Curr Genet (1994) 25: 107–113

Current Genetics

Differentiation of species and strains of entomopathogenic fungi by random amplification of polymorphic DNA (RAPD)

Michael J. Bidochka, Melanie A. McDonald, Raymond J. St. Leger, Donald W. Roberts

Insect Pathology Resource Center, Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY 14853, USA

Received: 21 November 1992 / Accepted: 2 August 1993

Abstract. Polymerase chain reaction (PCR)-based technology, involving random amplification of polymorphic DNA (RAPD), was used to assess the genomic variability between 24 isolates of deuteromycetous fungi (*Metarhizium anisopliae, Metarhizium flavoviride*, unidentified strains of *Metarhizium* and *Beauveria bassiana*) which were found to infect grasshoppers or locusts. *M. flavoviride* showed little intraspecific variability in PCR-amplified fragments when compared to *M. anisopliae*. The high level of variability in PCR-amplified fragments contained within *M. anisopliae* was similar to the total variability between *B. bassiana, M. anisopliae* and *M. flavoviride*, and suggests that *M. anisopliae* may include a number of cryptic species. Four polymorphic RAPD fragments were used to probe the genomic DNA of the various species and strains. On the basis of these probes the fungi can be grouped into *M. flavoviride, M. anisopliae*, or *B. bassiana*. According to PCR-amplified fragments, previously-unidentified *Metarhizium* strains were characterized as *M. flavoviride*. There was little evidence that these fungi, all isolated from, or virulent towards, grasshoppers or locusts, showed host-selection in PCR-amplified fragments. Nor was geographical origin a criterion for commonalty based on PCR-amplified fragments. PCR-fragment-pattern polymorphisms and the construction of probes from one or more of these fragments may provide a useful and rapid tool for identifying species and strains of entomopathogenic fungi.

Key words: *Metarhizium anisopliae* – *Metarhizium flavoviride* – *Beauveria bassiana* – Polymerase chain reaction – Polymorphism – RAPD markers – Entomopathogenic fungi – Acrididae

Introduction

Several Deuteromycetous fungi, including *Metarhizium anisopliae*, *Metarhizium flavoviride* and *Beauveria bassiana*, are currently considered as commercially-important pathogens of many insect species. However a major obstacle in population studies of entomopathogenic fungi, and in attributing insect infection to a particular fungal isolate, has been the lack of useful genetic markers in such fungi. Genetic markers are also needed to analyze the genetic resources of entomopathogenic fungi to assist in the construction of recombinant strains. Isoenzyme analysis of isolates of *Metarhizium sp.* and *Beauveria sp.* showed considerable genetic variation, but the production of some isozymes may be correlated with catabolite-repression events which are dependent on the growth medium (St. Leger et al. 1992 a, b). DNA fingerprinting techniques, such as restriction fragment length polymorphisms (RFLP) and the polymerase chain reaction (PCR), provide an alternative approach to distinguish genotypic variants (Meyer et al. 1991).

Welsh and McClelland (1990) and Williams et al. (1990) described a simple method for assessing genomic variability based on the amplification of random DNA segments with size primers of arbitrary nucleotide sequence. The genetic polymorphisms observed have been termed RAPD (random amplification of polymorphic DNA) markers (Williams et al. 1990) or arbitrary primed polymerase chain reaction (AP-PCR) fingerprinting (Welsh and McClelland 1990). RAPD markers and RFLP have been successfully applied to assess genomic variability in several fungi (Williams et al. 1990; Crowhurst et al. 1991; Kusters-van Someran et al. 1991; Cenis 1992).

We are interested in the population structure and taxonomy of *Metarhizium* and *Beauveria* (St. Leger et al. 1992 a, b) in determining the genetic basis of virulence and variability, and in establishing a rapid and rational approach for differentiating isolates of entomopathogenic fungi. We chose to begin our analysis by examining several species and strains of fungi which infect grasshoppers and locusts. Recently, the field application of several strains of these fungi showed promising results in the biological control of grasshoppers and locusts (Bidochka and Khachatourians 1991; Johnson et al. 1992; Lomer et al. 1992).

