Random amplified polymorphic DNA markers reveal a high degree of genetic diversity in the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae*

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*Metarhizium anisopliae* isolates from several insect hosts and from various sugar cane growing areas of Queensland, were examined for genetic diversity using random amplified polymorphic DNA (RAPD) markers. Thirty isolates of *M. anisopliae* var. *anisopliae* and one isolate of *M. anisopliae* var. *majus* were examined. Ten randomly chosen 10mer or 11mer primers were used and RAPD banding patterns were compared. Thirty distinct genotypes could be distinguished amongst the 31 isolates tested on the basis of RAPD patterns. Six of the isolates classified as *M. anisopliae* var. *anisopliae* exhibited closer similarity to the *M. anisopliae* var. *majus* isolate than to other *anisopliae* strains tested. Isolates exhibiting similar (> 80% similarity) RAPD profiles tended to be isolated from the same geographic area and evidence for the persistence of particular fungal genotypes in specific geographical localities was obtained. Pathogenicity assays suggested that, in some instances, RAPD groupings may also indicate insect host range. The mean similarity amongst isolates measured by band sharing in all pairwise comparisons was 41% and the most distinct pair of isolates shared only 9% of their RAPD bands. We conclude that the isolates tested belonging to the species *M. anisopliae*, as assessed on morphological grounds, represent a very diverse genetic group. The results also suggest that RAPD markers may be useful for the tracking of specific biocontrol strains in the field.

**Introduction**

The genus *Metarhizium* is one of the most ubiquitous entomopathogenic fungal genera and contains three recognized species, *M. anisopliae*, *M. flavoviridae* and *M. albin* (Tulloch, 1976; Romback et al., 1987). *M. anisopliae* is a facultative insect pathogen attacking greater than 200 insect species (Fargues et al., 1975). Two varieties of *M. anisopliae* have been described; the shortspored *M. anisopliae* var. *anisopliae* and the long-spored *M. anisopliae* var. *majus* (Tulloch, 1976). *M. anisopliae* var. *anisopliae* shows a greater host range and a wider geographic distribution than the long-spored variant (Tulloch, 1976). Individual isolates of *M. anisopliae* var. *anisopliae* produce colonies that may be one of many shades of green and have cylindrical to oval conidia, usually truncate at both ends, measuring 3.5–9.0 μm long (usually 5.0–8.0 μm long) (Tulloch, 1976). *M. anisopliae* var. *anisopliae* has been examined extensively as a possible control agent of insect pests of crops (Charnley, 1989; Samuels et al., 1989, 1990) and has been used as a commercial biocontrol agent in Brazil since 1970 to control the sugar cane spittle bug *Mahanarca postictata* (Charnley, 1989).

*M. anisopliae* var. *anisopliae* is a natural parasite of insect pests of sugar cane in Australia and has potential as a biological control agent in this region. As the use of *M. anisopliae* as a biocontrol agent increases, a more accurate means of identifying particular strains will become important (Yip et al., 1992). For example, it may be necessary to provide ‘DNA fingerprints’ of proprietary biocontrol strains. In addition it may be possible to correlate particular genotypes defined by DNA markers with particular pathogenicity groups. However,
at present DNA markers have not been used extensively to assess genetic variation in *M. anisopliae* var. *anisopliae*.

PCR-based methods for detecting genomic variability and for use in molecular diagnostics have been developed recently (Welsh & McClelland, 1990; Williams et al., 1991a). Random amplified polymorphic DNA (RAPD) markers (Williams et al., 1991a) have been applied to the study of genomic variation of various fungal species (Crowhurst et al., 1991; Guthrie et al., 1992; McClelland et al., 1990; Kersulyte et al., 1992; Leung et al., 1992; Mills et al., 1992; Smith et al., 1992; Williams et al., 1991a, b). The RAPD technique relies on the presence of priming sites for a single primer on the genome in an inverted orientation and close enough to permit PCR amplification. No prior knowledge of the genome to be analysed is required. In plants, RAPD markers have been demonstrated to provide a quantitative assessment of genetic relationships and similarities of genotypes at the sub-specific level which is consistent with established phenotypic schemes (Kazan et al., 1993b). Because of the paucity of morphological markers capable of distinguishing subspecies of *M. anisopliae*, RAPDs were explored as a potential means of assessing genetic variation in this asexual fungus.

