European Journal of Plant Pathology (2005) 111:317–326

Analysis of molecular diversity in *Crinipellis perniciosa* with AFLP markers

Randy C. Ploetz[1], Raymond J. Schnell[2], Zhentu Ying[1], Qi Zheng[1], Cecile T. Olano[2], Juan C. Motamayor[3] and Elizabeth S. Johnson[2]
[1]*Department of Plant Pathology, Tropical Research and Education Center, University of Florida, 18905 SW 280 Street, Homestead, FL 33031-3314, USA (Phone: +1-305-246-7000; Fax: +1-305-246-7003; E-mail: rcp@.ifas.ufl.edu);* [2]*National Germplasm Repository, USDA-ARS, 13601 Old Cutler Road, Miami, FL 33158, USA;* [3]*MasterfoodsUSA, c/o USDA-ARS, 13601 Old Cutler Road, Miami, FL 33158, USA*

Accepted 23 September 2004

Key words: AFLP, *Crinipellis perniciosa*, disease resistance, genetic diversity, haplotype, molecular diversity, molecular markers

Abstract

Crinipellis perniciosa causes a serious disease of cacao known as witches' broom (WB). Heritable resistance to witches' broom has been used in cacao improvement programs. 'SCA6' and 'SCA12' are highly resistant and are the most commonly used parents in the breeding schemes. However, SCA hybrids are not resistant to witches' broom in all production areas. Presumably, different populations of *C. perniciosa* cause these variable responses. Amplified fragment length polymorphism (AFLP) markers were used to assess variation and population structure in this pathogen. We examined 40 isolates of *C. perniciosa* and one isolate of *Melanotus subcuneiformis*. Nine of 64 primer pairs produced consistent and informative DNA amplification, and were used to screen all isolates. Fifteen haplotypes (AFLP fingerprints) were detected with 186 polymorphic markers. Cluster analysis grouped isolates of the C biotype (pathogenic on cacao) from Bolivia, Brazil, Ecuador and Trinidad together in a major cluster that was distinct from isolates of the S biotype (pathogenic on solanaceous hosts) and *M. subcuneiformis*. Isolates of the C biotype were divided further into well supported, country-specific groups. Segregation of AFLP alleles was not observed among basidiospore isolates from the same basidiome, broom, tree or field, supporting previous reports that the fungus did not outcross. The results corroborated prior conclusions that *C. perniciosa* was probably introduced into the Bahia state of Brazil from the Amazon basin. Representative isolates from the genetically distinct groups that were revealed will be used to examine pathogenic specialization in *C. perniciosa* and differential responses that have been reported in 'SCA6'-derived germplasm.

Introduction

Seeds of cacao (*Theobroma cacao*), which are commonly known as beans, are the source of chocolate and a major export commodity in the tropics. In 2002, global production was estimated at more than 2.8 million metric tons (FAO, 2003).

Bean production is threatened by several diseases worldwide (Bowers et al., 2001). In the western tropics, witches' broom (WB), caused by *Crinipellis perniciosa* is a major constraint that can reduce production by as much as 75% (Purdy and Schmidt, 1996). Direct losses of beans occur when pods are affected and indirect losses result from flower infections and reduced host vigor. Affected vegetative tissues become grossly distorted with reduced internode lengths, misshapen leaves and swollen or otherwise deformed stems (i.e., brooms).

Basidiospores are the only infective propagule of *C. perniciosa* (Purdy and Schmidt, 1996). They

are produced on basidiomes (basidiocarps) that develop from saprotrophic mycelium in necrotic host tissue. Soon after infection the pathogen establishes a biotrophic relationship with its host, during which the fungus is homokaryotic, intercellular and lacks clamp connections. Gradually, hyphae become dikaryotic, clamped and saprotrophic, and this coincides with the death of host tissue and the end of the biotrophic phase. The production of basidiomes on these tissues completes the disease cycle. Single basidiospores are capable of inducing symptoms, but multiple infections of a meristem are thought to be possible. *Crinipellis perniciosa* is homomictic (nonoutcrossing and primary homothallic) (Griffith and Hedger, 1994). Thus, multiple infections by genetically distinct basidiospores could provide an opportunity for heterokaryosis between biotrophic hyphae.

Although its origins are still debated, it is generally believed that cacao evolved in the western Amazon basin in an area defined by present-day eastern Ecuador (the Oriente region), northeastern Peru and southcentral Colombia (headwaters of the Caqueta and Putumayo Rivers) (Figure 1) (Pound, 1938; Cheesman, 1944; Motamayor et al., 2002). In the same region, *C. perniciosa* is presumed to have co-evolved with its cacao host (Purdy and Schmidt, 1996; Evans, 2002).

