



Detection of *Metarhizium anisopliae* var. *anisopliae* within infected sugarcane borer *Diatraea saccharalis* (Lepidoptera, Pyralidae) using specific primers

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Abstract

In order to construct specific primers for the detection and identification of the entomopathogenic fungus *Metarhizium* within infected sugarcane borer (*Diatraea saccharalis*) larvae we analyzed the ITS1 -5.8S- ITS2 rDNA regions of strains and varieties of *M. anisopliae*, *M. album* and *M. flavoviride*. The PCR amplification of these regions yielded a unique fragment of approximately 540 bp for *M. anisopliae* variety *anisopliae* strains E₉, B/Vi and C (isolated in Brazil), 600 bp for *M. a. anisopliae* strain 14 (isolated in Australia), 650 bp for the *M. album* and 600 bp for *M. flavoviride* strains. The PCR products were digested with different restriction endonucleases (*Afa* I, *Alu* I, *Dde* I, *Hae* III, *Hpa* II and *Sau* 3A) and the PCR-RFLP profiles showed clear differences between the species. Sequencing of the ITS-5.8S rDNA regions allowed us to design one specific primer (ITSMet: 5' TCTGAATTTTTTATAAGTAT 3') for the Brazilian *M. a. anisopliae* strains (E₉, B/Vi and C) and another specific primer (ITSMet14: 5' GAAACCGGGAC TAGGCGC 3') for the Australian strain (strain 14). Amplification was not observed with *M. album*, *M. flavoviride* and *Beauveria bassiana* strains. DNA extracted from larvae infected with the Brazilian or Australian strains were tested using the specific primers designed by us to identify the fungal strains with which the larva had been infected. The correct fungal strain was successfully detected within 48 h of the insect having been infected, showing that this molecular technique allows rapid and secure detection and identification of *M. anisopliae*.

Key words: *Metarhizium anisopliae*, entomopathogenic fungi, PCR-RFLP, ITS region.

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Introduction

About 80% of the etiologic agents involved in insect diseases are fungi, encompassing 90 genera and more than 700 species. Several research groups have verified the entomopathogenicity of the Deuteromycete fungi *Metarhizium anisopliae*, which has become an important biocontrol agent used in the microbial control of insect pests.

The sugarcane spittlebug (*Mahanarva posticata*) (Homoptera, Cercopidae) causes serious losses in sugarcane crops but has been successfully controlled in northeastern Brazil using biocontrol by *M. anisopliae*, which has also been used in the biocontrol of other spittlebug genera (*Aenolamia*, *Deois* and *Zulia*) infesting pasture grasses (Onofre *et al.*, 2002). Biological control of the sugarcane borer (*Diatraea saccharalis*) (Lepdoptera, Pyralidae) by

various *M. anisopliae* and *Beauveria bassiana* isolates has been achieved by Alves *et al.* (1984; 1985).

The production of mycoinsecticides is very simple, but their use as biocontrol agents depends critically on the standardization of the production runs and the stability of the mycoinsecticide under field conditions where the formulation must allow the fungus to maintain its virulence. It is also important to monitor how mycoinsecticides disseminate and survive in the environment after application (Hegedus and Khachatourians, 1996a).

There is a need for specific and sensitive systems for the detection and identification of fungi which can be used to evaluate the dispersion and environmental persistence of mycoinsecticides. Commercial implications such as the identification of existing or new fungal isolates, quality control and patent protection must also be considered because several researcher groups and commercial companies are developing production, formulation and application methods involving entomopathogens for use in the control of insect pests of agricultural and public health importance.

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The classical methods for the identification of entomopathogenic fungi are based on spore morphology, biochemical characteristics and immunological properties, but PCR and DNA sequence analysis are also being intensively used as standard tools for the detection, identification and phylogenetic analysis of many fungal species. These techniques are particularly valuable for the large number of species which are incapable of growth in the laboratory under artificial conditions and from which pure DNA is not easily obtained (Bindslev *et al.*, 2002).

Several molecular genetic methods have been suggested as potential systems for the identification and monitoring of entomopathogenic fungi. Techniques which use DNA probes to create restriction fragment length polymorphism (RFLP) fingerprints have been used to distinguish between species (Hegedus and Khachatourians, 1993a) or individual isolates (Hegedus and Khachatourians, 1993b), although direct DNA probing methods may not exhibit the desired degree of sensitivity required for detection under field conditions (Hegedus and Khachatourians, 1996b). Other methods based on the polymerase chain reaction (PCR), such as the random amplified polymorphic DNA (RAPD) method, also exhibit the ability to discriminate between entomopathogenic fungal isolates (Jensen *et al.*, 2001; Freire *et al.*, 2001; Alves *et al.*, 2001; Urtz and Rice, 1997; Fungaro *et al.*, 1996; Leal *et al.*, 1994; Bidochka *et al.*, 1994) but the use of random oligonucleotide primers in the design of these systems is not useful in the detection of fungi within environmental samples which can contain a significant amount of DNA from indigenous biotic materials.

