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TEMPORAL GENETIC CHARACTERIZATION OF ISOLATES OF *Moniliophthora perniciosa* IN SOUTHEAST OF BAHIA, BRAZIL

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Random amplified polymorphism DNA (RAPD) markers were used to characterize temporal genetic variability of *Moniliophthora perniciosa* populations in Bahia, Brazil, over four consecutive years (2001 to 2004). Five primers (OPH20, OPH13, OPI19, OPI14, and OPL7) showing reproducible polymorphism and clear banding patterns were used to amplify the genomic DNA of 89 isolates, generating a total of 42 bands. The matrix of RAPD data revealed the occurrence of genetic differentiation among years. Most of the genetic diversity was attributed to the differences between 2004 and the remaining years. The principal component analysis and AMOVA ($P < 0.001$) confirmed the presence of populations related to the year of collection. Overall, isolates from the same year shared the same coordinates. Weir and Cockerham's theta was used to estimate the degree of genetic differentiation between populations. The 2001 and 2002, and 2002 and 2003 populations were the most similar (è = 0.18 and è = 0.11; respectively, p<0.001), while the 2002 and 2004 populations differentiated the most (è = 0.51, p<0.001). The shift in the genetic composition of the populations studied seems to be correlated with the the increase of planting areas with Scavina descendant genotypes.

Key words: *Theobroma cacao*, witches' broom, RAPD, genetic variability.

Caracterização genética temporal de isolados de *Moniliophthora perniciosa* na Região Sudeste da Bahia, Brasil. Marcadores RAPD (Randon Amplified Polymorphic DNA) foram utilizados para caracterizar a variabilidade genética temporal de populações de *Moniliophthora perniciosa* na Bahia, Brasil, ao longo de quatro anos consecutivos (2001 a 2004). Cinco primers (OPH20, OPH13, OPI19, OPI14 e OPL7) mostrando polimorfismo reproduzível e padrões de bandas bem definidos foram utilizados para amplificar o DNA genômico de 89 isolados, gerando um total de 42 bandas. A matriz de dados de RAPD revelou variação genética entre os anos. A maior parte da diversidade genética foi atribuída às diferenças entre 2004 e os anos restantes. As análises de componente principal e AMOVA (P <0,001) confirmaram a presença de populações relacionadas ao ano de coleta. No geral, isolados do mesmo ano compartilharam as mesmas coordenadas. A estimativa de è (Theta Weir e Cockerham) foi utilizada para verificar o grau de diferenciação genética entre as populações. As populações de 2001 e 2002, e de 2002 e 2003, foram as mais similares geneticamente (è = 0,18 e è = 0,11; respectivamente, p <0,001), enquanto que as populações de 2002 e 2004 apresentaram uma maior diferenciação genética (è = 0,51, p <0,001). A mudança na composição genética das populações estudadas parece estar correlacionada com o aumento do plantio de áreas com genótipos Scavina descendentes.

Palavras-chave: *Theobroma cacao,* vassoura-de-bruxa, RAPD, variabilidade genética.

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Introduction

Moniliophthora (=*Crinipellis*) *perniciosa* (Stahel) Aime & Phillips-Mora (Aime and Phillips-Mora, 2005), belongs to the Kingdom Mycota subdivision Basidiomycotina, order Agaricales and family Marasmiaceae. The pathogen exhibits two phases in its life cycle, the biotrophic and the necrotrophic phase (Evans, 1980, 1981; McGeary and Wheeler, 1988). After alternating dry and wet periods, fruiting bodies, the basidiocarps, are produced on dry brooms and/or on infected fruits, releasing basidiospores (Rocha and Wheeler, 1982). In nature, *M. perniciosa* basidiospores are the only known infectious propagules, germinating on young cocoa tissues such as apical and axillary meristems, young leaves, developing flowers, and fruits (Purdy and Schmidt, 1996), subsequently penetrating plant tissues directly or entering through stomata (Frias and Purdy, 1991; Sreenivasan and Dabydeen, 1989).