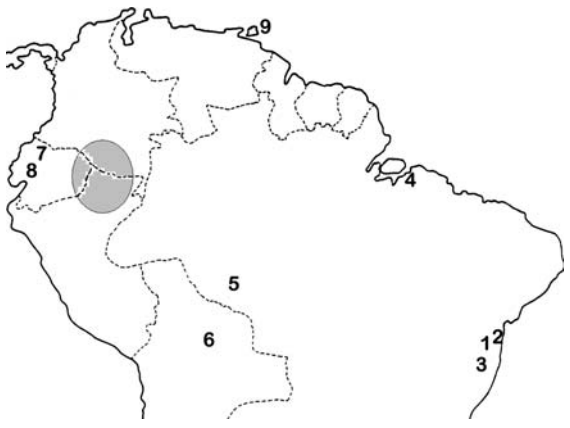

Figure 1. Isolates of *Crinipellis perniciosa* originated from: Brazil, (1) Ilheus (CEPEC), Bahia, (2) Itabuna, Bahia, (3) Camacan, Bahia, (4) Belém, Para, and (5) Rondônia; Bolivia, (6) Yungas; Ecuador, (7) Pichilingue, and (8) 20 km outside Pichilingue; and (9) Trinidad. Shaded area approximates the putative center of origin for *Theobroma cacao* and *C. perniciosa* (see text).

Witches' broom is now found in Panama, Trinidad and throughout South America. In the late 1980s, it appeared in the important cacao-producing areas in the Bahia state of Brazil, thereby affecting the last major cacao-producing region in South America that was free of the disease. In little more than a decade, the disease reduced production in Bahia from over 392,000 to less than 172,000 metric tons. Witches' broom would have a devastating impact on production in West Africa and Southeast Asia were it to spread to these areas.

Four biotypes of *C. perniciosa* are recognized based on host range. The C biotype affects *T. cacao* and cacao relatives in the Sterculiaceae, in particular species of *Theobroma* and *Herrania*, whereas the B, L and S biotypes affect, respectively, *Bixa orellana*, and plants in the Bignoniaceae and Solanaceae (Griffith and Hedger, 1994). Diverse genetic and phenetic characters have been used to study isolates of the different biotypes (McGeary and Wheeler, 1988; Laker, 1989; Andebrhan and Furtek, 1994; Yamada et al., 1998. Andebrhan et al., 1999; Arruda et al. 2003a, b; Rincones et al., 2003).

The outbreak of WB in Bahia in the late 1980s has been examined in some of the above studies. With RAPDs, Andebrhan et al. (1999) studied 46 isolates from Bahia (13 counties) and the Amazon basin (three counties in the states of Amazonas and Rondônia) in Brazil. Isolates of the C biotype clustered into two groups, one of which contained isolates from Bahia and Rondônia, and the other of which contained isolates from Bahia and Amazonas. Based on these data, the authors concluded that two different introductions occurred into Bahia. Recently, Arruda et al. (2003a) distinguished two populations of the C biotype in Bahia with ERIC-PCR fingerprints. Although these clusters were supported by low bootstrap values (51% and 61%), each contained isolates from the Amazon region.

Amplified fragment length polymorphism (AFLP) markers have been used to genetically characterize plant, nematode, bacterial, and fungal populations (Majer et al., 1996; Tooley et al., 2000). AFLPs are thought to be selectively neutral, are usually inherited in a Mendelian fashion, and

are more reproducible between laboratories than RAPDs (Jones et al., 2002).

Resistance to WB exists in a number of cacao genotypes (Marita et al., 2001; Queiros et al., 2003). 'SCA6' and 'SCA12' are tolerant, in that they display few or no symptoms after infection. They have been used extensively in breeding programs and impart resistance to progeny in Brazil and Trinidad, but not in Ecuador. The presence in Ecuador of isolates of *C. perniciosa* that affect 'SCA6' hybrids may explain these results (Wheeler and Mepsted, 1988).

Witches' broom resistance is a primary target for the cacao improvement program at the National Germplasm Repository (USDA-ARS) in Miami. In partnership with the University of Florida in Homestead, accessions and hybrids in this program can be screened against isolates of *C. perniciosa* from any region. This is not possible in cacao-producing countries, and allows disease reactions to be determined against diverse populations of the pathogen. In this regard, we were interested in assessing genetic variation and population structure in this pathogen. Genetically distinct isolates could be used to screen accessions for resistance, as well as examine pathogenic diversity in this fungus.

AFLP markers were used to assess genetic variation in the pathogen. AFLP markers were also used to examine variation among isolates of *C. perniciosa* from single basidiomes, brooms, trees and fields, since it may shed light on the epidemiology of WB and whether heterokaryosis, as suggested by Griffith and Hedger (1994), is possible.

