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Molecular fingerprinting suggests two primary outbreaks of witches' broom disease (*Crinipellis perniciosa*) of *Theobroma cacao* in Bahia, Brazil

Teklu Andebrhan[1], Antonio Figueira[2,*], Milton M. Yamada[3], Julio Cascardo[4] and Douglas B. Furtek[5]
[1]*Virginia State University, Agricultural Research Station, PO Box 9061, Petersburg, VA 23806, USA;* [2]*Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, CP 96, Piracicaba, 13400-970, São Paulo, Brazil;* [3]*MARA/CEPLAC/CEPEC, CP 7, Itabuna, 45600-000, Bahia, Brazil;* [4]*Almirante Centro de Estudos de Cacau, CP 55, Itajuipe, Bahia, 45630-000, Brazil;* [5]*Malaysian Cocoa Board, RRIM Experiment Station, Biotechnology Division, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia;* [*]*Author for correspondence (E-mail: figueira@cena.usp.br)*

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Abstract

Crinipellis perniciosa (Stahel) Singer is the causal agent of witches' broom disease in the Sterculiaceae, Solanaceae, and Bixaceae families. The disease is endemic to the Brazilian Amazon, and was first reported infecting *Theobroma cacao* (cocoa) in the State of Bahia, Brazil, in 1989. Random amplified polymorphic DNA (RAPD) analyses were performed on 46 isolates of *C. perniciosa* from cocoa that were collected from 15 counties in Bahia and the Brazilian Amazon. A total of 258 RAPD loci from 20 primers and three mixed primers were analyzed. Of these loci, 108 (42%) were polymorphic, with an average of 4.7 polymorphic loci per primer produced. Genetic similarities were estimated using Nei and Li's index and UPGMA clustering. Bootstrap analysis divided the phenogram into four significantly different clusters: two groups contained isolates from Ariquemes and from Ouro Preto, Rondônia, and the other two separated the isolates from Bahia into two major groups of *C. perniciosa*, classified as Group 1 (G1) and Group 2 (G2). The two groups of isolates from Bahia differed for their genetic similarity with the isolates from the Brazilian Amazon. The geographic distribution of the groups in Bahia suggests two independent focal points of introduction. Ongoing programs to screen for resistant cocoa genotypes should consider both groups of isolates.

Introduction

Crinipellis perniciosa (Stahel) Singer is a basidiomycete that causes witches' broom disease in members of the plant families Sterculiaceae, Solanaceae (Bastos and Evans, 1985), and Bixaceae (Bastos and Andebrhan, 1986). This fungus can also secondarily colonize a variety of substrates as a saprotroph (Evans, 1978; Hedger et al., 1987). The pathogen is hemibiotrophic with two types of mycelia. In green infected tissues, the mycelium is wide (5–8 μm), biotrophic and non-clamped, growing intercellularly. The brooms die for reasons not yet understood, colonized inter- and intracellularly by a narrower (1.5–3 μm) mycelium with clamp connections (Delgado and Cook, 1976; Evans, 1980; Griffith and Hedger, 1994). This saprophytic mycelium can readily be isolated on agar medium, but it is unable to infect cocoa (Evans, 1980), and does not produce basidiocarps in culture plates (Purdy and Smith, 1996). The formation of clamp connections on the hyphae of single basidiospore cultures (Baker and Crowdy, 1943), dikaryotization from monokaryotic hyphae derived from single uninucleate basidiospore (Delgado and Cook, 1976),

and the production of basidiocarps from single spore culture (Purdy et al., 1983) indicate that *C. perniciosa* is primarily homothallic (Griffith and Hedger, 1994).

The economic impact of the pathogen is mainly on *Theobroma cacao*. Basidiospores infect meristematic tissues (shoots, flower cushions, single flowers, and developing fruits), inducing a range of symptoms depending on the organ infected and stage of development (Purdy and Smith, 1996). Hypertrophic growth of infected buds ('brooms') are the most dramatic symptoms. Flower cushion infection usually leads to the production of vegetative shoots and abnormal flower development. Pod infections can directly result in seed losses approaching 90% of potential annual production in some areas and seasons (Andebrhan, 1984). Pod set is further reduced indirectly by the infection of flower cushions and the general debilitation of the tree. The life cycle of the pathogen is completed by the production of basidiocarps on necrotic brooms and dried pods releasing basidiospores, the unique source of inoculum (Purdy and Smith, 1996).

