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Development of a PCR-based diagnostic assay for the specific detection of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum*

Susan C. ENTZ[1], Dan L. JOHNSON[1] and Lawrence M. KAWCHUK[2]

[1] *University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada.*
[2] *Agriculture and Agri-Food Canada Research Centre, Lethbridge, Alberta T1J 4B1, Canada.*
E-mail: *susan.entz@uleth.ca*

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The entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* is registered as a mycopesticide for acridid control in Africa and Australia. Traditionally, identification of *M. anisopliae* var. *acridum* infection in grasshoppers and locusts has relied upon development of fungal growth in infected cadavers. Conventional methods of detection of this entomopathogen in the environment and non-target organisms have been based on culture and bioassay. A PCR-based method for the detection of *M. anisopliae* var. *acridum* was developed. Sequence data from the distinct ITS rDNA regions facilitated the design of PCR primers that were used in PCR-based diagnostic assays for the detection of fungal DNA. The amplified sequence was 420 bp in length and specific to *M. anisopliae* var. *acridum*. Isolates of *M. anisopliae* var. *anisopliae* and *M. flavoviride* produced no PCR product with these primers. Other fungal entomopathogens, plant pathogens, mycopathogens, and soil saprophytes were also not detected by the pathogen-specific primers. The assay was also effective for the detection of *M. anisopliae* var. *acridum* DNA in the presence of soil DNA extracts and in infected grasshoppers.

INTRODUCTION

Prior to the release of a biocontrol agent, the ability to identify and to monitor its impact on the target pest, persistence, and fate in the environment should be demonstrated (Bidochka 2001). A further issue requiring consideration is differentiation of the introduced organism from native populations.

Metarhizium anisopliae var. *acridum* is a hyphomycetous fungus that is pathogenic to grasshoppers and locusts. It has been commercialized as Green Muscle® in Africa (Douthwaite, Langewald & Harris 2000) and as Green Guard® in Australia (Milner & Hunter 2001) for the control of acridids. Registration has also been procured in Madagascar (Lomer *et al.* 2001), and field tests have been conducted in Brazil (Magalhães *et al.* 2001).

Traditionally, identification in the genus *Metarhizium* has been through the observation of morphological features on culture media, microscopic examination of spores and associated structures, and bioassay of target hosts, resulting in initial recognition of two species (*M. anisopliae* and *M. flavoviride*) with *M. album* later restored as a third. Concern, however, over the demonstration of considerable overlap in ranges of spore sizes and other features raised uncertainty over the taxonomic relationships among *Metarhizium* species (Bridge *et al.* 1997). Difficulties in identification are frequently encountered as different morphologies can be exhibited under varying environmental and physiological conditions. Spore morphology was previously observed to vary within the same culture and between isolates of the genus *Metarhizium*. Conidia and blastospores can be of variable size and shape (Glare, Milner & Beaton 1996). There may also be differences in colony morphology between isolates of the same variety (Milner *et al.* 2003). Lomer *et al.* (2001) noted that *M. anisopliae* var. *acridum* cannot be distinguished from other *M. anisopliae* varieties on the basis of spore size and shape.

Earlier, Bridge *et al.* (1997) had used molecular characterization to propose recognition of those isolates associated with acridoid hosts as a single distinctive genotype denoted as *M. flavoviride* Group 3. However, a high level of genetic diversity demonstrated by sequence data at the ITS and 28S rDNA D3 regions in this genus indicated a more complex resolution at the specific and varietal levels (Driver, Milner & Trueman 2000). Recognition of *M. album* as a separate species was supported by morphologically based taxonomy and molecular data; however, polymorphisms in the ITS region for *M. anisopliae* and

M. flavoviride suggested further refinement at the infraspecific level for these two species. Based on ITS sequence data, representative isolates were assigned to ten separate clades, four of these varieties of *M. anisopliae* and five varieties of *M. flavoviride*, while *M. album* was retained in a distinct separate clade. The acridoid isolates, most previously identified as *M. flavoviride* on the basis of conidial and phialide morphology, clustered as their own distinct clade and were described as *Metarhizium anisopliae* var. *acridum*. The work by Driver *et al.* (2000) was one of the first major indications within *Metarhizium* that the ovoid (rather than cylindrical) conidia, more clavate (rather than cylindrical) conidiogenous cell were not altogether dependable characters for species-level taxonomy in this genus (Richard A. Humber, pers. comm.).

Bioassay of target hosts has served as a sensitive method for the detection of *M. anisopliae* var. *acridum*, although caution must be exercised in the identification of isolates derived by this technique since acridids can serve as hosts to *M. anisopliae* other than *M. anisopliae* var. *acridum*. Moreover, in general, bioassays may fail in the detection of an isolate due to unfavourable temperature or target host, and thus may suggest artificially low levels of the pathogen. Notwithstanding, bioassay has been the method of choice for detection of *M. anisopliae* var. *acridum* in field trials (Lomer *et al.* 1993, Caudwell & Gatehouse 1996, Delgado *et al.* 1997a, Langewald *et al.* 1997, Lomer 1997, Milner *et al.* 1997, Magalhães *et al.* 2000) and for surveys and screening of virulent isolates (Zimmerman *et al.* 1994, Bateman *et al.* 1996, Thomas, Gbongboui & Lomer 1996, Shah *et al.* 1998).

Comparative studies of nucleotide sequences of rRNA genes have provided significant data for analysis of phylogenetics and taxonomy. White *et al.* (1990) introduced the use of PCR methods for the amplification of the ITS regions in nuclear rDNA of the fungal genome. The ITS sequences are an ideal target for the development of species-specific primers because they evolve relatively rapidly and are highly variable in length and nucleotide content between closely related species, and sometimes within a species as has been demonstrated for the genus *Metarhizium*. The objective of this study was to use sequence data from the ITS rDNA regions to develop a PCR-based assay for the highly specific detection of *M. anisopliae* var. *acridum*. An objective diagnostic assay was required that would facilitate the differentiation of an introduced strain of *M. anisopliae* var. *acridum* from native populations of *M. anisopliae* and *M. flavoviride* in environmental samples.

