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Development of molecular markers based on retrotransposons for the analysis of genetic variability in *Moniliophthora perniciosa*

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Abstract *Moniliophthora perniciosa* is a fungus that causes witches' broom disease (WBD) in the cacao tree (*Theobroma cacao*). The *M. perniciosa* genome contains different transposable elements; this prompted an evaluation of the use of its retrotransposons as molecular markers for population studies. The inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) techniques were used to study the variability of 70 *M. perniciosa* isolates from different geographic origins and biotypes. A total of 43 loci was amplified. Cluster analysis of different geographical regions of C biotype revealed two large groups in the state of Bahia, Brazil. Techniques using retrotransposon-based molecular markers showed advantages over previously used molecular techniques for the study of genetic variability in *M. perniciosa*.

Keywords Retrotransposon · *Moniliophthora perniciosa* · Genetic variability · IRAP · REMAP

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Introduction

The cacao tree (*Theobroma cacao* L.) is affected by several destructive diseases that result in major economic losses. Worldwide, brown rot caused by *Phytophthora* spp. is the most common disease in cacao because it occurs in all cacao-producing countries. However, in Brazil, witches' broom disease (WBD) caused by *Moniliophthora* (= *Crinipellis*) *perniciosa* (Stahel) is the most devastating disease of cacao (Aime and Phillips-Mora 2005), causing losses of up to 90 % of cacao production.

WBD was first reported in Surinam in 1895, and although there had been an endemic form of WBD in the Amazon region since the 19th century, it was only described in southern Bahia, the main cacao-producing region of Brazil, in 1989 (Pereira et al. 1989). Brazil used to be the second-largest exporter of cacao in the world but since the spread of WBD to the main areas

of cacao production, Brazil has become an importer of this product causing serious socio-economic problems in the affected regions (SUFRAMA 2003).

After nearly 20 years of experience with WBD, Brazilian cacao farming began to show signs of recovery, due to the use of resistant cultivars. However, most of these cultivars descended from a single source of resistance, the Scavina-6 clone. The narrow genetic base of the new cultivars represents a risk to the control of WBD achieved thus far. Depending on the genetic variability of the pathogen population, resistance may be supplanted and production lost again. Therefore, it is necessary to study and monitor the variability of *M. perniciosa* at the sites of genetic improvement programs and at the sites where resistant cultivars will be introduced (Neto et al. 2005).

Physiological, cultural and genetic variations among *M. perniciosa* isolates have been detected between individuals of different origins. This variability can be detected in mycelial growth in culture media, types of reaction to some biochemical tests, somatic compatibility and pathogenicity to cacao and other hosts (Oliveira and Luz 2005). The wide host range of *M. perniciosa* has led many researchers to utilize a classification system based on original host: the C biotype infects *Theobroma* and *Herrania* species (Malvaceae), the S biotype affects several members of the Solanaceae, the L biotype affects *Arrabidaea* spp (Bignoniaceae), the H biotype infects *Heteropterys acutifolia* (Malpighiaceae) and the B biotype infects *Bixa orellana* (Bixaceae). Biotypes C and S cause the characteristic symptoms of WBD in their hosts (Bastos and Anderbrahn 1986; Griffith and Hedger 1994a; Griffith et al. 2003).

Analyses of *M. perniciosa* isolates using restriction fragment length polymorphism (RFLP) markers of mitochondrial DNA, and DNA sequences of the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions of the ribosomal DNA region have revealed high genetic variability (Griffith and Hedger 1994b). Other techniques, such as random amplification of polymorphic DNA (RAPD) (Anderbrhan and Furtek 1994), enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) (De Arruda et al. 2003) and molecular karyotyping (Rincones et al. 2003) have also been used. Furthermore, microsatellite markers have been developed by Gramacho et al. (2007) and Silva et al. (2008). In general, the variability among biotype C isolates from the Amazon is greater than that among isolates from Bahia, which supports the hypothesized Amazonic origin of the individuals introduced in southern Bahia (De Arruda et al. 2003).

One of the processes known to generate variability in phytopathogenic fungi is the activity of transposable elements (Daboussi and Capy 2003; Ikeda et al. 2001). Due to their abundance, mode of amplification and insertion into the genome, retrotransposons have characteristics that can be used to discriminate between species or genotypes (Hansen and Heslop-Harrison 2004). Each transposition event generates an insertion polymorphism that can be identified using a range of molecular techniques. Transposons present in numerous copies can generate hundreds or thousands of tags. The presence or absence of a transposon at a given site can be used as a molecular tag for genotype fingerprinting, linkage mapping and the study of diversity (Grzebeleus 2006).

The structure and replication strategy of retrotransposons give them advantages as molecular markers. They cause large insertions by their transpositional activity and they contain conserved domains from which PCR primers can be designed (Kalendar et al. 2011). Much of the knowledge about genome complexity has resulted from the study of transposons in fungi (Novikova et al. 2007; Bouvet et al. 2008; Crouch et al. 2008). Currently, new fingerprinting techniques based on retrotransposons have been used to study genetic diversity, such as sequence-specific amplified polymorphism (S-SAP) (Waugh et al. 1997), inter-retrotransposon amplified polymorphism (IRAP) (Kalendar et al. 1999), retrotransposon-microsatellite amplified polymorphism (REMAP) (Kalendar et al. 1999), inter-MITE polymorphism (IMP) (Chang et al. 2001) and retrotransposon-based insertion polymorphism (RBIP) (Flavell et al. 1998), among others. Among these techniques, the three most frequently used as tools for diversity studies are S-SAP (Waugh et al. 1997), IRAP and REMAP (Kalendar et al. 1999). In fungi, IRAP and REMAP can be used as molecular markers to describe the profile of a population (Murata et al. 2008), identify specific races of pathogens (Pasquali et al. 2007) and to analyze population diversity (Chadha and Gopalakrishna 2005).