Correspondence to: M. J. Bidochka

Table 1. Origins of acridid-infecting *Metarhizium* and *Beauveria* strains

Species	Isolate[a]	Origin	Host
Metarhizium flavoviride	IMI 324673	Tanzania	*Zonocercus elegans*
	IMI 330189	Niger	*Ornithacris cavroisi*
	ARSEF 2023	Galapagos	Unidentified acridid
	IIBC I91-609	Benin	*Zonocercus variegatus*
Metarhizium anisopliae	IMI 168777ii	Ethiopia	*Schistocerca gregaria*
	IMI 298059	Papua New Guinea	*Scapanes australis*[b]
	IMI 299982	Trinidad	*Aeneolomia varia*
	IIBC I90-574	Pakistan	*Acrotylus sp.*
	IIBC I91-613	USA	*Curculio caryae*[b]
	IIBC I91-614	Thailand	*Patanga succincta*
	IIBC I91-625	Oman	*Pseudosphingonotus savignyi*
	IIBC I91-633	Oman	Unidentified cricket
	IIBC I91-676	Pakistan	*Oxya multidentata*
Metarhizium sp.	ARSEF 324ss	Australia	*Austracris guttulosa*
	IIBC I91-646	Benin	*Kraussaria angulifera*
	IIBC I91-647	Benin	*Hieroglyphus daganensis*
	IIBC I91-671	Benin	*K. angulifera*
	IIBC I91-672	Guinea Bissau	*Z. variegatus*
	IIBC I91-673	Tchad	*Diabolocatantops axillaris*
	IIBC I91-674	Tchad	*Acrida bicolor*
	IIBC I91-675	Mali	*Z. variegatus*
Beauveria bassiana	IIBC I91-612	Crete	*Anacridium aegyptium*
	IMI 331275	USA	Unidentified acridid
	Mycotech[c]	USA	*Melanoplus sp.*

[a] ARSEF = Agricultural Research Service Entomopathogenic Fungus collection; IMI = International Mycological Institute; IIBC = International Institute of Biological Control

[b] These species are beetles (Coleoptera) but the *M. anisopliae* isolated from them have been shown to infect acridids (C. Prior personal communication). All other species are grasshoppers or locusts (Orthoptera:Acrididae)

[c] Strain from Mycotech Corporation, Butte, Mont., USA

Materials and methods

Fungal isolates. The isolates used in this study were collected from diseased grasshoppers and locusts. The species, origin, and host insect of the strains is shown in Table 1. Several of the strains could only be identified to the genus level on the basis of morphological criteria.

Preparation of DNA. Isolates were grown with shaking (180 rpm) in 15 ml of 2% glucose, 1% peptone medium, for 2 days at 25 °C. After 2 days, the cultures were supplemented with 10 ml of 2% glucose, 1% peptone, 0.1% yeast extract medium, and allowed to grow for an additional 24 h. The mycelia were collected by centrifugation (5 k for 15 min) and incubated with shaking in 10 ml of 0.02 M Tris-HCl (pH 7.5), 10 mM dithiothreitol, at 37 °C, for 15 min. Following centrifugation, mycelia were washed with 10 ml of water and shaken (20 rpm) in 5 ml of 0.8% NovoZym 234 (Novo Biolabs, Bagsvaerd, Denmark) in 1.2 M sorbitol, 10 mM Tris-HCl (pH 7.0) for 3 h at 21 °C (Goettel et al. 1990). The resulting protoplasts were collected by centrifugation (2 k for 15 min) and then burst in 5 ml of 0.01 M Tris, 2% SDS, 1 mM EDTA. Preparations were treated sequentially with 100 µl of RNAse A (Sigma Chemical Co. St. Louis, Mo.; 10 mg/ml) and 100 µl of proteinase K (Sigma; 10 mg/ml; 2 h at 37 °C). The suspension was centrifuged and the supernatant extracted twice with equal volumes of phenol-chloroform. To the aqueous phase 0.1 vol of 3 M sodium acetate was added and the solution centrifuged at 4 k for 10 min. The supernatant was retained and DNA precipitated with an equal volume of isopropanol. The DNA was washed twice with 70% ethanol, dissolved in 250 µl of sterile distilled water and stored at –20°C.

PCR-amplification conditions. Oligonucleotide primers and sequences were: primer A, 5′-TTATGTAAAACGACGGCCAGT (universal M13); primer B, 5′-$(GACA)_4$ and primer C, 5′-CGACTGTCGG. Primers were dissolved in water and adjusted to 1 µg/ml. Amplification reactions were performed in 10 mM of Tris-HCl (pH 8.3), 50 mM KCl, 7 mM $MgCl_2$, 0.001% gelatin containing 100 µM each of dATP, dCTP, dGTP, and dTTP (Perkin-Elmer-Cetus, Connecticut, USA), 10 ng of primer, 0.5 µl of *Taq* DNA polymerase (Perkin-Elmer-Cetus) and approximately 10 ng of genomic DNA in a final volume of 50 µl. The samples were overlaid with 90 µl of mineral oil. Amplification was performed in a Perkin-Elmer-Cetus DNA Thermal Cycler programmed for: 97 °C for 5 min, cooled to 25 °C at which time the *Taq* polymerase was added, then brought to 94 °C for 3 min, followed by 45 cycles of 94 °C for 1 min, 37 °C for 1,5 min, and 72 °C for 2 min. Amplified fragments were electrophoresed in 1.2% TAE agarose gels and detected by staining with ethidium bromide (Sambrook et al. 1989).

