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Polymorphism in *Metarhizium anisopliae* var. *anisopliae* (Hypocreales: Clavicipitaceae) based on internal transcribed spacer-RFLP, ISSR and intron markers

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ABSTRACT. Isolates of entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* were characterized using internal transcribed spacer-RFLP, ISSR and intron splice site primers. Thirty-seven isolates were studied, most of which were obtained from the sugar cane pest, *Mahanarva fimbriolata* (Hemiptera: Cercopidae) from Tangará da Serra, Southwest Mato Grosso State, Brazil. Internal transcribed spacer-RFLP did not differentiate the isolates of *M. anisopliae* var. *anisopliae*, while ISSR and intron primers identified three distinct groups. Variability among these groups was 96% for $(GTG)_5$ and 100% for the other primers. We found considerable genetic variability, even among isolates from the same geographical origin and host.

Key words: *Metarhizium anisopliae*; *Mahanarva fimbriolata*; ITS-RFLP; ISSR; Intron

INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin is used in fighting a number of pests, particularly the sugar cane root spittlebug, *Mahanarva fimbriolata* Stal (Hemiptera: Cercopidae), which causes severe damage to this crop in the Southeast, Central-West and Northeast regions of Brazil (Dinardo-Miranda et al., 2006). According to Peixoto et al. (2009), for every nymph per linear meter there is a relative loss of 2.3% ton of sugar cane per hectare.

The genetic variability of entomopathogenic fungi can be considered one of the main advantages in the microbial control of insects (Alves, 1998) and can be observed by way of molecular markers. These can detect differences in DNA that will help in understanding the genetic diversity and the structure of fungal populations (Ferreira and Grattapaglia, 1998). Molecular markers based on the polymerase chain reaction (PCR) technique, such as ITS-RFLP (internal transcribed spacer-restriction fragment length polymorphism), ISSR (inter-simple sequence repeats) and intron splice site primer are used in characterizing inter- and intraspecific variations of *Metarhizium* and *Beauveria* (Márquez et al., 2006; Estrada et al., 2007; Velásquez et al., 2007).

ITS-RFLP enables the differentiation of species and isolates of *Metarhizium* based on the presence or absence of restriction sites in ribosomal DNA (Pipe et al., 1995). This technique, given the polymorphism observed, was used in designing specific primers for *M. anisopliae* var. *anisopliae* (Destéfano et al., 2004) and *M. anisopliae* var. *acridum* Driver, Milner & Trueman (Entz et al., 2005). The ISSR technique was used in studying the genetic variability of two varieties of *M. anisopliae* from different localities (Lima, 2005) and *Beauveria bassiana* (Balsamo) Vuillemin in China (Wang et al., 2005), Asia (Aquino de Muro et al., 2005) and Japan (Takatsuka, 2007). Group I introns, found in rDNA genes of eukaryotes, usually have irregular distribution, being present in some isolates and absent in others, which can become a source of genetic variability (Mavridou et al., 2000). Markers such as RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) have also been deployed in genetic variability studies of isolates of *M. anisopliae* from different hosts and geographic origins (Velásquez et al., 2007; Inglis et al., 2008; Freed et al., 2010).

The objective of this study was to characterize isolates of *M. anisopliae* var. *anisopliae* using ITS-RFLP, ISSR and intron splice site primer to analyze polymorphism among isolates of the same species and to evaluate different molecular markers, in providing an informative system for the DNA fingerprinting of these isolates and helping their detection in laboratory and field work.

MATERIAL AND METHODS

Isolates of *Metarhizium anisopliae* var. *anisopliae* and DNA extraction

Thirty-seven isolates of *M. anisopliae* var. *anisopliae* were studied; they were obtained from the sugar cane root spittlebug *M. fimbriolata*, with exception of isolates of URM6106 and URM6112, which were obtained from soil samples in the municipality of Tangará da Serra, Southwest Mato Grosso State, Brazil. The isolate URM6098 was obtained from *M.*

fimbriolata, in the municipality of Aripuanã, North of Mato Grosso State. These isolates were then deposited in the Culture Collection - Micoteca URM, of the Mycology Department of the Federal University of Pernambuco, Recife, PE, Brazil. The isolate of *M. anisopliae* var. *acridum* URM4412 was used as the outgroup.

