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

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ABSTRACT.

*Metarhizium anisopliae* var. *anisopliae* was 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*
INTRODUCTION

*Metarhizium anisopliae*

used in fighting a number of pests, particularly the sugar cane root spittlebug, *Mahanarva fimbriolata*

intraspecific variations of *Metarhizium* 

*Beauveria*

*Metarhizium*

M. anisopliae 

*anisopliae* 

M. anisopliae 

*acridum* 

M. anisopliae 

Beauveria bassiana

(random amplified polymorphic DNA), SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) have also been deployed in genetic variability studies of

*M. anisopliae*

*M. anisopliae* 

*anisopliae*

formative 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**

*M. anisopliae* 

*anisopliae* 

*M. fimbriolata* 

*M.*
Metarhizium anisopliae var. anisopliae

fimbriolata

M. anisopliae var. acridum

um filtration and washed using sterile distilled water. The wet weight was determined for extrac

Amplification of the ITS region of rDNA

Amplification reactions were performed in a final volume of 25 µL containing reaction

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

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

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 Pst Msp Eco Hae Hin Bsh in specific restriction buffer. Each reaction was incubated at 37°C for 3 h. The fragments ob

ISSR

The amplification reactions were performed in a final volume of 25 µL containing reac

0.25 µM primers GACA (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 , 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
a final extension at 72°C for 5 min (1 cycle). The amplification products were separated by

**Intron splice site primer**

Amplification reactions were performed in a final volume of 25 µL containing re

(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

a final extension at 74°C for 5 min (1 cycle). Amplification products were separated using

**Analysis of the molecular data**

using Jaccard’s coefficient. After similarity was determined, a dendrogram was generated us

. The Arlequin 3.11 program (Excoffier et al.,

**RESULTS**

The product of the amplification of locus ITS1-5.8S-ITS2 of rDNA, using primers

M. anisopliae aniso-

M. anisopliae

Bsh

M. anisopliae

Msp

Bsh

Alu

M. anisopliae

acridum

URM4412. The amplified ITS

restriction profile different from those of M. anisopliae

their differentiation in laboratory and field work.
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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.

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*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*. 

*Metarhizium anisopliae* var. *anisopliae*
Figure 2.
Genetic similarity dendrogram of 37 isolates of *Metarhizium anisopliae* var. *anisopliae* based on the primer’s amplification products (GACA). URM4412: *M. anisopliae* var. *acridum*. r = 96.10%. Group 1: URM6132, URM6115; Group 2: URM6035, URM6129, URM5947, URM6110; Group 3: URM5946, URM5948, URM6034, URM5949, URM6096, URM5952, 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). 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.
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**Figure 4.** *Metarhizium anisopliae* anisopliae amplification products M13. URM4412: *M. anisopliae* acridum

**Figure 5.** *Metarhizium anisopliae* anisopliae primer’s amplification products EI1. URM4412: *M. anisopliae* acridum
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)\textsuperscript{5} and 100% for the other primers. However, if we ignored the groupings formed and compared isolates of \textit{M. anisopliae} \textit{var. anisopliae} and the outgroup \textit{M. anisopliae} \textit{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 \textit{M. anisopliae} \textit{var. anisopliae} and eight exclusively in \textit{M. anisopliae} \textit{var. acridum}.

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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Groups</th>
<th>F\textsubscript{ST}</th>
<th>F\textsubscript{SC}</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>2</td>
<td>0.8980</td>
<td>0.1604</td>
</tr>
<tr>
<td>All</td>
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<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>GACA</td>
<td>a</td>
<td>0.8832</td>
<td>0.2288</td>
</tr>
<tr>
<td>GACA</td>
<td>b</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>GACA</td>
<td>c</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>GTG</td>
<td>a</td>
<td>0.9352</td>
<td>0.1377</td>
</tr>
<tr>
<td>GTG</td>
<td>b</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>GTG</td>
<td>c</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>Intron</td>
<td>a</td>
<td>0.9818</td>
<td>0.1466</td>
</tr>
<tr>
<td>Intron</td>
<td>b</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>Intron</td>
<td>c</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>M13</td>
<td>a</td>
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<td>0.0185</td>
</tr>
<tr>
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<td>b</td>
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<td>c</td>
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DISCUSSION

Among the 37 isolates, the genetic variability observed was dependent on the marker used. ITS-RFLP did not differentiate the isolates of \textit{M. anisopliae} \textit{var. anisopliae} when the locus ITS1-5.8S-ITS2 of rDNA was digested using the enzymes Dra\textsubscript{I}, Pst\textsubscript{I}, Msp\textsubscript{I}, Alu\textsubscript{I}, Eco\textsubscript{RI}, Hae\textsubscript{III}, Hin\textsubscript{fI}, and Bsh\textsubscript{1236I}. Velásquez et al. (2007) studied 39 isolates of \textit{M. anisopliae} from different regions of Chile and observed 12 genotypes after digestion of the ITS1 region with the enzymes Hha\textsubscript{I}, Hin\textsubscript{fIII}, Msp\textsubscript{I}, Tru\textsubscript{9I}, Alu\textsubscript{I}, Hae\textsubscript{III}, and Rsa\textsubscript{I} and nine genotypes after digestion of the ITS2 region using the same enzymes, except Rsa\textsubscript{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\textsubscript{HI}, Hin\textsubscript{dIII}, Pst\textsubscript{I}, and Xho\textsubscript{I}, that isolates of \textit{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 \textit{B. bassiana} \textit{bassiana} (Coates et al., 2002; Gaitan et al., 2002; Aquino de Muro et al., 2005), \textit{B. brongniartii} (Saccardo) Petch (Wada et al., 2003).
Polymorphism in *Metarhizium anisopliae* var. *anisopliae* 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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REFERENCES
Polymorphism in *Metarhizium anisopliae* var. *anisopliae*