MATERIALS AND METHODS

Fungal isolates and cultivation

The fungal isolates studied are listed in Table 1. All were propagated and maintained on potato dextrose agar (PDA). *M. anisopliae* var. *acridum* (IMI 330189; commercialized as Green Muscle® by the Lutte Biologique Contre les Locustes et Sauteriaux 'LUBILOSA' programme) was obtained from the International Institute of Tropical Agriculture (IITA, Benin). *M. anisopliae* var. *anisopliae* isolates 421 and 4450 and other fungi coded as UAMH were obtained from the University of Alberta Microfungus Collection and Herbarium, Edmonton. *Metarhizium* isolates coded as ARSEF were obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA. Those coded as LRC and *Isaria fumosorosea* (PFR-97) were obtained from the Lethbridge Research Centre (LRC), Lethbridge. *Metarhizium anisopliae* var. *acridum* SP9 and *Beauveria bassiana* (GHA 726) were previously obtained from Mycotech Corporation, Butte, MT. *M. anisopliae* var. *acridum* FI-985 (commercialized as Green Guard®) was procured from Bio-Care Technology, Somersby, Australia.

Fungal DNA isolation

The procedure of Cenis (1992) was used for fungal DNA extraction. Briefly, hyphae were used to inoculate 500 µl of potato dextrose broth in a 1.5 ml Eppendorf tube. Following 3–5 d incubation at 25 °, the mycelial mat was pelleted by centrifugation for 5 min at 16 000 ***g***, washed with 500 µl 10 mM Tris-HCl, 1 mM EDTA, pH 8 (TE), and pelleted again. The TE was decanted and 300 µl of 200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS extraction buffer added. The mycelial mat was hand-ground for 1–2 min with a conical microtube pestle. Following homogenization, 150 µl of 3 M sodium acetate, pH 5.2, was added. The suspension was briefly vortexed and placed at −20 ° for 10 min. The microtube was then centrifuged as previously described, and the supernatant transferred to a new tube. An equal volume of isopropanol was added and after incubation at *ca* 20 ° for approximately 10 min, the precipitated DNA was pelleted by centrifugation. The supernatant was removed, and the pellet washed with 70% ethanol. After another centrifugation and removal of the supernatant, the pellet was dried before being resuspended in 50 µl of TE and stored at −20 °. Estimates of DNA quantities were obtained by electrophoresis in 0.9% TAE (40 mM Tris acetate, pH approx. 8.3, containing 1 mM EDTA) agarose gels containing 10 µg ml^{-1} ethidium bromide (Sambrook, Fritsch & Maniatis 1989). PCR amplification with general fungal primers TW81 and AB28 and *M. anisopliae* var. *acridum*-specific Mac-ITS-spF and Mac-ITS-spR primers was performed on 50 ng DNA.

A positive control was generated by cloning the PCR product resulting from amplification of *M. anisopliae* var. *acridum* IMI 330189 DNA with the TW81 and AB28 primers in vector pGEM®-T Easy using the pGEM® and pGEM®-T Easy Vector Systems

Table 1. List of isolates studied.

Isolate code[a]	Name[b]	Host	Country of origin
***Metarhizium* spp.**			
IMI 330189	*M. anisopliae* var. *acridum*	*Ornithacris cavroisi* (*Orthoptera*: *Acrididae*)	Niger
SP9	*M. anisopliae* var. *acridum*	*Locusta migratoria capito* (*Orthoptera*: *Acrididae*)	Madagascar
FI 985	*M. anisopliae* var. *acridum*	*Austracris guttulosa* (*Orthoptera*: *Acrididae*)	Australia
ARSEF 3391	*M. anisopliae* var. *acridum*	*Zoonocerus elegans* (*Orthoptera*: *Pyrgomorphidae*)	Tanzania
ARSEF 6421	*M. anisopliae* var. *acridum*	*Kraussaria angulifera* (*Orthoptera*: *Acrididae*)	Senegal
ARSEF 437	*M. anisopliae* var. *anisopliae*	*Teleogryllus commodus* (*Orthoptera*: *Gryllidae*)	Australia
ARSEF 727	*M. anisopliae* var. *anisopliae*	Unidentified tettigonid (*Orthoptera*: *Tettigoniidae*)	Brazil
UAMH 421	*M. anisopliae* var. *anisopliae*	Unidentified insect larvae	USA
UAMH 4450	*M. anisopliae* var. *anisopliae*	Soil	Canada
S54	*M. anisopliae* var. *anisopliae*	Soil	Canada
6W-2	*M. anisopliae* var. *anisopliae*	Soil	Canada
11S-1	*M. anisopliae* var. *anisopliae*	*Galleria mellonella* (*Lepidoptera*: *Pyralidae*)	Canada
ARSEF 1184	*M. flavoviride* Gams & Rozsypal	*Otiorhynchus sulcatus* (*Coleoptera*: *Curculionidae*)	France
ARSEF 2023	*M. flavoviride* var. *minus*	Unidentified acridid (*Orthoptera*: *Acrididae*)	Galapagos Islands
Other isolates			
GHA 726	*Beauveria bassiana*	*Melanoplus sanguinipes* (*Orthoptera*: *Acrididae*)	USA
UAMH 4756	*Colletotrichum gloeosporioides* (telomorph *Glomerella cingulata*)	*Laeliocattleya* sp.	Canada
UAMH 1656	*Emericella nidulans*	Feed	Canada
LRC 2111	*Fusarium oxysporum*	Soil	Canada
LRC 2087	*Clonostachys rosea* f. *catenulata*	Soil	Canada
UAMH 772	*Hydropisphaera peziza*	Soil	Canada
UAMH 2876	*Isaria farinosa*	Soil	Canada
PFR 97	*Isaria fumosorosea*	*Phenacoccus solani* (*Homoptera*: *Pseudococcidae*)	USA
LRC 2176	*Penicillium bilaii*	Soil	Canada
LRC 2391	*Rhizopus* sp.	Soil	Canada
LRC 2524	*Trichoderma reesei*	Soil	Canada
LRC race 1	*Verticillium albo-atrum*	*Solanum tuberosum*	Canada

[a] IMI, International Mycological Institute, Egham; ARSEF, Agriculture Research Service Entomopathogenic Fungus Collection, Ithaca, NY; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton; and LRC, Lethbridge Research Centre, Lethbridge, Alberta.