The fungi were cultivated in minimum liquid medium (Pontecorvo et al., 1953), which was continuously stirred at 35 rpm at 28°C for 120 h. The mycelium was then collected by vacuum filtration and washed using sterile distilled water. The wet weight was determined for extraction of DNA and stored at -20°C. DNA was extracted using the Kuramae-Isioka (1997) method.

Amplification of the ITS region of rDNA

Amplification reactions were performed in a final volume of 25 µL containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP (Invitrogen, Carlsbad, CA, USA), primers ITS4 and ITS5, 0.2 µM each (Bioneer, Daedeok-gu, Daejeon, South Korea), 0.04 U/µL Taq DNA polymerase (Invitrogen) and 25 ng DNA, and the reactions were based on a modification of the method of Lima (2005). Amplification was according to the following program: an initial denaturation at 95°C for 4 min (1 cycle); 92°C for 1 min, 55°C for 1 min, and 72°C for 2 min (40 cycles), and a final extension at 72°C for 5 min (1 cycle). The amplification products of the locus ITS1-5.8S-ITS2 of rDNA were separated by electrophoresis on an agarose gel (1%), at 3 V/cm in 1X TBE buffer, pH 8.0, and using a 100-bp molecular weight marker (Invitrogen). The gel stained with ethidium bromide was visualized on an ultraviolet transilluminator and photographed using a digital camera.

ITS-RFLP - locus ITS1-5.8S-ITS2 of rDNA

The enzymatic digestion was carried out by mixing 4 µL PCR product from the ITS regions of rDNA with 16 µL restriction mix containing 0.1 U of the restriction enzymes: *Dra*I, *Pst*I, *Msp*I, AluI, *Eco*RI, *Hae*III, *Hin*fI, and *Bsh*1236I (Fermentas, Vilnius, Lithuania) in specific restriction buffer. Each reaction was incubated at 37°C for 3 h. The fragments obtained were separated by electrophoresis on an agarose gel (2%), according to the procedures described above.

ISSR

The amplification reactions were performed in a final volume of 25 µL containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM dNTP (Invitrogen), 0.25 µM primers $GACA_4$/GTG_5 (Bioneer), 0.04 U/µL Taq DNA polymerase (Invitrogen), and 25 ng DNA, and the reactions were based on a modification of the method of Lima (2005). Amplification was according to the following program: an initial denaturation at 93°C for 5 min (1 cycle); 93°C for 20 s, 55°C for 45 s, and 72°C for 1 min 30 s (40 cycles), and a final extension at 72°C for 6 min (1 cycle). For the primer M13, the reactions were carried out in a final volume of 25 µL containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.0 mM $MgCl_2$, 0.25 mM dNTP (Invitrogen), 0.5 µM primer M13 (Bioneer), 0.04 U/µL Taq DNA polymerase (Invitrogen) and 25 ng DNA, and reactions were based on a modification of the method of Lima (2005). The following program was applied: an initial denaturation at 94°C

for 4 min (1 cycle); 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min 30 s (35 cycles), and a final extension at 72°C for 5 min (1 cycle). The amplification products were separated by electrophoresis on an agarose gel (1.5%), at 3 V/cm in 1X TBE buffer, pH 8.0, and 1-kb molecular weight marker (Invitrogen). The gel stained with ethidium bromide was visualized on an ultraviolet transilluminator and photographed using a digital camera.

Intron splice site primer

Amplification reactions were performed in a final volume of 25 µL containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM dNTP (Invitrogen), 0.5 mM primer EI1 (Bioneer), 0.04 U/µL Taq DNA polymerase (Invitrogen) and 25 ng DNA, and reactions were based on a modification of the method of Lima (2005). Amplification was carried out using the following program: an initial denaturation at 94°C for 3 min (1 cycle); 94°C for 1 min, 44.5°C for 1 min, and 74°C for 30 s (40 cycles), and a final extension at 74°C for 5 min (1 cycle). Amplification products were separated using electrophoresis on an agarose gel (1.5%) as described above for the ISSR marker.

