

Contrastant banana accessions for resistance to the burrowing nematode, based on molecular markers RAPD

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Abstract This work aimed to proceed molecular characterization of seven banana accessions (Borneo, Grand Naine, 1304-06, 4249-05, 0337-02, 0323-03 and 4279-06) resistance to the nematode *Radopholus similis*. These accessions were selected taking in account the reproduction factor (RF) among 26 banana genotypes from a working collection belonging to Embrapa Mandioca e Fruticultura Tropical.

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The genomic DNA of the seven accessions was extracted, and 36 decamere primers had been used to obtain RAPD markers. The resulting markers were converted into a matrix of binary data. From that matrix the genetic distance between the accessions were estimated, for further clustering and graphic dispersion analyses. From a total of 521 RAPD markers generated, 420 (81%) were polymorphic, including 140 (27%) potentially promising for application on works related to genetic mapping of the resistance to *R. similis*. OPE-15, OPH-17, and OPG-09 were the primers that contributed to the highest number of bands promising for genetic mapping of resistance (12, 8, and 8, respectively). The genetic distances between accessions ranged from 0.106 to 0.455, with the longest one observed between cv. Borneo and the genotype 4279-06, considered as highly susceptible and resistant, respectively, to the nematode according to the RF. The graphic dispersion distinguished three groups of accessions, and most of resistant genotypes clustered together in the same group. The most contrastant genotypes for resistance (Borneo and 4249-05) were separated by a genetic distance of 0.374, and possessed a total of 114 polymorphic bands promising for genetic mapping of resistance. In addition, the results of pathogenicity tests were congruent with those obtained by RAPD analyses.

Keywords Genetic diversity · *Musa* ·
Molecular marker · *Radopholus similis*

Introduction

Banana is among the most popular fruits worldwide, and it is grown in the majority of the tropical countries. Brazil ranks as second place in world banana production, although near to 100% of fruit production is destined to the domestic market, where the preference is for cultivars in the group AAB, subgroup “Prata”.

Banana plant is suitable host for important species of plant-parasitic nematodes, such as the burrowing nematode, *Radopholus similis* (Cobb 1893) Thorne, 1949; the spiral nematode, *Helicotylenchus multicinctus* (Cobb 1893) Golden, 1956; the lesion nematode, *Pratylenchus coffeae* (Zimmermann 1898) Filipjev and Schuurmans Stekhoven, 1941; the root-knot nematode, *Meloidogyne* spp., and the reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, 1940. Especially, *R. similis*, *H. multicinctus*, *P. coffeae* and *Meloidogyne* spp. are limiting factors in various banana growing areas due to difficult and onerous nematode control.

The use of plant resistance has been proved to be an efficient and low cost method of disease control. Therefore, the possibility of using genetic resistance to control plant-parasitic nematodes in bananas has been explored by several authors (Fogain and Gowen 1997; Costa et al. 1998; Marin et al. 1998; Elsen et al. 2002; Vilas Boas et al. 2002; Costa 2004). Search for resistance to plant-parasitic nematodes is dependent on the genetic variability of the host, as well as, on the genetic variability among nematode populations. The genetic basis for banana resistance to *R. similis* remains to be understood, although there is evidence that the resistance is controlled by one or more dominant genes (Pinochet 1988). For banana breeding programs is of capital importance to understand the kind of resistance and the mechanisms of nematode resistance in resistant accessions of bananas (Giebel 1982). The accomplishment of these studies needs a strong support from works planned to determine the degree of resistance or tolerance to the pathogen, and from studies based on molecular markers applicable on characterization and evaluation of genetic variability in bananas (Bhat and Jarret 1995; Pillay et al. 2000).

The advent of techniques possible to detect polymorphisms at DNA level has generated a large number of molecular markers for genetic testing and

fingerprinting of individuals (Jeffreys et al. 1985). Molecular markers were used in the characterization and assessment of genetic variability in *Musa*, including RFLP (Restriction Fragment Length Polymorphism) (Fauré et al. 1994), RAPD (Random Amplified Polymorphic DNA) (Bhat and Jarret 1995; Pillay et al. 2000; Sawazaki et al. 2002); AFLP (Amplified Fragment Length Polymorphism) (Loh et al. 2000) and microsatellites or SSRs (Simple Sequence Repeats) (Kaemmer et al. 1997; Crouch et al. 1998; Grapin et al. 1998; Lagoda et al. 1998; Creste et al. 2001, 2003; Bonamico et al. 2004).