[b] Name as received.

cloning kit (Promega, Madison, WI). Standard protocols were used for plasmid DNA isolation, buffers, and electrophoresis techniques (Sambrook *et al.* 1989). Correct nucleotide sequence of the cloned product was confirmed by sequencing (University Core DNA and Protein Services, University of Calgary) and comparison to the published sequence for *M. anisopliae* var. *acridum* (AF137062; Driver *et al.* 2000).

Spiking of a simulated soil DNA pool with Metarhizium anisopliae *var.* acridum *DNA*

A simulated soil DNA pool was constructed using fungal DNA from above, exempting that from *Metarhizium anisopliae* var. *acridum* and *M. flavoviride* var. *minus*, at a final concentration of 100 ng μl^{-1}. The pool consisted of equal proportions of *Metarhizium* spp. DNA versus non-*Metarhizium* spp. DNA. The pool was spiked by addition of 100 ng *M. anisopliae* var. *acridum* DNA (concentration 100 ng μl^{-1}) to 900 ng soil DNA pool. Four ten-fold dilutions were made of the spiked DNA pool using the simulated soil DNA pool as diluent, representing final concentrations of 1 ng, 100 pg, 10 pg, and 1 pg μl^{-1} *M. anisopliae* var. *acridum* DNA. PCR amplifications using the Mac-ITS-spF and Mac-ITS-spR primers were performed with 1 µl of each spiked sample.

Inoculation of soil

Spores of *Metarhizium anisopliae* var. *acridum* were applied at various concentrations to a local southern