Three introductions of witches' broom disease into major cocoa-producing regions occurred since the disease was first reported in Surinam in 1895 (Lass, 1987): in the Western coastal plains of Ecuador in 1918 (Pound, 1938); Trinidad in the late 1920s (Pound, 1938); and in the State of Bahia, Brazil, in the late 1980s (Pereira et al., 1989). In Bahia, the disease was first reported in a farm in the county of Uruçuca (Locale 13 in Figure 1) (Pereira et al., 1996). Infected tissues were found on 112 trees. Assumed at the time to be the only focal point of the disease, the affected area plus a surrounding buffer zone (ca. 140 ha), along with the shade trees, was destroyed. Six months later, another outbreak was discovered in the county of Camacan (Locale 3, Figure 1), 120 km south of Uruçuca, affecting a larger area, and appeared to have existed before the Uruçuca focal point. The appearance of the disease at two distinct and distant sites, right in the center of the cocoa region, suggests that more than one introduction occurred, probably from the Brazilian Amazon, where the disease is endemic, and possibly through human intervention (Rocha et al., 1993; Pereira et al., 1996).

Determining the genetic variability of the pathogen is an important component in the selection of resistant or tolerant genotypes that could be used in breeding programs. Classification of various isolates of *C. perniciosa* has been based on pathogenicity studies (Fonseca et al., 1984; Hedger et al., 1987; Bastos et al., 1988; Wheeler and Mepsted, 1988), and biochemical