Bhat and Jarret (1995) demonstrated the application of RAPD markers on the characterization of germplasm of *Musa* spp., and it has been possible to distinguish clones that were morphologically identical.

Jesus et al. (2006) working with the techniques of microsatellite and RAPD obtained similar responses with the two techniques, for the separation of genotypes according to the genomic group and origin of hybrids. This shows that the technique of RAPD has considerable reliability, since it repeats results generated by other techniques. This fact justifies the use of RAPD technique to obtain markers that can lead to the mapping of resistance genes. Furthermore, for the required saturation of genetic maps it is interesting the use of different types of molecular markers, including RAPD.

The objective of this study was to use molecular markers RAPD, to carry out the molecular characterization of seven genotypes of *Musa* contrasting to the phenotypes of resistance to the nematode *R. similis*, aiming to identify promising primers and RAPD markers for upcoming studies on mapping of genetic resistance.

Materials and methods

The banana genotypes Borneo, Grand Naine, 1304-06, 4252-03, 8694-15, 1304-04, Pipit, 5854-03, 1318-01, 4285-02, N118, Tjau Lagada, Calcutta, 1319-01, Pa Rayong, Birmanie, Vitoria, Pisang Nangka, Thap Maeo, 4223-06, Jaran, Yangambi Km 5, 4279-06, 0323-03, 0337-02 and 4249-05, from the banana germplasm bank of Embrapa Mandioca e Fruticultura Tropical, were evaluated for reaction of susceptibility

Table 1 Accession of banana analyzed expressing phenotype of resistance or susceptibility to *Radopholus similis* based on the reproduction factor

Number	Accession	Reproduction factor	Phenotype
1	Borneo	21.31	HS
2	Grand Naine	11.30	S
3	1304-06	11.00	S
4	4249-05	0.01	HR
5	0337-02	0.11	R
6	0323-03	0.40	R
7	4279-06	0.41	R

HS highly susceptible, S susceptible, HR highly resistant, R resistant

or resistance to *R. similis*, under greenhouse conditions, according to Santos (2007).

After evaluation of the reproduction factor of *R. similis* in the 26 accessions of banana under greenhouse, seven genotypes were selected contrasting in relation to susceptibility/resistance to the nematode (Table 1). The three genotypes more susceptible according to the reproduction factor were Borneo, Grand Naine and 1304-06, while the four genotypes with the lowest reproduction factor, indicating a phenotype of resistance, were 4279-06, 0323-03, 0337-02 and 4249-05.

Samples of genomic DNA of the seven banana accessions were extracted using the method of CTAB (Doyle and Doyle 1990) with modifications (Faleiro et al. 2003). After extraction, DNA concentration was estimated by spectrophotometry at 260 and 280 nm (Sambrook et al. 1989). The visual appearance of total genomic DNA bands separated by electrophoresis in gel of agarose 0.8% was used as an indicator of integrity and purity of the DNA extracted. After the quantification, all DNA samples were diluted to the concentration of 10 ng/ μ L.

A total of 48 primers belonging to the kits OPD, OPE, OPF, OPG and OPH (Operon Technologies Inc., Alameda, CA) were tested. The reactions of amplification were performed in a total volume of 13 μ L, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 100 mM of each deoxyribonucleotide (dATP, dTTP, and dCTP, dGTP), 0.4 mM of a primer (Operon Technologies Inc., Alameda, CA), a unit of the enzyme Taq polymerase, and ~15 ng DNA.

The amplifications were performed in thermocycler programmed for 40 cycles, each one consisting of the following sequence: 15 s at 94°C, 30 s at 35°C and 90 s at 72°C. After the 40 cycles, there was a step of final extension of 6 min at 72°C, finally, the temperature was reduced to 4°C.

After amplification, were added to each sample, 3 μ L of a mixture of blue Bromophenol (0.25%) and glycerol (60%) in water. The samples were applied to agarose gel (1.2%), stained with ethidium bromide (0.2 mg/mL), submerged in TBE buffer (90 mM Tris-borate, 1 mM EDTA) to separate the microsatellite fragments. The electrophoretic separation was for about 4 h, at 85 V. At the end of the electrophoretic running, the gels were photographed under UV light.