An alternative approach is to use ribosomal DNA (rDNA), an important molecular marker widely used in the identification and differentiation of species. The rDNA

operon of eukaryotes can be present in multiple copies per genome, with each unit consisting of regions coding for rRNA, 18S, 5.8S and 28S genes as well as the two internal spaces (ITS 1 and ITS 2) between these regions, each rDNA unit being separated by one intergenic space (IGS). The rDNA unit presents sequence variations which may be used in systematic studies at different taxonomic levels (Fouly *et al.*, 1997; Argentina, 1999). The rDNA 18S and 28S regions are the most conserved units, and may be used in differentiating genera and species, while the ITS and IGS spacer regions have accumulated more variability and are better used to differentiate species or strains within the same species (Esteve-Zarzoso *et al.*, 1999).

In the study reported in this paper we sequenced the ITS1 –5.8S- ITS2 region of various *Metarhizium* strains and designed specific primers for the detection and identification of *M. anisopliae* within infected *D. saccharalis* larvae.

Material and Methods

Fungal strains

The *Metarhizium album* strain was supplied by Dr. Myrian Tigano-Milani (Cenargen-EMBRAPA, Brasília, DF, Brazil) while the other *Metarhizium* and *Beauveria bassiana* strains were obtained from the Germplasm Bank of the Laboratório de Genética de Microrganismos Entomopatogênicos (Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas - UNICAMP, Campinas, SP, Brazil). All strains are listed in Table 1.

Each fungal strain was individually grown on rice medium (50g of food grade rice plus 40 mL distilled water, sterilized at 121 °C for 20 min) for ten days at 28 °C.

Table 1 - Fungal strains used in this study and their insect hosts.

Fungal Strain	Host insect	Source	GenBank accession n.
<i>Metarhizium anisopliae</i> variety <i>anisopliae</i>			
E ₉	<i>Deois flavopicta</i> (Homoptera: Cercopidae)	Brazil	AY 373632
B/Vi (E ₉ auxotrophic mutant ty ^a)		Brazil	AY 373633
C	<i>Mahanarva posticata</i> (Homoptera: Cercopidae)	Brazil	AY 373634
14	unknown	Australia	AY 375445
<i>Metarhizium album</i>			
201 (ARSEF 2082)	<i>Cofana spectra</i> (Homoptera: Cicadellidae)	Indonesia	AY 375446
<i>Metarhizium flavoviride</i>			
204 (ARSEF 2024)	<i>Otiorynchus sulcatus</i> (Coleoptera: Curculionidae)	France	AY 375447
209 (ARSEF 2133)	<i>Ceutorhynchusmacula alba</i> (Coleoptera: Curculionidae)	Czechoslovakia	AY 375449
<i>Beauveria bassiana</i>			
ARSEF 959	<i>Spodoptera frugiperda</i> (Lepdoptera: Noctuidae)	Brazil	
ARSEF 2253	<i>Autographa gamma</i> (Lepdoptera: Noctuidae)	France	
ARSEF 2629	<i>Diatraea saccharalis</i> (Lepdoptera: Pyralidae)	Brazil	

ARSEF: Agricultural Research Service of Entomopathogenic Fungi.

Host insects and infection procedure

Third instar sugarcane borer (*Diatraea saccharalis*) larvae (produced from stock cultures maintained in CTC/COPERSUCAR, Piracicaba, SP, Brazil) were infected by allowing them to walk over the sporulating fungi for 10 min so that each larva received a potential inoculum of about 3.5×10^6 conidia. Each infected larva was placed in a plastic plate containing a small piece of sugarcane stalk and the plates kept at 28 °C and 80-100% relative humidity. Twenty-four larvae per treatment were collected 48 h post-infection, sacrificed immediately by freezing at -20 °C for 4 h and transferred to -80 °C. This procedure was repeated for each fungal strain.

To remove external fungi, the surface of the dead larvae were carefully washed, with gentle agitation, three times in 2.5% (v/v) sodium hypochloride solution for 10 min, three times in 0.1% (v/v) Tween 80 solution and a further three times with 0.85% (w/v) sodium chloride solution and the individual larva placed on sterilized filter paper and dried in a laminar flow chamber. To confirm the effectiveness of the surface disinfection process 0.1 mL of the effluent from the last wash was inoculated onto agar plates of the complete medium of Pontecorvo *et al.* (1953) and the plates incubated for up to ten days at 28 °C. Infection of the larvae was confirmed by placing some washed larvae onto the surface of the same medium and incubating the plate under the same conditions.

The dead, washed, cleaned and dried larvae were stored in Petri dishes in an ultra-freezer at -80 °C. Non-infected sugarcane larvae were washed and stored in the same way and the extracted DNA used as a control.

DNA extraction and amplification

Genomic DNA was extracted from fungal mycelia, non-infected larvae and infected larvae (48 h post-infection) as described by Garber and Yoder (1983). The ITS regions were amplified using the ITS1 and ITS4 primers (White *et al.*, 1990). The PCR reactions were carried out using a PTC 200 DNA Thermal Cycler (MJ Research) using 25 µL reaction volumes, each containing 100 ng of template DNA, 1X *Taq* buffer, 0.4 mM of each primer ITS1 and ITS4, 200 mM of dNTP mix, 0.1% bovine serum albumin (BSA) and 1U of *Taq* DNA polymerase (Genotaq-GENON) using one cycle of 3 min at 95 °C followed by 32 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C with a final extension of 3 min at 72 °C.