Materials and methods

Isolates of Crinipellis perniciosa

Thirty-nine isolates of the C biotype of *Crinipellis perniciosa* were analyzed (Table 1). Three mass isolates were obtained from broom material that RJS collected in the Yungas region in Bolivia, six isolates from Ecuador were received as mycelial cultures from Dr. Carmen Suarez-Capello (Instituto Nacional Autónomo de Investigaciones Agropecuarias, INIAP, Pichilingue), and two isolates from Trinidad were collected as basidiospores by ESJ. The remaining 28 isolates from Brazil were either recovered from brooms collected by RCP from two research plots of the Centro de Pesquisas do Cacau (CEPEC) in Ilheus, or were received as mycelial cultures from Dr. Roberto Barreto (University of Viçosa), Dr. Karina Gramacho (CEPEC), Dr. Edna Luz (CEPEC) and Dr. Alan Pomella (M&M Mars, Itajuípe). Isolates of the S biotype, TCP 24-1 (recovered from *Solanum lipocarpum*), and of *Melanotus subcuneiformis*, TCP 54-1 (an agaric recovered from a cacao broom), were used as outgroups.

Mass hyphal isolates were obtained in Florida by culturing 2–3 mm^3 pieces of tissue from the interior portions of brooms on a selective medium that was described by Griffith and Hedger (1994). The epidermis on brooms was removed and tissue pieces were excised, surface-disinfested in 70% ethanol (30 s) and 0.5% $NaClO_3$ (2 min) before placing them in empty, sterile 9-cm-dia Petri dishes. Molten medium (48 °C) was then poured over the pieces and allowed to solidify. After incubating plates in the dark for 3 weeks at room temperature (ca. 22 °C), mycelial plugs were recovered from the margins of colonies and transferred to V8 agar. V8 agar consisted of 163 ml of V8 juice (Campbell Soup Company, Camden, NJ, USA) and 3 g of $CaCO_3$ mixed on a magnetic stirrer for 10 min and centrifuged for 25 min at 1000 g. The supernatant and 20 g of agar (Becton, Dickson and Co., Sparks, MD, USA) were diluted with deionized H_2O to a final volume of 1 liter and autoclaved prior to use.

Brooms were induced to form basidiomes by soaking them for 1 h in a 1% solution of benomyl (Sigma Chemical Co., St. Louis, MO, USA), and then hanging them in an illuminated (12:12 diurnal light) cabinet in which a mister sprayed brooms with deionized H_2O for 1 min every 24 h. Basidiospores were harvested by excising the caps of basidiomes and attaching their top surface to the inside of a Petri dish lid with petroleum jelly, such that the gill side was exposed, and suspended overnight over a beaker of sterile 16% glycerol in MES buffer, pH 6.1, on a magnetic stirrer (Frias et al., 1995). Single or multiple basidiospore isolates were recovered by diluting the basidiospore suspension the following morning with MES buffer and streaking it on V8 agar. Single, germinated spores were harvested the following day with a sharpened, sterile spatula under a microscope and lawns of germinated spores were recovered for

Table 1. Isolates used in AFLP analyses [a]

Isolate	Haplotype[b]	Donor[c]	Origin; source/type; original accession
TCP 1	6	EL	Brazil; mass mycelial; CAMACAN
TCP 2	10	EL	Brazil; mass mycelial; CPILHEUS
TCP 3-1	11	RJS	Yungas, Bolivia; mass mycelial, TCP 3 broom; none
TCP 3-2	12	RJS	Yungas, Bolivia; mass mycelial, TCP 3 broom; none
TCP 3-3	12	RJS	Yungas, Bolivia; mass mycelial, TCP 3 broom; none
TCP 10	5	KG	Rondônia, Brazil; mass mycelial; FA 153
TCP 14	8	RB	Belém, Para, Brazil; mass mycelial; FA 35
TCP 15	9	KG	Brazil; mass mycelial; CSNES 128
TCP 16	6	KG	Brazil; mass mycelial; CP28
TCP 18	7	KG	Brazil; mass mycelial; CP52
TCP 24-1	15	RB	Brazil; single basidiospore from *Solanum lipocarpum* (= S biotype); Loberira
TCP 33-1	10	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, TCP 33-1 basidiocarp, TCP 33 broom, tree, field 1; none
TCP 33-2	10	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, TCP 33-2 basidiocarp, TCP 33 broom; none
TCP 33-3	10	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, TCP 33-3 basidiocarp, TCP 33 broom; none
TCP 33-4	10	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, TCP 33-4 basidiocarp, TCP 33 broom; none
TCP 41-1	10	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, TCP-41-1 basidiocarp, TCP 41 broom, TCP 33 tree; none
TCP 41-2	10	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, TCP-41-2 basidiocarp, TCP 41 broom; none
TCP 41-3	10	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, TCP-41-3 basidiocarp, TCP 41 broom; none
TCP 41-5	10	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, TCP-41-5 basidiocarp, TCP 41 broom; none
TCP 46-1	6	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, single basidiocarp, TCP 46 broom and tree, field 2; none
TCP 46-1-1	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-46-1 basidiocarp; none
TCP 46-1-2	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-46-1 basidiocarp; none
TCP 46-1-3	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-46-1 basidiocarp; none
TCP 46-1-4	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-46-1 basidiocarp; none
TCP 46-3	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-46-3 basidiocarp; none
TCP 46-3-1	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-46-3 basidiocarp; none
TCP 46-3-2	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-46-3 basidiocarp; none
TCP 46-3-3	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-46-3 basidiocarp; none
TCP 50-4	6	RCP	CEPEC, Ilheus, Bahia, Brazil; multiple basidiospores, single basidiocarp, TCP 50 broom and tree, field 2; none
TCP 50-4-1	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-50-4 basidiocarp; none
TCP 50-4-2	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-50-4 basidiocarp; none
TCP 50-4-4	6	RCP	CEPEC, Ilheus, Bahia, Brazil; single basidiospore, TCP-50-4 basidiocarp; none
TCP 54-1	1	RJS	Yungas, Bolivia; mass mycelial, TCP 3 broom, *Melanotus subcuneiformis*[d]