Analysis of the molecular data

The data obtained with molecular markers was analyzed by the NTSYSpc 2.1 program (Numerical Taxonomy System of Multivariate Programs) according to Rohlf (2000). These data were introduced in the form of binary variables and a similarity matrix was built, using Jaccard's coefficient. After similarity was determined, a dendrogram was generated using the UPGMA (unweighted pair group method with arithmetical average) grouping method, where a cophenetic matrix was also calculated. The Arlequin 3.11 program (Excoffier et al., 2005) was used for molecular variance analysis (AMOVA)

RESULTS

The product of the amplification of locus ITS1-5.8S-ITS2 of rDNA, using primers ITS4 and ITS5, showed a fragment of about 600 bp for isolates of *M. anisopliae* var. *anisopliae* and approximately 700 bp for *M. anisopliae* var. *acridum* URM4412. The amplified ITS fragment did not show a restriction site for the enzymes *Dra*I, *Pst*I and *Alu*I for all isolates of *M. anisopliae* var. *anisopliae* (Figure 1A, C). Enzymes *Msp*I, *Eco*RI, *Hae*III, *Hin*fI, and *Bsh*1236I generated monomorphic fragments, not differentiating isolates of *M. anisopliae* var. *anisopliae* (Figure 1B, D, E, F, and G). These enzymes generated fragments of different sizes: *Msp*I (370, 130 and 100 bp), *Eco*RI (290, 310 bp), *Hae*III (450, 150 bp), *Hin*fI (280, 100 bp), and *Bsh*1236I (380, 120, 100 bp). Fragments under 100 bp were not visualized on the gel and therefore not taken into account. The ITS fragments of the *M. anisopliae* var. *acridum* URM 4412 isolate did not show a restriction site for the enzymes *Dra*I and *Pst*I, but did display a restriction profile different from those of *M. anisopliae* var. *anisopliae* isolates when the enzymes *Alu*I (450, 250 bp), *Eco*RI (300 bp), *Hae*III (500, 150 bp), *Hin*fI (280, 200, 100 bp), and *Bsh*1236I (470, 130, 100 bp) were used. ITS-RFLP marker showed the same banding pattern for all isolates of *M. anisopliae* var. *anisopliae*, and it is not recommended in studies aimed at their differentiation in laboratory and field work.

