Molecular Characterization of Brazilian *Dicyma pulvinata* Isolates*

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(Aceito para publicação em 04/11/2003)

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TAVARES, E.T., TIGANO, M.S., MELLO, S.C.M., MARTINS, I. & CORDEIRO, C.M.T. Molecular characterization of Brazilian *Dicyma pulvinata* isolates. Fitopatologia Brasileira 29:148-154. 2004.

ABSTRACT

Forty-nine Brazilian *Dicyma pulvinata* isolates were examined by morphological traits and RFLP, RAPD and AFLP analyses. This fungus is a mycoparasite of *Microcyclus ulei*, the causal agent of the most devastating rubber (*Hevea brasiliensis*) disease, known as "South American Leaf Blight" (SALB). These isolates were compared with an Indian isolate from *Cercosporidium* sp., and a French isolate from *Cladosporium fulvum*. They were also compared with *Dicyma ampullifera* from Papua New Guinea. The morphological parameters analyzed confirmed the identification of the Brazilian isolates. The graphic representations of the distance matrices of each molecular marker showed similar results. *Dicyma pulvinata* isolates from *M. ulei* were closely related, whereas the reference isolates examined were dispersed. Among the *D. pulvinata* isolates obtained from *M. ulei*, a significant pairwise distance was obtained, for all the molecular markers, between the isolates from the areas favorable to the occurrence of SALB (North and Northeast of Brazil) and the region of escape for the disease (Mato Grosso State).

Additional keywords: fungi, molecular markers, biological control.

RESUMO

Caracterização molecular de isolados brasileiros de *Dicyma pulvinata*

Quarenta e nove isolados brasileiros de *Dicyma pulvinata* foram analisados quanto a características morfológicas, análises de RFLP, RAPD e AFLP. Este fungo é um micoparasita do *Microcyclus ulei*, o agente causal da mais importante doença da seringueira (*Hevea brasiliensis*), o mal-das-folhas. Estes isolados foram comparados com isolados indianos obtidos a partir de lesões de *Cercosporidium* sp., com um isolado proveniente da França obtido a partir de lesões de *Cladosporium fulvum* e com um isolado de *D. ampullifera* proveniente de Papua-Nova Guiné. A análise de parâmetros morfológicos confirmou a identificação dos isolados brasileiros. As representações gráficas das matrizes de distância de cada marcador molecular mostraram resultados semelhantes. Os isolados de *D. pulvinata* obtidos de *M. ulei* mostraram-se intimamente relacionados. Por outro lado, os isolados – referência, incluídos nestes estudos, mostraram-se bastante dispersos. De acordo com a análise de variância molecular, considerando todos os marcadores, os isolados de *D. pulvinata* obtidos de *M. ulei*, apresentaram diferença significativa entre isolados procedentes de áreas de cultivo de seringueira favoráveis à ocorrência do mal-das-folhas (regiões norte e nordeste do Brasil) e aqueles de áreas de escape da doença (Mato Grosso).

INTRODUCTION

The hyphomycete fungus *Dicyma pulvinata* (Berk. & Curt.) Arx [=*Hansfordia pulvinata* (Berk. & Curt.) Hughes] has been found parasitizing phytopathogenic fungi. *Cladosporium fulvum* Cooke and *Cercosporidium personatum* Earle, causal agents of tomato (*Lycopersicon esculentum* Mill.) leaf mould and late leaf spot of peanut (*Arachis hypogaea* L.), respectively, are some of the fungi colonized by this hyperparasite (Peresse & Picard, 1980; Mitchell *et al*., 1987). Therefore, *D. pulvinata* could be considered a potencial biocontrol agent for plant diseases.

South American Leaf Blight (SALB) of Hevea rubber (*Hevea brasiliensis*), caused by *Microcyclus ulei* (P. Henn.) Arx, is identified as one of the world's five most threatening plant diseases and it is still endemic to Central and South America. It was first recorded in 1900 on rubber tree in Brazil (Webster & Baulkwill, 1989). Presently the disease extends from Southern Mexico (18^0 latitude north) to the state of São Paulo in Brazil (24^0 latitude south) covering Brazil, Bolivia, Colombia, Peru, Venezuela, Guiana, Trinidad, Tobago, Haiti, Panama, Costa Rica, Nicaragua, Salvador, Honduras, Guatemala and Mexico. This disease has been the main constraint for the development of rubber cultivation in Latin America countries (Gasparotto *et al*., 1997).