Table 1. (Continued)

Isolate	Haplotype [b]	Donor[c]	Origin; source/type; original accession
TCP 88	4	CSC	INIAP, Pichilingue, Ecuador; mass mycelial; 2A
TCP 89	4	CSC	INIAP, Pichilingue, Ecuador; mass mycelial; Palma Chavez
TCP 90	4	CSC	INIAP, Pichilingue, Ecuador; mass mycelial; 7A pequeno
TCP 91	3	CSC	INIAP, Pichilingue, Ecuador; mass mycelial; 7A grande
TCP 94	2	CSC	INIAP, Pichilingue, Ecuador; mass mycelial; Iote Herrera
TCP 98	4	CSC	20 km outside Pichilingue, Ecuador; mass mycelial; Mocache 5
TRDC 14	14	EJ	Maracas, St Joseph, Trinidad; multiple basidiospores; none
TRDC 74	13	EJ	Shabali Tr., Mendez Village, Trinidad; multiple basidiospores; none

[a] All isolates except TCP 54-1 are of *Crinipellis perniciosa.*
[b] Haplotype as determined by AFLP fingerprint.
[c] Source: RJS = Raymond J. Schnell, USDA-ARS, Miami, FL, USA; CSC = Carmen Suarez-Capello, Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP), Pichilingue, Ecuador; KG = Karina Gramacho, Centro de Pesquisas do Cacau (CEPEC), Ilheus, Bahia, Brazil; EL = Edna Luz, CEPEC, Ilheus, Bahia, Brazil; RCP = Randy Ploetz, University of Florida, Homestead, FL, USA; RB = Roberto Barreto, University of Viçosa, Brazil; EJ = Elizabeth Johnson, Cacao Research Unit, University of the West Indies, Trinidad; AP = Alan Pomella, M&M Mars, Itajuípe, Bahia, Brazil.
[d] Identity as *Melanotus subcuneiformis* based on LSU DNA sequence data (personal communication: M.C. Aime, USDA ARS Systematic Botany and Mycology Lab, Beltsville, MD 20705, USA).

multispore isolates; both were transferred to V8 agar for future use.

Mycelium production

Mycelial plugs of isolates from actively growing V8 agar cultures were placed in 50 ml of V8 broth in 250 ml Erlenmeyer flasks. Flasks were incubated at room temperature on a rotary shaker at 100 rpm. After ca. 1 month, mycelial mats were harvested, washed three times with distilled water, and stored at −80 °C until they were lyophilized prior to DNA extraction (usually within 2 weeks).

DNA extraction

Genomic DNA was extracted from lyophilized mycelium with a modified CTAB procedure (Doyle and Doyle, 1990). About 200 mg of mycelium of each isolate were ground into powder with 0.5 g of washed sea sand (Fisher Scientific, Pittsburgh, PA, USA) in liquid nitrogen. The ground material was placed in 5 ml of extraction buffer (2% CTAB, 1% PEG-8000, 0.1 M Tris–HCl (pH 9.5), 0.02 M EDTA, 1.4 M NaCl and 0.2% β-mercaptoethanol). The homogenate was incubated at 65 °C for 30 min with occasional mixing, then cooled to room temperature, followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1), and centrifuged for 10 min at 12,000 × *g*. The supernatant was transferred to a clean tube. Genomic DNA was precipitated with an equal volume of isopropanol and washed twice with 70% ethanol. After air drying, the DNA was resuspended in 50 µl of TE, pH 8.0, and quantified by gel electrophoresis.

AFLP procedure

An AFLP microbial fingerprinting kit (Applied Biosystems, Foster City, CA, USA) was used, with slight modifications.. The restriction-ligation reaction for each sample was conducted at 25 °C for 16 h, and amplification was in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA).