Probes and DNA manipulations. Amplified PCR fragments were excised from low-melting-temperature gels (Sambrook et al. 1989). The criteria on which the fragments were chosen were that the fragments were (1) conserved within a portion of the same species (2) characterised by major bands which indicated that they were strongly amplified fragments and (3) clearly resolved from other bands. PCR-amplified fragments were labeled with ^{32}P-dCTP (NEN du Pont, Boston, Mass., USA) using a random primer labeling kit (Gibco BRL, Gaithersberg, Md., USA). DNA isolated from several of the fungal strains was digested with *Eco*R1 or *Sal*1 and digested DNA was electrophoresed in 0.8% TAE agarose gels. Gels were denatured and blotted onto nylon membrane according to the manufacturer's instructions. Genomic DNA dot-blots were executed according to manufacturer's instructions (NEN du Pont). Membranes were prehybridized and hybridized at 42 °C in 50% formamide, 6 x SSPE, 5 x Denhardts solution, 1% SDS and 1% Salmon sperm DNA (10 mg/ml). The membranes were washed in 2 x SSC-1% SDS at 65 °C for 30 min and exposed to X-Omat and X-ray film (Eastman Kodak Co., Rochester, N.Y., USA) for 24 h at –70 °C.

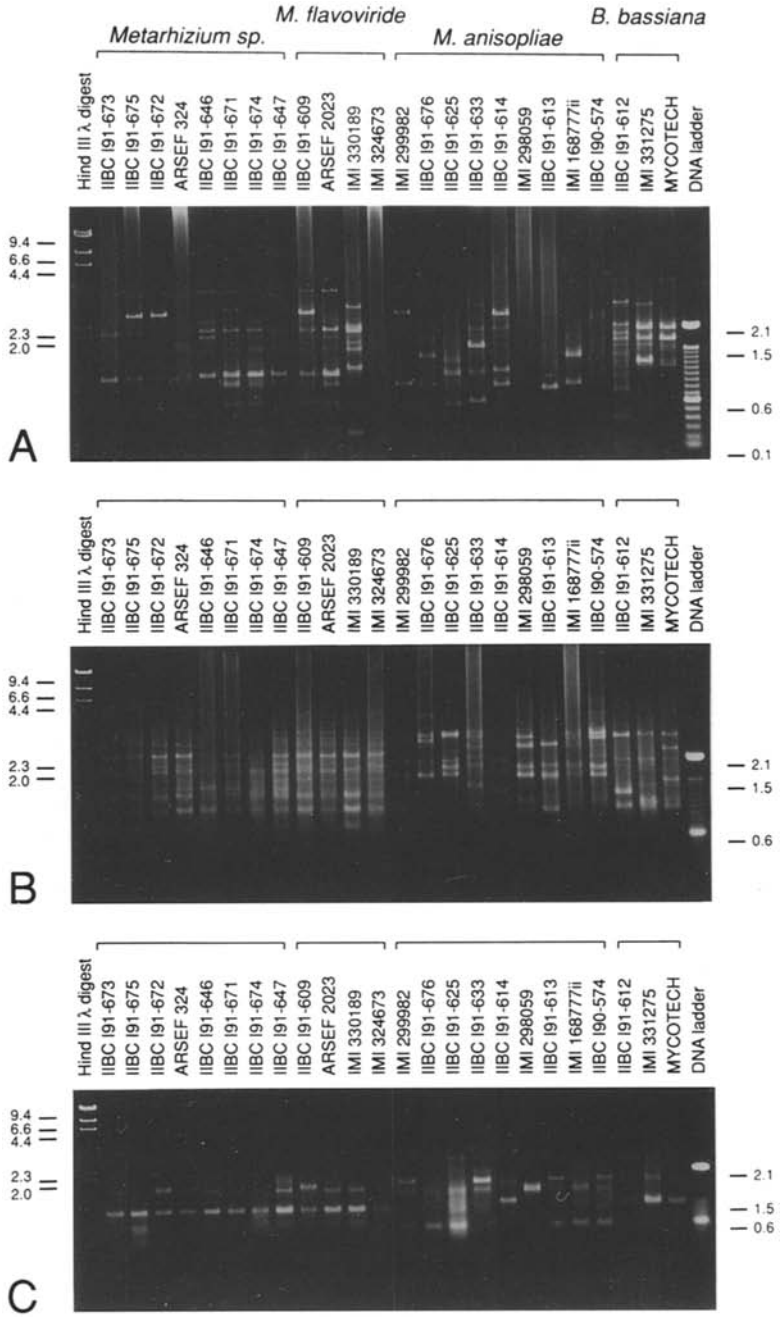

Fig. 1 A–C. Random amplification of polymorphic DNA from 24 fungal isolates using the 21-mer primer A (**A**), the 16-mer primer B (**B**) and the 10-mer primer C (**C**). Isolate numbers and species are indicated above each lane. Selected molecular-weight size markers are indicated in kilobases to the left and right of the gels

Phylogenetic analysis. Sizes of the PCR-amplified fragments from each strain were determined by measuring the distances of the bands from the well and relating this to the marker standards. This was repeated at least twice. Inter-run results always concurred. Similarity coefficients (F) were calculated for all pairwise combinations by means of the following formula: $F = 2n_{XY}/(n_X + n_Y)$, in which n_X and n_Y are the numbers of PCR-amplified fragments in strains X and Y, respectively, whereas n_{XY} is the number of fragments shared by the two strains (Nei and Li 1979).

Results

RAPD marker patterns were assessed in DNA from 21 isolates of *Metarhizium spp.* and three isolates of *B. bassiana* using three primers. On the basis of amplification fragment patterns three fungal species were differentiated; *M. flavoviride*, *M. anisopliae* and *B. bassiana*. The previously unspeciated *Metarhizium* strains (Table 1) had PCR fragment patterns typical for *M. flavoviride* (Fig. 1) and will be further treated as such.