The fungus is a pathogen of *Theobroma cacao* L., the chocolate tree, causing the known witche's broom disease of cocoa (WBD). This disease is one of the most important diseases in Latin America. It was first reported in 1895, in Surinam, where it caused severe losses (Stahel, 1915). Currently, the disease is present in Bolivia, Brazil, Colombia, Ecuador, Guyana, Panama, Peru, Trinidad, Suriname and Venezuela (Luz et al., 1997). Losses in yield up to 90% have been associated to this disease (Andebrhan, 1985). In Bahia, Brazil, *M. perniciosa* was introduced in 1989 (Pereira et al., 1996). The rapid increase and spread of the disease throughout extensive areas of the southeastern Bahia was due primarily to the favourable climatic conditions and the continuous cocoa monoculture of over 600,000 ha.

Despite the efforts the methods of disease control proposed to cocoa farmers (chemical, biological, cultural) were not sufficiently effective in reducing losses, either because they have little effect in controlling WBD or calls for a heavy cost input by farmers. The 'genetic control' component in integrated disease is perhaps the most durable method, but judicious development and deployment of resistant genotypes is predicated on a sound understanding of the population structure of the pathogen. Understanding the genetic diversity of the pathogen and spatiotemporal changes in population structure is vital to the success of any breeding program (Leung et al., 1993; McDonald and Linde, 2002). Research efforts on the population structure of *M. perniciosa* sought to (i) investigate the introduction and spread of the pathogen in Bahia (Andebrhan et al.,1999), (ii) determine the current level of genetic diversity in populations of *M. perniciosa* from different countries (Ploetz et al., 2005), and (ii) analyze how this diversity is distributed within and between a country (Moreira, 2006). Further studies in Brazil showed that *M. perniciosa* strains occurring in the main source of resistance (Scavina) – used to develop resistant varieties recommended for planting – are genetically different from the ones collected from local genotypes (Pires, 2003). Also, temporal studies on witches'broom disease incidence have shown a significant increase in disease severity in Scavina or derived-Scavina progenies recommended to farmers (Pires, 2003, Gramacho et al., 2003 a, b, c). Both elements considered together show that the fungus is adapting to and overcoming the Scavina's resistance.

However, these studies surveyed only spatial distribution of genetic variation among plantations and don't provided information on the temporal dynamics of population structure for this pathogen. Temporal stability of witches´ broom resistance is a major concern for intensively managed plantations, since pathogenic variation toward resistant families has been reported (Wheeler and Mepsted, 1988). Since the introduction of RAPD analysis (Williams et al, 1990), this technique has been proved to be a very powerful tool in genetic analysis and has been applied to a wide range of organisms. It was first applied to *M. perniciosa* by Andebrhan et al. (1999), who showed, that two populations of this fungus were present in Bahia. The objective of this research was to determine the level of temporal genetic diversity during the years 2001-2004 in the main agroecological areas of Southeastern of Bahia using RAPD markers. Understanding the genetics of *M. perniciosa* populations will allow predicting how populations will change throughout the years. This knowledge would benefit disease control, particularly breeding for resistance, considering that new cocoa genotypes can be challenged by isolates more representative of the pathogen population.

Materials and Methods

Fungal isolate collections. From 2001 to 2004, single vegetative broom isolates of *M. perniciosa* were obtained from commercial plantations in the three main agroecological zones of Bahia (Figure 1); a total of 25 isolated were collected in each year and region (the set of isolates collected annually was considered as an population). Trees were marked and GPS points registered. Every year isolates were collected on the same tree. Small tissue pieces taken from the edges of diseased tissues were incubated in Petri dishes on potato dextrose agar (PDA) medium amended with 0.5 g L^1 of streptomycin at 20-25°C. Then growing mycelium was transferred to PDA. A total of 89 isolates were obtained (Table 1). Fungal cultures were grown in Malt 50% for 12 days. Mycelia were filtered through a Whatman N°1 filter paper, washed twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and then lyophilized.