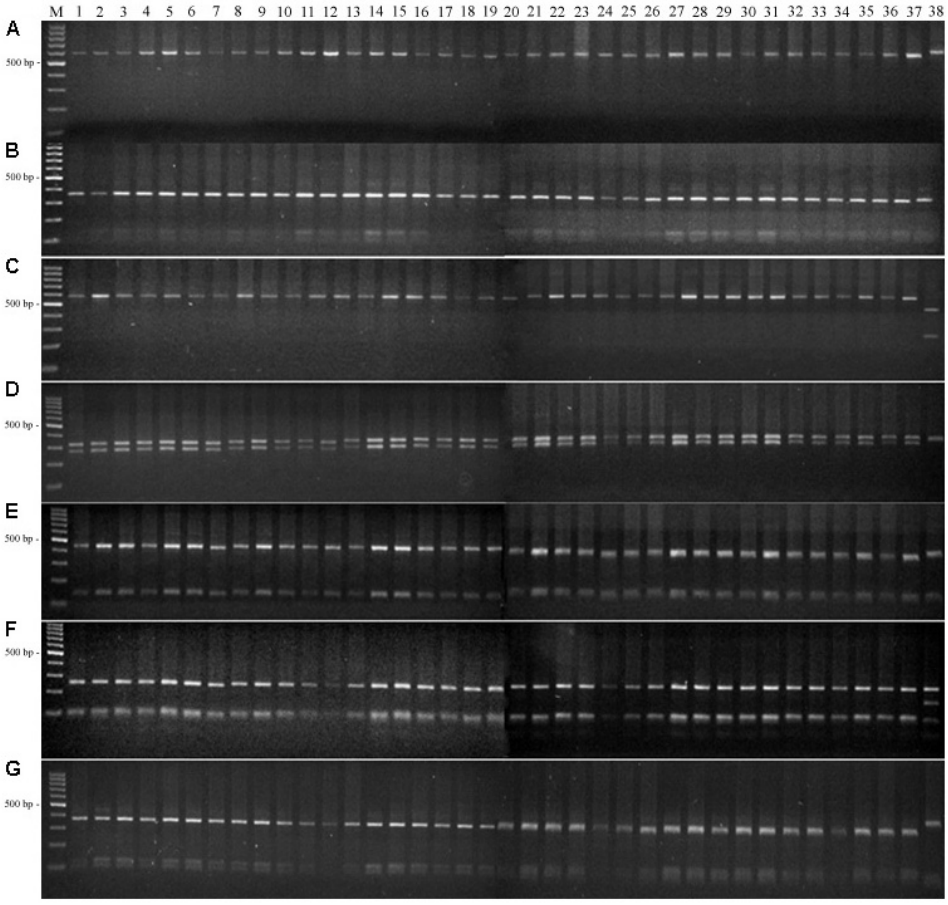

Figure 1. DNA fragment restriction profiles of the ITS region of rDNA obtained with the enzymes: **A.** *Dra*I, *Pst*I; **B.** *Msp*I; **C.** *Alu*I; **D.** *Eco*RI; **E.** *Hae*III; **F.** *Hin*fI; **G.** *Bsh*1236I. *Lane M* = 100-bp molecular weight marker. *Lanes 1* to *37* = isolates of *Metarhizium anisopliae* var. *anisopliae*. *Lane 38* = *M. anisopliae* var. *acridum* URM4412.

The dendrograms generated apparent similarities in fragment sizes, at the 100% level, forming three distinctive groups for ISSR and intron primers (Figures 2-5). Primer $(GACA)_4$ showed a non-grouping of isolates URM6098, URM6104, URM6106, URM6107, URM6109, URM6112, URM6131, URM6133, and URM6210 (Figure 2). For $(GTG)_5$, non-grouping of isolates URM6035, URM6104, URM6106, URM6108, and URM6112 (Figure 3) was demonstrated and for EI1 only isolate URM6133 (Figure 5). Among the isolates that did not group, URM6104, URM6106 and URM6112 stood out as displaying different band profiles, using two of the four primers tested, with the last two having been obtained from soil samples. Intron and ISSR markers showed differences among *M. anisopliae* var. *anisopliae* isolates, being higher for the $(GACA)_4$ primer and lower for the M13 primer (Figure 4). The latter showed lower polymorphism, since 84% of the isolates clustered in group 1. No coincident groups were noted among the dendrograms; however, isolates URM5948, URM5949, URM5951, URM5952, URM6034, URM6100, URM6102, URM6103, URM6105, URM6113, URM6114, and URM6128 remained grouped in every dendrogram.