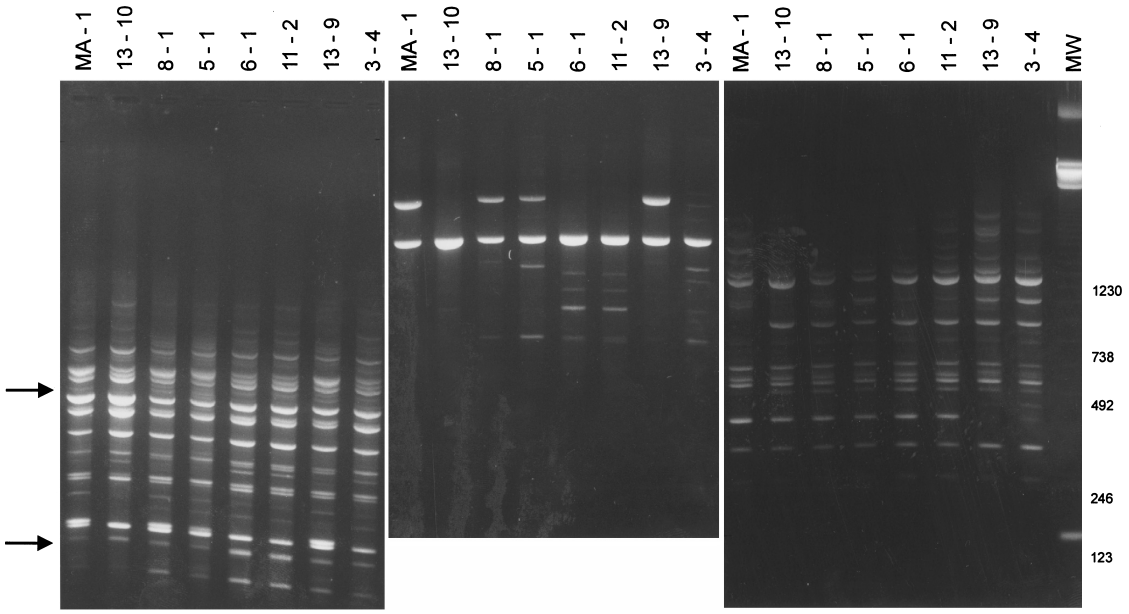

Figure 1. Examples of RAPD amplification of DNA from various *Crinipellis perniciosa* isolates using primers OPA19; OPC17; and OPC16, from left to right. Arrows indicate G1 or G2 specific bands. Isolates and grouping: MA-1 (lane 1); 13-10/G1 (lane 2); 8-1/G2 (lane 3); 5-1/G2 (lane 4); 6-1/G1 (lane 5); 11-2/G1 (lane 6); 13-9/G2 (lane 7); and 3-4/G1 (lane 8). MW is the 123 bp ladder (Gibco BRL Life Technologies Inc.)

and somatic incompatibility tests on the saprotrophic stage of the pathogen (Andebrhan, 1987; McGeary and Wheeler, 1988; Griffith and Hedger, 1994). Recently, Andebrhan and Furtek (1994) demonstrated the utility of the random amplified polymorphic DNA (RAPD) fingerprinting procedure for distinguishing *C. perniciosa* isolates on different hosts. They showed that geographical proximity may be more important than host species for determining the relative genetic similarities among isolates. Here we report studies on the genetic diversity of *C. perniciosa* isolates from several locations in the state of Bahia, in relation to the original appearance of two geographically separated disease foci representing two possible independent introductions of the disease, and discuss the results with the historical accounts on the introduction and spread of the pathogen.

Materials and methods

Crinipellis perniciosa *isolates*

Cultures of *C. perniciosa* were obtained from Fazenda Almirante (Km 2, road from BR 101 to Barro Preto, Itajuipe, Bahia, Brazil) and from the culture collections of the Commission for the Development of Cocoa (CEPLAC) in Belém, Pará (SEPES) and Itabuna, Bahia (CEPEC) (Table 1; Figure 2). The Almirante isolates were collected from March 1994 through May 1994 on sites used for cocoa crop forecast, distributed over the Bahia cocoa region. Isolates from CEPLAC-CEPEC were collected on various farms from May 1989 through January 1995.

Mycelial cultures were established either from multiple basidiospores derived from single basidiocarps from dried brooms or pods (see Table 1), as described by Andebrhan and Furtek (1994), or isolated directly from infected vegetative tissues. Cultures were maintained in potato dextrose agar medium (PDA; Difco, Detroit, MI, USA).

DNA extraction

DNA was extracted from lyophilized mycelia as described by Zolan and Pukkila (1986). Briefly, ground mycelia was mixed with extraction buffer (1% CTAB; 0.7 M NaCl; 20 mM Tris-Cl, pH 8.0; 10 mM EDTA; 1% β-mercaptoethanol) for 20 min at room temperature. The DNA was precipitated from the aqueous phase after a chloroform : isoamyl alcohol (24 : 1) extraction, with isopropanol. The DNA pellet was resuspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA), and treated with ribonuclease A at 37 °C for 30 min, followed by a chloroform : isoamyl alcohol (24 : 1) extraction, and re-precipitation with 95% ethanol. The DNA pellet was then washed in 70% ethanol, dried, re-dissolved in TE, and quantified fluorometrically.

PCR of ribosomal spacer DNA

Ribosomal RNA internal transcribed spacer (ITS) and non-transcribed spacer (NTS) regions from each isolate were amplified using two sets of primers (Georgiev et al., 1981; Walker and Doolittle, 1983) synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA): ITS forward: 5′GTTTCCGTAGTGAACCTGCGG 3′; ITS reverse: 5′CATATGCTTAAGTTCAGCGGG 3′; NTS forward: 5′CTGAACGCCTCTAAGTCAGAA 3′; NTS reverse: 5′AGTCCTATGGCCGTGGAT 3′. PCR reaction mixes were as described by Andebrhan and Furtek (1994). The total reaction volume was 50 µl, containing enzyme buffer; 50 ng of genomic DNA; 150 ng of each primer; 250 µm each of dATP, TTP, dCTP, dGTP; 1 $\mu g\,l^{-1}$ bovine serum albumin; and 2.5 units of *AmpliTaq* DNA polymerase (Applied Biosystems, Norwalk, CT, USA). Amplifications were conducted in a GeneAmp PCR System 9600 (Applied Biosystems, Norwalk, CT, USA) for 35 times by denaturing at 94 °C for 45 s, annealing at 55 °C for 2 min, and extending at 72 °C for 3 min, ending with a 7 min extension. One to two (1–2) µl aliquots of the reaction mix were analyzed in a 1.2% agarose gel as described by Andebrhan and Furtek (1994). Fragment sizes were compared with fragments from confirmed isolates of *C. perniciosa* in our collection.