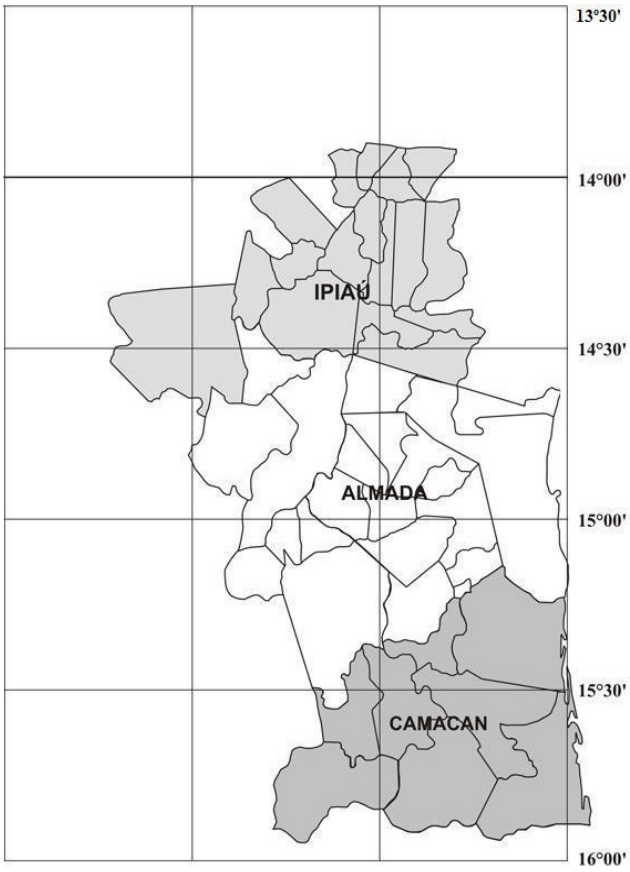

Figure 1. Geographic origin of *Moniliophthora perniciosa* isolates collected from agroecological zones in the Southeast of Bahia.

Table 1. Isolates of *Moniliophthora perniciosa* collected in each surveyed agroecological zone and year used in this study

Year of collection	Agroecological zones			
	Almada	Camacan	Ipiaú	**Total**
2001	7	12	5	24
2002	7	12	5	24
2003	7	12	5	24
2004	6	6	5	17
Total	**-**	**-**		**89**

Study sites. The agroecological zones Almada, Ipiaú and Camacan are, among the fourteen agroecological zones that compose the cacao production region of Bahia, the main regions in terms of cacao planted area and production (Silva and Leite, 1988). The soil and climate conditions of Almada and Camacan regions are considered exceptional for growing cocoa. On the other hand, Ipiaú agroecological zone is considered a transition zone for cacao plantation due to lower average annual rainfall varying from 1,100 mm to 1,200 mm. This area is also apt for cattle.

DNA Extraction. Total DNA was extracted as described by Zolan and Pukila (1986). DNA amount was quantified by electrophoresis (1% of BET agarose gel). DNA concentration of all samples was adjusted to 10 ng μl^{-1} and stored at -20°C for further use. Samples were treated with RNAse according to the manufacturer recommendation.

RAPD analysis. Twenty-four primers (Operon kits), obtained from Operon Technologies (Alameda, California), were tested on a small sub-set of isolates (one from each year) to identify polymorphic primers. Primers that generated reproducible polymorphism and clear banding patterns were used to amplify DNA from all isolates. Five primers satisfying these criteria were identified: OPH20, OPH13, OPI19, OPI14, and OPL7. RAPD analysis with each of the primers was repeated twice and only consistent and reproducible banding patterns were scored. PCR reactions were performed in 25 uL containing 10 mM of Tris-HCl (pH 8.3), 2.4

mmoL. L^{-1} of $MgCl_2$, 0.25 mM. L^{-1} of each dNTP, 0.4 µM of each primer, 0.5 uL of Taq DNA polymerase and approximately 30 ng of genomic DNA. Amplifications were conducted in a GeneAmp PCR System 9600 (Perking-Elmer) under following conditions: 30 cycles of 1 min at 92°C, 1 min at 35°C, for 2 min at 72°C. Amplification products were separated by electrophoresis at 110 V for 2 h on BET-stained 1.2% agarose gel in 1X Tris-borate EDTA buffer, and visualized under UV light.

Genetic Analysis. Amplified DNA fragments were scored as either present (1) or absent (0). For each isolate, all scorable quantified polymorphic and monomorphic fragments were included for the computation of similarity coefficients and the data were processed as a diploid model with two alleles per locus (Lynch and Milligan, 1994). The genetic relationship among isolates was estimated and a phylogenetic tree was constructed. The genetic similarity between two isolates was calculated based on Dicés coefficient using the Darwin5 program (http://darwin.cirad.fr/darwin). The similarity matrix was used to construct a dendogram based on hierarchical clustering (Ward minimum variance method, 1963). In addition, factorial analyses were used to assigned isolates to RAPD groups (clusters); each defined group of isolates was considered as a separate population allowing examining the distribution of isolates based on the year of collection. For each population, the number of polymorphic loci (P), the distribution of genetic diversity, the Nei's genetic identity (h) (1973) within and across populations, and the Shannon index (I) of phenotypic diversity (King and Schaal, 1989) were computed with the PopGene32 software (Yeh et al., 2000) assuming that all loci were dominant and in Hardy-Weinberg equilibrium.