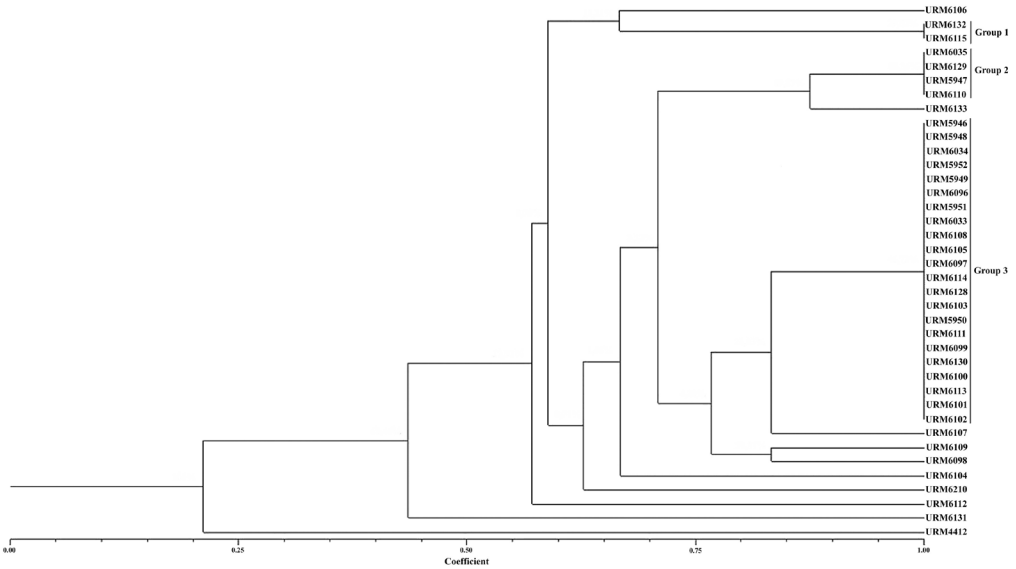

Figure 2. Genetic similarity dendrogram of 37 isolates of *Metarhizium anisopliae* var. *anisopliae* based on the primer's amplification products $(GACA)_4$. URM4412: *M. anisopliae* var. *acridum*. r = 96.10%. Group 1: URM6132, URM6115; Group 2: URM6035, URM6129, URM5947, URM6110; Group 3: URM5946, URM5948, URM6034, URM5952, URM5949, URM6096, URM5951, URM6033, URM6108, URM6105, URM6097, URM6114, URM6128, URM6103, URM5950, URM6111, URM6099, URM6130, URM6100, URM6113, URM6101, URM6102.

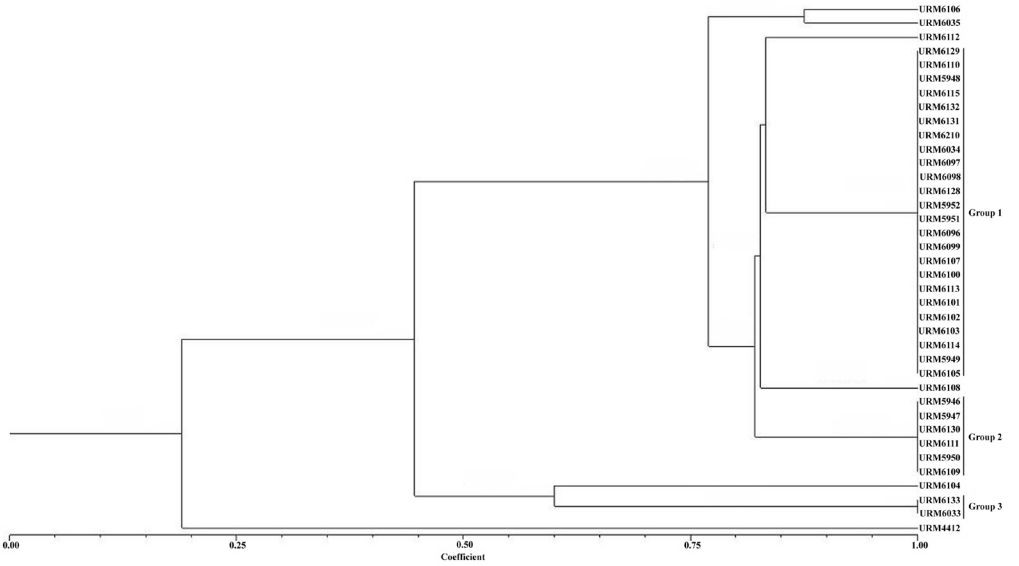

Figure 3. Genetic similarity dendrogram of 37 isolates of *Metarhizium anisopliae* var. *anisopliae* based on the primer's amplification products $(GTG)_5$. URM4412: *M. anisopliae* var. *acridum*. r = 96.39%. Group 1: URM6129, URM6110, URM5948, URM6115, URM6132, URM6131, URM6210, URM6034, URM6097, URM6098, URM6128, URM5952, URM5951, URM6096, URM6099, URM6107, URM6100, URM6113, URM6101, URM6102, URM6103, URM6114, URM5949, URM6105; Group 2: URM5946, URM5947, URM6130, URM6111, URM5950, URM6109; Group 3: URM6133, URM6033.