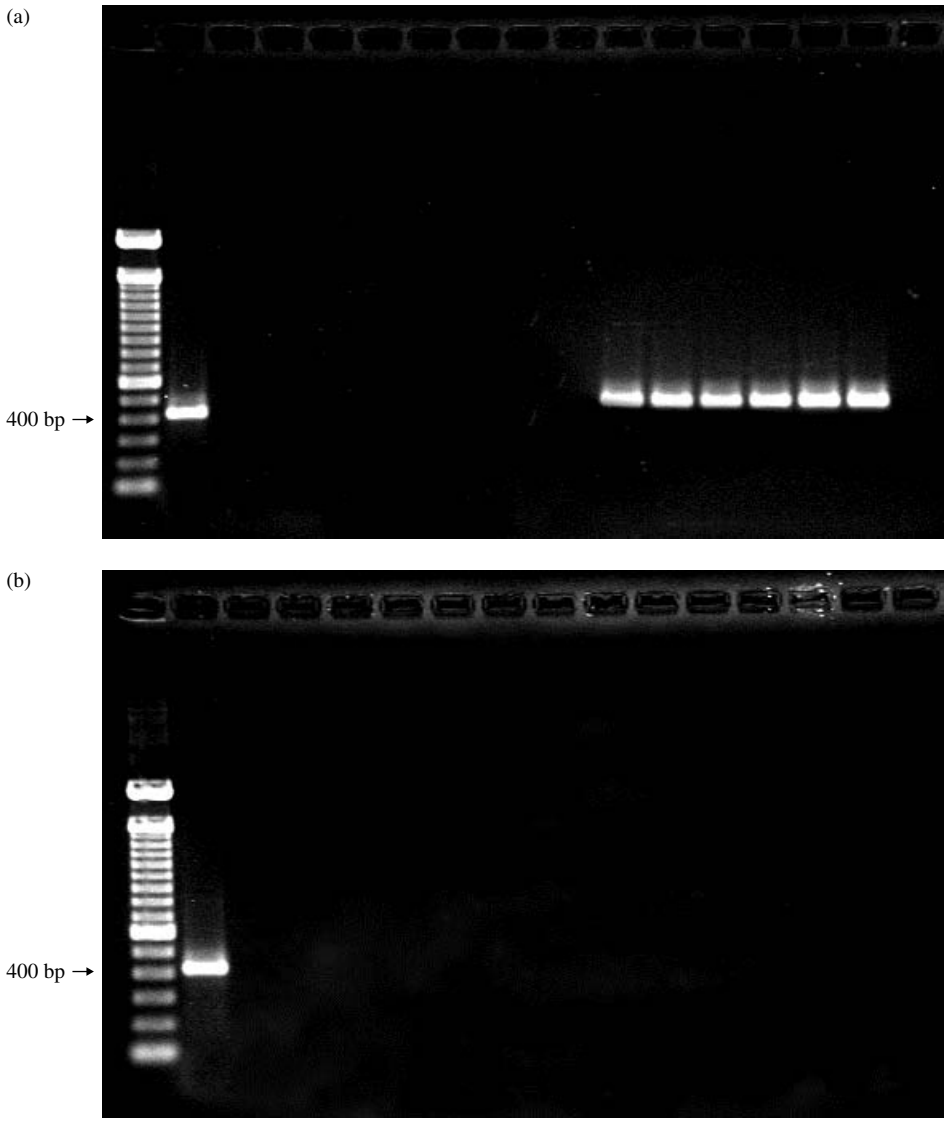

Fig. 1. Specificity determination of the PCR assay using the Mac-ITS-spF and Mac-ITS-spR primers and genomic DNA from various fungal isolates. (a) Lane 1, 100 bp ladder; Lane 2, Positive control (cloned *M. anisopliae* var. *acridum*); Lane 3, *M. anisopliae* var. *anisopliae* UAMH 421; Lane 4, *M. anisopliae* var. *anisopliae* UAMH 4450; Lane 5, *M. anisopliae* var. *anisopliae* S54; Lane 6, *M. anisopliae* var. *anisopliae* 6W-2; Lane 7, *M. anisopliae* var. *anisopliae* 11S-1; Lane 8, *M. anisopliae* var. *anisopliae* ARSEF 437; Lane 9, *M. anisopliae* var. *anisopliae* ARSEF 727; Lane 10, *M. flavoviride* Gams & Rozsypal ARSEF 1184; Lane 11, *M. flavoviride* var. *minus* ARSEF 2023; Lane 12, *M. anisopliae* var. *acridum* IMI 330189; Lane 13, *M. anisopliae* var. *acridum* SP9; Lane 14, *M. anisopliae* var. *acridum* FI 985; Lane 15, *M. anisopliae* var. *acridum* ARSEF 3391; Lane 16, *M. anisopliae* var. *acridum* ARSEF 6421; and Lane 17, Water. (b) Lane 1, 100 bp ladder; Lane 2, Positive control (cloned *M. anisopliae* var. *acridum*); Lane 3, *Beauveria bassiana*; Lane 4, *Isaria farinosa*; Lane 5, *I. fumosoroseus*; Lane 6, *Verticillium albo-atrum*; Lane 7, *Colletotrichum gloeosporioides*; Lane 8, *Clonostachys rosea* f. *catenulata*; Lane 9, *Trichoderma reesei*; Lane 10, *Fusarium oxysporum*; Lane 11, *Penicillium bilaii*; Lane 12, *Emericella nidulans*; Lane 13, *Hydropisphaera peziza*; Lane 14, *Rhizopus* sp.; and Lane 15, Water.

Alberta soil (clay-loam). Prior to inoculation, the soil was examined for *Metarhizium* spp. according to Rath, Koen & Yip (1992). Moist soil equivalent to 20 g oven-dried weight of the soil sample was added to 200 ml of sterile Ringer's solution (Oxoid, Ogdensburg, NY), the suspension shaken on an orbital shaker at 150 rpm for 30 min at *ca* 20 °, and then spread-plated as 0.1 ml of neat or 10^{-1} dilutions in Ringer's solution onto a 100×15 mm Petri dish containing selective media consisting of 3.5% mycological agar (Difco, Franklin Lakes, NJ) with 10 μg/ml dodine (Cyprex 65-W, American Cyanamid, Wayne, NJ), 50 μg/ml chloramphenicol (Sigma-Aldrich, St Louis, MO), and 200 μg ml^{-1} cycloheximide (Sigma-Aldrich) (Liu *et al.* 1993). Each dilution was plated as five replicates. Plates were incubated at 25 ° for 15 d before examination for colonies of *Metarhizium* spp. Also prior to inoculation, DNA was extracted from 0.25 g of the soil using the Ultra Clean Soil DNA kit (MoBio, Carlsbad, CA). Following extraction, the