RFLP analysis of PCR amplified DNA

The PCR products (3 µL) were individually digested with restriction endonucleases *Afa* I, *Alu* I, *Dde* I, *Hae* III, *Hpa* II and *Sau* 3A I under conditions specified by the manufacturer (Amersham Biosciences) and the restriction fragments separated by electrophoresis in 3% agarose gels using 1X TAE buffer (Sambrook *et al.*, 1989). The gels

were stained with ethidium bromide and then visualized under UV. The molecular weights of the fragments were determined by comparison with a 100 bp DNA ladder (Amersham Biosciences).

Sequence analysis

The ITS1 – 5.8S – ITS2 amplified products (about 540 bp) were purified with the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences) and sequenced in an automated system (ABI 377, Perkin Elmer, Foster City, CA, USA). The sequences were aligned using the ClustalW (<http://www.ebi.ac.uk/clustalw>) program and compared with those available in the GenBank data base for *M. a. anisopliae* (accession numbers AF516295 and AF134150), *M. album* (AF137067), *M. flavoviride* (AF138269) and *Beauveria bassiana* (BBA345090) using the Genetic Data Environment (GDE) software version 2.2 and phylogenetic trees constructed using the Neighbor-joining method (gopher://megasun.bch.umontreal.ca:70/11/GDE).

Primer design and PCR conditions for *Metarhizium anisopliae*

We found that the sequenced regions of the products from the Brazilian *M. a. anisopliae* strains E₉, B/Vi and C differed from those of the Australian *M. a. anisopliae* strain 14 and this allowed us to design a specific primer for each of these distinct groups based on the location of the differences in the ITS1 – 5.8S – ITS2 sequences. Two Oligonucleotide primers were designed: a 20 mers oligonucleotide primer (5' TCTGAATTTTTTATAAGTAT 3'), which we named ITSMet, for the E₉, B/Vi and C strains and an 18 mers primer (5' GAAACCGGGACTAGGCGC 3'), which we named ITSMet14, for strain 14. These primers were used in PCR reactions with DNAs from the different *Metarhizium* strains, the ITS4 oligonucleotide being used as the reverse primer and primer specificity checked by comparison with available GenBank/EMBL/DDBJ data base sequences.

The DNAs from *M. a. anisopliae* strains E₉, B/Vi and C and 14 were used as targets in PCRs performed in 25 µL reaction mixtures containing 1X *Taq* buffer, 0.6 mM of the ITSMet primer, 0.4 mM of the ITS4 primer, 0.1% BSA (w/v), 0.2 mM of dNTP mix and 1U of *Taq* DNA polymerase (Genotaq-GENON). The ITSMet/ITS4 set primer amplification reaction conditions consisted of 3 min at 95 °C followed by 32 cycles of 1 min at 94 °C, 1 min at 46 °C and 1 min at 72 °C with a final extension of 3 min at 72 °C. The reaction conditions for the ITSMet14/ITS4 primer set were 3 min at 95 °C followed by 32 cycles of 1 min at 94 °C, 1 min at 61 °C and 1 min at 72 °C with a final extension of 3 min at 72 °C. The products were visualized under UV light in 1% agarose gels stained with ethidium bromide.

Detection of *Metarhizium anisopliae* within infected insects

The ability of the PCR-based system to identify *M. anisopliae* within infected larvae was determined using total DNA extracted from infected larvae 48 h after inoculation with *M. a. anisopliae*. PCR was conducted either with the ITSMet/ITS4 or ITSMet14/ITS4 primer sets according to the strain involved, DNAs from pure cultures of the different *Metarhizium* strains were used as controls and DNA from non-infected larvae was also tested. The presence of *Metarhizium* strains within infected larvae was confirmed by restriction profiling (using the endonucleases *Afa* I, *Alu* I, *Dde* I, *Hae* III, *Sau* 3A I and *Taq* I) of the PCR products obtained from infected larvae and pure cultures of the fungi.

Results

PCR-RFLP of the ITS1 – 5.8S – ITS2 sequence

The amplification of the ITS region resulted in a single product for all isolates. The size of the product was about 540 bp for *M. a. anisopliae* strains E₉, B/Vi and C, 600 bp for *M. a. anisopliae* strain 14, 650 bp for the *M. album* strain and 600 bp for *M. flavoviride* strains. The PCR products digested with the restriction enzymes *Afa* I, *Alu* I, *Dde* I, *Hae* III, *Hpa* II and *Sau* 3A I (Figure 1, Table 2) showed distinct RFLP patterns for different strains. Digestions with *Afa* I generated fragments of from 100 to 600 bp while *Alu* I yielded fragments of from 100 to 650 bp. Digestions with *Hae* III produced fragments ranging from 100 to 450 bp and *Dde* I fragments of from 150 to 450 bp, but with slightly different profiles for different strains. The *Hpa* II digestions revealed fragments ranging from 100 to 370 bp, showing clear differences between *M. anisopliae*, *M. album* and *M. flavoviride*. Fragments from 100 to 410 bp were obtained in the *Sau* 3A I digestions and differentiation between the three *Metarhizium* species was again evident. The Australian strain of *M. a. anisopliae* (strain 14) showed a distinct profile in comparison with the other strains of the same species with all restriction enzymes tested, representing a separate group.