Molecular markers based on transposable elements are advantageous in detecting large changes in the genome. Moreover, restriction fragment length polymorphism (RFLP), single nucleotide polymorphism (SNP) and analyses based on amplified fragment length polymorphism (AFLP) detect single nucleotide changes with very high reversion frequency. Molecular markers based on microsatellites generally detect the gain or loss of 20 nucleotides. Microsatellite alelles differ in the number of simple sequence repeats (SSR) they contain and, the same way as the single-nucleotide changes, suffer from homoplasy because the number of SSRs can increase or decrease in a reversible way, making it impossible to distinguish ancestral and derived states (Kalendar and Schulman 2006).

With the completed sequencing of the *M. perniciosa* genome, supported by the *M. perniciosa* Genome Project (http://www.lge.ibi.unicamp.br), it is possible to find the sequences of representatives of different groups of transposable elements (Mondego et al. 2008). One of these elements has been termed *MpSaci* and belongs to the group *Gypsy/Ty-3*, the main group of transposable elements found in phytopathogenic fungi (Pereira 2005; Pereira et al. 2007).

The aim of this study was to test the feasibility of using different polymorphic tags related to retrotransposon-based molecular markers for the analysis of *M. perniciosa* genetic variability. For this purpose, we used the banding profiles generated by two molecular techniques, IRAP and REMAP (Kalendar et al. 1999).

Materials and methods

Source of *Moniliophthora perniciosa* isolates and total DNA extraction

A total of 36 *M. perniciosa* isolates from the culture collection of the Universidade Federal de Viçosa (UFV) and 34 isolates from the Universidade Federal do Recôncavo Baiano (UFRB) culture collection were used. The isolates were collected from different regions of Brazil between 2001 and 2003 (Table 1).

Total DNA extraction from isolates was performed using the UltraClean™ Microbial DNA Isolation Kit from MO BIO Laboratories, Inc. The quality and quantity of the total DNA were assessed with a spectrophotometer and confirmed by electrophoresis in a 0.8 % agarose gel. The purified DNA was used immediately or stored at −20 °C.

IRAP and REMAP

The IRAP and REMAP techniques (Kalendar et al. 1999) were used to obtain different polymorphic markers for the C, S and L biotypes of *M. perniciosa*. For both techniques, primers were designed using Primer 3 (Rozen and Skaletsky 2000) for the conserved regions of the long terminal repeats (LTRs) of the *MpSaci* retrotransposon. For IRAP, two primers complementary to the *MpSaci* LTR region were used. For REMAP, primers complementary to the LTR were used in combination with primers that annealed to a simple sequence repeat (SSR); therefore, it was possible to detect transposon insertions near the SSRs. The microsatellite primers were obtained from Gramacho et al. (2007). The primer-pair combinations that were used in REMAP were: CPLTR1/F and MS2; CPLTR1/R and MS4; CPLTR2/R and MS1 (Table 2).

The IRAP and REMAP reactions were performed in a reaction volume of 25 μl containing 1X Thermophilic DNA Polymerase Buffer (Promega), 2.0 μmol/l $MgCl_2$ (Promega), 100 μmol/l of each dNTP, 0.2 μmol/l of each primer, 40 ng of DNA and 1 unit of Taq DNA polymerase (Promega). PCRs were performed in a PTC-100 thermal cycler (MJ Research) programmed to undergo an initial denaturation step of 2 min at 94 °C, 6 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C. Twenty-four cycles were added to these 6 initial cycles, with 30 s added to the extension time (at 72 °C) every 6 cycles. The final extension step was 10 min at 72 °C.

Analyses of the PCR amplicons

Differences in the patterns of amplicons from the isolates were visually assessed on a two-percent agarose gel. The bands corresponding to each combination of primers used in the selective amplification were identified by the numbers 1 (presence) and 0 (absence). Bands common to all of the isolates were incorporated into the analysis. The reproducibility of the DNA band profile was tested by repeating the PCR with each of the selected primers. Only reproducible bands were considered for analysis.