To control the disease, it is recommended that *H. brasiliensis* be planted in escape areas, characterized by a well–defined dry season. Escape areas occur in Goiás, Mato Grosso do Sul, Pernambuco, Maranhão, Espírito Santo, Rio de Janeiro, Paraná, São Paulo, and Minas Gerais, all are non-traditional areas for rubber tree cultivation. Recent studies, however, have indicated the existence of an ecophysiologic variability among *M. ulei* isolates. If the information about rapid adaptation of the pathogen to environment is confirmed, the control of this disease will be very difficult (Junqueira & Gasparotto, 1991).

*Part of the Master Dissertation of the first author. Universidade de Brasília (2001)

**Capes fellowship

Efforts have been made to control this disease, including the use of *D. pulvinata* (Bettiol, 1991). This mycoparasite was first reported as colonizing black spots produced by *M. ulei* in the Amazon Region (Junqueira & Gasparoto (1991). It has subsequently spread to different geographic areas (Mello *et al*., 1999). At present, little is known about the variability among *D. pulvinata* isolates obtained from *M. ulei* lesions. Thus, to better exploit the potential of *D. pulvinata* as a biocontrol agent, the correct identification and characterization of these isolates is critical to better understand the pathogen-host relationship.

Molecular markers have been used to resolve taxonomic problems and to address questions about the genetic variability of fungi. The objectives of this study were to examine the molecular variation of the *D. pulvinata* isolates obtained from lesions produced by *M. ulei* on rubber tree leaves from different regions of Brazil; and to compare these isolates with typical *D. pulvinata* isolates from two other fungal hosts, as well as with an unrelated *D. ampullifera* isolate. Genomic DNA of *Dicyma* spp. was subjected to molecular analyses based on AFLPs (Zabeau & Vos, 1993), RAPDs (Welsh & McClelland, 1990; Williams *et al*., 1990) and RFLPs.

MATERIALS AND METHODS

Fungal isolation

From late February to late December of 1999, a survey was carried out in several rubber producing areas of Brazil. Leaves with *M. ulei* lesions colonized by fungi, were placed in a paper bag, inside a plastic bag and transported in a cooler. Spores were harvested from lesions, and plated on potato dextrose agar (PDA). After five days, pure cultures were prepared from single conidia and maintained as stock cultures on PDA slants. The isolated fungi were initially identified based on their conidial morphology and growth characteristics.

Fungal culture and DNA extraction

Monoconidial cultures of 49 *D. pulvinata* isolates collected from different locations in several rubber production areas of Brazil and two others, originating from India and France, were used in this study (Table 1). One isolate of *D. ampullifera* (CG 704), obtained from soil in Papua New Guinea, was also included as an outgroup. Cultures were maintained on PDA slants, and stored at 8 ºC in the dark until needed. The mycelia were grown in liquid cultures of spores using Richard medium broth (Tuite, 1978), with shaking at 150 rpm, 26 ºC, for seven days. Mycelium was harvested by filtration through filter paper (Whatman No. 1) and stored at -80 ºC. Genomic DNA was obtained from mycelium ground in liquid nitrogen by using the CTAB extraction method (Rogers & Bendich, 1988).

Morphological analyses

Morphological analyses were conducted with cultures of *D. pulvinata* grown on Richard medium. Four replicate plates were inoculated with 0.5-cm diameter mycelial plugs cut from the margin of seven-day-old colonies of each isolate. Cultures were incubated at 28 ºC and exposed to a 12-h photoperiod under white light of wavelengths between 350 and 750 nm. Colony aspect and conidial morphology were recorded using 20-day-old cultures. Fifty conidia were examined for each replicate.

IGS region amplification and restriction digestion

The IGS region of ribosomal DNA was amplified by PCR using the primers CNL 12 (CTGAACGCCTCTAA GTCAG) and CNS 1 (GAGACAAGCATATGACTACTG) (Appel & Gordon, 1995). The reactions were conducted in a final volume of 50 µl in a microcentrifuge tube containing: 1x *Taq* DNA polymerase buffer (GIBCO-BRL), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.22 µM of the primer CNL 12, 0.28 µM of the primer CNS 1, 20 ng of genomic DNA and 2.5 units of *Taq* DNA polymerase (GIBCO-BRL). Amplifications were performed using a PTC-100 thermocycler (MJ Research) programmed for 40 cycles of 1 min at 94 ºC, 45 s at 50 ºC, and 2 min at 72 ºC with a final extension of 5 min at 72 ºC. The reaction mixture (5 µl) was run on 1.5% agarose gels, stained with ethidium bromide and visualized under UV illumination to estimate the size of the amplified IGS fragment based on comparison to size markers. Restriction digestions were carried out with 10 µl of the PCR reaction using the restriction enzymes *Alu* I, *Ava* II, *Cfo* I, *Mbo* I, and *Taq* I, according to the manufacturers' directions (GIBCO-BRL). Digested DNA was separated on 2.3% agarose gels, stained with ethidium bromide and visualized under UV light.