This study was undertaken to determine the genetic variability among isolates of *M. anisopliae* var. *anisopliae* infecting insect pests of sugarcane in Queensland, Australia, using RAPD genetic markers. The results of this study suggest that considerable genetic diversity exists. This diversity, in some instances, may be related to
geographical location and pathogenicity groupings of isolates.

Methods

Fungal isolates. The 30 isolates of *Metarhizium anisopliae* var. *anisopliae* and one isolate of *M. anisopliae* var. *majus* (isolate 9) used in this study were isolated from diseased insects collected from sugar cane growing areas of Queensland, Australia, except for one European isolate (isolate 30). The geographic origins and original insect hosts of isolates are presented in Table 1. In many cases severe fungal disease made identification of the insect host impossible. In these cases, the original insect host was inferred from the insect species in the immediate sampling area from which the isolate was taken. To provide completely distinct genotypes (outgroups) for comparison of RAPD markers to the *M. anisopliae* isolates used in this study, isolates of *Colletotrichum gloeosporioides* (Braithwaite et al., 1990), *Phytophthora megasperma* f. sp. *glycinea* (Whisson et al., 1992) and *Uromyces appendiculatus* (Braithwaite et al., 1991) were used.

Isolation of DNA and RAPD analysis. Isolates of *M. anisopliae* were grown for 3-5 d in Sabouraud dextrose broth (40 g dextrose l\(^{-1}\), 10 g mycological peptone l\(^{-1}\), pH 5.6) and the mycelium harvested through Miracloth (Calbiochem). DNA was extracted from the mycelium using the method of Yoon et al. (1991). DNA concentrations were estimated using a fluorimeter (Model TKO 100, Hoefer Scientific Instruments). Duplicate extractions of DNA were made from independent cultures of each isolate.

A total of 10 oligonucleotide primers were used for RAPD analysis (Table 2). These primers were obtained from either Operon Technologies (Alameda) or from the Queensland Institute of Medical Research (Brisbane, Australia). Amplification reactions were carried out in a 25 \(\mu\)l volume containing 67 mM-Tris/HCl (pH 8.8), 16.6 mM-(NH\(_4\))\(_2\)SO\(_4\), 0.45% (v/v) Triton X-100, 200 \(\mu\)g gelatin ml\(^{-1}\), 3.5 mM-MgCl\(_2\), 125 \(\mu\)M-dNTPs, 0.2 \(\mu\)M-primer, 40-70 ng genomic DNA and 1.4 units Taq polymerase (Biotech International). Reactions were performed using a Perkin Elmer-Cetus thermal cycler for 40 cycles after initial heat denaturation at 94°C for 5 min. Each cycle consisted of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C. The 40 cycles were followed by 5 min final extension at 72°C. Amplification products were resolved by electrophoresis at 8 V cm\(^{-1}\) through 0.5 cm thick, 1.3% agarose gels using Tris Acetate EDTA (TAE) buffer (Sambrook et al., 1989) for 2.5 h and visualized by staining with ethidium bromide. Control reactions containing no added fungal DNA, and amplified by PCR as above, showed no DNA fragments. For each primer, some isolates were run on a number of gels to enable accurate band comparison among all isolates between gels.

Data Analysis. Polymorphisms between isolates were scored from gel photographs (see Fig. 1). Amplified fragments ranging in size between approximately 100 and 2000 bp were scored. Products not appearing in both RAPD amplifications carried out on the duplicate samples of genomic DNA of each isolate were disregarded.

The proportion of shared RAPD products between isolates (\(F\)) was calculated using the formula proposed by Nei & Li (1979): \(F = 2m_m + m_m\), where \(m_m\) and \(m_m\) are the number of amplification products produced by isolates and \(m_m\) is the number of products shared by the isolates. Dissimilarity values (1-\(F\)) were obtained using data pooled from all 10 primers, analysed using an unweighted pair group method with arithmetic mean (UPGMA) procedure and a dendrogram produced from the data (see Fig. 2).