Six primers were originally assessed for RAPD-PCR and three of these were used based on their ability to produce consistent and distinguishable fragment patterns. Each primer produced different amplification fragment patterns (Fig. 1). The greatest number of PCR fragments was found with primer C (37 total bands from 24 isolates) followed by primer B (34 bands) and primer A (23 bands). In these cases the larger the primer the fewer PCR frag-

ments. Primer C PCR-amplified fragments were generally smaller than 2.7 kb while the primer A and primer B fragments were up to 4 kb.

Many of the strains showed identical PCR fragments for one or two of the primers but no two strains were identical for all three. For example, strains I91-673 and I91-672 showed identical patterns for primers B and C but not for primer A. Strains of *M. flavoviride*, I91-672, I91-674, ARSEF 324, 330189, 324673 and 2023, showed identical patterns for primers A and B but not for primer C. Several of the major PCR fragments were shared by all isolates of *M. flavoviride*. The isolates morphologically characterized as *M. anisopliae* produced a heterogeneous array of banding patterns. None of the major bands generated by any one of the three primers were common throughout *M. anisopliae*. By contrast the three *B. bassiana* strains shared several PCR fragments. For example the 2.62-, 1.76- and 1.12-kb bands from primer A, the 3.09-, 2.03-, 1.88- and 0.94-kb bands for primer B and the 4.25-, 2.02-, 1.71-, 1.18-, 0.82- and 0.54-kb bands from primer C, were common to the three *B. bassiana* strains analyzed.

The similarity between isolates of *M. flavoviride* was not due to geographical origin. Strains I91-674, I91-672, 324673 and 330189 were isolated in West Africa while strains ARSEF 2023 and ARSEF 324 were isolated in the Galapagos and Australia, respectively. These strains which span three continents showed remarkably similar banding patterns. Of the *B. bassiana* strains, two were isolated in the USA and one in Crete and they too showed similar banding patterns. Of the *M. anisopliae* strains which did show some similarities in banding patterns, i.e., strains I90-574, I91-613 and 168777ii, one was isolated in the USA, another in Pakistan and the third in Ethiopia. *M. anisopliae* strains I91-633 and I91-625 were isolated from Oman but showed distinctly-different PCR-amplified fragment patterns. Three *M. anisopliae* isolates from Benin, strains I91-646, I91-647 and I91-671, showed less similarity with each other than with isolates which were more-widely geographically separated.