Random amplified polymorphic DNA (RAPD)

PCR was performed in 15-µl volumes, using a PTC-100 thermocycler (MJ Research). The amplifications were carried out using 20 ng/reaction of each template DNA and the following reaction mix: 4 mM $MgCl_2$, 0.1 mM of each dNTP, 1 µM primer, 1 U of *Taq* DNA polymerase (GIBCO-BRL) and 1x *Taq* DNA polymerase buffer (GIBCO-BRL). The temperature program for the reactions was 94 ºC for 3 min, followed by 40 cycles of 94 ºC for 1 min, 35 ºC for 1 min, 72 ºC for 2.5 min, with a final extension at 72 ºC for 7 min (Tigano-Milani *et al*., 1995a). Eleven 10-mer random primers were used in this study: OPB-01, OPB-04, OPB-08, OPB-09, OPB-11, OPB-15, OPB-18, OPD-01, OPD-08, OPD-11 and OPD-20 (Operon Technologies Inc.). PCR products were separated on 1.5 % agarose gel, stained with ethidium bromide and visualized under UV light. All amplifications were repeated.

Amplified fragment length polymorphism (AFLP)

Genomic DNA (500 ng) was digested with *Eco* RI (5 U) and ligated to *Eco* RI adaptors (50 pmol) in a single reaction at 37 ºC for 16 h using standard molecular biology techniques (Sambrook *et al*., 1989). Ammonium acetate (3 M, pH5.2) and 95% ethanol were added to the reaction mixture, and incubated at room temperature for 30 min. Anchor-annealed *Eco* RI fragments were precipitated by centrifugation at 13,000 g for 10 min. The pellet was washed with 70 % ethanol, dried, and resuspended in 50 µl of 0.1x TE buffer (Boucias *et al*., 2000b). A series of four 19-mer, comprised of the *Eco* RI adapter

sequence plus three 3' selective nucleotides (Boucias *et al*., 2000a) were used for PCR amplifications. The reactions were carried out in 25 µl final volume containing: 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of single primer, 3 µl anchor-annealed *Eco* RI fragment, 1 U *Taq* DNA polymerase (GIBCO-BRL) and 1x *Taq* DNA polymerase buffer(GIBCO-BRL). Amplifications were performed using a PTC-100 thermocycler (MJ Research) programmed with an initial step of 1 min at 95 ºC followed by 35 cycles of 1 min at 94 ºC, 1 min at 56 ºC, 2.5 min at 72 ºC and a final extension of 5 min at 72 ºC. Amplification products were run on 2% agarose-synergels (Diversified Biotech), stained with ethidium bromide and visualized under UV light.

Analysis of Data

Results obtained from molecular markers IGS, AFLP, and RAPD analyses were used to construct binary matrices. Each of the isolates had been scored for the presence (1) or absence (0) of fragments. A dissimilarity matrix of genetic distances between isolates was calculated based on the method of Nei and Li (1979). Multidimensional scaling (MDS) model was adjusted for each genetic distance matrix using PROC MDS (SAS, 1999) to generate graphic representations of these matrices. Associations among genetic distances for each marker type were analyzed by the Mantel test (Manly, 1997). This procedure gives the product moment correlation (r) and a statistical test to measure the degree of relatedness between two matrices. The relationship among individuals using the molecular markers was also evaluated according to hierarchical structure, using Arlequin v. 2000 (Schneider *et al.* 1997), considering Brazilian populations of *D. pulvinata* from the different regions and individuals within regions. The estimate of the variance components associated with the different hierarchical levels was obtained with the aid of AMOVA (Analysis of Molecular Variance).