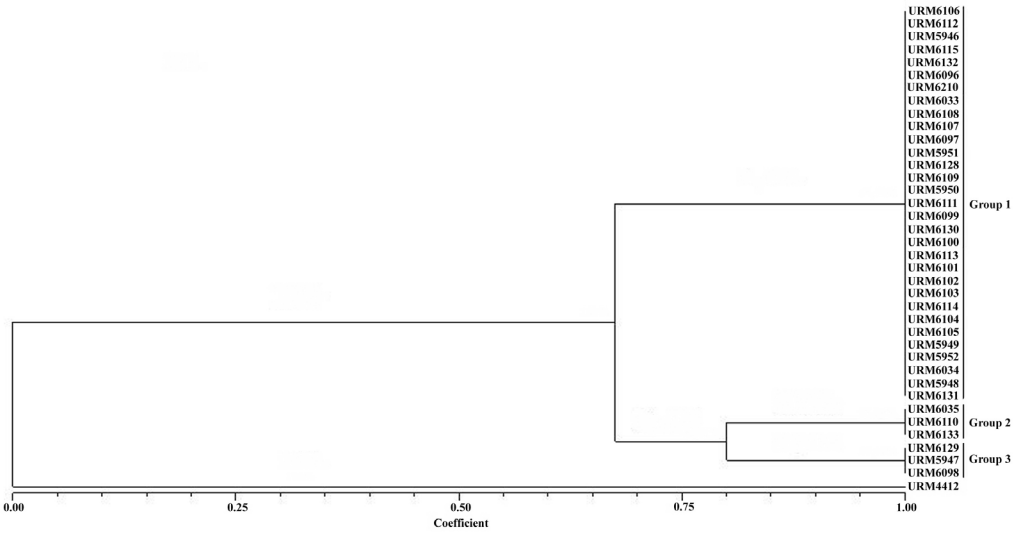

Figure 4. Genetic similarity dendrogram of 37 isolates of *Metarhizium anisopliae* var. *anisopliae* based on the primer's amplification products M13. URM4412: *M. anisopliae* var. *acridum*. r = 98.80%. Group 1: URM6106, URM6112, URM5946, URM6115, URM6132, URM6096, URM6210, URM6033, URM6108, URM6107, URM6097, URM5951, URM6128, URM6109, URM5950, URM6111, URM6099, URM6130, URM6100, URM6113, URM6101, URM6102, URM6103, URM6114, URM6104, URM6105, URM5949, URM5952, URM6034, URM5948, URM6131; Group 2: URM6035, URM6110, URM6133; Group 3: URM6129, URM5947, URM6098.

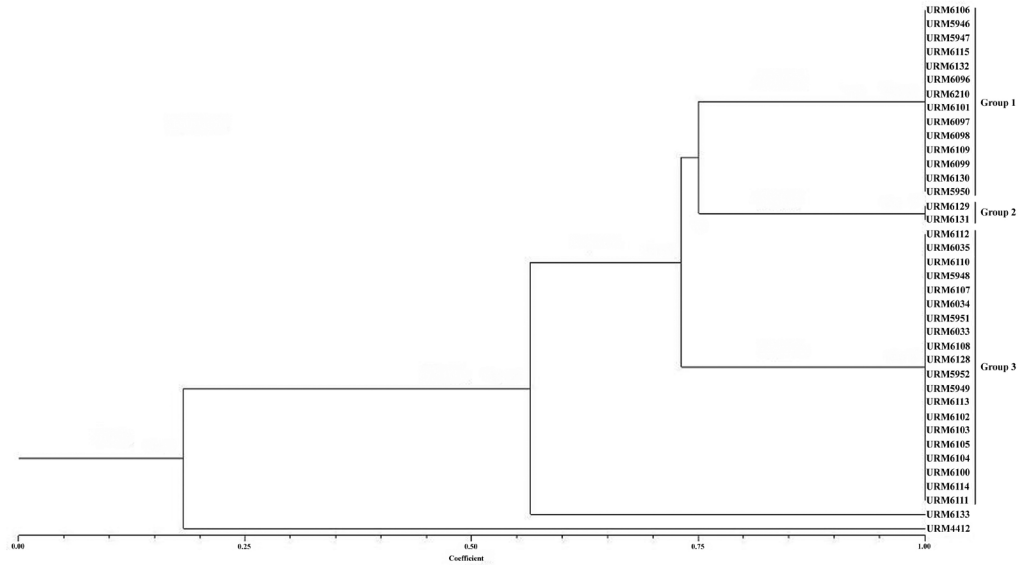

Figure 5. Genetic similarity dendrogram of 37 isolates of *Metarhizium anisopliae* var. *anisopliae* based on the primer's amplification products EI1. URM4412: *M. anisopliae* var. *acridum*. r = 98.25%. Group 1: URM6106, URM5946, URM5947, URM6115, URM6132, URM6096, URM6210, URM6101, URM6097, URM6098, URM6109, URM6099, URM6130, URM5950; Group 2: URM6129, URM6131; Group 3: URM6112, URM6035, URM6110, URM5948, URM6107, URM6034, URM5951, URM6033, URM6108, URM6128, URM5952, URM5949, URM6113, URM6102, URM6103, URM6105, URM6104, URM6100, URM6114, URM6111.