An analysis of molecular variance (AMOVA; version 1.55) was performed on *M. perniciosa* populations to partition the total variance into hierarchical components (among populations, among subpopulations, and within subpopulations) (Excoffier et al., 1992).

To measure the temporal population differentiation between the four *M. perniciosa* populations, the Weir and Cockerhams theta (è), which is comparable to Wright's F_{st} (Weir & Cockerham, 1984), was calculated among pairs of populations using the software Multilocus 2.2. This method estimates the difference in allele frequency distributions, independently of the number of populations and number of individuals sampled in each population (Weir & Cockerham 1984). An Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendogram was constructed using Nei's genetic distance (1978) among populations. Genetic distances among populations were calculated using PopGene 32.

Results

To test the extent of genetic variation in the genome of *M. perniciosa*, RAPD profiles were generated for the 89 isolates using five RAPD primers. These primers generated simple banding patterns that revealed DNA polymorphisms among *M. perniciosa* isolates. Examples of banding profiles of DNA of some isolates using the primer OPH20 are shown in Figure 2. The primers used resulted in the detection of 144 reproducible amplification products (RAPD markers), of which 75 were polymorphic. Primer OPL7 was the most polymorphic. All markers were present in every population (Table 2).

Dendrograms analysis constructed with UPGMA Ward minimum variance clustering method revealed the existence of genetic differentiation in sample among isolates from the different years (Figure 3).

Cluster analysis of RAPD markers (Multiple correspondence) revealed the presence of distinct clusters according to the year in which the isolates were collected. The distribution of the 89 isolates in the factorial planes (1, 2, 3) is presented in Figure 4. Each of the first three axes explains a limited part of the total variability: 26.85%, 17.62%, and 8.4% for the X, Y and Z axis, respectively. Most of the groups contained isolates of the same year, e.g., isolates from 2004 always merged around the axis 3.

Therefore, isolates were grouped by the year of collection to define population for further analyses. AMOVA analysis revealed that the genetic variation within populations accounted for 68.0 % of the total variation ($P < 0.001$) and that the differentiation among the populations was significant ($P < 0.001$) (Table 3). Computation of gene diversity also indicated the same trend: adjusted values within populations (H_S) ranged between 0.21 to 0.24 and accounted for 75% of the total genetic diversity ($H_T = 0.33$) (Table 4).