Random amplified polymorphic DNA (RAPD) analysis

Ten base synthetic primers were obtained from John Carlson (University of British Columbia, Vancouver, Canada) and from Operon Technologies, Inc. (Alameda, California, USA). The primers used (Table 2) were selected based on amplification profile reproducibility as compared to our previous studies with *C. perniciosa* (Andebrhan and Furtek, 1994; unpublished data). The total reaction volume was 50 µl, containing 50 ng of genomic DNA; 300 ng of 10-mer

Table 1. Source of isolates of *Crinipellis perniciosa*

Map[1] code	Origin[2]	Year	Sample size[3]	MBS[4]	Broom[5]	Pod[6]	Collector
1	Almadina	1995	1	1	—	—	Almirante[7]
2	Buerarema	1994, 1995	3	1	2	—	Alm and CEPEC
3	Camacan	1990, 1991, 1993, 1994	7	3	2	2	Alm and CEPEC
4	Floresta Azul	1991	1	—	1	—	CEPEC
5	Gongogi	1991	1	—	1	—	CEPEC
6	Ibicaraí	1994	1	1	—	—	Almirante
7	Ilhéus	1991, 1992, 1994	4	2	2	—	Alm and CEPEC
8	Itabuna	1994	1	1	—	—	Almirante
9	Itacaré	1990, 1991	4	—	4	—	CEPEC
10	Itajuípe	1994, 1995	2	1	1	—	Alm and CEPEC
11	Juçari	1994	2	2	—	—	Almirante
12	Pau Brasil	1994	4	1	1	2	Alm and CEPEC
13	Uruçuca	1989, 1990, 1991, 1994	10	4	4	2	Alm and CEPEC
AR	Ariquemes, Rondônia	1988	2	2	—	—	SEPES
OPR	Ouro Preto, Rondônia	n.a.[8]	2	2	—	—	SEPES
MA	Manaus, Amazonas	1988	1	1	—	—	SEPES

[1]Refers to the locations shown on the map (Figure 2) and phenogram (Figure 3).
[2]Province/County (1–13 are in the State of Bahia).
[3]Number of samples collected.
[4]Culture initiated from multiple basidiospores.
[5]Culture initiated directly from a green broom.
[6]Culture initiated from an infected pod.
[7]Almirante = Fazenda Almirante.
[8]n.a. = not available.

primer; 250 μM each of the four dNTPs; and 2.5 units of *AmpliTaq* DNA polymerase (Applied Biosystems, Norwalk, CT, USA). These reaction conditions had been optimized as described previously (Andebrhan and Furtek, 1994). Amplifications were conducted in a GeneAmp PCR System 9600 (Applied Biosystems, Norwalk, CT, USA) for 42 cycles by denaturing at 94 °C for 30 s, annealing at 36 °C for 1 min, and extending at 72 °C for 2 min, ending with a 5 min extension. The agarose gel electrophoresis was as described by Andebrhan and Furtek (1994).

Data analysis

RAPD products (major and minor band intensity), which have shown to be consistently amplified in various experiments (Andebrhan and Furtek, 1994), were scored visually for presence or absence (Figure 1). Variation among isolates was evaluated from pairwise comparisons of the proportion of shared fragments among samples, i.e., two times the number of shared fragments divided by the total number of fragments (Nei and Li, 1979). Relationships among samples were evaluated with a phenetic cluster analysis using unweighted pair-group arithmetic average (UPGMA) clustering (Sneath and Sokal, 1973), and plotted in a phenogram using NTSYS-pc version 1.70 (Exeter Software, Setauket, NY, USA). Bootstrap analysis was performed using the WinBoot program (Yap and Nelson, 1996), with 1000 repetitive samplings of the RAPD data to compute bootstrap *P* values.