The RAPD markers generated were converted into a matrix of binary data, from which were estimated genetic distances between each pair of accessions, based on the similarity coefficient of Nei and Li (1979) (Table 2).

The matrix of genetic distances was used for the clustering analysis to generate a dendrogram using the method UPGMA (Unweighted Pair Group Method with Arithmetic Mean) as a criterion for grouping, and for the graphical dispersion based on multidimensional scales using the method of principal coordinates.

Results

Among the 48 decamere primers tested, 36 were selected which generated sharp and polymorphic molecular markers. The 36 primers (Table 2) generated a total of 521 RAPD markers, making an average of 14.5 markers per primer. As an example, we can highlight the pattern of amplification generated by the primer OPE-16 (Fig. 1), being possible to see clearly the presence of polymorphic markers originated.

Among the primers used only two (OPH-04 and OPF-20) did not generate bands in resistant and absent in all susceptible genotypes. Therefore, 96% of primers generated at least one promising band for genetic mapping. The primers OPE-15, OPH-17 and OPG-09 were those that conferred a larger number of promising bands (12, 8 and 8, respectively), standing out among the others (Table 2).

Table 2 Primers selected to obtain the RAPD markers, and the respective numbers of bands polymorphic, monomorphic, and those promising for works on genetic mapping of resistance to the nematode *Radopholus similis*

Primers	Sequence 5' → 3'	No. of polymorphic bands	No. of monomorphic bands	No. of promising bands
OPE-18	GGAAGTCGCA	6	6	1
OPF-08	GGGATATCGG	14	2	4
OPF-14	TGCTGCAGGT	4	6	1
OPG-05	CTGAGACGGA	15	1	3
OPG-09	CTGACGTCAC	17	1	8
OPH-04	GGAAGTCGCC	8	2	0
OPH-12	ACGCGCATGT	14	3	2
OPH-17	CACTCTCCTC	18	1	8
OPF-19	CCTCTAGACC	15	1	7
OPG-13	CTCTCCGCCA	6	4	2
OPD-11	AGCGCCATTG	9	2	1
OPH-02	TCGGACGTGA	11	0	4
OPG-18	GGCTCATGTG	9	1	1
OPH-06	ACGCATCGCA	7	2	1
OPH-08	GAAACACCCC	8	4	3
OPH-15	AATGGCGCAG	14	0	7
OPE-19	ACGGCGTATG	6	4	4
OPE-15	ACGCACAACC	19	0	12
OPE-16	GGTACTGTG	15	4	6
OPE-17	CTACTGCCGT	4	4	2
OPF-03	CCTGATCACC	9	1	1
OPF-12	ACGGTACCAG	10	5	3
OPF-20	GGTCTAGAGG	9	3	0
OPG-08	TCACGTCCAC	10	6	2
OPE-07	AGATGCAGCC	12	2	7
OPG-12	CAGCTCACGA	14	6	4
OPG-17	ACGACCGACA	15	2	4
OPG-15	ACTGGGACTC	10	5	3
OPG-11	TGCCCGTTCGT	16	3	5
OPE-09	CTTCAACCGA	12	3	4
OPE-05	TCAGGGAGGT	12	3	5
OPE-02	GGTGCGGGAA	11	4	5
OPE-11	GAGTCTCAGG	16	5	5
OPD-01	ACCGCGAAGG	18	1	7
OPD-02	GGACCCAACC	17	1	4
OPD-05	TGAGCGGACA	10	3	4

Of the 521 markers, 101 (19%) were monomorphic, 420 (81%) were polymorphic and 140 (27%) were promising for works on the genetic mapping of resistance to *R. similis*.