Phylogenetic analysis of the ITS sequence

The alignments of the nucleotide sequences of the ITS1 – 5.8S – ITS2 regions of the strains investigated by us with GenBank ITS sequences for the same region and species of fungi are shown in Figure 2. The alignments and phylogenetic analysis (Figure 3) confirmed the taxonomic identity of the strains used in our study, with *M. album* strain 201 grouping with the GenBank *M. album* sequence AF137067 while *M. flavoviride* strains 204 and 209 grouped with the GenBank *M. flavoviride* sequence AF138269, although strain 204 was phylogenetically more distantly related. The *M. a. anisopliae* ITS sequences from strains E₉, B/Vi and C were phylogenetically identical to

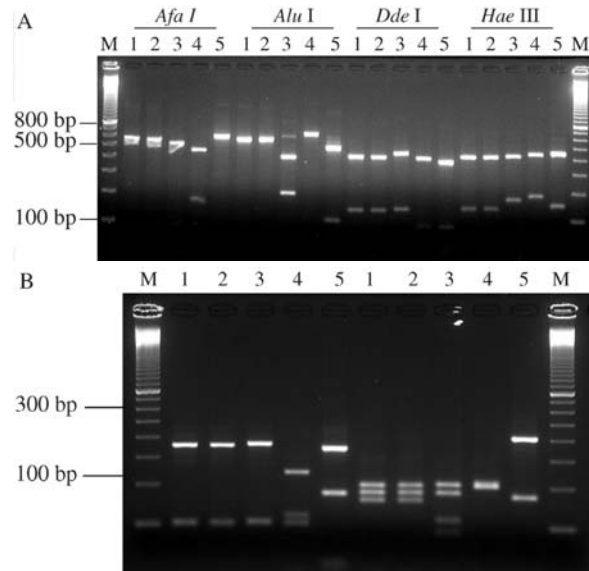


Figure 1 - PCR-RFLP of the ITS1 – 5.8S – ITS2 region digested with (A) *Afa* I, *Alu* I, *Dde* I and *Hae* III, and (B) *Hpa* II e *Sau* 3A I. 1 = *Metarhizium anisopliae* var. *anisopliae* strain E₉; 2 = *M. a. anisopliae* strain C; 3 = *M. a. anisopliae* strain 14; 4 = *Metarhizium album* strain 201; (5) *Metarhizium flavoviride* strain 204. M = 100 bp marker.

Table 2 - RFLP profiles produced by digestions of the ITS-PCR products with different restriction enzymes.

Restriction enzymes	Fragments (bp)	Fungal strains
<i>Afa</i> I	540	<i>M. anisopliae</i> : E9, B/Vi, C
	100, 500	<i>M. anisopliae</i> : 14
	180, 450	<i>M. album</i> : 201
	600	<i>M. flavoviride</i> : 209
<i>Alu</i> I	540	<i>M. anisopliae</i> : E9, B/Vi, C
	200, 400	<i>M. anisopliae</i> : 14
	650	<i>M. album</i> : 201
	100, 450	<i>M. flavoviride</i> : 204 and 209
<i>Dde</i> I	150, 400	<i>M. anisopliae</i> : E9, B/Vi, C
	180, 430	<i>M. anisopliae</i> : 14
	190, 450	<i>M. album</i> : 201
	160, 450	<i>M. flavoviride</i> : 204 and 209
<i>Hae</i> III	120, 410	<i>M. anisopliae</i> : E9, B/Vi, C
	130, 450	<i>M. anisopliae</i> : 14
	100, 410	<i>M. album</i> : 201
	100, 390	<i>M. flavoviride</i> : 204 and 209
<i>Hpa</i> II	100, 360	<i>M. anisopliae</i> : E9, B/Vi, C
	100, 370	<i>M. anisopliae</i> : 14
	100, 120, 250	<i>M. album</i> : 201
	100, 160, 340	<i>M. flavoviride</i> : 209
<i>Sau</i> 3A I	170, 190, 210	<i>M. anisopliae</i> : E9, B/Vi, C
	100, 120, 190, 2	<i>M. anisopliae</i> : 14
	10	<i>M. album</i> : 201
	210	<i>M. flavoviride</i> : 209
	180, 410	

each other and to GenBank *M. a. anisopliae* sequences AF516295 and AF134150, while *M. a. anisopliae* strain 14 from Australia formed a separate group, supporting the high level of polymorphy detected in the PCR-RFLP experiments. The *B. bassiana* strains grouped with the GenBank sequences (Figure 3).