This study provided no evidence of host clustering. *M. flavoviride* strains (I91-675, I91-672, I91-609 and 324673) which were isolated in Africa from the grasshopper genus *Zonocerus* showed one band in common (2.48 kb from primer C). But in other cases no correlation between host species and PCR-amplified fragments was found.

Probes were made from primer A PCR-amplified fragments excised from agarose gels. Sources were *B. bassiana* strain Mycotech (1.12 kb fragment), *M. anisopliae* strain I90-574 (0.63 kb fragment), and *M. flavoviride* strain I91-647 (0.94 kb fragment), and a 1.6-kb primer C amplified fragment from *M. flavoviride* strain IMI 330189. Dot-blots containing genomic DNA were probed with each of the four fragments (Fig. 2). The strain I91-647-amplified-fragment probe hybridized to all DNA from the genus *Metarhizium* but not to that of the *B. bassiana* strains. This probe is a *Metarhizium* genus-specific probe. The strain IMI 330189-amplified-fragment probe hybridized only to the DNA from *M. flavoviride* strains and is a species-specific probe. Similarly, the strain I90-574-amplified-fragment probe hybridized only to the DNA from *M. anisopliae* strains. Even though the *M. anisopliae* and *M. flavoviride* probes were derived from amplified fragments which were not common throughout the *Metarhizium* strains they nonetheless hybridized to all strains. This indicates that the genomic DNA fragment is common to the strains but present in a differing arrangement within the genome. The *B. bassiana* probe specifically hybridized to DNA from the three *B. bassiana* strains.

In order to determine the iteration frequency of one of the PCR-amplified sequences, the 1.6-kb primer C-amplified fragment from *M. flavoviride* strain 330189 was excised from agarose gels. This fragment was used as a hybridization probe in Southern analysis of DNA from *M. flavoviride* strains 330189, I91-609, I91-647 and I91-671, *M. anisopliae* strain I91-613, and *B. bassiana* strain Mycotech. The probe hybridized to one major fragment in both restriction digests (data not shown) to all strains of *M. flavoviride* but not to the strains of *B. bassiana* and *M. anisopliae*. The single hybridization band in the *M. flavoviride* DNA digests indicated that this RAPD fragment most probably corresponds to a single-copy sequence.

Table 2 shows the PCR-fragment similarity indices between each fungal strain. Table 3 shows the average similarity indices between and within species of *M. flavoviride*, *M. anisopliae* and *B. bassiana* on the assumption that the previously-unidentified *Metarhizium* species are *M. flavoviride*. The *B. bassiana* and *M. flavoviride* strains showed the highest intraspecific similarity indices based on PCR fragments. In comparison the *M. anisopliae* strains showed the greatest diversity. The intraspecific diversity found within *M. anisopliae* was comparable to the inter-

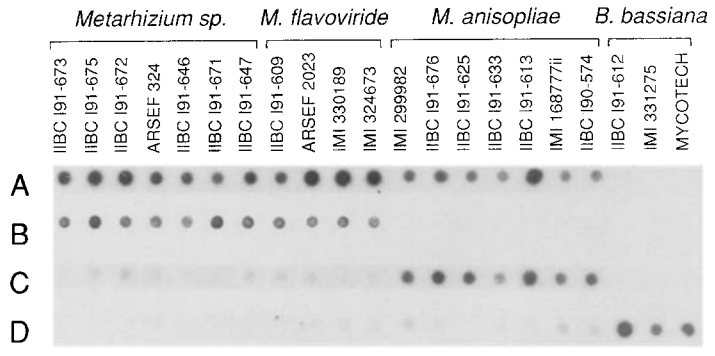

Fig. 2 A–D. Genomic DNA (100 ng) from a representative sample of fungi was blotted and probed with PCR-amplified fragments from *M. flavoviride* strain I91-647 (0.94-kb primer A-amplified fragment) (**A**) *M. flavoviride* strain IMI 330189 (1.6-kb primer C-amplified fragment (**B**) *M. anisopliae* strain I90-574 (0.63-kb primer A-amplified fragment) (**C**), and *B. bassiana* strain Mycotech (1.12-kb primer A-amplified fragment) (**D**). Isolate numbers and species are indicated above each lane

Table 2. Coefficients of similarity based on PCR fragment patterns for strains of *M. flavoviride*, *M. anisopliae* and *B. bassiana*. Values are calculated from three RAPD primers