Table 1 *Moniliophthora perniciosa* isolates used in this study

Isolates	Identification in the collection	Biotype	Collection Site	Host	Institution
1	DOA 100	C	–	*Theobroma cacao*	–
2	Santo Amaro	C	Santo Amaro/ BA	*Theobroma cacao*	UNB
3	Belmont	C	Belmont/BA	*Theobroma cacao*	UFB
4	ALF 276	C	Itabuna/BA	*Theobroma cacao*	FAC
5	ALF 42	C	Itabuna/BA	*Theobroma cacao*	FAC
6	ALF 301	C	–	*Theobroma cacao*	FAC
7	ALF 305	C	–	*Theobroma cacao*	FAC
8	ALF 278	C	Itabuna/BA	*Theobroma cacao*	FAC
9	SABA	C	Santo Amaro/BA	*Theobroma cacao*	UFLA
10	Ilhéus	C	IIhéus/BA	*Theobroma cacao*	UFB
11	FA 281	C	Aiquara/BA	*Theobroma cacao*	FAC
12	FA 553	C	Ilhéus/BA	*Theobroma cacao*	FAC
13	FA 317	C	Ilhéus/BA	*Theobroma cacao*	FAC
14	FA 42	C	Itabuna/BA	*Theobroma cacao*	FAC
15	CP02	C	Itabuna/BA	*Theobroma cacao*	UESC
16	1734 D-W	C	Gandú/BA	*Theobroma cacao*	CEPLAC
17	676 G D-W	C	Floresta Azul/BA	*Theobroma cacao*	CEPLAC
18	896 FD-W	C	Jaquaquara/BA	*Theobroma cacao*	CEPLAC
19	606 G D-W	C	Itabuna/BA	*Theobroma cacao*	CEPLAC
20	948 F D-W	C	–	*Theobroma cacao*	CEPLAC
21	ALF1577	C	Mucuri/BA	*Theobroma cacao*	FAC
22	ALF 1578	C	Mucuri/BA	*Theobroma cacao*	FAC
23	ALF 1579	C	Mucuri/BA	*Theobroma cacao*	FAC
24	ALF 321	C	Ilhéus/BA	*Theobroma cacao*	FAC
25	ALF 1140	C	Itabuna/BA	*Theobroma cacao*	FAC
26	FA 277	C	Itabuna/BA	*Theobroma cacao*	FAC
27	FA 563	C	Ilhéus/BA	*Theobroma cacao*	FAC
28	DOA102	C	–	*Theobroma cacao*	–
29	ALF 110	C	–	*Theobroma cacao*	FAC
30	MP 227	C	–	*Theobroma cacao*	UFRB
31	MP 1181	C	Gandú/BA	*Theobroma cacao*	UFRB
32	MP 1105	C	–	*Theobroma cacao*	UFRB
33	MP 1102	C	Itajuipé/BA	*Theobroma cacao*	UFRB
34	MP 1158	C	Ibirapitanga/BA	*Theobroma cacao*	UFRB
35	MP 553	C	Itajuipé/BA	*Theobroma cacao*	UFRB
36	MP 1160	C	Itajuipé/BA	*Theobroma cacao*	UFRB
37	MP 310	C	Itagí/BA	*Theobroma cacao*	UFRB
38	MP 1138	C	Itajuipé/BA	*Theobroma cacao*	UFRB
39	MP 307	C	–	*Theobroma cacao*	UFRB
40	MP 1170	C	Ilhéus/BA	*Theobroma cacao*	UFRB
41	MP 1146	C	Itajuipé/BA	*Theobroma cacao*	UFRB
42	MP 1180	C	Gandú/BA	*Theobroma cacao*	UFRB
43	MP 1172	C	Ilhéus/BA	*Theobroma cacao*	UFRB
44	MP 1159	C	Itajuipé/BA	*Theobroma cacao*	UFRB

Table 1 (continued)

Isolates	Identification in the collection	Biotype	Collection Site	Host	Institution
45	MP 1140	C	Itajuipé/BA	*Theobroma cacao*	UFRB
46	MP 1185	C	Gandú/BA	*Theobroma cacao*	UFRB
47	MP 1096	C	Itajuipé/BA	*Theobroma cacao*	UFRB
48	MP 1183	C	Gandú/BA	*Theobroma cacao*	UFRB
49	MP 314	C	Itajuipé/BA	*Theobroma cacao*	UFRB
50	MP 316	C	Pau-Brasil/BA	*Theobroma cacao*	UFRB
51	MP 179	C	–	*Theobroma cacao*	UFRB
52	MP 1178	C	Ipiaú/BA	*Theobroma cacao*	UFRB
53	MP 1191	C	Itajuipé/BA	*Theobroma cacao*	UFRB
54	MP 1173	C	Ilhéus/BA	*Theobroma cacao*	UFRB
55	MP 1169	C	Ilhéus/BA	*Theobroma cacao*	UFRB
56	MP 285	C	Ubaitaba/BA	*Theobroma cacao*	UFRB
57	MP 1157	C	Itajuipé/BA	*Theobroma cacao*	UFRB
58	MP 320	C	Buerarema/BA	*Theobroma cacao*	UFRB
59	MP 277	C	Itajuipé/BA	*Theobroma cacao*	UFRB
60	MP 283	C	Lomanto Jr/ BA	*Theobroma cacao*	UFRB
61	MP 1193	C	Ouro Preto/RO	*Theobroma cacao*	UFRB
62	MP 1194	C	Jarú/RO	*Theobroma cacao*	UFRB
63	FA 551	C	Tabatinga/AM	*Theobroma cacao*	FAC
64	ALF 551	C	Tabatinga/AM	*Theobroma cacao*	FAC
65	LA17	L	San Carlos/Equador Carlos/Equador	*Arrabidaea verrucosa*	UW
66	SCL4	L	Pichilingue/Equador	*Arrabidaea verrucosa*	UW
67	FA 607	S	Coimbra/MG	*Solanum lycocarpum*	FAC
68	FA 609	S	Poços de Caldas/MG	*Solanum* sp	FAC
69	RWB 500	S	Rio Pomba/MG	*Solanum cernum*	UFV
70	RWB 551	S	Juiz de Fora/ MG	*Solanum lycocarpum*	UFV