Design of new *M. anisopliae* primers

As pointed out in the Material and Methods section, the presence of sequence differences in the PCR products of the ITS-5.8 S rDNA regions meant that the specificity of the system to generate primers capable of recognizing regional differences in the ITS sequences of the strains could be increased by sequencing the PCR products from the *M. a. anisopliae*, *M. album* and *M. flavoviride* strains and comparing the sequences to each other. We observed some differences in the sequenced regions of the products from the Brazilian *M. a. anisopliae* (strains E₉, B/Vi and C) and the Australian *M. a. anisopliae* strain 14 which allowed us to develop a specific primer for each of these distinct

groups, ITSMet for the Brazilian strains and ITSMet14 for Australian strain.

With all the *M. a. anisopliae* strains studied by us the ITSMet/ITS4 primer set yielded a specific 440 bp PCR product. Although this set has been developed only to Brazilian strains, the Australian strain 14 manifested a low-intensity band in the gels. The ITSMet14/ITS4 primer set produced a 490 bp fragment only from strain 14. Neither of these primers produced amplified products with any of the *M. album*, *M. flavoviride* or *Beauveria bassiana* strains (Figure 4).

Detection of *Metarhizium anisopliae* within infected insects

The presence of *Metarhizium* strains within infected larvae was confirmed by the restriction profiles of the PCR

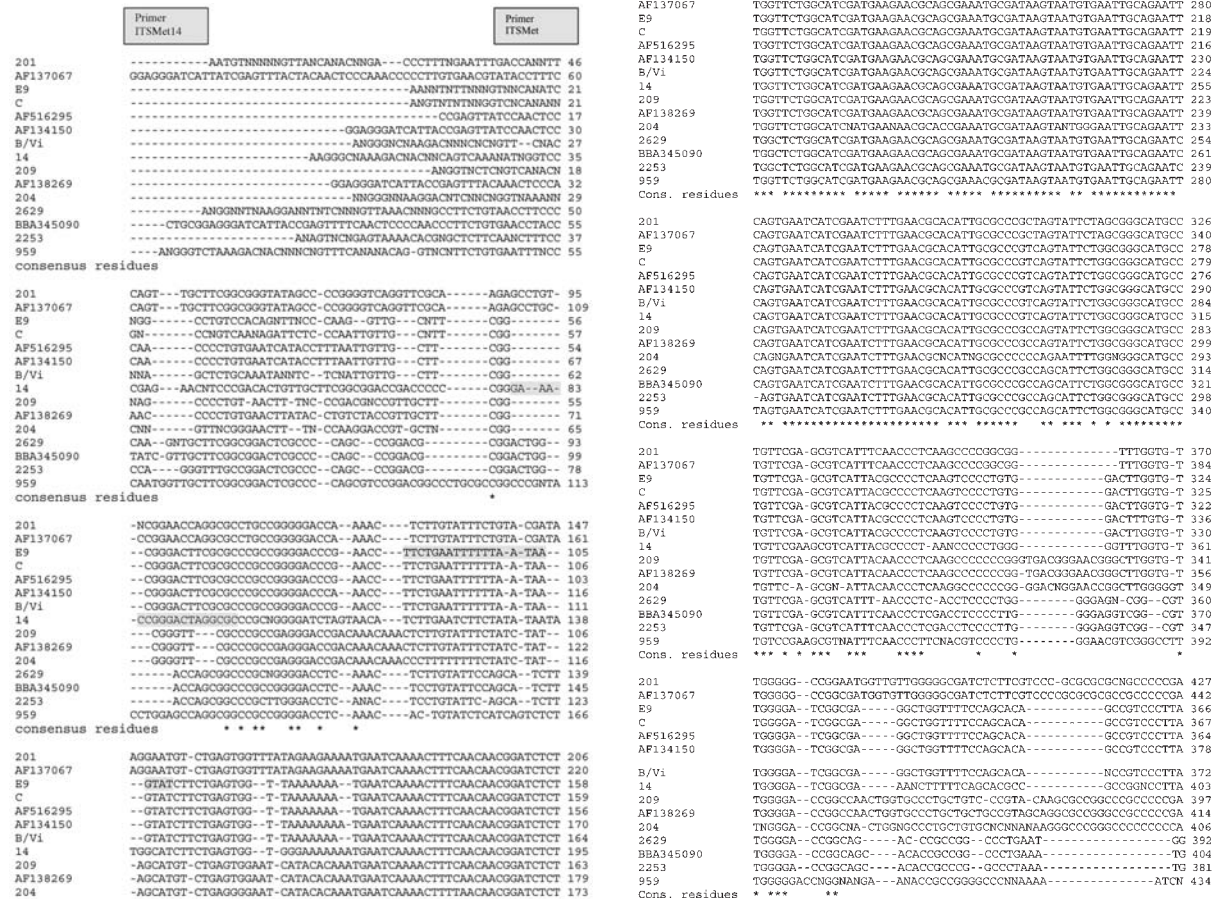


Figure 2 - Alignment of the nucleotide sequences of the ITS1 – 5.8S – ITS2 region of the strains investigated by us (*Metarhizium anisopliae* var. *anisopliae* strains E₉, B/Vi, C and 14; *Metarhizium album* strain 201; *Metarhizium flavoviride* strains 204 and 209; *Beauveria bassiana* strains 959, 2253 and 2629) with GenBank ITS sequences for *M. a. anisopliae* (AF516295 and AF134150), *M. album* (AF137067), *M. flavoviride* (AF138269) and *B. bassiana* (BBA345090). The specific primers ITSMet and ITSMet14 developed by us are indicated by gray boxes. *consensus residues.