		IIBC I91-673	IIBC I91-675	IIBC I91-672	ARSEF 324	IIBC I91-646	IIBC I91-671	IIBC I91-674	IIBC I91-647	IIBC I91-609	ARSEF 2023	IMI 330189	IMI 324673	IMI 299982	IIBC I91-676	IIBC I91-625	IIBC I91-633	IIBC I91-614	IMI 298059	IIBC I91-613	IMI 168777ii	IIBC I90-574	IIBC I91-612	IMI 331275
M. flavoviride	IIBC I91-675	.77																						
	IIBC I91-672	.82	.87																					
	ARSEF 324	.76	.76	.82																				
	IIBC I91-646	.42	.44	.42	.42																			
	IIBC I91-671	.68	.68	.73	.73	.71																		
	IIBC I91-674	.70	.65	.70	.75	.73	.85																	
	IIBC I91-647	.70	.70	.76	.81	.49	.91	.84																
	IIBC I91-609	.70	.75	.76	.76	.49	.77	.74	.80															
	ARSEF 2023	.71	.71	.76	.71	.70	.86	.88	.80	.76														
	IMI 330189	.70	.65	.65	.65	.17	.64	.60	.60	.65	.67													
	IMI 324673	.67	.67	.73	.73	.29	.60	.67	.67	.73	.63	.67												
M. anisopliae	IMI 299982	.26	.35	.32	.39	.23	.32	.35	.35	.47	.41	.35	.37											
	IIBC I91-676	.90	.31	.35	.35	.24	.33	.38	.38	.31	.38	.38	.24	.54										
	IIBC I91-625	.27	.30	.27	.27	.24	.27	.25	.25	.25	.27	.25	.12	.29	.25									
	IIBC I91-633	.22	.20	.16	.22	.15	.14	.15	.15	.20	.13	.20	.18	.18	.13	.30								
	IIBC I91-614	.25	.23	.12	.31	.22	.26	.23	.23	.23	.25	.23	.29	.35	.30	.23	.23							
	IMI 298059	.16	.14	.16	.16	.00	.13	.14	.14	.14	.12	.14	.19	.18	.20	.14	.29	.09						
	IIBC I91-613	.19	.18	.19	.26	.23	.21	.24	.24	.18	.15	.18	.22	.36	.23	.29	.35	.14	.36					
	IMI 168777ii	.00	.12	.07	.20	.18	.16	.24	.24	.24	.16	.12	.08	.30	.32	.30	.06	.21	.19	.37				
	IIBC I90-574	.18	.28	.36	.24	.22	.25	.17	.17	.28	.29	.11	.14	.27	.21	.33	.28	.00	.25	.33	.14			
B. bassiana	IIBC I91-612	.15	.14	.15	.15	.14	.17	.14	.14	.14	.25	.19	.11	.05	.11	.19	.23	.21	.07	.32	.17	.10		
	IMI 331275	.05	.10	.05	.11	.14	.18	.09	.10	.10	.17	.10	.06	.00	.06	.10	.24	.22	.31	.21	.06	.05	.73	
	MYCOTECH	.15	.09	.10	.15	.13	.21	.13	.18	.18	.16	.18	.11	.11	.11	.18	.14	.15	.06	.21	.05	.10	.64	.71

M. flavoviride *M. anisopliae* *B. bassiana*

Table 3. PCR-amplified fragment similarities within and between strains of *M. flavoviride*, *M. anisopliae* and *B. bassiana*

Strains or species compared	Similarity index		No. of comparisons
	X	SD	
M. flavoviride[a]	0.685	0.133	66
B. bassiana	0.693	0.047	3
M. anisopliae	0.246	0.100	36
M. flavoviride and *M. anisopliae*	0.237	0.108	116
M. flavoviride and *B. bassiana*	0.133	0.045	36
M. anisopliae and *B. bassiana*	0.139	0.840	27

[a] Previously unidentified *Metarhizium sp.* (see Table 1) grouped with *M. flavoviride*

specific diversity found between the three fungal species. However, *M. flavoviride* and *M. anisopliae* were more similar to each other than to *B. bassiana*.