Pathogenicity assays. A subset of isolates (isolates 1-6, 8-12, 18-23, 27-29) were tested for pathogenicity on specific hosts. Isolates were tested for pathogenicity towards grubs of *Lepidoptera* spp., *Antitrogus consanguineus* and *Dermolepida albihirtum* collected from sugar cane fields. Isolates were screened for pathogenicity by rolling 10 grubs per isolate in sporulating cultures on Sabouraud dextrose agar supplemented with 1% (w/v) malt extract. Individual grubs were transferred to 200 ml polypropylene cups filled with soil. A piece of sugar cane was provided as food. Untreated grubs were used as controls. The grubs were held at 25°C with a diurnal day/night cycle of 12 h:12 h. Grubs were visually checked at 4 and 8 weeks for mortality and infection. The sugar cane was replaced after 4 weeks. Some isolates were tested further by placing grubs in soil containing 10\(^{6}\) spores g\(^{-1}\), holding them at 25°C and checking for mortality or infection every 4 weeks for 16 weeks in the case of *A. consanguineus* or for 24 weeks in the case of *D. albihirtum* and *L. negatoria*. In this second pathogenicity test, isolates 2, 3, 5, 6, 11 and 28 were tested against all three grub species, isolates 1, 8, 9 and 10 against *A. consanguineus* and *D. albihirtum*, and isolates 11 and 21 against *D. albihirtum* only. Grubs were held at 25°C and observed for mortality or infection on week 4 and then every four weeks until 12 weeks had elapsed in the case of *A. consanguineus* and 24 weeks in the case of *D. albihirtum* and *L. negatoria*.

Results

RAPD analysis of *M. anisopliae* DNA

All primers (Table 2) used in this investigation successfully amplified multiple bands from all isolates of *M. anisopliae* (e.g. Fig. 1). A total of 314 unique bands were scored using the ten primers. Although amplification patterns were extremely reproducible it was occasionally difficult to score faint bands. This problem was overcome by making two independent DNA preparations from separate cultures of each isolate for RAPD analysis. In this way bands were only scored if they were amplified from both DNA preparations. An example of the reproducibility between replicate samples for each isolate and the diverse banding patterns obtained among isolates using a single primer is shown in Fig. 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Code*</th>
<th>Primer (5'-3')</th>
<th>Mean 1-(F)†</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>F06</td>
<td>GGGATTCGG</td>
<td>0.774</td>
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<tr>
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<td>F07</td>
<td>CCGATATCC</td>
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<td>F08</td>
<td>GGGATTCGG</td>
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<td>4</td>
<td>F10</td>
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</tr>
<tr>
<td>5</td>
<td>H01</td>
<td>GGTCCGAAGA</td>
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</tr>
<tr>
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<td>H02</td>
<td>TCCGAGTGA</td>
<td>0.653</td>
</tr>
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</tr>
<tr>
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<td>CK09</td>
<td>TCACGATGCA</td>
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</tr>
<tr>
<td>9</td>
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<td>CGACTTCCAG</td>
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</tr>
<tr>
<td>10</td>
<td>CK16</td>
<td>ATCGATCGG</td>
<td>0.566</td>
</tr>
</tbody>
</table>

* Primers 1-6 were obtained from Operon Technologies (Alameda), primers 7-10 are CSIRO primer bank numbers.
† Mean 1-\(F\) value from the pairwise comparison of the total number of bands scored when comparing the 33 isolates, for each primer.
The mean 1–F value obtained for all pairwise comparisons among the 30 isolates of *M. anisopliae* var. *anisopliae* was 0.58. The highest 1–F value (0.91) observed within this sub-species was between isolates 6 and 26, whilst the lowest value was obtained with isolates 8 and 9 which exhibited identical RAPD banding patterns with all 10 primers.

From a total of 465 pairwise comparisons, 386 revealed mean 1–F values > 0.4, indicating that considerable genetic diversity existed among the collection of isolates studied. One isolate (isolate 7) of *M. anisopliae* var. *majus* was included originally in this collection to provide a taxonomic outgroup. However, the mean 1–F value obtained between isolate 7 and all other isolates was 0.74, and isolate 7 was not the most genetically different isolate among the 31 isolates of *M. anisopliae* studied.

Each primer revealed a high frequency of RAPDs within all pairwise comparisons (Table 2) and mean 1–F values for each primer ranged between 0.39 and 0.77. This suggests some difference in the ability of specific primers to detect RAPDs, but not to the extent that overall RAPD comparisons were skewed by the use of any particular primer and thus pooled data were used for cluster analysis.

**Analysis of RAPD data**

A dendrogram was constructed from the 1–F values obtained in pairwise comparisons among all isolates using data pooled from all 10 primers (Fig. 2). The dendrogram was divided into 11 clusters, comprising at least two major groupings of isolates, group A (isolates 1–7) and group B (isolates 8–31) separating at a branch point of 1–F = 0.8. Some clusters branched very deeply within each group, for example isolate 7 representing *M. anisopliae* var. *majus* formed a part of group A although it was quite distinct within this group (Fig. 2). Of the 11 clusters (outlined on Fig. 2) which were separated at branch points of 1–F values of > 0.4, only 4 were represented by more than one isolate (clusters 1, 3, 5 and 7). A 1–F value of 0.2 further distinguished two individual isolates [branches 3a(ii) and 5b] within cluster 3 and one isolate within cluster 5 (branch 5b).