Four isolates of *C. perniciosa* were used to screen primers, three of the C biotype (TCP 3-2, TCP-10 and TCP-14), and one of the S biotype, TCP-24-1 (Table 1). Sixty-four primer combinations from the AFLP kit were screened. PCR products were separated individually on an automated capillary electrophoresis apparatus (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). Nine of the 64 primer pairs generated easily resolved products and were selected to characterize the entire collection (Table 2).

Data analysis

Data files for each sample were created with GeneScan analysis software (3.1 version, Applied

Table 2. Selected AFLP primer pairs and the number of polymorphic peaks and total number of peaks that they generated

Primer pairs	Total number of peaks	Number of polymorphic peaks
*Eco*RI-AA JOE + *Mse*I-CC	60	36
*Eco*RI-AA JOE + *Mse*I-CT	64	22
*Eco*RI-AC FAM + *Mse*I-A	88	9
*Eco*RI-AC FAM + *Mse*I-CA	59	24
*Eco*RI-AC FAM + *Mse*I-CG	36	13
*Eco*RI-AC FAM + *Mse*I-CT	38	30
*Eco*RI-AG JOE + *Mse*I-CC	45	19
*Eco*RI-AT NED + *Mse*I-CA	61	18
*Eco*RI-AC NED + *Mse*I-CG	47	15
Total	498	186

Biosystems) and analyzed using Genotyper software (2.1 version, Applied Biosystems) for each primer pair. Nonoverlapping peaks above a threshold value of 25 relative fluorescent units were used. Amplification products of each isolate were scored for presence (1) or absence (0) of peaks. Peaks common to all isolates were not included in the analysis. A binary character matrix was used to generate a pairwise similarity matrix among haplotypes (Nei and Li, 1979). The similarity coefficient was determined as follows:

$$S_{ij} = 2a/(2a + b + c),$$

where S_{ij} is the similarity between two individuals i and j, a is the number of positive peaks shared by both individuals, b is the number of peaks present in i and absent in j, and c is the number of peaks present in j and absent in i. The SAHN clustering program in NTSYS pc was then used to group the entries based on similarity coefficients with the unweighted pair-group method using arithmetic average (UPGMA) (NTSYS, Exeter Software, Setauket, NY). A phenetic tree was constructed and support for the inferred clusters was estimated using bootstrapping in the program Winboot with 5000 replications (Yap and Nelson, 1996).

An analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was applied to the polymorphic AFLP markers and the variation was partitioned within and among populations from each country. Input files for dominant marker data were prepared using AMOVA-Prep 1.01 (Miller, 1998). The analysis was only performed on isolates of the C biotype; isolates of *M. Subcuneiformis*, TCP 54-1 and the S biotype, TCP 24-1, were excluded from the analysis.

Results

No variation in haplotype (AFLP fingerprint) was observed among replicates of the nine selected primer combinations on four test isolates. Additionally, the AFLP procedure was repeated for all isolates, excluding those already duplicated by multiple, within-broom/basidiocarp extractions (samples TCP 33, 41, 46 and 50). A total of 498 markers was scored, 186 (37.4%) of which were polymorphic. The average number of polymorphic markers per primer pair was 21. 15 haplotypes were identified.

Single-basidiospore and multiple-spore isolates from the same basidiocarp had identical haplotypes (TCP 46-1, 46-3 and 50-4 series; Table 1). In addition, multispore isolates that were recovered from the same tree but eight different basidiomes were also identical (TCP 33 and 41 series; Table 1). Variation in haplotype was observed between but not within two experimental plantings at CEPEC (single- and multiple-basidiospore isolates). Thus, variation was not observed among isolates from single basidiomes, brooms, trees and sites at CEPEC. However, minimal variation was detected among three mass isolates from a single broom in Bolivia: TCP 3-1 produced a 412-bp product with the *Eco*RI-AA JOE and *Mse*I-CC primer pair that was not produced by TCP 3-2 and TCP 3-3.

Genetic similarity coefficients among the 15 haplotypes averaged 0.74, and the similarity coefficient between the S biotype and C biotype isolates was 0.53. Similarities among isolates of the C biotype from Brazil ranged from 0.83 to 0.99 (mean = 0.88), for those from Ecuador from 0.98 to 0.99, and for those from Trinidad 0.84. The similarity coefficient between the two Bolivian haplotypes was 0.99, whereas those between these and that for an isolate of *M. subcuneiformis* were, respectively, 0.41 and 0.42. Average similarity among isolates of the C biotype was 0.82.