To determine whether PCR-amplified fragments of the same sizes are homologous we performed Southern-hybridization controls with seven isolates. Two probes were constructed from primer A-amplified fragments of *M. flavoviride* strain I91-671; an 0.9-kb fragment and an 0.6-kb fragment. An 0.9-kb fragment was found in *M. flavoviride* I91-671, I91-674, 2023, and *M. anisopliae* I91-614, 299982 but not in *M. anisopliae* I91-633 or *B. bassiana* 331275. The primer A-PCR fragments from these seven strains were probed with the 0.9-kb fragment in a Southern analysis. The 0.9-kb probe hybridized only to the five strains showing the 0.9-kb fragment and not to the other strains (Fig. 3A). The probe also hybridized to a slightly larger fragment in the *M. flavoviride* strains. The 0.6-kb fragment, which is unique to the three *M. flavoviride* strains tested, hybridized only to fragments found in those strains (Fig. 3B). Here, multiple bands of hybridization occurred. A fragment slightly larger than 0.6 kb was found in I91-633 but did not show homology to the probe. These controls provide evidence that fragments of the same size are homologous. In addition to this, fragments of other sizes within a single PCR fragment pattern may share partial homologies.

Discussion

Up to now the identification of entomopathogenic fungi has been based largely on morphological details regarding the production of conidia (Rombach et al. 1987). However, the taxonomy remains questionable and now that several entomopathogenic fungi are being commercially

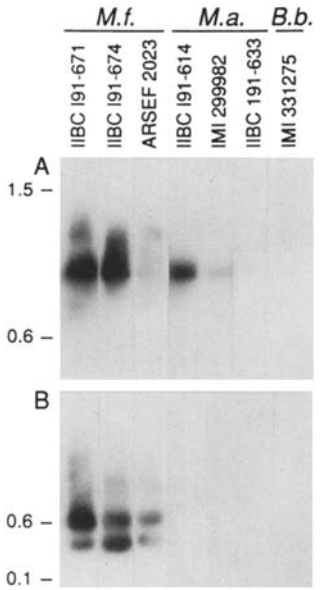

Fig. 3 A–B. Southern analysis of primer A PCR-amplified fragment patterns from several fungal strains probed with either the 0.9-kb primer A-amplified fragment (**A**) or the 0.6-kb amplified fragment from *M. flavoviride* strain I91-671 (**B**). Isolate numbers and species are indicated above each lane. Selected molecular-weight markers are indicated in kilobases to the left of the gels

produced, new, more objective criteria are needed. We used a PCR-based technique employing single primers of arbitrary nucleotide sequence to examine amplified fragment pattern differences. The differences which were observed resulted from the amplification of polymorphic regions of genomic DNA. We were able to identify several unidentified isolates of *Metarhizium* as belonging to *M. flavoviride*. Previously, several isolates of *Metarhizium* had morphological characteristics intermediate to *M. anisopliae* or the *M. flavoviride* group. For example, *Metarhizium sp.* strain ARSEF 324 has conidial dimensions intermediate between *M. flavoviride* and *M. anisopliae* (St. Leger et al. 1992 a) but is otherwise morphologically characterized as *M. anisopliae*. Allozyme data revealed that strain ARSEF 324 clustered with an isolate of *M. flavoviride* (St. Leger et al. 1992 a). The PCR-amplified banding patterns confirm that ARSEF 324 is *M. flavoviride*.

Although the strains of *M. anisopliae* used in this study showed morphological as well as host-species similarities, and, in some cases, geographical proximity, they showed extreme diversity in PCR-amplified fragments to such a degree that less than 25% of the bands were common among the strains. Substantial inter-isolate variability was also reflected in allozyme polymorphism (St. Leger et al. 1992 a). It is not surprising that the results concur since in both cases the underlying assumption is that the markers or primers represent polymorphic regions of selectively-neutral traits. By contrast, *M. flavoviride* isolates showed an average of 69% similarity (including the previously-unidentified *Metarhizium* isolates). The differences in inter-isolate variability of the species is not due to geographical distribution. The geographical distribution of *M. flavoviride* strains used in this study was as wide as that for *M. anisopliae*. In plant pathogenic fungi, inter-isolate variability in RAPD patterns was not correlated with geographical origin in two races of *Fusarium solani* (Crowhurst et al. 1991).

We found no evidence of host clustering in *M. anisopliae*. Plant pathogenic fungi may show strong host clustering which can be differentiated using RFLP or PCR techniques (Boccara et al. 1991; Forster and Coffey 1992). The lack of host clustering among isolates of *M. anisopliae* may stem from the wide host range of this entomopathogenic fungus, or because the grasshopper or locust from which these fungi were isolated also represent an evolutionary diverse group within the Orthoptera.

Why does *M. anisopliae* show such wide-ranging variation while *M. flavoviride* does not? The results suggest that strains of *M. anisopliae* may belong to cryptic species represented by genetically-distinctive strains which have undergone divergent evolution from a common ancestor. By contrast, *M. flavoviride* shows genetic evidence for a strongly clonal origin for the various strains found around the world. The clonal nature of this species suggests a common origin and perhaps, a recent evolutionary history, in comparison to *M. anisopliae*.