RESULTS AND DISCUSSION

For all *D. pulvinata* isolates the conidial size and other morphological characteristics such as type of conidiogenous cells, conidiophores, colony appearance and mycelial growth rates in the culture corresponded to the description of the species (Hughes, 1951). Colonies of *D. pulvinata* isolates varied from white to grey/greenish, becoming black. Isolates obtained from *M. ulei* produced exudates that varied in coloration. Mycelium was immersed and superficial. Conidiophores appeared smooth, dark brown or olivaceous brown, bearing a number of primary branches on the upper part. Conidiogenous cells were on branches, polyblastic, discrete, sympodial and deticulate. *D. pulvinata* conidia were subspherical, aseptate, with a basal scar, olive brown and solitary (data not shown). The mean conidial size for the *D. pulvinata* isolates obtained from *M. ulei* varied from 4.3±0.2 µm (CG 766) to 7.3±0.2 µm (CG 777) in diameter, while the means for non-*M. ulei* hosts isolates, CG 826 and CG 678, varied between 4.2±0.2 µm and 3.6±0.1 µm, respectively.

Like other fungi, the morphological parameters of *D. pulvinata* do not produce information sufficiently clear for differentiating isolates among the species. In order to determine the genetic variability of *D. pulvinata* isolates obtained from *M. ulei* in different Brazilian regions, three genetic methods (RFLP, RAPD and AFLP) were used in this study.

Dicyma pulvinata isolates differed in the size of their IGS region amplified by PCR with primers CNS 1 and CNL 12. The isolates obtained from *M. ulei* amplified a single double-stranded product of approximately 1.6 kb. However, the isolates collected from different hosts (CG 678 and CG 826) showed a product of approximately 2.0 kb. Although the size of IGS products is the same at the species level for other fungi (Appel & Gordon, 1995; Chillali *et al*., 1998), polymorphism of the IGS size has already been observed among isolates of *Verticillium albo-atrum* Reinke & Berthold (Pramateftaki *et al*., 2000). The isolate of *D. ampullifera*, CG 704, produced IGS fragments of about 2.6 kb (Figure 1A).

Restriction digestions of IGS revealed RFLPs, reflecting variation in this region of the rDNA among the isolates with the

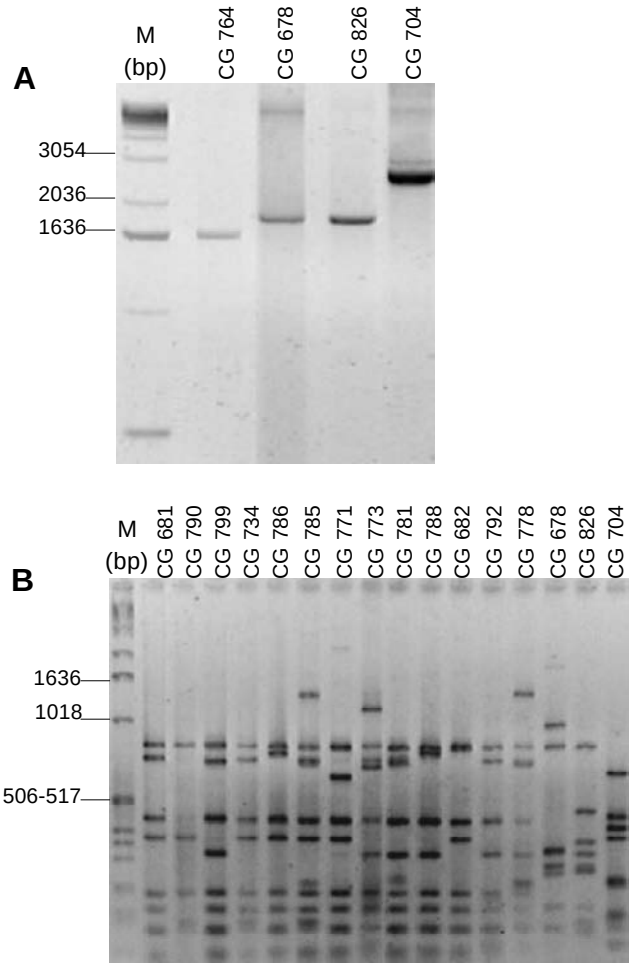

FIG. 1 - Ethidium bromide-stained 1.5% agarose gel containing the products obtained from *Dicyma* spp. isolates (A) for IGS region amplified with primers CNS1 and CNL12, (B) for the RFLP´s products resulting from the digestion of the IGS region with *Taq* I. M = molecular size standards (1kb DNA ladder).