The UPGMA tree clearly separated isolates of the S biotype and *M. subcuneiformis* (respectively, haplotypes 15 and 1) from isolates of the C biotype (Figure 2). In turn, isolates of the C biotype formed a single major cluster with four, country-specific subgroups that contained: six isolates from

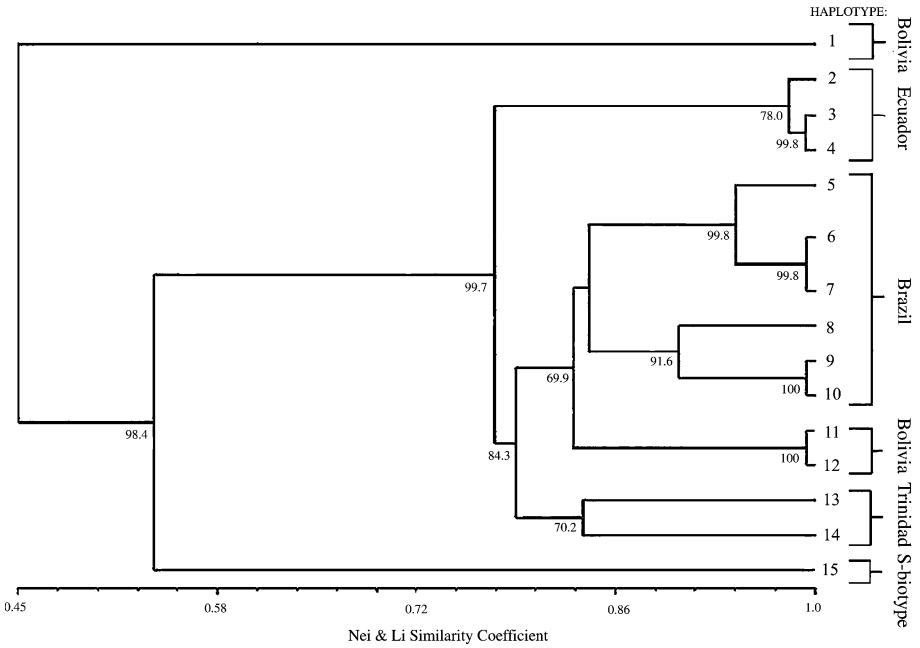

Figure 2. UPGMA-based dendrogram for 15 haplotypes that were generated with 186 AFLP markers. The numerical scale indicates the degree of similarity among haplotypes. Bootstrap values are based on 5000 replications.

Ecuador with haplotypes 2–4; three isolates from Bolivia with haplotypes 11 and 12; 29 isolates from Brazil with haplotypes 5–10; two isolates from Trinidad with haplotypes 13 and 14. The Brazilian isolates could be subdivided further into two distinct populations that were about 85% similar. Clusters were supported by bootstrap values ≥69.9%.

AMOVA was used to assess overall variation within and between populations. Fifty-five percent of the total genetic diversity was partitioned among populations whereas 45% was within populations. About 70.2% of the within-population variation was observed for the Brazilian isolates, which may reflect the relatively large sample size from this country; variation for isolates from Bolivia, Ecuador and Trinidad was far lower, respectively, 0.7%, 9.7% and 19.6% (Table 3). Based on a pairwise Bartlett test with 1000 iterations, distances between populations were significantly different ($P > 0.0001$) (data not shown).

Discussion

AFLPs were useful for examining population structure in *C. perniciosa* and for discriminating isolates of this important pathogen. AFLP markers

Table 3. Analysis of molecular variance (AMOVA) for 13 haplotypes for the C biotype of *Crinipellis perniciosa*[a]

Level of variation	Df	SSq	MSq	Variance	%	%[b]	P[c]
Among populations	3	119.500	39.833	10.534	55.34		< 0.001
Within populations	9	76.500	8.500	8.500	44.66		< 0.001
Bolivia	1	0.500	0.500			0.65	
Ecuador	2	7.333	3.665			9.68	
Brazil	5	53.667	10.733			70.15	
Trinidad	1	15.000	15.000			19.61	
Total	12	196.000					

[a] The analysis is based on AFLP phenotypes consisting of 186 peaks amplified with nine selective primer sets.
[b] % of within-population SSq contributed by each population.
[c] Levels of significance are based on 1000 random permutations.

revealed substantial variation among isolates of the C biotype, but remarkable similarity among isolates from the same geographical region. Distinct populations of the C biotype are clearly established in Bolivia, Brazil, Ecuador and Trinidad.

Isolates from regions that were not represented in this study should be used in future work on genetic and pathogenic diversity in *C. perniciosa*. Those from cacao's putative center of origin would be of special interest. If the commonly held assumption that this pathogen co-evolved with *T. cacao* in this region is correct, substantial progress could be made in the improvement of this crop with materials from this region. Presumably, cacao germplasm that resists populations of *C. perniciosa* from this region also would perform well in other locations that are affected by WB.

Cacao progeny derived from two 'Scavina' clones, 'SCA6' and 'SCA12', are resistant to WB in Brazil and Trinidad, but not in Ecuador. Wheeler and Mepsted (1988) distinguished two groups of *C. perniciosa* based on their virulence on 'SCA6'-derived seedlings. Isolates in group A were from Bolivia, Colombia and Ecuador and caused severe symptoms on these seedlings, whereas those in group B from Brazil, Trinidad and Venezuela did not.