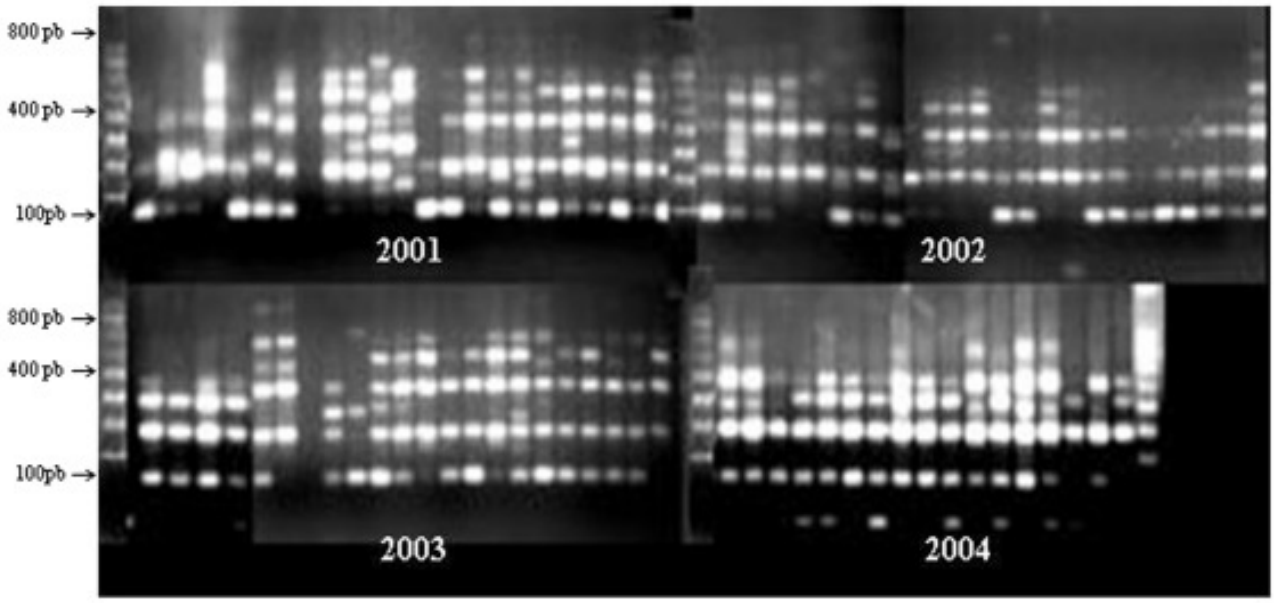

Figure 2. RAPD band profile of 89 isolates of *Moniliophthora perniciosa* from 2001 to 2004 obtained with the primer OPH20.

Table 2. Number of RAPD markers obtained per population (year of collection) and primer

Survey	OPH20	OPH13	OPI14	OPI19	OPL7	Total
2001	7	7	7	6	8	35
2002	7	7	9	9	9	35
2003	9	8	9	6	9	41
2004	7	8	6	5	9	33
Total	**30**	**30**	**31**	**26**	**35**	**144**

Table 3. Analysis of molecular variance (AMOVA) of RAPD data generated for *Moniliophthora perniciosa* population collected during 2001-2004. Populations are according to year of collection

Source of variation	d.f	Sum of squares	Variance Components	Percentage of variation	p-value
Among populations (Year of collection)	3	39.95	0.55	31.59	< 0.0001
Within populations	85	100.98	1.19	68.41	
Total	88	140.93	1.74		

Table 4. Genetic diversity in populations of *Moniliophthora perniciosa* in the Southeast

Population	2001 (N =24)	2002 (N = 24)	2003 (N = 24)	2004 (N = 17)	2001 to 2004 (N = 89)
H	0.23	0.24	0.21	0.21	0.33
I	0.35	0.36	0.34	0.31	0.49

H = Total genetic diversity (Nei 1973). I = Shannon's Information index (Lewontin 1972) N = Sample size