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201      AATAAATTGG -CGGCTCGCGGCGCC -TCCTCTGCGTANNAACATGTTGCCCTCC - 481
AF137067  AATGAATTG -CGGCTCGCGGCGGCC -TCCTCTGCGTAGTAACATGTTGCCCTCTCGC 498
E9       AATTAATTGG -CGGCTCGCGGCGGCC -TCCTCTGCGCA -GTA -GTAACAACACTCGC 420
C       AATTAATTGG -CGGCTCGCGGCGGCC -TCCTCTGCGCA -GTA -GTAACAACACTCGC 420
AF516295 AATTAATTGG -CGGCTCGCGGCGGCC -TCCTCTGCGCA -GTA -GTAACAACACTCGC 418
AF134150 AATTAATTGG -CGGCTCGCGGCGGCC -TCCTCTGCGCA -GTA -GTAACAACACTCGC 432
B/Vi    AATTAATTGG -CGGCTCGCGGCGGCC -TCCTCTGCGCAAGTA -GTAACAACACTCGC 427
14      AATTTATTGGGCGGCGCTCCCGGCGCTTCCCTCTGCGCAAGAA -GTAACCTCGCTCGC 460
209     AATGAATTGGGCGGCGCTCGCGGCGCT -CCCTCTGCGTAAGTA -ACACAATCTCGC 453
AF138269 AATGAA -TTGGGCGCTCGTGGGCGCT -CCCTCTGCGTA -GTA -GCACAATCTCGC 468
204     AAANAANTGGGGGCGCTTCCCGGCT -NCCCTTNGGAAAAAA -NNAAAATTTNCCA 462
2629    AAGTGGCGG -CC -GT - - -CGGCGGCA -CCTCTGCGTAGTA -TACAGCT -CGCAC 440
BBA345090 AAGTGGCGG -CCGCT - - -CGGCGGCA -CCTCTGCGTAGTA -TACAGCT -CGCAC 453
2253    AAGTGGCGG -CCGCT - - -CGGCGGCA -CCTCTGCGTAGNA -TACAGCTTGGCAC 431
959     AAGTNGGAACCCCTTNGCCGCGGAC -CTTTGNGNAGAAA -CTCAGNTTNNIC 489
Cons. residues * * * * *

201      AACAGAGCC -GGCGCG -C -ACTGNGTAAA -CCACC -ACTTTTC - -CAAGTT 529
AF137067 AACAGAGCCC -GGCGCG -CCCTGCGTAAACCC -ACTTTTTTCAGAGTT 553
E9       AACAGAGCCCCCGCGCGGCC -ACTGCGTAAACCC -CAACTTT -TTATA -GTT 473
C       AACAGAGCCC -GGCGCGTCCANTGCGGTAAACCC -CAACTTT -TTATA -GTT 474
AF516295 AACAGAGCCC -GGCGCGTCC -ACTGCGTAAACCC -CAACTTT -TTATA -G - 469
AF134150 AACAGAGCCC -GGCGCGTCC -ACTGCGTAAACCC -CAACTTT -TTATA -GTT 485
B/Vi    AACAGAGCC -GGCGCG -CC -ACTGCGNAAAACCC -CAACTTT -TTATAAGTT 479
14      AAGCGAGCCC -GGCGCGCA -NCTGCGAARAACCCCATCACTTTGTATCA -GTT 517
209     AAGTGGCGGCGCGGCG -CACTGCGTAAACG -CA -CAACTTT -TTATAAGTT 505
AF138269 AAGTGGCGGCGCGGCGG -CACTGCGTAAACG -CA -CAACTTT -TTATAAGTT 521
204     ACTTGANAGGGGGGGGGGCGTAAACCC -CCNCTTT -TTATA - 518
2629    CGG - -AACCCGACGCG -CCAC -GCGCT -AAAACCC -CAACTTC -TGAAGTT 489
BBA345090 CGG - -AACCCGACGCG -CCAC -GCGCT -AAAACCC -CAACTTC -TGAAGTT 501
2253    CGG - -AACCCGACGCG -GCAGCGCGTTAAACCC -CAACTTC -TGAAGTT 481
959     CGGGAAACCCGAGGNG -CNTNGCGA -AAAAGCC - -CANNTA -AAAACNTN 541
Cons. residues * * * * *