same size of the IGS fragment. The sum of the fragment sizes exceeded the size of the amplified IGS, indicating the existence of different IGS types in the same individual. Each rDNA repeating unit usually consists of a highly conserved region among various organisms and the IGS, which is highly variable in length and in the primary structure among organisms and individuals (Chou & Tsai, 1999). Length variation and sequence polymorphism in IGS have been reported in many fungi (Appel & Gordon, 1996; Kauserud & Schumacher, 2001). From the five enzymes used for the IGS digestion, *Taq* I produced the most variable patterns (Figure 1B). The RFLP analysis of the IGS region using five restriction enzymes produced 109 scorable characters, from which 107 were polymorphic. Of these selected bands, 42 were due to the inclusion of the two *D. pulvinata* isolates obtained from non-*M. ulei* hosts and the outgroup, *D. ampullifera* (CG 704). The MDS model of the restriction digests of IGS distance matrix data demonstrated the presence of a tight group of *D. pulvinata* isolates obtained from *M. ulei*. The two *D. pulvinata* isolates obtained from non-*M. ulei* hosts and the outgroup were dispersed from this group (Figure 2A). The digestion with restriction enzyme of this region of the rDNA showed the intraspecific variation among the isolates of *D. pulvinata*, even among those isolated from the same host in the same country. This method also showed the intraspecific variation within populations of *Fusarium oxysporum* Smyder & Hansen (Appel & Gordon, 1995).

The IGS region exhibits enough genetic diversity to distinguish intra and interspecies isolates. According to Appel & Gordon (1995), a mutation in an IGS repeat may spread to other spacer regions, leading to the homogenization of the IGS repeats in an individual and in populations that reproduce sexually throughout the parallel evolution mechanism. Thus, the diversity observed among *D. pulvinata* isolates suggests that this mechanism does not operate, reinforcing the suspicion that this species is an asexual fungus. Sequence data from the IGS region may provide additional information about the phylogeny of *D. pulvinata*.

The RAPD analysis by using 11 random 10-mer oligonucleotide primers produced a total of 224 scorable polymorphic bands. About 68 % of the bands detected were associated to *D. pulvinata* species isolated from *M. ulei*. An example of the patterns obtained with the RAPD analysis is shown in Figure 3. The MDS model for the RAPD data produced a diffuse group of *D. pulvinata* isolates obtained from *M. ulei* lesions. Like the RFLP data, *D. pulvinata* isolates collected from non-*M. ulei* hosts and the outgroup were also dispersed in this group (Figure 2B). Because of considerable concerns as to the reproducibility of RAPD profiles, all amplifications were repeated and the results were confirmed.

The amplification of the anchor-ligated *Eco* RI fragments with the four primers produced an array of scorable bands, shown in the example of fingerprintings in Figure 4. A total of 90 scorable characters was selected, all being polymorphic among isolates of *Dicyma* spp. As observed by RAPD analysis, much of the polymorphism detected with AFLP's was also associated with the *D. pulvinata* isolates obtained from *M. ulei* lesions. The MDS model of the AFLP data also produced one dispersed group for the *D. pulvinata* isolates obtained from *M. ulei*, while the *pulvinata* isolates collected from non-*M. ulei* hosts and the outgroup were dispersed (Figure 2C).

The three molecular markers analyzed proved to be powerful tools for analyzing the intraspecific variability among *D. pulvinata* isolates. The calculation of the product-moment correlation coefficients for the different combinations of the RFLP, RAPD and AFLP on the *Dicyma* spp. isolates