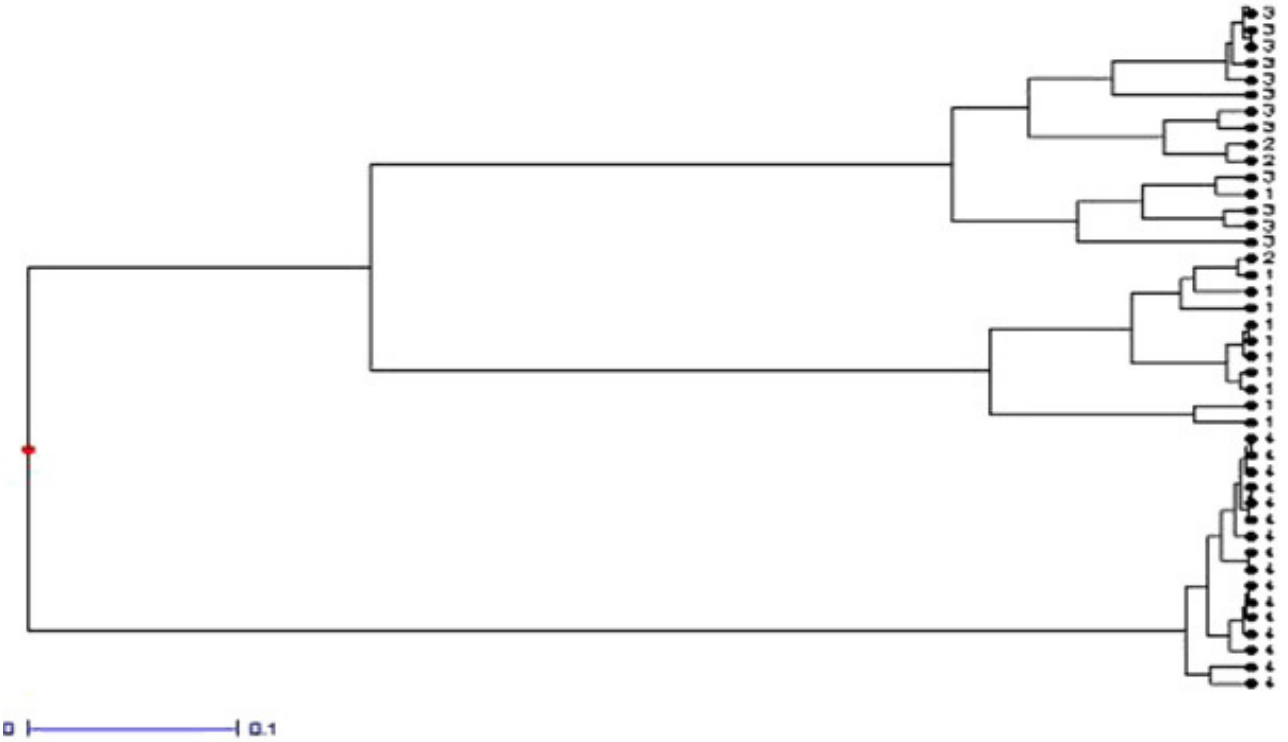

Figure 3. Dendrogram constructed with UPGMA Ward minimum variance clustering method among 89 isolates of *Moniliophthora perniciosa*. Similarities were computed from 75 random amplified polymorphic DNA loci. The scale in the dendrogram is the genetic similarity coefficient calculated according to Dice (1945).

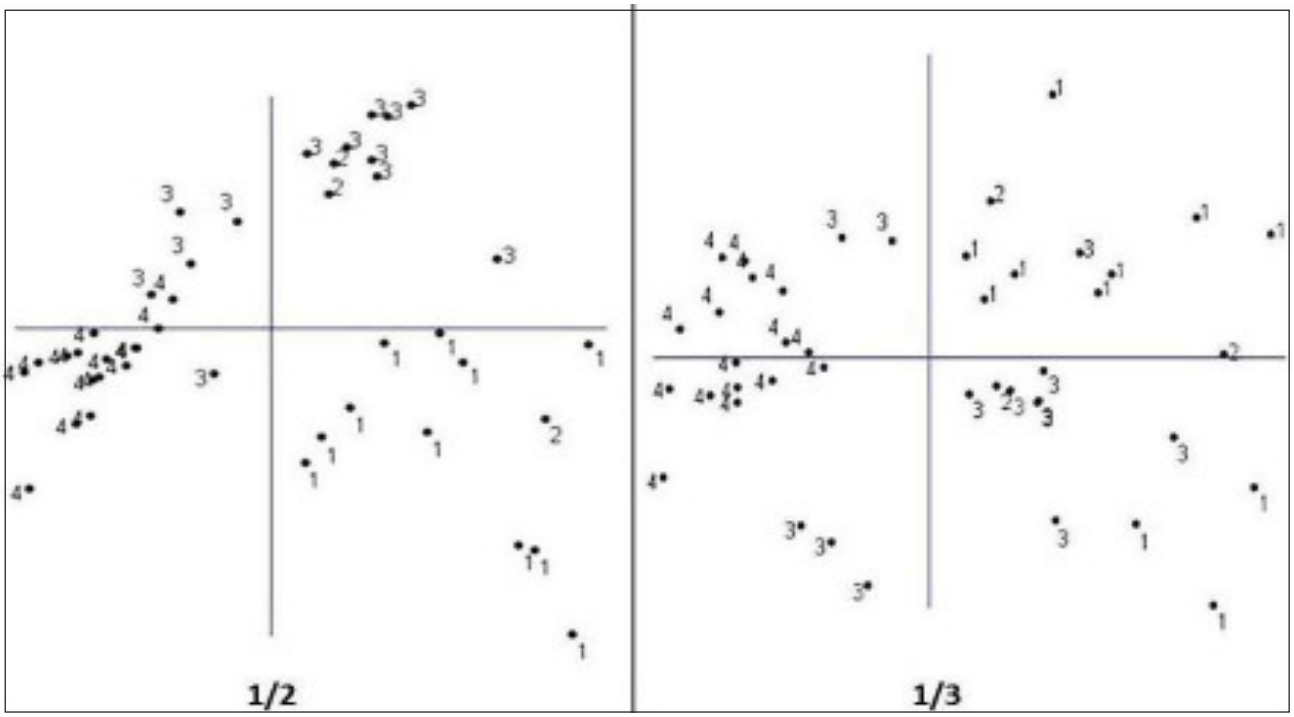

Figure 4. Principal coordinates showing axes 1/2 and 1/3 plot of 89 *Moniliophthora perniciosa* isolates based on differences in random amplified polymorphic DNA fingerprints.