The genetic distances between accessions ranged between 0.106 and 0.455 (Table 3). The smallest

genetic distance (0.106) was obtained between the genotypes 0337-02 and 4279-06, which reacted as resistant to the attack of the pathogen, followed by that observed among the genotypes Borneo and Grand Naine (0.287), which behaved as the most susceptible. The greatest genetic distance (0.455) was

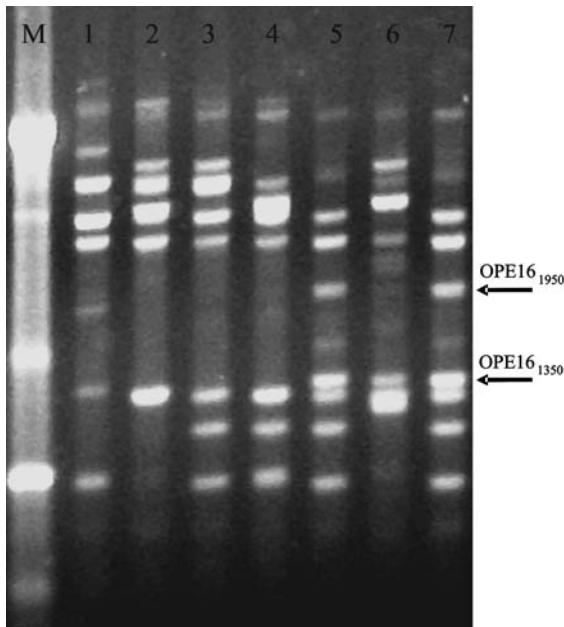


Fig. 1 Pattern of markers generated by the primer OPE-16 in the seven banana accessions. The markers indicated with *arrows* are promising for studies on genetic mapping. (M) Size markers (0.5–10 Kb); (1) Borneo; (2) Grand Naine; (3) 1304-06; (4) 4249-05; (5) 0337-02; (6) 0323-03; (7) 4279-06. (*HS* highly susceptible, *S* susceptible, *HR* highly resistant, *R* resistant)

observed between cultivar Borneo and the genotype 4279-06, classified as highly susceptible and resistant, respectively.

The cluster analysis performed on the basis of genetic distances allowed to divide the seven accessions in at least three groups of genetic similarity to a relative genetic distance of 0.327 (Fig. 2). The distances between accessions and their distribution in groups of similarity can also be seen in the dispersion graphic (Fig. 3). The more contrasting accessions for resistance (Borneo and 4249-05) had a

distance of 0.374 and a total of 114 polymorphic and promising bands for mapping (Tables 3, 4). The combination of Borneo with the resistant genotypes (4279-06, 0337-02 and 0323-03) also provided a high number of polymorphic bands (104, 111 and 106, respectively), as well as the intersection of 4279-06 with Borneo and 1304-06, and of these genotypes with 0323-03 (Table 4).

The analysis of dispersion based on genetic distances allowed the separation of most resistant accessions from the susceptible ones (Fig. 3).

Discussion

RAPD technique allowed to obtain large numbers of polymorphic markers, of which 140 appear to be promising for studies on genetic mapping of resistance to the nematode *R. similis*, i.e. were present in accessions resistant and absent in all susceptible accessions. The high average numbers of markers generated per primer and the low percentage of monomorphic markers demonstrate the high genetic variability among the accessions studied.

The high level of polymorphism, detected with the RAPD markers, is in agreement with that observed by Gomes et al. (2005). Jarret and Gawel (1995), using the technique of RAPD in the characterization of clones of plantains and the assessment of genetic diversity among diploids of *M. acuminata*, concluded that this technique can provide information on various aspects of genetic diversity in banana germplasm, such as the differentiation among clones morphologically identical.

The smallest genetic distance observed between the resistant genotypes 0337-02 and 4279-06, may be attributed to the fact the two diploid genotypes share Calcutta as common ascendant. The group consisting

Table 3 Matrix of genetic distances between seven accessions of banana susceptible and resistant to the nematode *Radopholus similis*, based on 521 RAPD markers

Genotypes	Borneo	G. Naine	1304-06	4249-05	0337-02	0323-03	4249-06
Borneo	0						
G. Naine	0.28712	0					
1304-06	0.31168	0.32579	0				
4249-05	0.37369	0.31954	0.33595	0			
0337-02	0.44034	0.37355	0.35080	0.36078	0		
0323-03	0.45089	0.39903	0.39256	0.37074	0.31006	0	
4279-06	0.45494	0.40758	0.38843	0.39271	0.10655	0.31049	0

