an alternate pattern (circa 721 bp) along with strains CaXc1 (coffee) and CspIc1 (citrus) from Costa Rica. Interestingly, the similarity between these two strains was substantiated by the presence of a unique pattern for the '272' product digested with enzyme *Mnl*I which was not observed for any other strain assayed in this study (Table 1). This concordance in grouping or differentiation of strains with different genetic loci, supports the possible use of these criteria to identify different strains or genotypes of the bacteria (Chen *et al*., 2005). The results suggest that the RST primer set product is reliable for distinguishing strains of *X. fastidiosa* from Brazil and Costa Rica-US when digested with enzymes *Alu*I, *Bsp*PI, *Bsp*68I, *Hin*1II, and *Psu*I.

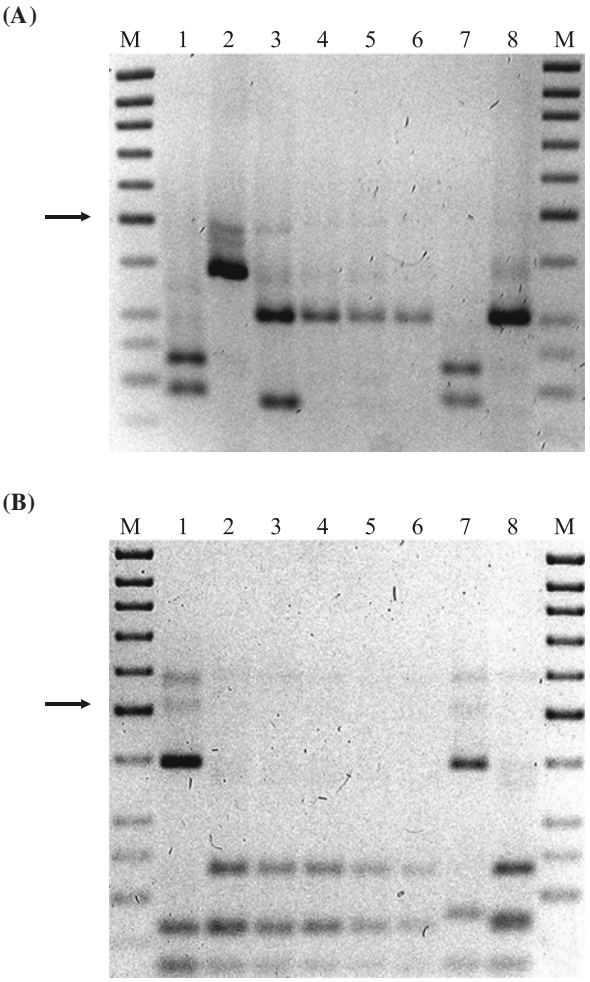

Fig. 4. Restriction patterns generated with enzyme *Mnl*I and the PCR-amplification product 272-1-int/272-2-int (A) and with enzyme *Hin*1II and the PCR-amplification product RST31/RST33 (B) for *X. fastidiosa* strains from different hosts. Lane M, 50-bp molecular marker, the arrow indicates the 500 bp band; 1, strain Lar20/11 (sweet orange, Brazil); 2, CspIc1 (sweet orange, Costa Rica); 3 to 6, strains CaIc1, CaIIIc3, CaVc1, CaVIIIc1 (coffee, Costa Rica); 7, strain Café20/11 (coffee, Brazil); and 8, strain ATCC 35868 (mulberry, US).

Dendrogram calculated from RFLP data

Generally, RFLP analysis (Fig. 5) showed a closer relationship between Costa Rican and US strains than between Costa Rican and Brazilian strains, consistent with previous results by VNTR analysis (Montero-Astúa *et al*., 2007). The