Nei's unbiased genetic distances among populations ranged from 0.043 to 0.31 (Table 5). The lowest similarity was found between 2001 and 2004. Weir and Cockerham's theta (è) was used to estimate the degree of differentiation between populations; theta varies from zero (no differentiation among populations) to one (populations completely differentiated). Based on nuclear DNA markers, the 2001 and 2002, and 2002 and 2003 populations were the most similar (è = 0.18 and è = 0.11; p<0.001 respectively), while the 2004 and 2002 populations differentiated the most (è = 0.51) (P<0.001) (Table 6).

Table 5. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among *Moniliophthora perniciosa* populations

Population	2001	2002	2003	2004
2001	***	0.96	0.92	0.73
2002	0.04	***	0.97	0.74
2003	0.08	0.03	***	0.77
2004	0.31	0.30	0.26	***

Table 6. Estimate the temporal Population differentiation between *Moniliophthora. pernicosa* populations based an Weir and Cockerham's Theta (è)

Population	2002	2003	2004
2001	0.18***	0.28***	0.36***
2002		0.11***	0.51***
2003			0.42*

* p < 0.1 ** p < 0.05 *** p < 0.001

Discussion

The purpose of this study was to determine the level of temporal genetic diversity on *M. perniciosa* from 2001 to 2004 using RAPD markers. Multiple correspondence analysis and AMOVA tests of RAPD profiles assigned isolates into two main groups defined by the year of collection; interestingly, the 2004 population was genetically more uniform than the other populations. This result is not surprising, since RAPD amplify the genomic DNA randomly and it is therefore possible that some RAPD markers have a selective value (e.g., if they represent DNA fragments within coding regions that are under selection, or if they are linked with such regions) while others are neutral. Therefore, RAPD markers provide useful information about the genome because they detect length polymorphisms arising from base sequence changes, insertions, deletions, substitutions or inversions, either at or between the priming sites (Williams et al., 1990; Grajal-Martin et al., 1993; Kolmer et al., 1995; Cooke et al., 1996). Furthermore, probable causes of these variations can be inferred from RAPD data.

Dendrograms, analysis of molecular variance and Nei's genetic distance revealed the existence of genetic differentiation in sample among isolates from the different years. Analysis of molecular variance showed that genetic variation was higher within a year (68%) than among years (32%). These results are consistent with previous observations that greater genetic variability was recorded between individual farms within a state (Gramacho et al., 2003a) and between states and within countries (Ploetz et al., 2005, Moreira, 2006, Solis, 2005).

Population differentiation was significantly different among the populations surveyed from different years, mostly between populations collected in 2004 and the others ones. The differences in frequency of some RAPD markers between the populations could have been maintained by selections, migrations, genetic drift, or by a combination of these processes. Observations using molecular markers (RAPD and SSRs) in studies of 40 isolates (strains) of the fungus, collected from brooms from five resistant and two susceptible genotypes, showed a clear genetic differentiation between fungal isolates from resistant clones and those from susceptible ones (Pires, 2003, Gramacho et al., 2003 a,b,c). Also Pires (2003) in studying 65 genotypes of cacao with lesser level of witches´ broom severity, in the CEPEC germplasm collection over two periods 1995-1999 and 2002-2004, showed a significant increase in disease severity in progenies of the main source of resistance (Scavina), as well as in varieties recommended to farmers and derived from that source. Both elements considered together show that the fungus is adapting to and overcoming the Scavina's resistance.

Considering the increase in susceptibility in Scavina descendants, associated to the distinction of isolates from susceptible/resistant clones (Pires, 2003), it can be concluded that the temporal variability found might be the result of the increase in frequency of strains capable to overcoming the resistance of Scavina. In overcame of scavina's resistance, fact this has been already observed in some farms in Bahia (Pires, 2003). Nevertheless, pathogenicity tests using isolates from different years are required to confirm this hypothesis.

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