Assessment of phylogenetic relationships by RAPD analysis involves the assumption that bands of similar size are homologous. Obviously, data could be misinterpreted and faulty conclusions drawn if different DNA fragments have similar sizes. To minimize this effect we used three different primers which collectively allowed comparisons between a large number of bands. Also, Southern analysis using PCR-fragments as probes confirmed that at least two same-sized fragments were homologous in several fungal strains. The validity of using RAPD analysis in phylogenetic studies is further supported by the similarity in conclusions reached using this technique with those obtained previously using allozymes (St. Leger et al. 1992 a, b).

The pathogenic processes of these fungi toward insects is now being critically dissected. For example, the proteases of *M. anisopliae* and *B. bassiana* have been shown to be virulence factors (St. Leger et al. 1988; Bidochka and Khachatourians 1990) and have been cloned and sequenced (St. Leger et al. 1992 c); and it may be possible to screen the various fungi for the presence, absence, and iteration frequency of such a virulence determinant using PCR-based technology. In this way strains of fungi may be compared and grouped according to the presence or absence of pathogenicity genes, as described for phytopathogenic fungi (Boccara et al. 1991). PCR-based technology provides an additional means to resolve taxonomic problems in entomopathogenic fungi and also provides markers for genetic analysis and population studies in the field.

Acknowledgements. We thank Dr. C. Prior from IIBC, CAB International, Silwood Park, Ascot, UK, for the fungal strains; and K Hodge of ARS-USDA, Ithaca N.Y., and J. Jones of Ecoscience Corp., Worcester, Mass., for some of the primers screened in this study. This work was supported by IIBC; the Grasshopper Integrated Pest Management Program, APHIS, USDA, Boise, Idaho; and the Natural Sciences and Engineering Research Council of Canada.

References

Bidochka MJ, Khachatourians GG (1990) J Invert Pathol 56: 362–370

Bidochka MJ, Khachatourians GG (1991) Biocontrol Sci Technol 1: 243–259

Boccara M, Vedel R, Lala D, Lebrun M-H, Lafay JF (1991) Mol Plant-Microbe Interact 4: 293–299

Cenis JL (1992) Nucleic Acids Res 20: 2380

Crowhurst RN, Hawthorne BR, Rikkerink EH, Templeton MD (1991) Curr Genet 20: 391–396

Forster H, Coffey HD (1992) Mycol REs 96: 571–577

Goettel M, St. Leger RJ, Bhairi S, Jung KM, Oakley BR, Roberts DW, Staples RC (1990) Curr Genet 17: 129–132

Johnson DL, Goettel MS, Bradley C, van der Paauw H, Maiga B (1992) Field trials with the entomopathogenic fungus *Beauveria bassiana* against grasshoppers in Mali, West Africa, July, 1990 In: Lomer CJ, Prior C (eds) Biological control of locusts and grasshoppers. CAB International, Wallingford, England, pp 296–310

Kusters-van Someran MA, Samson RA, Visser J (1991) Curr Genet 19: 21–26

Lomer CJ, Bateman RP, Godonou I, Kooyman C, Kpindu D, Paraiso A, Prior C, Shah P (1992) Field trials against *Zonocercus variegatus* and *Schistocerca gregaria* with a mycopesticide formulation of *Metarhizium flavoviride*. 25th Annu Meeting, Soc Invert Pathol, Heidelberg, Germany, pp 77

Meyer W, Koch A, Niemann C, Beyermann B, Epplen JT, Borner T (1991) Curr Genet 19: 239–242

Nei M, Li W-H (1979) Proc Natl Acad Sie USA 76: 5269–5272

Rombach MC, Humber RA, Evans HC (1987) Trans Br Mycol Soc 81: 451–459

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

St. Leger RJ, Durrands PK, Charnley AK, Cooper RM (1988) J Invert Pathol 52: 285–293

St. Leger RJ, May B, Allee LL, Frank DC, Staples RC, Roberts DW (1992 a) J Invert Pathol 60: 89–101

St. Leger RJ, Allee LL, May B, Staples RC, Roberts DW (1992 b) Mycol Res 96: 1007–1015

St. Leger RJ, Frank DC, Roberts DW, Staples RC (1992 c) Eur J Biochem 204: 991–1001

Welsh J, McClelland M (1990) Nucleic Acids Res 18: 7213–7218

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) Nucleic Acids Res 18: 6531–6535

Communicated by H. Bertrand