Results and discussion

Fungal classification is based commonly on the morphology of reproductive structures, asexual resting structures, spores, and sporophores. Because *in vitro*-cultured *C. perniciosa* lacks suitable morphological markers and does not produce basidiocarps, a problem

Table 2. RAPD primers used to determine genetic similarities among *Crinipellis perniciosa* isolates

Primer code[1]	Sequence (5′-3′)	No. bands[2]	Poly[3]	Mono[3]
101	GCGGCTGGAG	11	3	8
103	GTGACGCCGC	12	0	12
104	GGGCAATGAT	15	13	2
503	ATCGTCCAAC	5	0	5
511	ATCGTCCAAC	13	7	6
514	CGGTTAGACG	10	3	7
515	GGGGGCCTCA	11	1	10
524	CGGTTACTAG	5	4	1
536	GCCCCTCGTC	11	6	5
543	CGCTTCGGGT	11	3	8
544	TAGAGACTCC	17	16	1
545	ACGTTGAGAC	9	1	8
604	GGCCCATTGC	16	1	15
OPA15	TTCCGAACCC	5	2	3
OPA19	CAAACGTCGG	21	13	8
OPC03	GGGGGTCTTT	14	3	11
OPC11	AAAGCTGCGG	9	5	4
OPC12	TGTCATCCCC	14	4	10
OPC16	CACACTCCAG	20	10	10
OPC17	TTCCCCCCAG	2	1	1
524 + OPC17	See above	8	3	5
544 + OPC17	See above	10	6	4
545 + OPC17	See above	9	3	6
TOTAL	23	258	108	150
Average loci	per primer	11.2	4.7	6.5

[1]Primers with alphanumeric and numeric codes were purchased from Operon Technologies and the University of British Columbia, respectively.
[2]Total number of RAPD bands observed for the 46 isolates.
[3]Poly and Mono are the number of polymorphic and monomorphic loci, respectively.

arises when attempting to establish the authenticity of isolates derived directly from infected tissues. The length of spacer regions within ribosomal RNA gene clusters (rDNAs), however, can often be used to distinguish fungal species and even strains (Hintz et al., 1985, 1989; Taylor et al., 1986; Arora et al., 1996). Fungal rDNA clusters consist of up to several hundred tandemly repeated units, each containing coding regions for the major ribosomal RNA subunits (18S, 5.8S, 26S), as well as internal transcribed spacer regions (ITS) and non-transcribed spacer regions (NTS) (also known as intergenic spacers [IGS]). In most eukaryotes, including fungi, these spacer regions, especially the NTS regions, evolve more quickly than the regions encoding the rRNAs, and thus they are more prone to vary in length and sequence (White et al., 1990).

Isolates whose DNA gave different ITS and NTS fragment sizes compared to the DNA from reference isolates were excluded from these studies. All isolates authenticated by basidiocarp morphology produced ITS and NTS bands of 740 and 1000 bp, respectively (not shown). The single excluded isolate produced ITS and NTS bands of 680 and 800 bp, respectively, and was isolated directly from infected tissues; thus its identity could not be confirmed by basidiocarp morphology. The amplified fragments were purified and sequenced (Andebrhan and Furtek, unpublished).