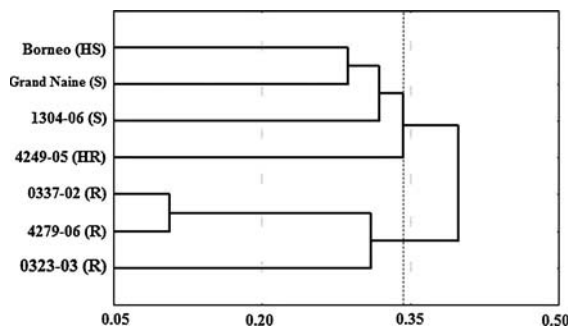


Fig. 2 Cluster analysis of seven accessions of banana based on the matrix of genetic distances calculated using 521 RAPD markers. The UPGMA method was used as a criterion for grouping. (HS highly susceptible, S susceptible, HR highly resistant, R resistant)

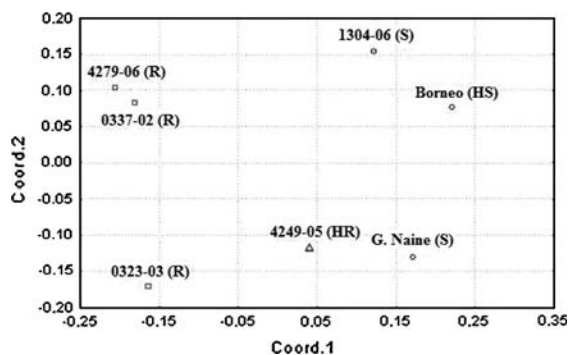


Fig. 3 Graphical dispersion of seven accessions of banana based on a matrix of genetic distances calculated using 521 RAPD markers. The matrix of genetic distances was displayed in a scatter plot based on the multidimensional scaling, using the principal coordinates analysis method. Accessions with the same symbol belong to the same group of similarity. (HS highly susceptible, S susceptible, HR highly resistant, R resistant)

of all resistant accessions, too is due to Calcutta, since this genotype is also an ascendant of 0323-03. The Diploid 4249-05 considered highly resistant, by the method UPGMA was closest to the susceptible accessions. Probably this genotype has part of its genetic material similar to that of the susceptible genotypes. This is an evidence that resistance to *R. similis* in banana can be determined by a few genes, as postulated by Pinochet (1988) that through preliminary studies demonstrated that resistance to *R. similis* can be controlled by one or more dominant genes.

The fact the more contrasting accessions for resistance (Borneo and 4249-05) have a total of 114 polymorphic and promising markers for genetic mapping, suggests that the crossing between these two genotypes could generate progenies of interest for understanding the inheritance of resistance to *R. similis*, and for future works on genetic mapping of resistance. The crossing of the diploid Borneo with any of the genotypes considered resistant (0337-02, 0323-03 or 4279-06) would also be of great importance, given the high number of promising markers found in these genotypes (111, 106 and 104, respectively).

Another promising analysis, would be that considering those markers observed in susceptible genotypes and absent in the resistant ones, where crosses between 4279-06 (resistant) and 1304-06 or Borneo (susceptible) could be made. Therefore, more accurate studies are necessary to determine the relationship between the markers and observed resistance genes in those plants, noting the occurrence of segregation of resistance in the crosses above indicated.

Table 4 Number of promising molecular markers for genetic mapping of pairing between resistant and susceptible accessions of banana to the nematode *Radopholus similis*

Genotypes	Borneo	G. Naine	1304-06	4249-05	0337-02	0323-03	4279-06
Borneo				114	111	106	104
G. Naine				83	85	83	85
1304-06				95	81	87	80
4249-05	65	56	76				
0337-02	92	76	93				
0323-03	96	83	103				
4279-06	103	87	108				

Values above diagonal are number of markers present in resistant genotypes and absent in susceptible ones and vice versa for values below diagonal

The cluster analyzes by the method UPGMA and the graphical dispersion based on multidimensional scales using the principal coordinates, showed clearly the separation of banana genotypes contrastant for resistance to the nematode *R. similis*. The fact that these tools allow a distinct separation between the majority of the resistant and susceptible genotypes, indicates the existence of differences in the genetic background of those accessions. Also, these genetic differences open windows for future studies on genetic mapping of resistance of banana to the burrowing nematode.

Conclusions

The RAPD technique generates a large number of polymorphic markers between contrastant genotypes, which generate promising bands for genetic mapping of banana genotypes. By the analysis of dispersion is possible to separate the genotypes in groups, where resistant genotypes group with each other, as well as the most susceptible also group by themselves. The results of pathogenicity tests are congruent with the results obtained by the analysis of RAPD.

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