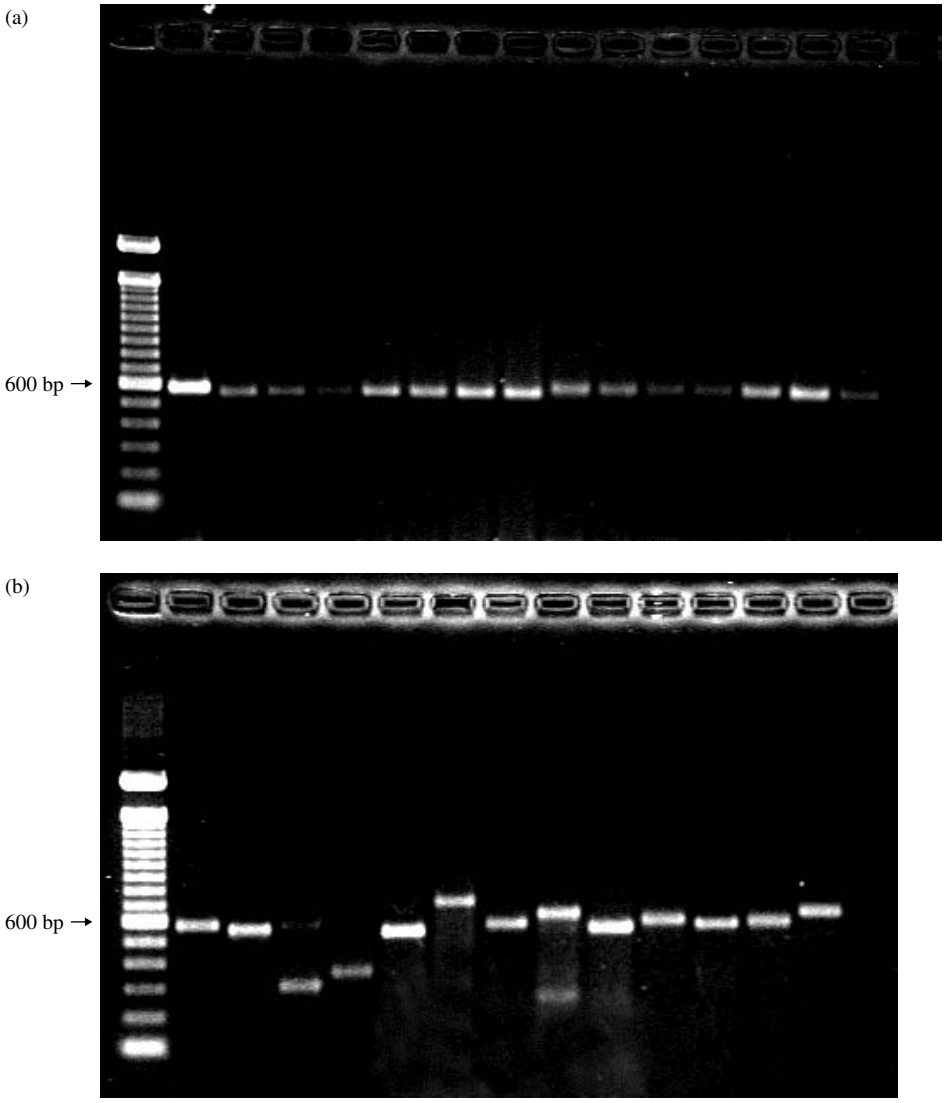

Fig. 2. Amplification of genomic DNA from various fungal isolates with general fungal primers TW81 and AB28. (a) Lane 1, 100 bp ladder; Lane 2, Positive control (cloned *M. anisopliae* var. *acridum*); Lane 3, *M. anisopliae* var. *anisopliae* UAMH 421; Lane 4, *M. anisopliae* var. *anisopliae* UAMH 4450; Lane 5, *M. anisopliae* var. *anisopliae* S54; Lane 6, *M. anisopliae* var. *anisopliae* 6W-2; Lane 7, *M. anisopliae* var. *anisopliae* 11S-1; Lane 8, *M. anisopliae* var. *anisopliae* ARSEF 437; Lane 9, *M. anisopliae* var. *anisopliae* ARSEF 727; Lane 10, *M. flavoviride* Gams & Rozsypal ARSEF 1184; Lane 11, *M. flavoviride* var. *minus* ARSEF 2023; Lane 12, *M. anisopliae* var. *acridum* IMI 330189; Lane 13, *M. anisopliae* var. *acridum* SP9; Lane 14, *M. anisopliae* var. *acridum* FI 985; Lane 15, *M. anisopliae* var. *acridum* ARSEF 3391; Lane 16, *M. anisopliae* var. *acridum* ARSEF 6421; and Lane 17, Water. (b) Lane 1, 100 bp ladder; Lane 2, Positive control (cloned *M. anisopliae* var. *acridum*); Lane 3, *Beauveria bassiana*; Lane 4, *Isasia farinosa*; Lane 5, *I. fumosorosea*; Lane 6, *Verticillium albo-atrum*; Lane 7, *Colletotrichum gloeosporioides*; Lane 8, *Clonostachys catenulata*; Lane 9, *Trichoderma reesei*; Lane 10, *Fusarium oxysporum*; Lane 11, *Penicillium bilaii*; Lane 12, *Emericella nidulans*; Lane 13, *Hydropisphaera peziza*; Lane 14, *Rhizopus* sp.; and Lane 15, Water.

DNA was then subjected to PCR amplification with the general fungal TW81 and AB28 primers of Curran *et al*. (1994) to confirm successful DNA extraction, and amplification with a set of primers (Mac-ITS-spF and Mac-ITS-spR) designed for the specific detection of *M. anisopliae* var. *acridum* DNA.

Spores of *M. anisopliae* var. *acridum* (IMI 330189) were scraped from a PDA plate and resuspended in 0.05% Tween 20. Spore concentration was estimated with a hemocytometer and concentrations adjusted to 10^2, 10^3, 10^4, and 10^5 spores, each in 200 µl of 0.05% Tween 20. The spore suspensions were each added to 0.25 g of soil, followed immediately by soil DNA extraction using the MoBio Ultra Clean Soil DNA kit. Extracted DNA (1 µl) was subsequently subjected to PCR amplification with the Mac-ITS-spF and Mac-ITS-spR primers.

Inoculation of grasshoppers

Nymphs (second and third instar) of a non-diapausing strain of *Melanoplus sanguinipes* (Pickford & Randell

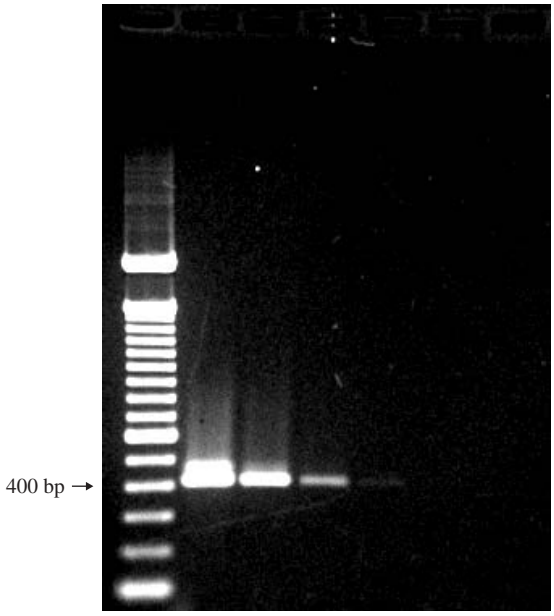

Fig. 3. Sensitivity determination of the PCR assay using the Mac-ITS-spF and Mac-ITS-spR primers and genomic DNA from *Metarhizium anisopliae* var. *acridum*. Lane 1, 100 bp ladder; Lane 2, 1 ng; Lane 3, 100 pg; Lane 4, 10 pg; Lane 5, 1 pg; Lane 6; 100 fg; and Lane 7, Water.

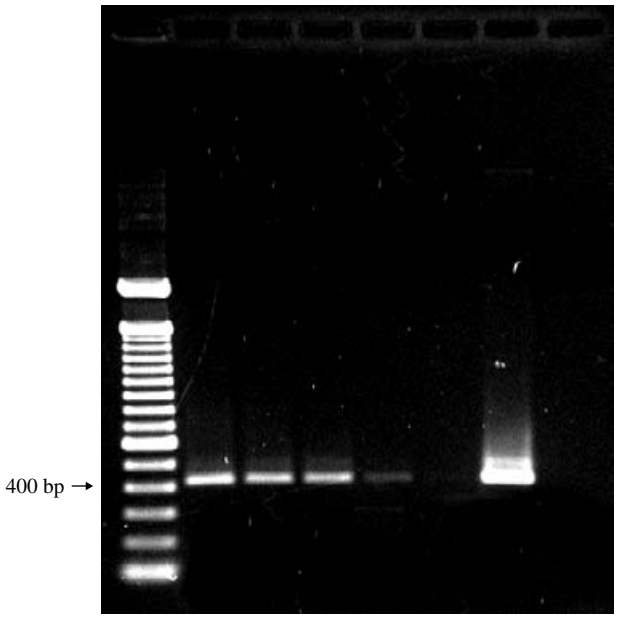

Fig. 4. Detection of *Metarhizium anisopliae* var. *acridum* DNA in a simulated soil DNA pool using PCR primers Mac-ITS-spF and Mac-ITS-spR. Lane 1, 100 bp ladder; Lane 2, 10 ng; Lane 3, 1 ng; Lane 4, 100 pg; Lane 5, 10 pg; Lane 6, 1 pg; Lane 7, Positive control (cloned *M. anisopliae* var. *acridum*); and Lane 8, Water.