FAC Fazenda Almirante Cacau, Itajuipé, Bahia, Brazil; *UESC* Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil; *UNB* Universidade de Brasília, Brasília, Distrito Federal, Brazil,; *UFLA* Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil; *UFRB* Universidade Federal do Recôncavo Baiano, Bahia, Brazil; *CEPLAC* Comissão Executiva do Plano da Lavoura do Cacau, Ilhéus, Bahia, Brazil; *UW* University of Wales, Aberysstwyth, U.K.—means unproven origin

The number of loci amplified, rate of polymorphism and number of haplotypes and singletons were calculated by the POPGENE program (Yeh et al. 1999) using all isolates (70 isolates). A dendrogram

Table 2 Primers used in IRAP and REMAP analyses of *Moniliophthora perniciosa* isolates

Technique	Identification	Sequence (5′–3′)
IRAP	CPLTR1/R	GTGCAAGCGACACACAAACT
	CPLTR2/R	GCAGTGTTTCTACAGCGAAG
REMAP	CPLTR1/F	CTCGCTTCTTCAGCTTGACC
	CPLTR1/R	GTGCAAGCGACACACAAACT
	CPLTR2/R	GCAGTGTTTCTACAGCGAAG
	MS1	GGCGGCGGCGGCGGCGGCGGCT
	MS2	ATGATGATGATGATGATGATGG
	MS4	CACACACACACACACACACAG

(bootstrap with 1,000 replicates) for isolates of the biotype C with proven origin was constructed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) hierarchical clustering algorithm using the R package (R Development Core Team 2007). The Arlequin program (Excoffier et al. 2006) was used to calculate the analysis of molecular variance (AMOVA).

The isolates of the biotype C with proven origin were grouped into 21 populations based on the municipality of origin (collection site) (Fig. 1). We calculated: total genetic diversity (H_T) and gene diversity within populations (H_S). The parameters for the measure of genetic diversity were calculated using the following formulas for Nei diversity (Nei 1973):

(i) $H_S = 1 - \sum_{i=1}^{h} x_{ij}^2$
(ii) $H_T = 1 - \sum_{i=1}^{k} x_{ij}^{-2}$

where h is the number of alleles and x_{ij} is the frequency of allele i in population j. H_T is the total genetic diversity within all populations, where k is the number of populations and x_{ij}^{-2} is the mean of the frequencies of i alleles in the populations.

Genotypic diversity (SH) was estimated by the Shannon-Wiener index, H' (Hill 1973), calculated as $SI_j = \sum_{i=1}^{k} p_{ij} X \ln p_{ij}$, where p_{ij} is the frequency of haplotype i in population j. For these analyses, we used the POPGENE program (Yeh et al. 1999). Evidence of random mating in *M. perniciosa* populations of the biotype C was inferred by the index of association (I_A) (Maynard-Smith et al. 1993), using the Multilocus 1.3 program (Agapow and Burt 2001).

Results

A total of 43 loci was amplified, 14 loci by IRAP and 29 by REMAP. 21 loci were clearly amplified for the biotype C. The number of PCR products obtained was specific for each primer set. The set of primers used was also able to generate amplicons in the biotypes L and S (Fig. 2c). Based on the combined electrophoretic patterns of IRAP (Fig. 2a) and REMAP (Fig. 2b), we determined that 20 loci were polymorphic for the biotype C. The combination of the CPLTR1/F and

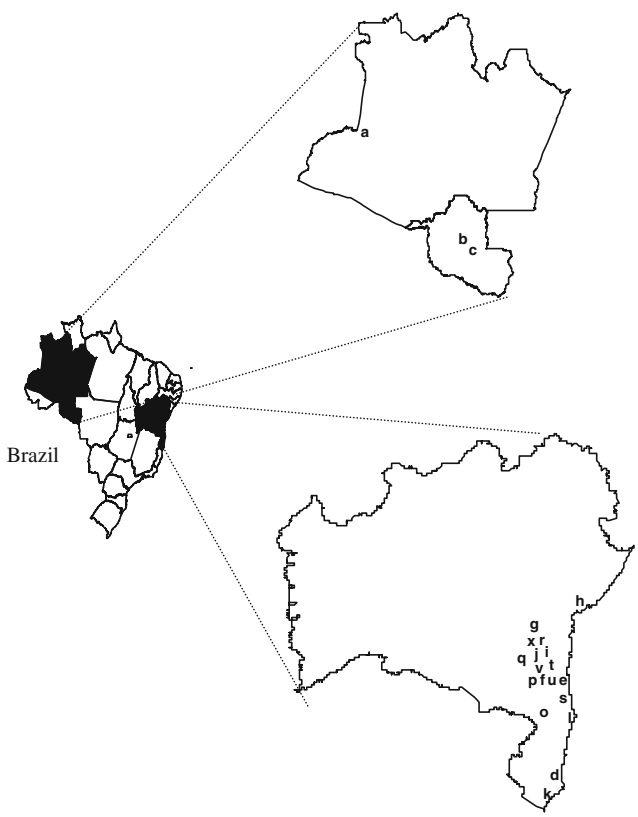

Fig. 1 Geographic origin and number of isolates (n) sampled by region (collection site)