The considerable number of high 1–F values among isolates posed the question of whether isolates might have been grouped together by chance by the fortuitous sharing of bands. To test this hypothesis, a subset of *M. anisopliae* isolates (isolates 5, 7, 9, 15, 24, 27, 28 and 29) were compared to individual isolates of *Colletotrichum gleosporioides, Phytophthora megasperma* f.sp. *glycinea* and *Uromyces appendiculatus* using the same 10 primers.
However, isolate 10 exhibited at least 10% difference in banding patterns ($1 - F > 0.1$; Fig. 2), indicating that genetically different isolates coexisted on the same insect species at the same time and location. The majority of the isolates in cluster 5a were taken from region 5 (7 of 9 isolates) although the genus of the insect host from which they were isolated varied. Cluster 7 consists of only 3 isolates which were isolated from 3 different regions and from 3 different host species.

**Pathogenicity assays**

Pathogenicity tests were made on 20 of the 30 *M. anisopliae* isolates to investigate the relative pathogenicity of selected isolates within the clusters defined by RAPD markers. Isolates pathogenic for *Lepidiota* spp. generally exhibited pathogenicity towards *A. consanguineus* but were at best weakly pathogenic for *D. albohirtum*; these isolates were designated pathogenicity group 1. Isolates non-pathogenic to *Lepidiota* spp. and exhibiting pathogenicity for *A. consanguineus* and *D. albohirtum* were designated pathogenicity group 2. Isolates non-pathogenic for *Lepidiota* spp., *A. consanguineus* and *D. albohirtum* were designated pathogenicity group 0. Of the 7 isolates belonging to pathogenicity group 1 all but one (isolate 28) belonged to RAPD group A (Table 1). All but one (isolate 6) of the isolates belonging to pathogenicity group 1 were isolated from an insect of the genus *Lepidiota* in region 1. Isolates of pathogenicity group 2 were contained in RAPD clusters 3, 5 and 7. The two non-pathogenic isolates (pathogenicity group 0), isolates 18 and 29, did not cluster together using RAPD markers; isolate 18 clustered in a group containing four members of pathogenicity group 2 (isolates 19-22) and isolate 29 was the only representative of RAPD cluster 9.

**Discussion**

RAPD markers represent a convenient means of scanning and comparing the genomes of individuals (Williams et al., 1991a). The accuracy of RAPD markers in predicting genetic relationships has been demonstrated in plant phylogenetic studies where groupings of individuals within several species on the basis of RAPDs have been shown to coincide with taxonomic systems based on morphological, genetic and agronomic criteria (Halward et al., 1991; Kazan et al., 1993a, b; Tao et al., 1993). Although less work has been undertaken with fungi, some recent studies have demonstrated the utility of RAPDs for the separation of biotypes, races and vegetative incompatibility groups in fungi (Crowhurst et al., 1991; Guthrie et al., 1992; Leung et al., 1992). The most important finding of the current investigation was
that isolates of *M. anisopliae* var. *anisopliae* are extremely
diverse when compared at the DNA level using RAPDs.
This indicates that the morphological markers employed
in the taxonomy of *M. anisopliae* var. *anisopliae* fail to
define a genetically uniform group, and isolates with
genomes exhibiting greater than 70% dissimilarity
(assessed by RAPD generated $I - F$ values) have been
included in the subspecies *M. anisopliae* var. *anisopliae*.
The observations that fungal species defined by mor-
phological characters are genetically very diverse when
assayed by molecular markers has recently been made
for other fungi, e.g. *Uromyces appendiculatus* and
*Phytophthora megasperma* (Maclean et al., 1993).

The findings with RAPDs in *M. anisopliae* var.*
anisopliae* support those of other workers using bi-
chemical markers. Allozyme analysis of *M. anisopliae*
var. *anisopliae* also shows the great diversity of this
subspecies (Riba et al., 1990; St Leger et al., 1992). St
Leger et al. (1992) calculated genetic differences ranging
from 0 to > 0.8 based on allelic frequencies at eight
biochemical loci. The 92 *M. anisopliae* isolates tested by
these authors fell into 40 distinct clusters.