1969) were collected at random from a laboratory colony at the Lethbridge Research Centre and placed individually in sterile 20 ml glass vials stoppered with a sterile polyurethane foam plug. The experiment involved a total of 174 insects (30 in the control group, 144 in the treated group) with approximately equal proportions of males and females in each group. On the day of inoculation, conidia of *M. aniospliae* var. *acridum* were harvested from a PDA culture (15–20 d of growth) and resuspended in sunflower oil (Safflo). Formulation of the inoculum has been previously described by Johnson *et al.* (2002). Briefly, the concentration of conidia was estimated with a hemocytometer and adjusted to 5×10^7 conidia ml^{-1}. Subsequently, 2 µl aliquots were pipetted onto lettuce-leaf wafers (0.7 cm diam), resulting in a dose of approximately 10^5 spores per insect (*via* handling and feeding). Each grasshopper was confined with one wafer for 24 h. Control grasshoppers were confined with wafers containing only sunflower oil. After 24 h confinement, all grasshoppers were removed and individually housed in 240-ml transparent plastic containers. Throughout the experiment, insects were exposed to a temperature regime of 24 °/16 ° day/night with a corresponding 16/8 h light/dark photoperiod under ambient relative humidity (40–55%). Nymphs were observed and fed daily with fresh wheat leaves. Cadavers were removed daily with sterile forceps and stored in sterile 1.5 ml Eppendorf vials at −20 ° prior to DNA extraction. All treated grasshoppers were dead by day 8; all remaining control grasshoppers were then killed at −20 °. Viability of conidia was determined by microscopic examination of germination following 48 h incubation at 25 ° of 2 × 10-µl replicate aliquots of the inoculum onto PDA blocks on a microscope slide.

Grasshopper DNA extraction

The method of Hegedus & Khachatourians (1993) was modified for the extraction of DNA from infected and noninfected grasshoppers. Individual nymphs were macerated in 500 µl of TE with a sterile microtube pestle for 2–3 min accompanied by vigorous vortexing. A 25 µl aliquot of the homogenate was removed and spread on a 60 × 15 mm Petri dish containing selective media for *Metarhizium* spp. as described above. Inoculated agar plates were incubated at 25 ° for confirmation of presence/absence of *M. anisopliae* var. *acridum* colonies (maximum 20 d). The remaining solution was extracted with an equal volume of phenol:chloroform (1:1, v/v) followed by a 10 min centrifugation at 16 000 ***g***. The upper aqueous phase was removed and extracted once more with chloroform:isoamyl alcohol (24:1, v/v), followed by addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and one volume of isopropanol to the aqueous phase. Following incubation at *ca* 20 ° for 10 min, the mixture was centrifuged, and the supernatant removed. The pellet was washed with 1 ml of ice-cold 70% ethanol, centrifuged, and the pellet dried briefly. The DNA was then resuspended in 500 µl of TE containing 2 µl RNase A (Sigma-Aldrich) and stored at −20 °. Quantitation of DNA was determined with use of

a spectrophotometer (Pharmacia Biotech, Piscataway, NJ) and 100 ng later subjected to PCR amplification.

PCR amplification

General fungal primers TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Curran *et al.* 1994) were used to amplify the region of the ribosomal repeat from the 3′ end of the 16S rDNA to the 5′ end of the 28S rDNA flanking the ITS1, the 5.8S rDNA, and ITS2 sequences, from total fungal DNA. PCR amplifications were performed in a total volume of 50 μl containing 10 mM Tris, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.05% Tween 20, 0.05% NP40, 0.4 μM of each primer, 25 μM of each dNTP (Invitrogen, Carlsbad, CA), 2.5 units *Taq* DNA polymerase (MBI Fermentas, Hanover, MD) and template DNA. Negative controls contained sterile water in place of DNA. DNA amplification was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) programmed as follows: initial denaturation 5 min at 94 °; 30 cycles of: denaturation 1 min at 94 °, annealing 1 min 30 at 55 °, extension 2 min at 72 °; with a final extension 5 min at 72 °. PCR products were analyzed on a 1.5% TAE agarose gel with a 100 bp DNA ladder (MBI Fermentas) included as a size marker.

Primers Mac-ITS-spF (5′-CTGTCACTGTTGCTTCGGCGGTAC-3′) and Mac-ITS-spR (5′-CCCGTTGCGAGTGAGTTACTACTGC-3′) were designed based on the ITS1 and ITS2 regions of the rDNA sequence data for *M. anisopliae* var. *acridum* (clade 7; Driver *et al.* 2000). Total fungal and soil DNA and grasshopper DNA from infected and noninfected insects were used in PCR assays with this primer combination. Amplifications were performed in a total volume of 50 μl containing 20 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.4 μM of each primer, 25 μM of each dNTP (Invitrogen), 2.5 units *Taq* DNA polymerase (MBI Fermentas) and template DNA. As previously noted, negative controls contained sterile water in place of DNA. DNA amplification was also performed in a GeneAmp® PCR System 9700 (Applied Biosystems) programmed as follows: initial denaturation 5 min at 94 °; 30 cycles of: denaturation 1 min at 94 °, combined annealing and extension 3 min at 72 °; with a final extension 5 min at 72 °. PCR products were analyzed as previously mentioned.

Nested PCR amplifications were carried out on grasshopper DNA from infected insects that initially produced weak products in a single amplification with the Mac-ITS-spF and Mac-ITS-spR primers. DNA from infected grasshoppers was amplified in an initial reaction with the TW81 and AB28 primers using conditions previously described. A second amplification was then performed with a 1-μl aliquot from the initial reaction and the Mac-ITS-spF/Mac-ITS-spR primer combination using conditions described above.