Amazonas - AM
a. Tabatinga, n= 2

Rondônia - RO
b. Jarú, n= 1
c. Ouro Preto, n= 1

Bahia - BA
d. Prado, n= 1
e. Ilhéus, n= 8
f. Itabuna, n= 8
g. Jaquaquara, n= 1
h. Santo Amaro, n= 2
i. Ibirapitanga, n= 1
j. Ipiaú, n= 1
k. Mucuri, n= 3
l. Belmont, n= 1
o. Pau-Brasil, n= 1
p. Itajuipé, n= 12
q. Itají, n= 1
r. Gandu, n= 5
s. Buerarem, n= 1
t. Ubaitaba, n= 1
u. Lomanto Jr, n= 1
v. Floresta azul, n= 1
x. Aiquara, n= 1

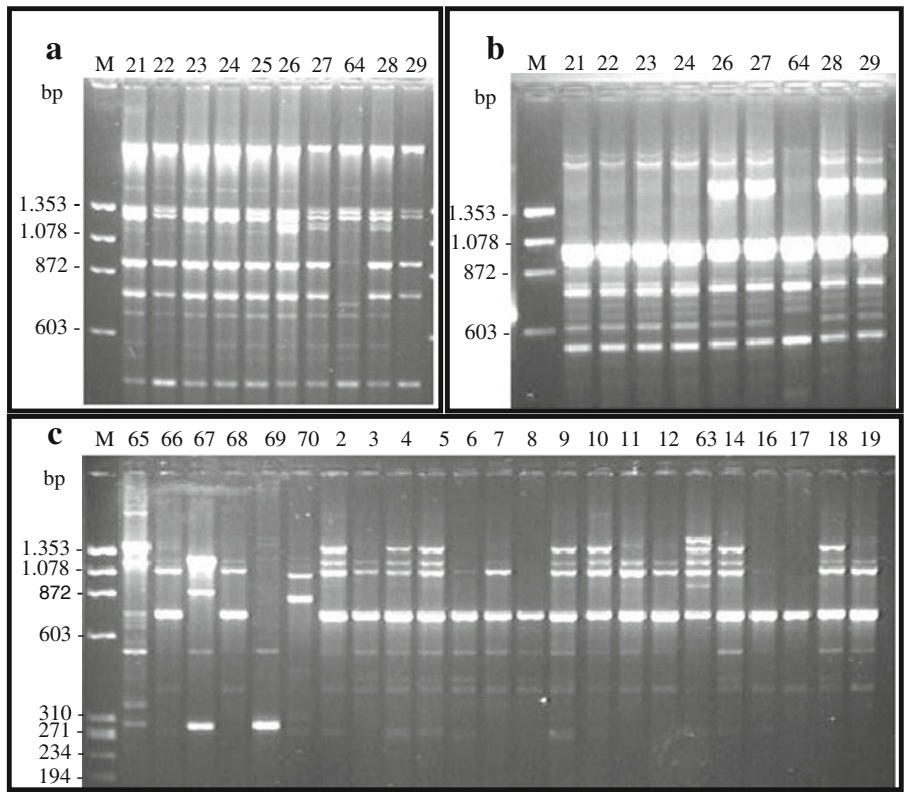

Fig. 2 Electrophoretic profile of *Moniliophthora perniciosa* DNA generated by IRAP (**a**) and REMAP (**b** and **c**). **b** the results of REMAP using the CPLTR2/R and MS1 primers; **c** the results of REMAP using the CPLTR1/F and MS2 primers. M indicates the HaeIII ΦX174 size marker. Numbers indicate the isolates in Table 1

MS2 primers generated the most pronounced rate of polymorphism; 80 % of the bands amplified by this primer combination were polymorphic (Fig. 2c). Altogether, 24 haplotypes were found in biotype C. Among the haplotypes sampled in Bahia, 14 were singletons.

The total diversity (H_T=0.06), the diversity within populations (H_S=0.01), and the genotypic diversity (SH=0.1) were low among the populations subdivided by geographical origin (collection site). Considering the isolates of the biotype C, the index of association (I_A) was greater than zero (I_A=6.40), and the hypothesis of random mating was rejected (P<0.001). An AMOVA was used to ascertain the variation found among and within populations subdivided by different geographic origins (collection site) that possessed haplotype numbers (n) ranging from 2 to 12 (Fig. 1). It was observed that 73.7 % of the genetic variation found between isolates is within local populations (Table 3).

Cluster analysis by collection site revealed the groups G1, G2, and G3 with bootstrap support of 70, 72, and 88 %, respectively. The first (G1) included the populations from Bahia and Rondônia (Ouro Preto) and the second (G2) consisted of Bahia (Fig. 3). The clusters G1 and G2 contain most of the collecting sites analyzed (16 of 20). However, in group G1 isolates from some sites have high genetic similarity as

Table 3 Analysis of molecular variance (AMOVA): variation attributed to differences among and within populations subdivided by different geographic origins (collection site) of *Moniliophthora perniciosa* biotype C