The present study shows that an isolate of *M.
anisopliae* var. *majus* fell within the range of genetic
diversity of isolates of *M. anisopliae* var. *anisopliae*,
suggesting that this varietal distinction does not reflect
overall genetic differences. At the level of 80% dis-
similarity ($I - F = 0.8$), the dendrogram produced from
RAPD data in the present study splits the 31 isolates into
two major groups, A and B (Fig. 2). Group A contains
seven isolates, one of which was the only *M. anisopliae*
var. *majus* isolate tested in the present study. Group B
comprised the remaining 24 isolates of *M. anisopliae* var.*
anisopliae*. Hence, isolates 1–6 (Fig. 2) were more closely
related to the isolate of *M. anisopliae* var. *majus* than to
the remaining *M. anisopliae* var. *anisopliae* isolates tested
in this study. Allozyme analysis (Riba et al., 1990; St
Leger et al., 1992) and pyrolysis–gas chromatography
(Messias et al., 1983) have also shown a lack of distinc-
tion between isolates of *M. anisopliae* var. *anisopliae*
and *M. anisopliae* var. *majus*. Riba et al. (1990) found
that two isolates of *M. anisopliae* var. *majus* did not
cluster together and that certain isolates of *M.
anisopliae* var. *anisopliae* were more closely related to
the var. *majus* isolates than to the other var. *anisopliae*
isolates used in their study. St Leger et al. (1992) included
13 isolates of *M. anisopliae* var. *majus* which were
scattered throughout the phenogram produced from
allozyme data. Messias et al. (1983) compared six var.*
anisopliae* isolates and a single var. *majus* isolate using
pyrolysis–gas chromatography. These workers also
found that certain var. *anisopliae* isolates were more
similar to the var. *majus* isolate than to other members of
the var. *anisopliae* variant.

Our results demonstrate that considerable genetic
diversity exists amongst isolates of *M. anisopliae* within
coastal regions of Queensland, Australia. St Leger et al.
(1992) studied 49 isolates from 14 sites throughout
Colombia and Brazil and discovered nine distinct
allozyme profiles, compared with the 31 isolates studied
here producing 30 distinct RAPD profiles. This greater
degree of differentiation using RAPDs may be indicative
of the ability of DNA markers to provide more
characters for analysis which allows the researcher to
look directly at genetic relationships rather than looking
indirectly by the use of biochemical characters. For
example, in the present study a total of 314 unique
RAPD bands were detected using only 10 primers; many
fewer bands would be detected with 10 isozyme systems.

Despite the great diversity of RAPD profiles identified
in this study, the grouping of isolates at the level of 20%
dissimilarity, and below, suggested some association of
clusters with geographical locations and provided some
information on persistence. However, there was not an
absolute correlation. Within cluster 1 (Table 1) five of the
six isolates originated from region 1; these isolates were
taken over a 5 year period, indicating persistence of
members of this group within the region. The only
exception in this cluster (isolate 6, originating in region
5) was distinguished from the other isolates (isolates 1–5)
at a $1 - F$ value of 0.09. Similarly, the majority of isolates
from cluster 5a (Table 1) were isolated from region 5 (five
of seven isolates) and again these isolates were taken over
a 5–6 year period. Riba et al. (1986) and St Leger et al.
(1992) using allozyme analysis were also able to assign
like isolates of *M. anisopliae* to restricted geographic
regions. However, in the present study it is not clear
whether the *M. anisopliae* isolates were associated with
a particular region or whether they were associated with
an insect species isolated from that region. For example,*L.
consobrina* is endemic only to region 1 (that is, the far
north of Queensland) and is a major pest of sugar cane
in this region; hence if isolates had *L. consobrina* as
preferred host they would be isolated only from this
region. This may be the case with isolates 1–5 of RAPD
cluster 1, which were isolated from a different insect host
to isolate 6 of cluster 1.

Some evidence that RAPDs may act as markers for
pathogenicity groupings for *M. anisopliae* var. *anisopliae*
was obtained in the present study, as all but a single
isolate of pathogenicity group 1 belonged to RAPD
cluster 1. Hence RAPDs could be useful for finger-
printing pathogenicity groups for commercial and
epidemiological studies, but a larger sample size than that
studied here would be necessary to assess the probability
of strain identification. The evidence obtained for the
persistence of particular RAPD genotypes in specific
regions (e.g. RAPD cluster 1 in region 1) suggests that
RAPD fingerprinting may be useful for tracking released biocontrol strains in the field.

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