201      GACCNC -AATCAGG -AAGAATCCCTTACTAGATT - - - - - 563
AF137067 GACCTCGAATCAGG -TAGGAAT - - - - - 574
E9       GACCTCGAATCAGG -TAGGACTACCGCTGAACCTAAGCATATCAATAGCGGAAA - 528
C       GACCTCGAATCAGG -TAGGACTACCGCTGAACCTA -GCATATCAATAGCNGAA - 528
AF516295 GACCTCGAATCAGG -TAGGACT - - - - - 506
AF134150 GACCTCGAATCAGG -TAGGACT - - - - - 533
B/Vi    GACCTCGAATCAGG -TAGGACTACCGCTGAACCTAAGCATATCAATAGCGGAAA - 533
14      GACCTCGAAT -AG -GANGACNCCC -CCNAACTTA - - -CTCATTAACCGGAAA - 563
209     GACCTC -AATCAGG -GANGAATACCCGCTGAACCTACATATCAN - 548
AF138269 GACCTCGAATCAGG -TAGGAAT - - - - - 542
204     GACCTCGAATCA - - - - - 501
2629    GACCTCGAATCAGG -TAGGACTACCGCTGAACCTAAGCATATCAATAGC - 550
BBA345090 GACCTCGAATCAGG -TAGGACTACCGCTGAACCTAAGCATATCAATAGC - 527
2253    GACCTCGAATCAGG -TAGGACTACCGCTGAACCTAAGCATATCAATAGC - 527
959     AACTTGAANTAGGAGGAGAAACCCCTGACACTTTGCTTTCNATACGGGGGA - 598
    
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Figure 2 (Cont).

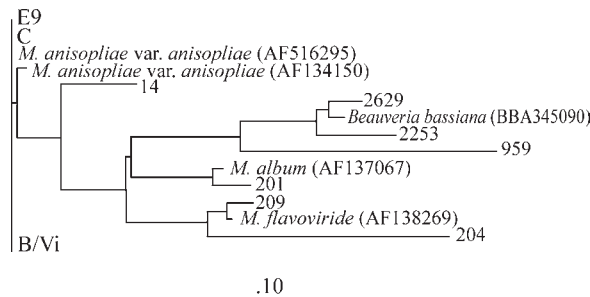
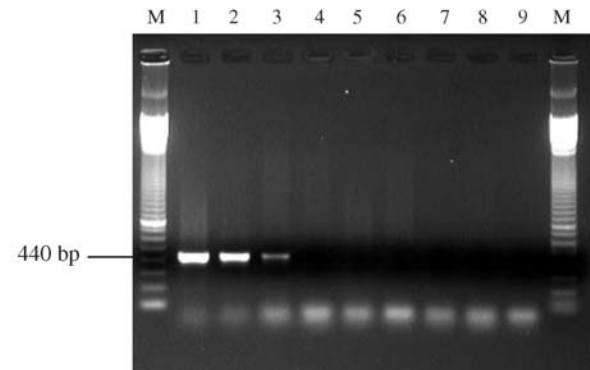


Figure 3 - Phylogenetic analysis of ITS1 – 5.8S – ITS4 rDNA sequences from *Metarhizium anisopliae*, *Metarhizium album*, *Metarhizium flavoviride* and *Beauveria bassiana* strains based on the Neighbor-joining method. GenBank accession numbers are listed after species names.



products obtained from infected larvae and pure cultures of the fungi (Figures 5 and 6).

Discussion

Our PCR-RFLP data showed that the variation in length and restriction sites in the ITS regions could be used to differentiate *M. a. anisopliae* from *M. album* and *M. flavoviride*. The six distinct restriction enzymes tested in this study were able to clearly distinguish the different *Metarhizium* species investigated. Ribosomal genes and their ITS and IGS spacer regions have been widely used for the identification and differentiation of species (Fouly *et al.*, 1997) as well as in taxonomic (Driver *et al.*, 2000), phylogenetic (Rakotonirainy *et al.*, 1994) and genetic diversity (Anderson *et al.*, 2001; Uetake *et al.*, 2002) studies, with ITS sequences having been reported as being useful for discriminating between different species of fungi (Neueglise *et al.*, 1994; Fouly *et al.*, 1997; Jensen *et al.*, 2001; Anderson *et al.*, 2001; Thomsen and Jensen, 2002).

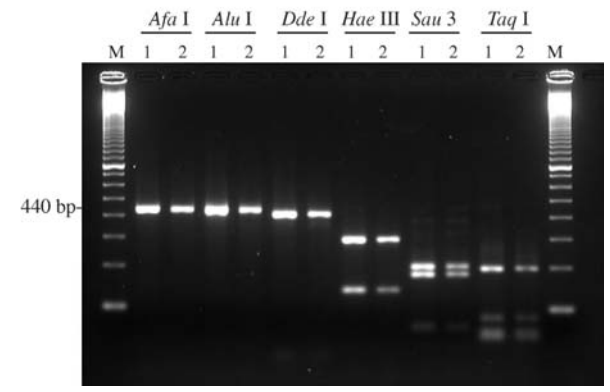


Figure 5 - PCR-RFLP profiles of *Metarhizium anisopliae* var. *anisopliae* strain E₉ using the ITSmet/ITS4 primer set with the restriction enzymes *Afa* I, *Alu* I, *Dde* I, *Hae* III, *Sau* 3 A I and *Taq* I. 1 = DNA from a pure culture of strain E₉; 2 = DNA from larvae infected with strain E₉. M = 100 bp DNA marker.