In the present study, genetically distinct populations were identified in Bolivia, Brazil, Ecuador and Trinidad (Figure 2). Whether pathogenic specialization occurs among these populations should be determined, since it would shed light on the results of Wheeler and Mepsted (1988) and on racial specialization in this pathogen. Phenetic or genetic markers that correlate with disease response would be most useful in cacao breeding programs (McGeary and Wheeler, 1988).

The level of discrimination and reproducibility that was observed for AFLP markers in the present study suggest that they may be suited for this purpose. We are currently testing the pathogenicity of representative isolates from the above populations on cacao, and have begun to import and propagate clonal lines of cacao for use as differentials, some of which have sources of resistance other than the 'Scavina' clones.

Andebrhan et al. (1999) used RAPD markers to identify two populations of *C. perniciosa* in Bahia, and to suggest that two introductions of *C. perniciosa* were responsible for the outbreak of witches' broom in that state of Brazil in the late 1980s. Their study included four isolates from the state of Rondônia (Ariquemes and Ouro Preto) and a single isolate from the state of Amazonas (Manaus). Isolates from Rondônia and Amazonas clustered separately within the two Bahian populations (similarity coefficients of 0.91–0.92 and 0.88).

Recently, Arruda (2003a) used ERIC-PCR markers to examine isolates from a wider area in Brazil, including the states of Amazonas, Bahia, Mato Grosso, Minais Gerais, Para and Rondônia. Similarities in ERIC fingerprints among isolates of the C biotype ranged from 69% to 100%, and there was clear separation among the isolates based on geographic origin. Two populations were distinguished in Bahia, each of which contained isolates from the Amazon basin [either Amazonas (Manaus) or Para (Belém)].

Results from the present study confirm the relationship of isolates of the C biotype from Bahia to those found in the Amazon region. An isolate from Rondônia, TCP 10 (haplotype 5), nested with 16 isolates from Bahia (haplotypes 6 and 7, similarity coefficients of 0.94 and 0.95), whereas another isolate from Para (Belém), TCP 14 (haplotype 8), nested with a second group of 10 isolates from Bahia (haplotypes 9 and 10, similarity coefficients of 0.90 and 0.91) (Table 1).

The Trans-Amazonian highway stretches from Rondônia in the west to Para in the east. Upon its completion in the 1970s, there was a rapid influx of farmers into the newly opened frontier. Cacao was promoted as a crop for the new colonists. This caused considerable anxiety among cacao producers in Bahia since increased cacao production in the Amazon basin would increase the likelihood that witches' broom would spread to Bahia (Evans, 2002). Obviously, these misgivings were warranted. To date, the available evidence supports the hypothesis that the outbreak of WB in Bahia was caused by populations of *C. perniciosa* from the Amazon basin (Andebrhan et al., 1999; Arruda et al. 2003a; this study).

With one exception, variation was not observed among isolates from single basidiomes, brooms, trees and sites. Although variation was observed among three mass isolates from a single broom from Bolivia (TCP 3 series), it is not clear whether the single difference that was observed indicates that two individuals infected this broom. A more

likely scenario may be that we detected a mutation in the original infecting strain (TCP 3-1, 3-2 and 3-3 were identical at 185 of 186 restriction sites). Thus, it is still not clear whether heterokaryosis in *C. perniciosa*, as was proposed by Griffith and Hedger (1994), could result from infections by genetically distinct individuals.

Acknowledgements

The authors thank the following individuals for isolates of *C. perniciosa* that were used in this work: Dr. Roberto Barreto, University of Viçosa, Brazil; Drs. Karina Gramacho and Edna Luz, Centro de Pesquisas do Cacau (CEPEC), Ilheus, Brazil; Dr. Alan Pomella, M&M Mars, Itajuípe, Brazil; and Dr. Carmen Suarez-Capello (Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP), Pichilingue, Ecuador. We are also grateful to Dr. M. Catherine Aime, USDA ARS Systematic Botany and Mycology Lab, Beltsville, MD, USA, who identified the isolate of *M. subcuneiformis* that was used in this study.

References

Alexopoulos CJ, Mims CW and Blackwell M (1996) Introductory Mycology, 4th edn. John Wiley and Sons, Inc. New York, USA.

Andebrhan T, Figueria A, Yamada M, Cascardo J and Furtek DB (1999) Molecular fingerprinting suggests two primary outbreaks of witches' broom disease (*Crinipellis perniciosa*) of *Theobroma cacao* in Bahia, Brazil. European Journal of Plant Pathology 105: 167–175.

Andebrhan T and Furtek DB (1994) Random amplified polymorphic DNA (RAPD) analysis of *Crinipellis perniciosa* isolates from different hosts. Plant Pathology 43: 1020–1027.

Arruda MCC, Ferreira MAS, Miller RNG and Felipe MSS (2003a) Comparison of *Crinipellis perniciosa* isolates from Brazil by ERIC repetitive element sequence-based PCR genomic fingerprinting. Plant Pathology 52: 236–244.