Variation Sources	d.f.	Sum of squares	Variance Components	Variation (%)
Among populations	5	16.6	0.4Va	26.3
Within Populations	32	34	1.0Vb	73.7
Total	37	50.6	1.4	100

df degree of freedom

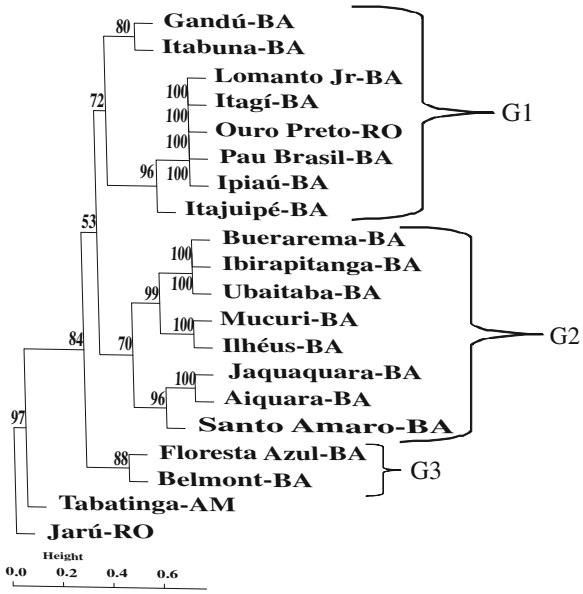

Fig. 3 Dendrogram (UPGMA) based on the analysis of IRAP and REMAP markers for populations of the C biotype. Populations of *M. perniciosa* were divided by collection site. G1, G2 and G3 represent three groups found in the cluster analysis. The numbers above each node indicate the percentage of times in which each branch appeared in a bootstrap analysis with 1,000 replicates

observed in Itagí-BA, Lomanto Jr-BA, Ipiaú-BA, Pau Brasil-BA and Ouro Preto-RO. In the third cluster (G3), the isolates belonging to Floresta Azul-BA and Belmont-BA have a high genetic diversity compared to other isolates belonging to Bahia. The Jarú population in Rondônia and the Tabatinga population in Amazonas appear with significant genetic divergence from the three groups (Fig. 3).

Discussion

Due to their ability to move within the host genome, transposable elements may influence the adaptation and evolutionary potential of their hosts through events such as insertion mutation, gene disruption, enhancement of gene expression and chromosomal rearrangements (Huan-Van et al. 2005). In this study, the IRAP and REMAP markers were able to differentiate between *M. perniciosa* isolates of the biotype C. These markers were also able to amplify the isolates of the biotypes L and S. As the sample was small these biotypes were included for comparative purposes. Thus, IRAP and REMAP can be used in future analyses of genetic diversity between and among biotypes of *M. perniciosa*. This is the first report on the use of these techniques in the study of *M. perniciosa* genetic variability. In our study, IRAP and REMAP identified 14 % more polymorphisms than were found by Anderbrhan and Furtek (1994) with RAPD markers, and we identified nine more haplotypes than were found by Ploetz et al 2005 (who used amplified fragment length polymorphism PCR [AFLP] for the analysis of *M. perniciosa* molecular diversity). Primers for SSR markers in *M. perniciosa* have been described (Gramacho et al. 2007; Silva et al. 2008). However, we identified 20 polymorphic loci for the biotype C, while Gramacho et al. (2007) and Silva et al. (2008) identified only 12 and nine loci polymorphic, respectively. Furthermore, homoplasy may occur in microsatellite loci due to different forward and backward mutations, which may cause underestimation of the genetic divergence. Another very common problem in microsatellite analysis is the stutter bands. These artifacts occur by slippage during DNA amplification by PCR (Kumar et al. 2009). Thus, we found a higher haplotype number than in other studies using different techniques, which shows that molecular techniques based on retrotransposons identify sufficient numbers of polymorphisms to differentiate between *M. perniciosa* populations and can be used for genetic studies of populations within this fungus.

Both techniques produced satisfactory numbers of bands for population analysis; however, the technique REMAP, particularly the primer combination CPLTR1/F and MS2, generated a large number of polymorphic bands. Generally, the REMAP technique is able to distinguish more closely related individuals better than IRAP by taking into consideration the design of primers for microsatellite regions that are known to be present in large numbers in the genome (Kalendar et al. 1999).

The combined data analyses, IRAP and REMAP, revealed intraspecific variation of *M. perniciosa* originating from *Theobroma cacao* from different geographical regions. In the dendrogram generated from our data (Fig. 3), two main populations were identified in the state of Bahia (clusters G1 and G2); the isolates from Ouro Preto (Rondônia) were grouped in the

cluster G1, suggesting that there was at least one separate introduction of *M. perniciosa* into the state of Bahia. However, the isolates belonging to Floresta Azul-BA and Belmont-BA have a high genetic diversity compared to other isolates belonging to Bahia, forming a third cluster (G3). Inclusion of the isolates from Floresta Azul-BA and Belmont-BA in a separate cluster may indicate a second introduction of *M. perniciosa* in to Bahia, whose origin could not be identified. These results are consistent with those found by Andebrhan et al. (1999).

The low genetic diversity detected confirms the results of Gramacho et al. (2007) and Silva et al. (2008). This is probably the result of the homothallic mating strategy (Silva et al. 2008). Similarly, the genetic diversity was low among populations subdivided by geographical origin. This means that there are many alleles that are shared among different collection sites. The AMOVA values for populations subdivided by geographical region demonstrate that isolate diversity was higher within collection site than among collection sites; an estimated 73.7 % of the total diversity was found to occur within each collection site. This is mainly due to the existence of several singletons within each collection site. The high value of the index of association (I_A) found within the biotype C shows that they are not in gametic equilibrium, and therefore deviate from random mating.