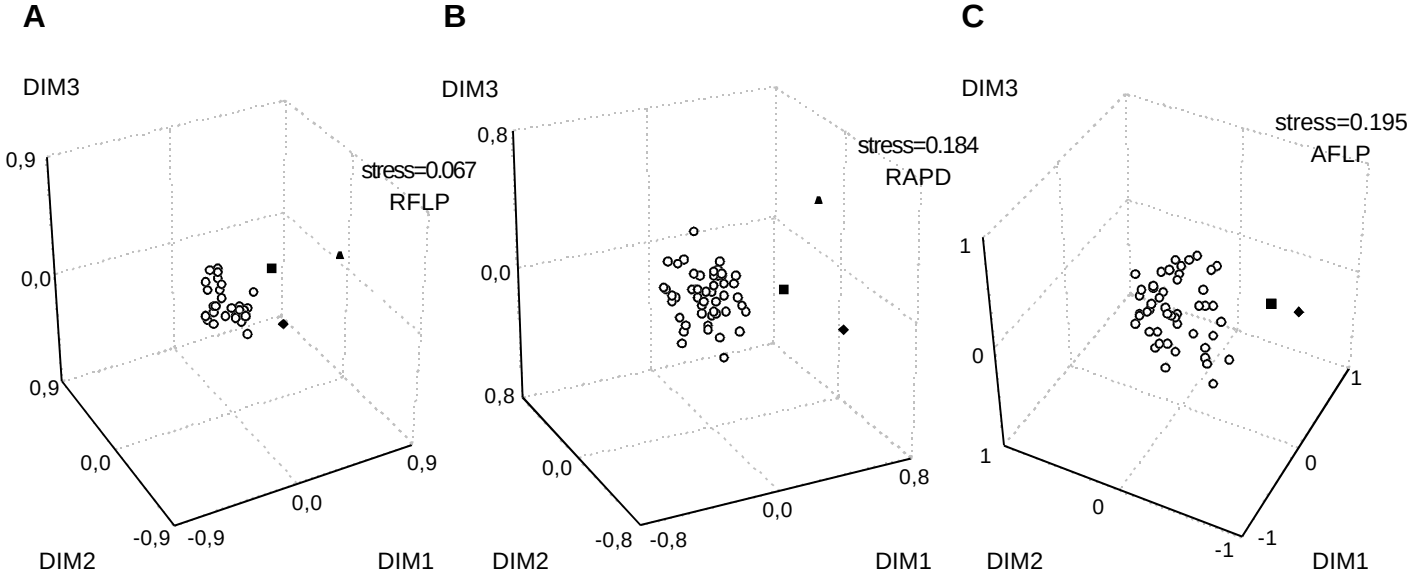

FIG. 2 - Multidimensional scaling model generated from distance matrices of (A) RFLP, (B) RAPD, and (C) AFLP data. The ○, ■, ◆, and ▲ represent *Dicyma pulvinata* isolates obtained from *Microcyclus ulei*, *D. pulvinata* isolate obtained from *Cercosporidium* sp. in India, *D. pulvinata* isolate obtained from *Cladosporium fulvum* in France, and *D. ampullifera* isolate, respectively.

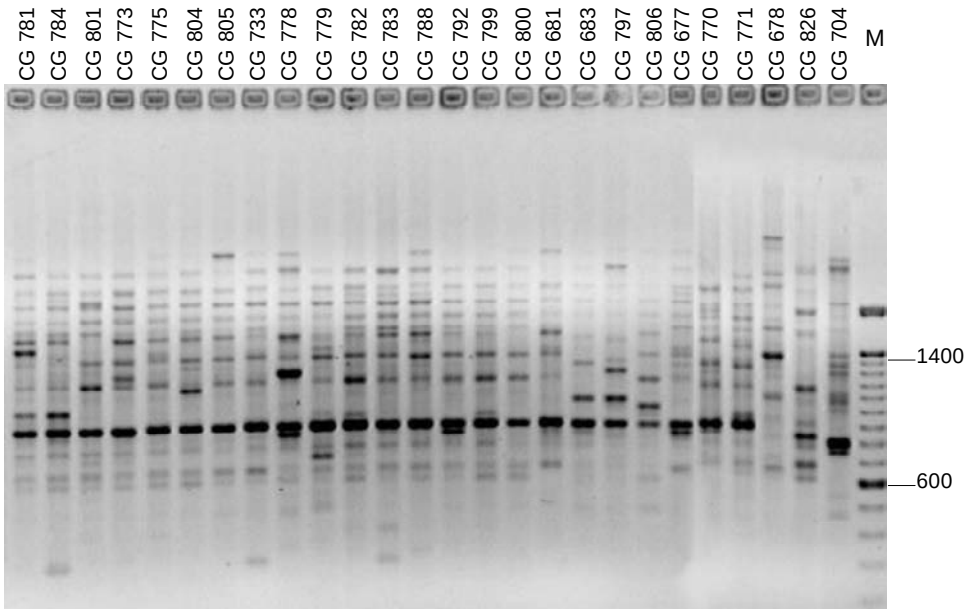

FIG. 3 - Example of ethidium bromide - stained 1.5% agarose gel showing the products from RAPD fingerprinting of *Dicyma* sp. isolates, with primer OPB-09. M = molecular size standards (1 kb DNA ladder).