Based on molecular variance analysis, it was possible to observe that variability was higher between the groups than within them (Table 1). Considering only the groups generated by the dendrogram, it was seen that variability between groups, apparently equal, was 96.27% for $(GTG)_5$ and 100% for the other primers. However, if we ignored the groupings formed and compared isolates of *M. anisopliae* var. *anisopliae* and the outgroup *M. anisopliae* var. *acridum*, it could be seen that variability between these two groups was 77.11 to 89.80% and that variability within them was 10.19 to 22.89%. Fifteen exclusive amplification products for both varieties were observed, with seven of these being present only in *M. anisopliae* var. *anisopliae* and eight exclusively in *M. anisopliae* var. *acridum*.

Table 1. Analysis of molecular variance (AMOVA) of ISSR and intron markers for 37 isolates of *Metarhizium anisopliae* var. *anisopliae* and 1 isolate of *M. anisopliae* var. *acridum*.

Primer	Number of groups	Bootstrap F_{ST}		F_{ST}	F_{SC}	P
		95%	99%			
All	2	0.8980	0.9165	0.8395	0.1604	0.0342
All	31	1.0000	1.0000	1.0000	0.0000	0.0000
$GACA_4$[a]	2	0.8832	0.9149	0.7711	0.2288	0.0234
$GACA_4$[b]	3			1.0000	0.0000	0.0000
$GACA_4$[c]	13	1.0000	1.0000	1.0000	0.0000	0.0000
GTG_5[a]	2	0.9352	0.9586	0.8622	0.1377	0.0254
GTG_5[b]	3	1.0000	1.0000	0.9627	0.0372	0.0000
GTG_5[c]	9	1.0000	1.0000	0.9719	0.0280	0.0000
Intron[a]	2	0.9818	1.0000	0.8533	0.1466	0.0205
Intron[b]	3			1.0000	0.0000	0.0000
Intron[c]	5	1.0000	1.0000	1.0000	0.0000	0.0000
M13[a]	2	1.0000	1.0000	0.8980	0.1019	0.0185
M13[b]	3			1.0000	0.0000	0.0000
M13[c]	4	1.0000	1.0000	1.0000	0.0000	0.0000

The percentage of variation between the groups (F_{ST}) and within the groups (F_{SC}) is shown, considering both markers together and separately. **a.** considering 2 groups: isolates (*M. anisopliae* var. *anisopliae*) and outgroup (*M. anisopliae* var. *acridum*); **b.** considering only groups with more than 2 isolates formed by the dendrogram: G1, G2 and G3; **c.** considering all groups formed by the dendrogram: G1, G2, G3, G4, ... Gn, outgroup.0. *P < 0.05; **P < 0.01.

DISCUSSION

Among the 37 isolates, the genetic variability observed was dependent on the marker used. ITS-RFLP did not differentiate the isolates of *M. anisopliae* var. *anisopliae* when the locus ITS1-5.8S-ITS2 of rDNA was digested using the enzymes *Dra*I, *Pst*I, *Msp*I, *Alu*I, *Eco*RI, *Hae*III, *Hin*fI, and *Bsh*1236I. Velásquez et al. (2007) studied 39 isolates of *M. anisopliae* from different regions of Chile and observed 12 genotypes after digestion of the ITS1 region with the enzymes *Hha*I, *Hin*fIII, *Msp*I, *Tru*9I, *Alu*I, *Hae*III, and *Rsa*I and nine genotypes after digestion of the ITS2 region using the same enzymes, except *Rsa*I. The marker ITS-RFLP differentiated a few isolates, but there was no association between the diversity observed and the geographic origin of these isolates. Nonetheless, Pipe et al. (1995) found, based on the digestion of rDNA with *Bam*HI, *Hin*dIII, *Pst*I, and *Xho*I, that isolates of *M. anisopliae* grouped according to their geographic origin, but there was no correlation with the host. ITS-RFLP was also used in intraspecific differentiation studies of *B. bassiana* (Coates et al., 2002; Gaitan et al., 2002; Aquino de Muro et al., 2005), *B. brongniartii* (Saccardo) Petch (Wada et al., 2003)