Genetic similarities among 46 isolates of *C. perniciosa* from *T. cacao* were estimated from RAPD banding patterns. Forty-one isolates were from Bahia and five were from the Brazilian Amazon. A total of 258 RAPD loci, amplified from 20 primers and three mixed primers were analyzed. Examples of typical

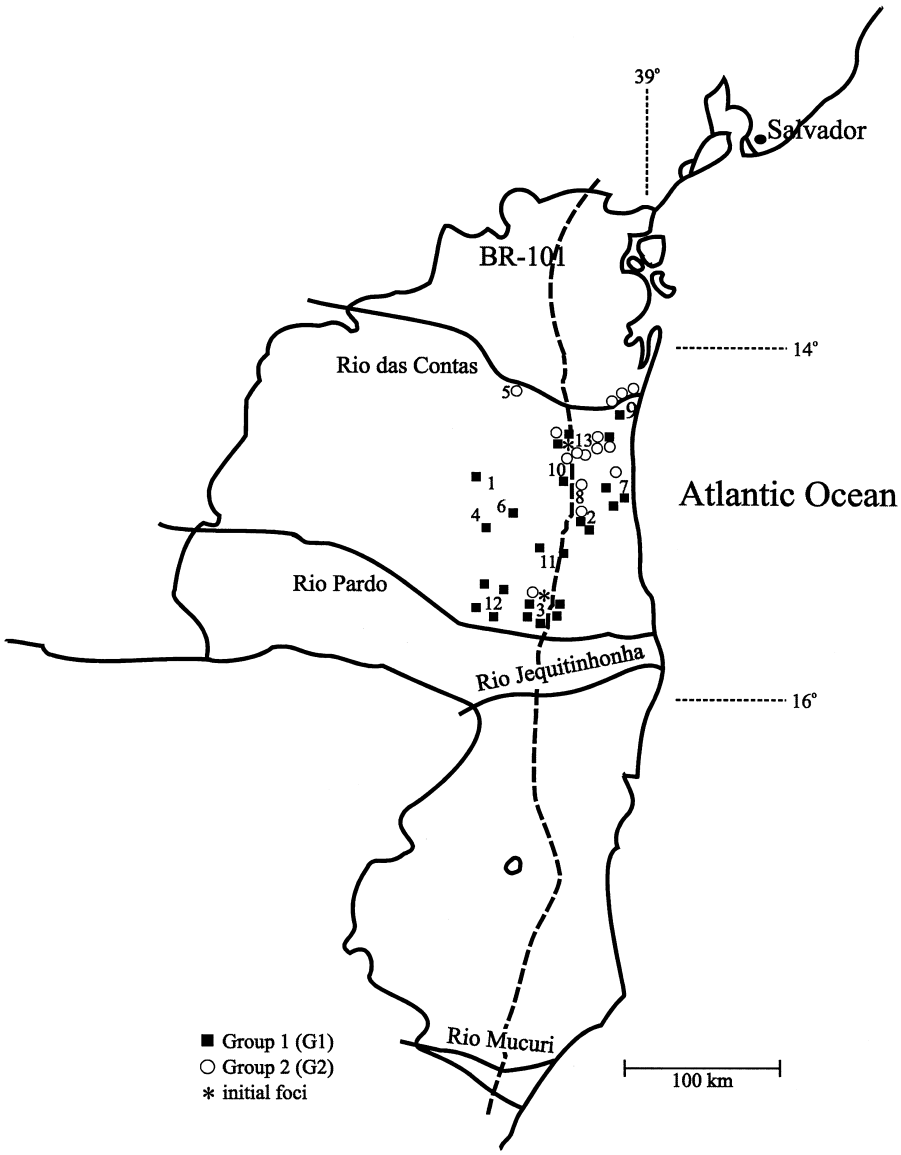

Figure 2. Locations where *C. perniciosa* isolates were collected. Refer to Table 1 for the province (map) codes. Filled squares designate G1 and empty circles designate G2 isolate families. Asterisks designate the two putative initial foci of Camacan (locale 3) and Uruçuca (locale 13).

RAPD amplification are shown in Figure 1. An average of 11.2 loci per primer were produced, and of these loci, 108 (42%) were polymorphic (Table 2). Estimated genetic similarities based on Nei and Li's (1979) index (S_{xy}) ranged from 0.837 [Figure 2, Map Codes 13-9 (Uruçuca, Bahia) and AR-1 (Ariquemes, Rondônia)] to 0.995 [Map Codes 3-2 and 3-3 (Camacan)] (matrix not shown).