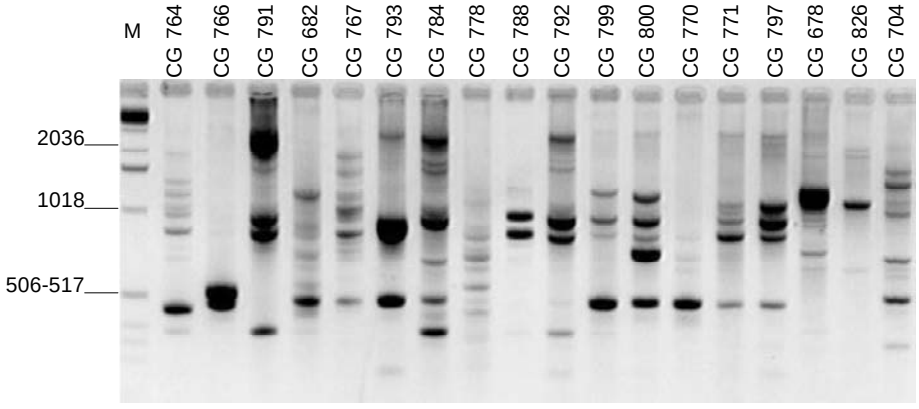

FIG. 4 - Example of ethidium bromide-stained agarose-synergel showing the products from AFLP fingerprinting of *Dicyma* sp. isolates, with primer DGB22. M = molecular size standards (1 kb DNA ladder).

demonstrated a significant correlation for the combination RFLP and RAPD (r=0.789). The association between RFLP and AFLP or RAPD and AFLP distance matrix produced lower r-values (0.560 and 0.598 respectively).

Spatial distribution of *Dicyma* spp. isolates based on a combination of genetic distances obtained from AFLP, RAPD and RFLP data sets, showed similar and separate results to the molecular markers. The isolates of *D. pulvinata* obtained from *M. ulei* lesions from different regions of Brazil form a group that is distinct from the non-*M. ulei* isolates and the outgroup (Figure 5). These results suggest a specialization of the isolates to the hosts.

Although the isolates obtained from *M. ulei* were grouped, the AMOVA tests showed that for each of the three types of molecular markers analyzed, there was a small but significant difference between the 25 isolates obtained from the humid regions, which are favorable to the development of SALB, North and Northeast Brazil, and the 24 isolates from the escape region of the disease, MT, Brazil (Tables 1 and 2). The variance components associated to the variation between these two regions were 0.67961 (P=0.00831), 0.85481 (P< 0.00001) and 0.50814 (P=0.00044) for RFLP, RAPD and AFLP, respectively.

Junqueira *et al.* (1987) have shown the genetic variability among isolates of the host *M. ulei* by isozymes analysis, and the correlation between virulence and the isoenzymatic patterns. Considering this, the distance between the two geographical populations of *D. pulvinata* occurring in lesions of *M. ulei* should be analyzed taking in consideration the variability of the host.

In spite of the differentiation between the two regions, variation was observed among individuals within regions,

TABLE 1 - Origin of *Dicyma pulvinata* isolates used in this study

	Host/ Isolate[1]	Geographic origin
	Microcyclus ulei	
1	CG 764	Brasiléia - AC
2	CG 766	Rio Branco - AC
3	CG 791	Rio Branco - AC
4	CG 763	Santo Antônio - AC
5	CG 682	Manaus - AM
6	CG 732	Manaus - AM
7	CG 734	Ituberá - BA
8	CG 761	Ituberá - BA
9	CG 762	Ituberá - BA
10	CG 767	Ituberá - BA
11	CG 772	Ituberá - BA
12	CG 786	Ituberá - BA
13	CG 793	Ituberá - BA
14	CG 795	Ituberá - BA
15	CG 796	Ituberá - BA
16	CG 765	Una - BA
17	CG 785	Una - BA
18	CG 798	Una - BA
19	CG 680	Itiquira - MT
20	CG 789	Itiquira - MT
21	CG 790	Itiquira - MT
22	CG 794	Itiquira - MT
23	CG 802	Itiquira - MT
24	CG 803	Itiquira - MT
25	CG 777	Nova Maringá - MT
26	CG 780	Nova Maringá - MT
27	CG 781	Nova MarIngá - MT
28	CG 784	Nova Maringá - MT
29	CG 801	Nova Maringá - MT
30	CG 773	Ponte de Lacerda MT
31	CG 775	Ponte de Lacerda - MT
32	CG 804	Ponte de Lacerda - MT
33	CG 805	Ponte de Lacerda - MT
34	CG 733	São José do Rio Claro - MT
35	CG 778	São José do Rio Claro - MT
36	CG 779	São José do Rio Claro - MT
37	CG 782	São José do Rio Claro - MT
38	CG 783	São José do Rio Claro - MT
39	CG 788	São José do Rio Claro - MT
40	CG 792	São José do Rio Claro - MT
41	CG 799	São José do Rio Claro - MT
42	CG 800	São José do Rio Claro - MT
43	CG 681	Belém - PA
44	CG 683	São Francisco - PA
45	CG 806	Ji-Paraná - RO
46	CG 677	Ouro Preto do Oeste - RO
47	CG 770	Ouro Preto do Oeste - RO
48	CG 771	Ouro Preto do Oeste - RO
49	CG 797	Ouro Preto do Oeste - RO
	Cercosporidium sp.	
50	CG 678	India
	Cladosporium fulvum	
51	CG 826	France