Figure 4 - Amplification of DNA from pure cultures and infected larvae using the primer sets ITSmet/ITS4 (A) and ITS14/ITS4 (B). Primer set A: 1 = strain E₉; 2 = larvae infected by strain E₉; 3 = strain 14; 4 = larvae infected by strain 14; 5 = strain 201; 6 = strain 204; 7 = strain 959; 8 = non-infected larvae; 9 = control reaction without DNA. Primer set B: 1 = strain 14; 2 = larvae infected by strain 14; 3 = strain E₉; 4 = larvae infected by strain E₉; 5 = strain 201; 6 = strain 204; 7 = strain 959; 8 = non-infected larvae; 9 = control reaction without DNA. M = 100 bp DNA marker.

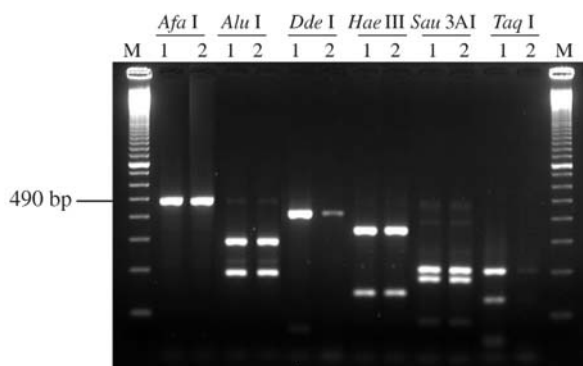


Figure 6 - PCR-RFLP profiles of *Metarhizium anisopliae* var. *anisopliae* strain 14 using the ITS_{Met14}/ITS₄ primer set with the restriction enzymes *Afa* I, *Alu* I, *Dde* I, *Hae* III, *Sau* 3A I and *Taq* I. 1 = DNA from a pure culture of strain 14; 2 = DNA from larvae infected with strain 14. M = 100 bp DNA marker.

Our ITS1 – 5.8S – ITS2 sequencing data showed variations which allowed us to design specific primers which could not only detect and identify *M. a. anisopliae* but also to differentiate between our Brazilian strains (E₉, B/Vi and C) and the Australian strain (strain 14) because the sequence variation of strain 14 was very different from that of the other members of the same species, these sequence variations allowing us to develop a specific primer for strain 14.

Isolation of PCR-amplifiable DNA from environmental samples is often difficult due to the co-isolation of compounds with the ability to inhibit the PCR (Hegedus and Khachatourians, 1996b). Samples of DNA from plants (Do and Adams, 1991) and fungi (Pfeifer *et al.*, 1993) may retain polysaccharides which can cause problems during amplification and insect cuticles may contain a number of compounds that can act as potent PCR inhibitors (Hackman, 1974). Our extraction protocol produced DNA from infected larva with sufficient purity for direct PCR amplification and although this DNA produced a reduced amount of product in comparison with that produced when DNA from pure cultures was used the amount of product was still sufficient for us to be able to identify the fungal species with which the larva had been infected.

Several fungi have been cited as being potential mycoinsecticides (Samson *et al.*, 1988), although only a few have been intensively investigated and examined at the molecular level (Hegedus and Khachatourians, 1996b). Commercial mycoinsecticides (mycopenicidines) require specific and sensitive methods for the identification of the specific fungal strain used during production, such methods being needed not only to ensure strain stability and protect patents but also for use under field conditions for such purposes as making environmental impact assessments.

Specific primers have been developed for the detection and identification of some entomopathogenic fungi such as *Beauveria bassiana* (Hegedus and Khachatourians, 1996b) and *Glucocladium catenulatum* (Paavananen-Huhtala

et al., 2000). In this study we developed a PCR-based primer set system specific for *Metarhizium anisopliae* var. *anisopliae* which can simply, rapidly and securely detect this fungus in *Diatraea saccharalis* larvae 48 h after infection without the need to use techniques such as probe hybridization or DNA sequencing.

Our study also showed that *M. a. anisopliae*, *M. album* and *M. flavoviride* can be clearly differentiated using PCR-RFLP of the ITS 1-5.8S-ITS 2 region, supporting the view of Curran *et al.* (1994) who used amplification and sequencing of the 5.8 S gene, intergenic regions ITS 1 and ITS 2 and phylogenetic analysis to show that *M. anisopliae* and *M. flavoviride* + *M. album* represent two separate evolutionary lines.

The fact that DNA extracted from infected insects can be rapidly analyzed using our set of primers means that the identification of the entomopathogen fungus *M. a. anisopliae* in laboratory and field studies can be used not only to evaluate the efficacy of mycopenicidines and indicate when re-application is necessary but also to study the environmental persistence of entomopathogens, such evaluations normally taking a long time because entomopathogenic fungi must be re-isolated and cultured under specific conditions in order for them to be identified by morphological and physiological tests. Our method offers an alternative approach for typing *M. a. anisopliae* strains within infected insects and reduces the need for time-consuming conventional methods. This technique also opens up the possibility of monitoring the environmental impact of *M. a. anisopliae* mycoinsecticides on pollinators and other non-target insects.

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