RESULTS

As expected, use of the Mac-ITS-spF and Mac-ITS-spR primers in a PCR assay successfully amplified a 420 bp DNA sequence from the total genomic DNA extracted from *Metarhizium anisopliae* var. *acridum* (Fig. 1a). *M. anisopliae* var. *minus* also produced a 420 bp amplification product. Isolates of *Metarhizium anisopliae* var. *anisopliae* and *M. flavoviride* produced no amplified product, nor did isolates of *Beauveria bassiana*, *Isaria fumosorosea*, *I. farinosa*, *Verticillium albo-atrum*, *Colletotrichum gloeosporioides*, *Emericella nidulans*, *Trichoderma reesei*, *Fusarium oxysporum*, *Clonostachys rosea* f. *catenulata*, *Penicillium bilaii*, *Hydropisphaera peziza*, or an isolate of *Rhizopus* sp. (Fig. 1b). In contrast, the TW81 and AB28 primers produced a varying range (most around 500–600 bp) of amplified products (Fig. 2a–b) in all isolates tested, thus confirming successful extraction of PCR-quality DNA from all fungal species.

The sensitivity of the *M. anisopliae* var. *acridum*-specific PCR assay was determined for genomic fungal DNA extracted from an axenic culture of *M. anisopliae* var. *acridum*. The assay was sensitive enough to detect approximately 1 pg of genomic DNA (Fig. 3).

The *M. anisopliae* var. *acridium*-specific PCR assay successfully detected *M. anisopliae* var. *acridum* DNA in the presence of a simulated soil DNA pool. A detection limit of 10 pg was observed, representing 0.001% of total DNA in the sample (Fig. 4).

M. anisopliae var. *acridum* spores were detected at a concentration of 10^4 spores per 0.25 g of soil (Fig. 5). Use of general fungal TW81 and AB28 primers in conjunction with specific Mac-ITS-spF and Mac-ITS-spR primers in a nested PCR assay increased the detection limits to 10^2 spores per 0.25 g of soil (data not shown).

The specific assay also successfully detected *M. anisopliae* var. *acridum* DNA in each of the 144 infected grasshoppers. Counts of viable conidia in the inoculum revealed a germination rate of >90% at 48 h after incubation at 25 °. Ecdysis was either completed or initiated by 65 of the treated nymphs prior to death; however, this did not inhibit detection of fungal DNA. Only 28 of the treated cadavers displayed the reddish discolouration of the cuticle associated with infection by *M. anisopliae* var. *acridum*. Fungal colonies with *M. anisopliae* var. *acridum* morphological features, namely dark green conidia, were observed on 134 agar plates for the treated group. No growth was observed on nine plates, and for another plate, overgrowth by *Rhizopus* sp. interfered with examination for colonies of *M. anisopliae* var. *acridum*.

No amplified products were observed with PCR assay of the control group, and no colonies of *M. anisopliae* var. *acridum* were isolated from any of the agar plates for the control nymphs. Fig. 6 shows the var. *acridum*-specific PCR amplification results for a representative group of infected and noninfected nymphs.

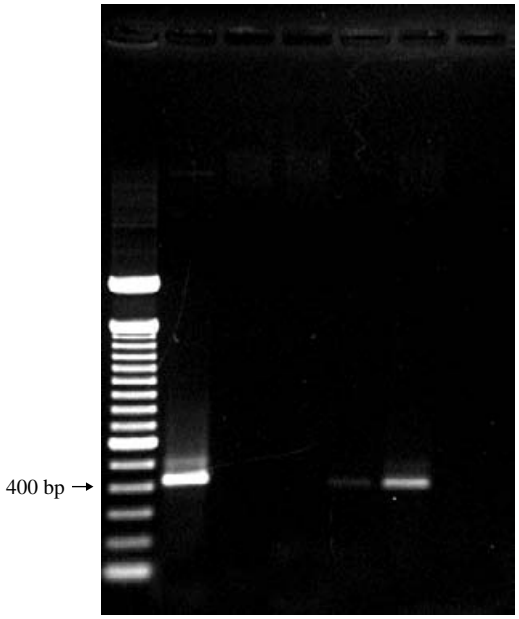

Fig. 5. Detection of *Metarhizium anisopliae* var. *acridum* spores (spore counts per 0.25 g soil) in soil using PCR primers Mac-ITS-spF and Mac-ITS-spR. Lane 1, 100 bp ladder; Lane 2, Positive control (cloned *M. anisopliae* var. *acridum*); Lane 3, 10^2; Lane 4, 10^3; Lane 5, 10^4; Lane 6, 10^5; and Lane 7, Water.

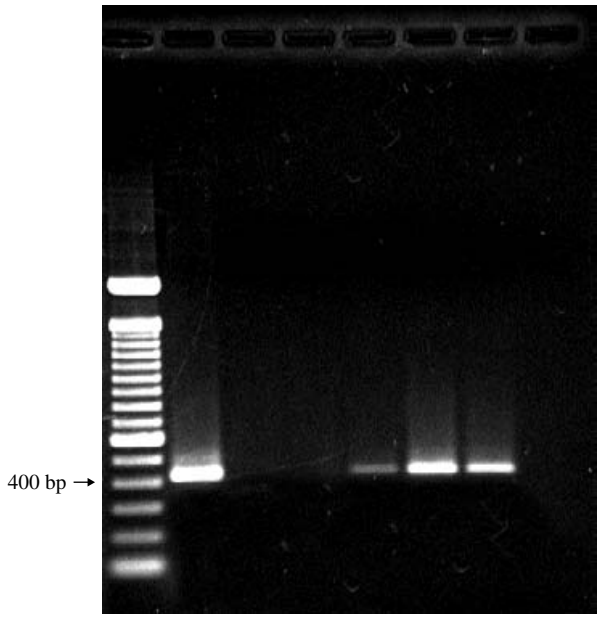

Fig. 6. Detection of *Metarhizium anisopliae* var. *acridum* DNA in infected grasshoppers using PCR primers Mac-ITS-spF and Mac-ITS-spR. Lane 1, 100 bp ladder; Lane 2, *M. anisopliae* var. *acridum* (positive control); Lane 3, DNA from uninfected grasshopper; Lane 4, DNA from uninfected grasshopper; Lane 5, DNA from grasshopper infected with *M. anisopliae* var. *acridum* (4 days post-infection); Lane 6, DNA from grasshopper infected with *M. anisopliae* var. *acridum* (5 days post-infection); Lane 7, DNA from grasshopper infected with *M. anisopliae* var. *acridum* (6 days post-infection); and Lane 8, Water.