The IRAP and REMAP techniques have the following advantages over other techniques already used for *M. perniciosa* population analysis: (i) versatility, as they allow for the combination of various primers that anneal to conserved regions of retrotransposons (IRAP) or microsatellites (REMAP); (ii) high reproducibility by using specific primers; (iii) easy handling because the techniques involve straightforward PCR; and (iv) lower cost and less labour than the AFLP and the microsatellite techniques, because IRAP and REMAP use agarose gels and inexpensive reagents but generate several different polymorphic markers to be used in studies of genetic variability. However, IRAP and REMAP have some disadvantages. These markers require prior knowledge of the genome to design primers, the amplifications are dependent on the number of copies of retrotransposons, and they are considered dominant markers. These molecular marker techniques based on transposons will allow a better understanding of *M. perniciosa* variability, which is of fundamental importance to disease control and success of the cacao breeding programs for resistance to this pathogen.

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References

Agapow, P.-M., & Burt, A. (2001). Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes, 1*, 101–102.

Aime, M. C., & Phillips-Mora, W. (2005). The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. *Mycology, 97*, 1012–1022.

Andebrhan, T., Figueira, A., Yamada, M. M., Cascardo, J., & Furtek, D. B. (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.

Anderbrhan, T., & Furtek, D. B. (1994). Random amplified polymorphism DNA (RAPD) analysis of *Crinipellis perniciosa* isolates from different hosts. *Plant Pathology, 43*, 1020–1027.

Bastos, C. N., & Anderbrahn, T. (1986). Urucum (*Bixa orellana*): nova espécie da Vassoura-de-Bruxa (*Crinipellis perniciosa*) do cacaueiro. *Fitopatologia Brasileira, 13*, 963–965.

Bouvet, G. F., Jacobi, V., Plourde, K. V., & Bernier, L. (2008). Stress-induced mobility of OPHO1 and OPHO2, DNA transposons of the Dutch elm disease fungi. *Fungal Genetics and Biology, 45*, 565–578.

Chadha, S., & Gopalakrishna, T. (2005). Retrotransposon-microsatellite amplified polymorphism (REMAP) markers for genetic diversity assessment of the rice blast pathogen (*Magnaporthe grisea*). *Genome, 48*, 943–945.

Chang, R.-Y., O'donoughue, L. S., & Bureau, T. E. (2001). Inter-MITE polymorphisms (IMP): a high throughput transposon-based genome mapping and fingerprinting approach. *Theoretical and Applied Genetics, 102*, 773–781.

Crouch, J. A., Glasheen, B. M., Giunta, M. A., Clarke, B. B., & Hillman, B. I. (2008). The evolution of transposon repeat-induced point mutation in the genome of *Colletotrichum cereale*: reconciling sex, recombination and homoplasy in an "asexual" pathogen. *Fungal Genetics and Biology, 45*, 190–206.

Daboussi, M. J., & Capy, P. (2003). Transposable elements in filamentous fungi. *Annual Review of Microbiology, 57*, 275–299.

De Arruda, M. C. C., Miller, R. N. G., Ferreira, M. A. S. V., & Felipe, M. S. S. (2003). Comparison of *Crinipellis perniciosa* isolates from Brazil by ERIC repetitive element sequence-based PCR genome fingerprinting. *Plant Pathology, 52*, 236–244.

Excoffier, L., Laval, G., & Schneider, S. (2006). Arlequin ver 3.01. An integrated software package for population genetics data analysis. Computational and molecular population

genetics Lab (CMPG). Institute of Zoology. University of Berne.

Flavell, A. J., Knox, M. R., Pearce, S. R., & Ellis, T. H. N. (1998). Retrotransposon-based insertion polymorphism (RBIP) for high throughput marker analysis. *The Plant Journal, 16*, 643–650.

Gramacho, K. P., Risterucci, A. M., Lanaud, C., Lima, L. S., & Lopes, U. V. (2007). Characterization of microsatellites in the fungal plant pathogen *Crinipellis perniciosa*. *Molecular Ecology Notes, 7*, 153–155.

Griffith, G. W., & Hedger, J. N. (1994a). The breeding biology of biotypes of the witches' broom pathogen of cocoa, *Crinipellis perniciosa*. *Heredity, 71*, 278–289.

Griffith, G. W., & Hedger, J. N. (1994b). Spatial distribution of mycelia of the liana (L-) biotype of the agaric *Crinipellis perniciosa* (Stahel) Singer in tropical forest. *New Phytologist, 127*, 243–259.

Griffith, G. M., Nicholson, J. N., Nenninger, A., Birch, R. N., & Hedger, J. N. (2003). Witches' brooms and frosty pods: Two major pathogens of cacao. *New Zealand Journal of Botany, 41*, 423–435.

Grzebeleus, D. (2006). Transposon insertion polymorphism as a new source of molecular markers. *Journal of Fruit and Ornamental Plant Research, 14*, 21–29.

Hansen, C. N., & Heslop-Harrison, J. S. (2004). Sequences and phylogenies of plant pararetroviruses, viruses and transposable elements. *Advances in Botanical Research, 41*, 165–193.

Hill, M. O. (1973). Diversity and evenness: A unifying notation and its consequences. *Ecology, 54*, 427–432.

Huan-Van, A., Ruzic, L., Maisonhaute, C., & Capy, P. (2005). Abundance, distribution and dynamics of retrotransposable elements and transposons: similarities and differences. *Cytogenetic and Genome Research, 110*, 426–440.

Ikeda, K., Nakayashiki, H., Takagi, M., Tosa, Y., & Mayama, S. (2001). Heat shock, cooper sulfate and oxidative stress active the retrotransposon MAGGY resident in the plant pathogenic fungus *Magnaporthe grisea*. *Molecular Genetics and Genomics, 266*, 318–325.