Bootstrap analysis divided the phenogram into four significantly different clusters, considering significant bootstrap *P* values above 90%. The first cluster (GAR, Figure 3) included only the isolates from Ariquemes, Rondônia (AR-1 and AR-2), while the second one (GOPR, Figure 3) contained the isolates OPR-1 and OPR-2, originally collected in Ouro Preto, Rondônia. The third cluster included isolates from 1-1 through

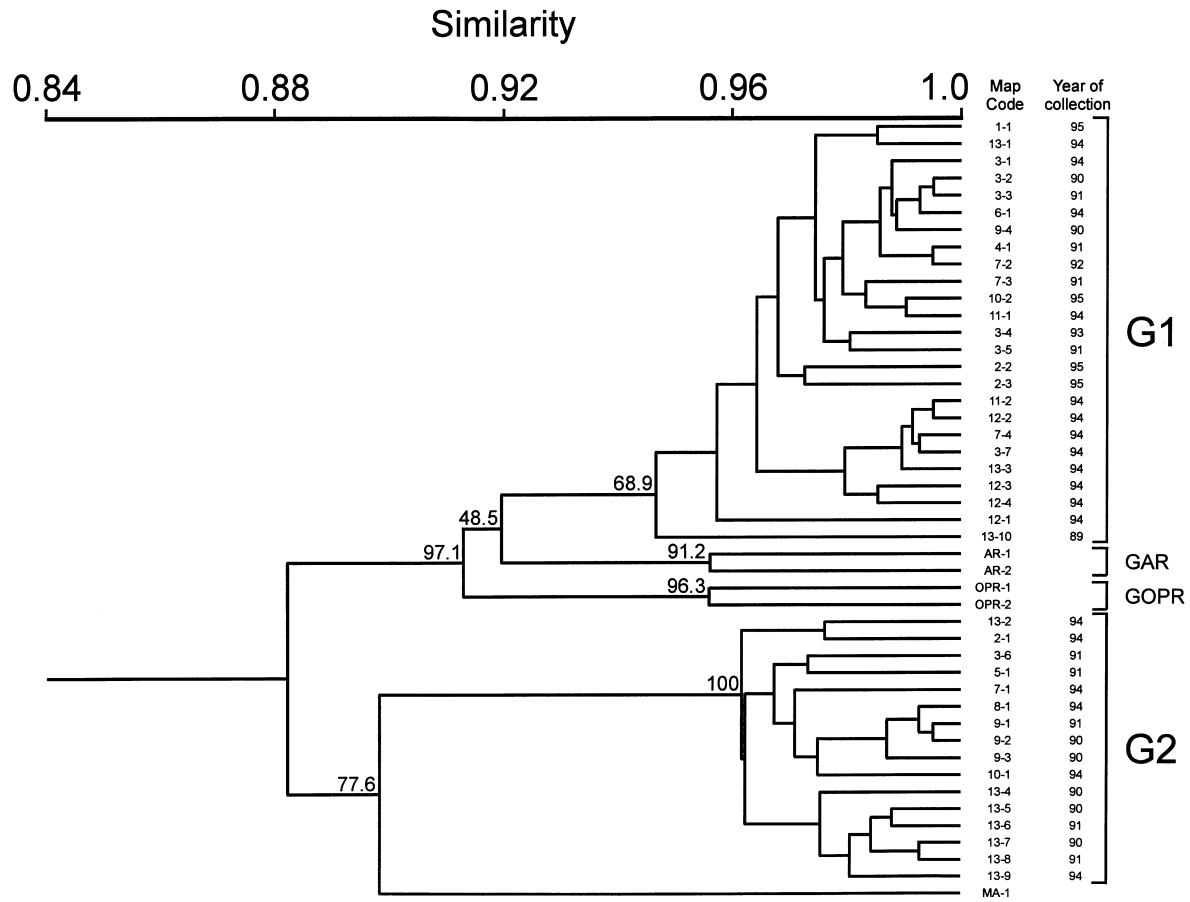

Figure 3. Phenogram of isolates, generated using UPGMA clustering based on RAPD polymorphism. Bootstrap *P* values are given at the corresponding node for each cluster. 'Map Code' refers to the collection sites of isolates listed in Table 1 and shown in Figure 2. The first number indicates the province and the second the collection number within the province.