DISCUSSION

The ITS sequences of the rDNA region of the fungal genome are an ideal target for molecular characterization due to their high copy number and divergence of sequences between taxa (Pipe *et al.* 1995). We exploited these features to design *Metarhizium anisopliae* var. *acridum*-specific DNA primers. The primers were used in a PCR-based assay to amplify a 420 bp sequence with genomic DNA extracted from *M. anisopliae* var. *acridum*. A 420 bp product observed after amplification of *M. flavoviride* var. *minus* DNA was also expected as this species has been recognized as *M. anisopliae* var. *acridum* by Driver *et al.* (2000). The ability to produce an amplified product specific to *M.* anisopliae var. *acridum* supports the concept of divergence between taxa and also corroborates the theme of divergent evolutionary lines within the genus *Metarhizium* (Driver *et al.* 2000). Although representatives from only two other clades of *Metarhizium* were evaluated, the high sequence variability of *M. anisopliae* var. *acridum* in comparison with other clades combined with the highly stringent composition of the synthesized sequences support the specificity of the Mac-ITS-spF and Mac-ITS-spR primers.

The fungal genera other than *Metarhizium* analyzed in this study encompassed a range of entomopathogenic, phytopathogenic, mycopathogenic, and soil saprophytic organisms. Several of the genera have previously been isolated from southern Alberta soils (Inglis *et al.* 1998). *Verticillium albo-atrum* and *Colletotrichum gloeosporioides* are phytopathogens (Domsch, Gams & Anderson 1980, Evans, Greaves & Watson 2001). Others, such as *Isaria* (syn. *Paecilomyces* p.p.), are entomopathogenic (Inglis *et al.* 2001). One of these other entomopathogens, *Beauveria bassiana*, was selected due to its nature as an acridid pathogen (Johnson & Goettel 1993). *Clonostachys* spp., *Trichoderma* spp., and *Fusarium* spp. have been identified as pathogens of fungi (Vey, Hoagland & Butt 2001). A pending survey of southern Alberta soils and insects necessitated analysis of these genera with the *M. anisopliae* var. *acridum*-specific primers to determine specificity of the PCR assay. Further, demonstration of successful amplification of *M. anisopliae* var. *acridum* DNA in the presence of other DNA, particularly from soil, was essential and has been demonstrated in this work.

Extraction of PCR-amplifiable DNA from insects is often difficult due to the number of PCR inhibitors in the form of tannic acids, quinones, polyphenols, chelators, etc. coisolated from the insect cuticle (Hackman 1974). We experienced some weak amplification products using the var. *acridum*-specific primers in a single amplification from infected grasshopper DNA but found that these products could be strongly amplified with a nested PCR assay using the TW81/AB28 primers for the first amplification and the

Mac-ITS-spF/Mac-ITS-spR primers for the second amplification. Inhibitory compounds were diluted to a negligible amount when 1 μl of the first amplification reaction was used as template for the second amplification. Molting did not interfere with the ability of the assay to detect *M. anisopliae* var. *acridum* DNA in infected grasshoppers that underwent ecdysis. This supports a previous observation by Milner & Prior (1994) that ecdysis did not interfere in the infection of the Australian plague locust with *M. anisopliae* var. *acridum*.

Studies have demonstrated that, depending on the dose, the majority of laboratory bioassay mortality in acridids infected with *M. anisopliae* var. *acridum* occurs between 4–6 d post-infection (Delgado *et al.* 1997b, Magalhães *et al.* 1997, Milner 1997, Lomer, Prior & Kooyman 1997). In this study, the *M. anisopliae* var. *acridum*-specific PCR assay amplified sequences from DNA extracted from treated nymphs that died 1–3 d post-inoculation. Presumably, the majority of nymphs at this stage died from complications due to contact with the sunflower oil component of the inoculum rather than from active fungal infection. Our diagnostic PCR assay is qualitative and not designed to determine activity levels of the target organism. However, confirmation of *M. anisopliae* var. *acridum* colony growth for 93.1% of the treated grasshoppers indicates that the presence of viable spores can be detected early post-infection. Moreover, the intensity of amplification products increased with DNA from cadavers from the later days of the experiment, thus suggesting a progressive increase in fungal mass in the infected host.

Surveys for natural incidence of *M. anisopliae* var. *acridum* have indicated that these levels are generally very low. In northern Benin, Shah *et al.* (1998) found levels of 0.3–1.7% and 1.2–3.2% at different sites, respectively. Also in Benin, Douro-Kpindou *et al.* (1995) detected fungal incidence at 15% in field trial plots before application of a formulation of *M. anisopliae* var. *acridum* for biocontrol of *Zonocerus variegatus*. The ability of our assay to detect levels of *M. anisopliae* var. *acridum* DNA as low as 0.001% of total DNA present demonstrates its suitability for detection of this fungus at low incidence.

Laboratory and field tests indicate differential impacts of weather affect the operation and efficacy of entomopathogens (Inglis *et al.* 1997). Further, spring temperature, overwintering conditions, and moisture strongly affect the target insect. Insect body temperature can be calculated (Lactin & Johnson 1998) and is largely a result of immediate weather factors; however, the probable impact of weather on the effectiveness of *M. anisopliae* var. *acridum* is largely unknown. Improved knowledge of the biology and ecology of this fungus in a natural setting is a prerequisite for the development of an effective long-lasting pest management strategy for the biological control of acridids. Our study offers a reliable, specific, and sensitive diagnostic PCR assay that can be performed on a number of templates including those with non-target DNA. We plan to use this molecular method to investigate the geographical extent of *Metarhizium* spp. in soils and native insects, to compare this distribution to possible future distributions under changing weather and climate, and to assess the opportunities for including *Metarhizium* spp. in integrated grasshopper management plans.

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