Arruda MCC, Miller RNG, Ferreira MAS, Resende MLV and Felipe MSS (2003b) Nuclear and mitochondrial rDNA variability in *Crinipellis perniciosa* from different geographic origins and hosts. Mycological Research 107: 25–37.

Bowers JH, Bailey BA, Hebbar PK, Sanogo S and Lumsden RD (2001) The impact of plant diseases on world chocolate production. Online at: http://www.apsnet.org/online/feature/cacao/top.html. Plant Health Progress doi:10.1094/PHP-2001-0709-01-RV.

Cheesman EE (1944) Notes on the nomenclature, classification and possible relationships of cacao populations. Tropical Agriculture 21: 144–159.

Doyle JJ and Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

Evans HC (2002) Invasive neotropical pathogens of tree crops. In: Watling R, Frankland J, Ainsworth M, Isaac S and Robinson C (eds) Tropical Mycology; Vol. 2, Micromycetes (pp, 83–112) CABI publishing, Wallingford, Oxon, UK.

Excoffier L, Smouse PE and Quatro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479–491.

FAO (2003) FAOSTAT online database at: <http://www.fao.org/default.htm>

Frias GA, Purdy LH and Schmidt RA (1995) An inoculation method for evaluating resistance of cacao to *Crinipellis perniciosa*. Plant Disease 79: 787–791.

Griffith GW and Hedger JN (1994) The breeding biology of biotypes of the witches' broom pathogen of cocoa, *Crinipellis perniciosa*. Heredity 72: 278–289.

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van de Wiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volcakaert G, Rueda J, Linacero R, Vazques A and Karp A (1997) Reproducibility testing of RAPD, AFLP, and SSR markers in plants by a network of European laboratories. Molecular Breeding 3: 381–390.

Laker HA (1989) Variation in the cultural characteristics of isolates of *Crinipellis perniciosa* in Trinidad. Turrialba 39: 501–505.

Majer D, Mithen R, Lewis BG, Vos P and Oliver RP (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycological Research 9: 1107–1111.

Marita, JM, Nienhus, J, Pires, JL and Aitken, WM (2001) Analysis of genetic diversity in *Theobroma cacao* with emphasis on witches' broom resistance. Crop Science 41: 1305–1316.

McGeary FM and Wheeler BEJ (1988) Growth rates and mycelial interactions between isolates of *Crinipellis perniciosa* from cocoa. Plant Pathology 37: 489–498.

Miller MP (1998) AMOVA-PREP 1.01 A program for the preparation of AMOVA input files from dominant-marker raw data. Northern Arizona Univ. Dept of Biological Sciences, mpm2@nauvax.ucc.nau.edu

Motamayor JC, Risterucci AM, Lopez PA, Ortiz CF, Moreno A and Lanaud C (2002) Cacao domestication I: The origin of the cacao cultivated by the Mayas. Heredity 89: 380–386.

Nei M and Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences (USA) 76: 5269–5273.

Pound FJ (1938) Cacao and Witches' Broom Disease of South America. Port-of-Spain, Trinidad

Purdy LH and Schmidt RA (1996) Status of cacao witches' broom: Biology, epidemiology, and management. Annual Review of Phytopathology 34: 573–594.

Rincones J, Meinhardt LW, Vidal BC and Pereira GAG (2003) Electrophoretic karyotype analysis of *Crinipellis perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*. Mycological Research 107: 452–458.

Queiros VT, Guimarães CT, Anhert D, Schuster I, Daher RT, Pereira MG, Miranda VRM, Loguercio LL, Barros EG and

Moreira, MA (2003) Identification of major QTL in cocoa (*Theobroma cacao* L.) associated with resistance to witches' broom. Plant Breeding 122: 268–272.

Schnell RJ, Brown JS, Olano CT, Power EJ, Krol CA, Kuhn DN and Motamayor JC (2003) Evaluation of avocado germplasm using microsatellite markers. Journal of the American Society for Horticultural Science 128: 881–889.

Tooley PW, O'Neill NRO, Goley ED and Carras MM (2000) Assessment of diversity in *Claviceps africana* and other *Claviceps* species by RAM and AFLP analyses. Phytopathology 90: 1126–1130.

Wheeler BEJ and Mepsted R (1988) Pathogenic variability among isolates of *Crinipellis perniciosa* from cocoa (*Theobroma cacao*). Plant Pathology 37: 475–488.

Yamada MM, Andebrhan T and Furtek DB (1998) Genetic variability among isolates of *Crinipellis perniciosa* from solanaceous hosts and their relationship to isolates from *Theobroma cacao*. Agrotrópica 10: 123–126.

Yap IV and Nelson RJ (1996) WinBoot; a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA- based dendrograms. IRRI Discussion Paper, Series No. 14, pp. 1–20.