[1] CG, Embrapa Genetic Resources and Biotechnology Culture Collection, Brasília, Federal District, Brazil

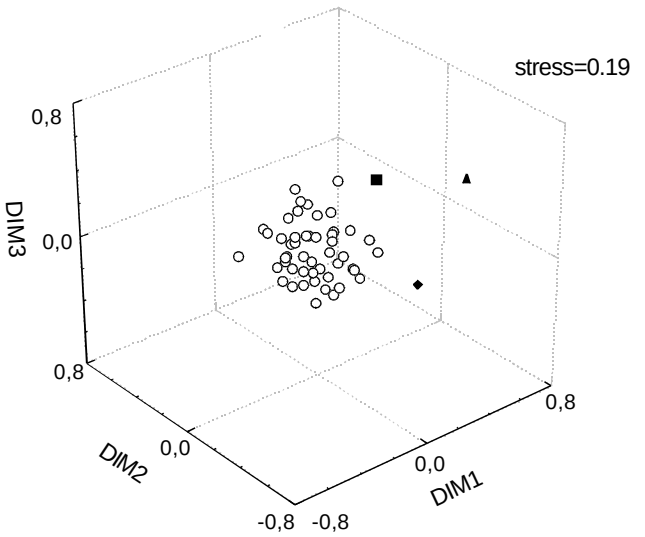

FIG. 5 - Spatial distribution of *Dicyma* spp. isolates based on combination of genetic distances obtained from AFLP, RAPD and RFLP data sets. The ◆, ■, ●, and ▲ represent *D. pulvinata* isolates obtained from *Microcyclus ulei* in Brazil, *D. pulvinata* isolate obtained from *Cercosporidium* sp. in India, *D. pulvinata* isolate obtained from *Cladosporium fulvum* in France, and *D. ampullifera* isolate, respectively.

TABLE 2 - Analysis of molecular variance (AMOVA) for 49 *Dicyma pulvinata* isolates grouped into two regions. Region 1 contains the North and Northeast isolates; region 2 contains the isolates from Mato Grosso State

Molecular marker/ Variance component	d.f.	Variance	% of variation	P-value[1]
RFLP				
Among regions	1	0.67961	10.07	0.00831
Among individuals/within regions	47	6.06908	89.93	
RAPD				
Among regions	1	0.85481	5.82	0.00000
Among individuals/within regions	47	13.821152	94.18	
AFLP				
Among regions	1	0.50814	6.74	0.00044
Among individuals/within regions	47	7.03411	93.26	

[1]P-value is the probability of obtaining a larger or equal variance by chance alone under the null hypothesis that the variance is zero. P-value is estimated from 16.000 sampling permutation.

indicating the existence of relevant genetic diversity between haplotypes within these populations. This heterogeneity might be important to ensure that the population fits to environment and host variation. In addition, this diversity might be necessary to effectively control the disease by interacting with the parasite, and adapting to changes in host resistance recognition genes. Tigano-Milani *et al.* (1995b) suggested, for entomopathogenic fungi, that it would be necessary for more than one haplotype to start and maintain epizootics under field conditions. Strongman & MacKay (1993) identified two varieties of the entomopathogenic fungus *Hirsutella longicolla* Strongman, Eveleigh & Royama, one associated to the first stage of the insect development, and the other associated to the sixth instar of the plague.

The results presented in this study demonstrated the utility of three molecular markers for the characterization of different populations of *D.* pulvinata.

ACKNOWLEDGEMENTS

Grateful thanks are expressed to William Sihler and Ana Maria C. L. de Barros for technical support; to Bergmann Morais Ribeiro for critical review of the manuscript; to Fundo Nacional do Meio Ambiente (FNMA) and Embrapa for partial support; and to Capes for the award of a scholarship to E. T. Tavares.

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