Kalendar, R., & Schulman, A. H. (2006). IRAP and REMAP for retrotransposon-based genotyping and fingerprint. *Nature Protocols, 1*, 24782484.

Kalendar, R., Grob, T., Regina, M., Suoniemi, A., & Schulman, A. H. (1999). IRAP and REMAP: Two new retrotransposon-based DNA fingerprinting techniques. *Theoretical and Applied Genetics, 98*, 704–711.

Kalendar, R., Flavell, A. J., Ellis, T. H. N., Stakste, T., Moisy, C., & Schulman, A. H. (2011). Analysis of plant diversity with retrotransposon-based molecular markers. *Heredity, 106*, 520–530.

Kumar, P., Gupta, V. K., Misra, A. K., Modi, D. R., & Pandey, B. K. (2009). Potential of molecular markers in plant biotechnology. *Plant Omics Journal, 2*, 141–162.

Maynard-Smith, L., Smith, N. H., O'rourke, M., & Spratt, B. G. (1993). How clonal are bacteria? *Proceedings of the National Academy of Sciences, 90*, 4384–4388.

Mondego, J. M. C., Carazzolle, M. F., Costa, G. G. L., Formighieri, E. F., Parizzi, L. P., Rincones, J., et al. (2008). A genome survey of *Moniliophthora perniciosa* gives new insights into witches' broom disease of cacao. *BMC Genomics, 9*, 548.

Murata, H., Babasaki, K., Saegusa, T., Takemoto, K., Yamada, A., & Ohta, A. (2008). Traceability of Asian Matsutake, specialty mushrooms produced by the ectomycorrhizal basideomycetes *Tricholoma matsutake*, on the basis of retroelement-based DNA markers. *Applied and Environmental Microbiology, 74*, 2023–2031.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA, 70*, 3321–3323.

Neto, A. D., Corrêa, R. X., Monteiro, W. R., Luz, E. D. M. N., Gramacho, K. P., & Lopes, U. V. (2005). Characterization of a cocoa population for mapping of genes of resistance to Witches' Broom and *Phytophthora* pod rot. *Fitopatolologia Brasileira, 30*, 380–386.

Novikova, O. S., Fet, V., & Blinov, A. G. (2007). Homology-dependent inactivation of LTR retrotransposons in *Aspergillus fumigatus* and *A. nidulans* genome. *Molecular Biology, 41*, 886–893.

Oliveira, M. L., & Luz, E. D. M. N. (2005). *Identificação e manejo das principais doenças do cacaueiro no Brasil*. Ilhéus: CEPLAC/CEPEC/SEFIT. 132p.

Pasquali, M., Dematheis, F., Gullino, M. L., & Garibaldi, A. (2007). Identification of race 1 of *Fusarium oxysporum* f. sp. *lactucae* on lettuce by inter-retrotransposon sequence-characterized amplified region technique. *Phytopathology, 97*, 987–996.

Pereira, J. F. (2005). Caracterização, distribuição e estudo da atividade de elementos transponíveis em *Crinipellis perniciosa*, agente causal da vassoura-de-bruxa no cacaueiro (*Theobroma cacao*). Dissertation, Universidade Federal de Viçosa, Brazil

Pereira, J. L., Ram, A., Figueiredo, J. M., & Almeida, L. C. C. (1989). Primeira ocorrência de vassoura-de-bruxa na principal região produtora de cacau do Brasil. *Agrotópica, 1*, 79–81.

Pereira, J. F., Ignacchiti, M. D. C., Araújo, E. F., Brommonshenkel, S. H., Cascardo, J. C. M., Pereira, G. A. G., et al. (2007). PCR amplification and sequence analyses of reverse transcriptase-like genes in *Crinipellis perniciosa* isolates. *Fitopatologia Brasileira, 32*, 373–381.

Ploetz, R. C., Schnell, R. J., Ting, Z., Zheng, Q., Olano, C. T., Motamayor, J. C., et al. (2005). Analysis of molecular diversity in *Crinipellis perniciosa* with AFLP markers. *European Journal of Plant Pathology, 111*, 317–326.

R Development Core Team. (2007). *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Rincones, J., Meinhardt, L. W., Vidal, B. C., & Pereira, G. A. G. (2003). Electrophoretic karyotype analysis of *Crinipellis perniciosa,* the causal agent of witches' broom disease of *Theobroma cacao*. *Mycological Research, 107*, 452–458.

Rozen, S. & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics methods and protocols: Methods in molecular biology*, *132*, 365–386.

Silva, J. R. Q., Figueira, A., Pereira, G. A. G., & Albuquerque, P. (2008). Development of novel microsatellites from *Moniliophthora perniciosa*, causal agent of the witches' broom disease of *Theobroma caçao*. *Molecular Ecology Resources, 8*, 783–785.

SUFRAMA—Superintendência da Zona Franca de Manaus, (2003). Potencialidades regionais – Estudo da viabilidade econômica – Cacau- p 34.

Waugh, R., Mclean, K., Flavell, A. J., Pearce, S. R., Kumar, A., Thomas, B. B., et al. (1997). Genetic distribution of *BARE-1*-like retrotransposable elements in the barley genome revealed by sequence-specific amplification polymorphisms (S-SAP). *Molecular Genetics and Genomics, 253*, 687–694.

Yeh, F. C., Yang, R., & Boyle, T. (1999). *POPGENE. Microsoft window-based freeware for population genetic analysis. Release 1.31*. Edmonton: University of Alberta, Canada.