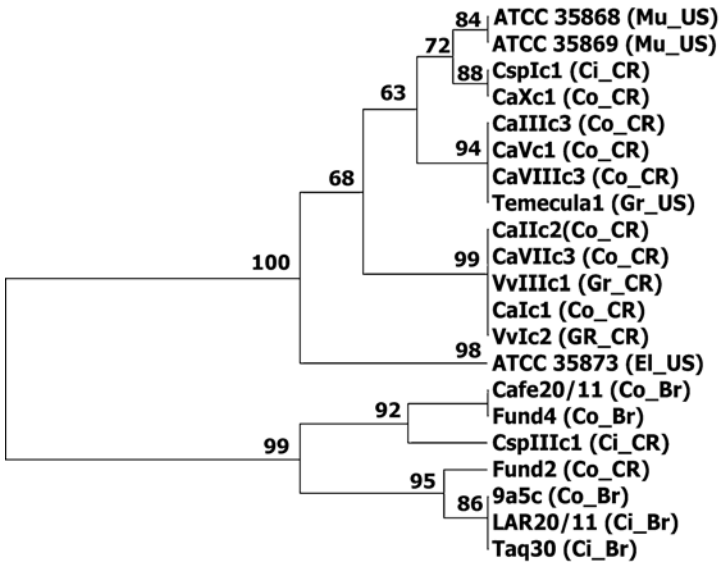

Fig. 5. Dendrogram based on computed similarity data from RFLP analysis with the unweighted pair group arithmetic mean average (UPGMA) algorithm for *X. fastidiosa* strains used in this study. Ci, sweet orange; Cf, coffee; El, elm; Gr, grapevine, Mu, mulberry; Br, São Paulo (Brazil); CR, Costa Rica and US, United States of America. Numbers at each node indicate the bootstrap percentages (1,000 permutations).

data also suggest variability among coffee strains from Costa Rica, which presented a mixture of patterns observed with Brazilian and US strains for several enzymes. The dendrogram generated with the combined restriction digest data sets has two well resolved clusters, one consisting of strains from the US and Costa Rica and the other including all Brazilian strains and one citrus strain, CspIIIc1 from Costa Rica (Fig. 5). Coffee strains within the US-Costa Rica cluster are separated in two subgroups (suggested by a 68% node, 1,000 permutations). One of these subgroups includes the sweet orange strain CspIc1. Another sweet orange strain from Costa Rica, CspIIIc1, clusters with other sweet orange strains from Brazil, and thus may represent an introduction of a Brazilian strain into Costa Rica. The results presented herein suggest that coffee strains of *X. fastidiosa* from Costa Rica do not belong to the proposed *X. fastidiosa* subsp. *pauca* (Schaad *et al*., 2004) which includes strains from sweet orange from Brazil. Although coffee strains from Brazil were not included in the study by Schaad *et al*. (2004), all other evidence is consistent with the idea that they should be placed within the proposed *X. fastidiosa* subsp. pauca (De Lima *et al*., 1998; Li *et al*., 2001).

The area from northern Nicaragua to northern Colombia, including Costa Rica, is considered a connection and a biotic interchange area between two major biogeographic realms, Neoarctica (North America), and Neotropica (South America). Thus, much of the biotic diversity found in the area reflects an intermixing of elements from North and South America (Rich and Rich, 1983). This statement is true for many of the macro-organisms, and it is hypothesized that it is also true for micro-organisms as well. The diversity observed in Costa Rican *X. fastidiosa* strains would be an example of such intermixing if the *X. fastidiosa* sampledin Costa Rica represented a native population rather than recent human-assisted introductions from either North or South America. Earlier reports from Costa Rica (Goheen *et al.*, 1979), Mexico (Raju *et al*., 1980) and Brazil as well as current research in Central America [PROMECAFE 2003 (http://www.iica.org.gt/promecafe/boletines/boletin96.pdf), Universidad Nacional Autónoma De Honduras (http://www.cra.unah.edu.hn/microbiologia/inves.htm)], suggests that *X. fastidiosa* is present throughout the Americas. The diseases known to be caused by the bacterium may be the result of adaptation of the bacterium to new horticultural hosts from native plants that do not become diseased following infection by *X. fastidiosa* (Chen *et al*., 2002; Schuenzel *et al*., 2005). Such host range extensions would be facilitated by non-specific feeding preference of the insect vectors, which appear to be abundant in Costa Rica (Godoy *et al.*, 2006; Godoy and Villalobos, 2006).

X. fastidiosa was confirmed to be present in coffee plants in Costa Rica by isolation, ELISA, morphological observations, and PCR-based techniques. The bacterium is associated with "crespera" coffee disease symptoms in Costa Rica. "Crespera" is very similar to the "requeima do café" or coffee leaf scorch described in Brazil, but the strains of *X. fastidiosa* isolated in Costa Rica are for the most part readily distinguished from those from Brazil. The RFLP data suggest the "crespera"-associated strains of *X. fastidiosa* are more closely related to US strains of *X. fastidiosa*-from other hosts than to either coffee or sweet orange strains from Brazil. It is very interesting to hypothesize the convergent evolution of diverse strains of the bacterium to infect and cause similar diseases in the same host species in two different geographic regions. This hypothesis, and its relation to *Xylella* host selection and evolution, has not been studied and deserves further attention.

Acknowledgements

This work was supported by the Fundación Costa Rica-USA (CRUSA); the Universidad de Costa Rica (UCR); and the Fruit Laboratory, Agriculture Research Service (ARS), United States Department of Agriculture (USDA).

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