and *Entomophthora muscae* (Cohn) Fresenius (Jensen et al., 2001). Isolates of *B. bassiana*, obtained from different hosts and geographic regions, showed the same band pattern after digestion of the locus ITSl-5.8S-ITS2 of rDNA with *Hae*III, *Hpa*II, *Eco*RI, *Pst*I, *Alu*I, and *Mbo*I (Aquino de Muro et al., 2003). In other cases, isolates of *B. brongniartii* (Neuvéglise et al., 1994) and *E. muscae* (Jensen et al., 2001) obtained from the same host showed the same band pattern.

Higher polymorphism was observed for $(GACA)_4$ and $(GTG)_5$ markers due to the non-grouping of some isolates that showed a different banding pattern. The three groups formed by ISSR and intron markers were considered to be distinctive groups according to AMOVA, since variability was higher between the groups than within them. ISSR $(GACA)_4$, $(GTG)_5$ and M13 and intron EI1 primers were used in studying polymorphism in fifteen isolates of *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum* from different areas and hosts (Lima, 2005). This author observed that $(GACA)_4$ primer was more sensitive in detecting the intraspecific variability among different *M. anisopliae* isolates. Four isolates of *M. anisopliae* var. *anisopliae* obtained from the spittlebug *M. posticata* showed 100% similarity with $(GTG)_5$ and EI1 primers. There was no correlation between groups and host or geographic origin. SSR markers (Ma097, 099, 142, 145, 164, 165, 195, 210, 307, 325, 327, and 375) were used in studying polymorphism in isolates of *M. anisopliae* from soil samples (80%) and insects (20%) from different regions of Chile (Velásquez et al., 2007) and soil samples (forest and agriculture) from countries in Asia and Europe (Freed et al., 2010). The authors observed the formation of three groups and a slight variation among the populations (18.5 and 19.35%), and there was no association between genetic diversity and the collection sites for the different isolates.

ISSR markers detected a high level of polymorphism for isolates of *B. bassiana*, which were grouped according to geographic origin, but there was no clear correlation between those isolates and their insect hosts (Aquino de Muro et al., 2005; Wang et al., 2005; Estrada et al., 2007) and for isolates of *E. muscae* (Lihme et al., 2009) and *Pandora neoaphidis* (Remaudiére & Hennebert) Humber (Tymon and Pell, 2005). Wang et al. (2005) observed, among 36 *B. bassiana* isolates, genetic similarity that ranged from 0.651 to 0.972. Estrada et al. (2007) studied 11 isolates and found that seven isolates showed exclusive bands and that ISSR primer 873 was able to distinguish all the isolates. Studies with respect to introns of group I have sought to identify them and check for polymorphism among the isolates of *M. anisopliae* by way of phylogenetic analysis (Mavridou et al., 2000; Márquez et al., 2006). This technique was also successfully applied in polymorphism studies of *B. bassiana* (Wang et al., 2003) and *B. brongniartii* (Neuvéglise et al., 1997).

Studies on the genetic diversity of entomopathogenic fungi aimed at determining the association between isolates and their point of origin or host still show ambiguous results. According to Rehner (2005), the association of a genotype to a given group of insects, could develop on a local geography scale. The association of genotype (number and sizes of fragments) of *M. anisopliae* with the spittlebug *M. fimbriolata*, for the 37 isolates used in this study, was more evident using the ITS-RFLP technique, since all isolates showed the same banding pattern. For the other markers (ISSR and intron), a differentiation was observed, indicating the existence of genetic variability among isolates of the same origin and host, probably resulting from mutations and/or parasexual recombination. Genetic variability is an important feature for biological control of *M. fimbriolata*, because among the isolates studied some may be effective in controlling this insect.

The results indicate that ISSR and intron markers allow the characterization and differentiation of isolates of *M. anisopliae*, especially for $(GACA)_4$ and $(GTG)_5$ markers, which provide DNA fingerprinting for some isolates. These markers can be successfully used in studies targeting their detection in laboratory and field work, enabling the monitoring of isolates after their application in the field.

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