13-10 (G1, Figure 3). The fourth cluster included the other isolates [Bahian from 13-2 through 13-9 (G2, Figure 3)]. Based on the bootstrap analysis, the isolates from Bahia can be classified into two significantly different main groups: one containing mostly (14 out of 25) isolates from the southern region (G1), and the other composed mainly (14 out of 16) with isolates collected in the northern region (G2). The region G2 was less diverse. Some primers amplified group-specific bands. A 1.8 kbp fragment was amplified with primer OPC17 only from isolates of G2 (Figure 1). The same was observed for a 150 bp fragment amplified with primer OPA19 (Figure 1), while using the same primer, a 0.5 kbp fragment was present only on isolates from G1. The isolates from Ouro Preto, Rondônia (OPR-1 and OPR-2) formed a significantly different group (bootstrap P value = 97.1) from the group containing isolates from Ariquemes, Rondônia (AR-1 and AR-2) and the Bahian isolates from G1. The grouping between isolates AR-1 and AR-2 and G1 was not so robust (bootstrap P value = 48.5), suggesting that the isolates from G1 in Bahia were more similar to the isolates from Ariquemes, than from Ouro Preto, Rondônia. The isolate from Manaus (MA-1) was significantly different from G2 (bootstrap P value = 77.6). Sixty-one percent (61%) and 39% of the isolates from Bahia belonged to G1 and G2, respectively.

The geographic overlap of isolates within each group might have derived from the time of isolate sampling. A survey of disease incidence in Bahia in 1991 revealed two major concentrations of infected trees: one centering around Camacan (G1) in the south and accounting for 60% of the recorded infected trees, and the other centering around Uruçuca (G2) in the

north and accounting for only about 1% of infected trees (Rocha et al., 1993). One year later, the areas of disease spread from each site were starting to overlap (Pereira et al., 1996). Most isolates used in the present study were sampled after 1992. Since the disease outbreak at Camacan preceded the one in Uruçuca, according to Pereira et al. (1996), and since eradication was not attempted at Camacan, it is likely that inoculum from this focus had spread at faster rate than from Uruçuca. This may explain the occurrence of a large number of isolates from G1 in northern locations. Griffith and Hedger (1994) suggested that multiple infection by genetically distinct basidiospores might provide an opportunity for heterokaryosis in the cocoa biotype of *C. perniciosa*.

Because basidiospores are the only identified infectious propagules to witches' broom disease and are short-lived in air (Baker and Crowdy, 1943), the pathogen is highly unlikely to have survived wind dispersal of many hundreds of kilometers from hosts in the Amazon basin to southern Bahia, where cocoa had been cultivated without previous incidence of the disease for 200 years (Rocha et al., 1993). Similarly, the discovery of the disease at two widely separated locations, in the center of the cocoa region, strongly suggests that the disease was introduced into Bahia, as infected living or necrotic cocoa tissue, by human intervention (Rocha et al., 1993).

The two groups of isolates from Bahia differed for their genetic similarity with the isolates from the Brazilian Amazon. The isolates from the more homogeneous G2 (39% of the isolates from Bahia) were significantly different from any of the isolates tested from the Amazon (OPR, AR and MA), while the isolates from G1 were not. Results of studies on the structure of populations of the cocoa biotype of *C. perniciosa* have demonstrated the occurrence of geographically widespread clones, as assessed by somatic compatibility reactions, that suggests a non-outcrossing breeding strategy in this biotype (Griffith, 1989; McGeary and Wheeler, 1988). Therefore, *C. perniciosa* being homothallic, our data suggest that two independent introductions of the pathogen occurred in Bahia.

Our study demonstrates the existence of two genetically diverse lineages of isolates of *C. perniciosa* in Bahia, and programs to screen cocoa trees for resistance should take these two lineages into consideration.

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