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PCR Applications to the Taxonomy of Entomopathogenic Fungi

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8.1 Introduction

The extent of biodiversity within fungi is often underestimated: there are over 72,000 described species and the total number of species in the world may be as many as 1.5 million (Hawksworth, 1995). It is more widely known that insects are a highly diverse component of the animal kingdom with large numbers of undescribed species and it is estimated that a total of 10 million species of insect exist. Given that 5% of these insects are attacked by a specific fungus then it might be expected that there are 500,000 species of fungi as symbionts, commensals or pathogens of insects (Hawksworth, 1991). The diversity of association between insects and fungi has recently been reviewed by Lawrence and Milner (1996) and Murrin (1996). While over 750 species of fungi have been described as being pathogens of insects, only a small proportion of these have been intensively studied, mainly because they attack pest insects and are, therefore, of interest as biological control agents. The different types of fungi which are pathogenic on insects and the detailed ecology of some of these have been reviewed by Glare and Milner (1991).

There are two basic approaches to the use of pathogens for biological control of insects. A pathogen can be introduced into an area where it does not naturally occur in the hope of achieving long-term classical biological control. The other strategy is to mass-produce the pathogen for repeated use in an area as a biological insecticide. In both cases, it is important to know the genetic make-up of the isolate of the fungus being released into the field. Increasingly scientists are finding that PCR methodologies are useful in these applied programmes. This increased knowledge of the genetic make-up of

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isolates is being applied to track the distribution of artificially released isolates in the field, to understand the host specificity profile of an isolate and for protection of intellectual property. The obvious implications for a greater understanding of the taxonomy of the pathogens involved is often a side-benefit, and not a prime objective of the research.

In this chapter, we review the use of PCR for study of the taxonomy of entomopathogenic fungi and use as a case study our own research on *Metarhizium*. Finally we offer some conclusions and outline some of the future directions for this research. For a more general treatment of the applications of molecular biology to entomopathogenic fungi the reader is referred to Khachatourians (1996), and the application of biotechnology to the development of mycoinsecticides has been covered by Hegedus and Khachatourians (1995).

8.2 Recent Studies on PCR of Entomophthorales

The largest and most successful group of insect pathogenic fungi is the 150 or so species within the order *Entomophthorales* of the subdivision *Zygomycetina*. Most of these fungi were once placed in a single large genus, *Entomophthora*, but now some nine genera are generally recognized: *Conidiobolus*, *Entomophaga*, *Entomophthora*, *Eryniopsis*, *Neozygites*, *Erynia*, *Zoophthora*, *Strongwellsea* and *Massospora*. Some species can be grown on simple media and have been investigated as biological insecticides but most are too fastidious for this application. Unfortunately the species most effective as biological control agents are very widely distributed (along with their hosts) and so opportunities for introductions are rare.

Nagahama *et al*. (1995) examined the phylogenetic relationships between the entomophthoran genera, *Zoophthora*, *Entomophthora* and *Conidiobolus* and other zygomycetes such as *Mucor*, *Glomus*, *Smittium* and *Basidiobolus* by comparing sequences of the 18S RNA gene. Phylogenetic trees were developed using neighbour-joining (Saitou and Nei, 1987) or parsimony (Swofford, 1993) and the robustness of the branches was assessed using bootstrap analysis (Felsenstein, 1985). The two trees both showed that the three insect pathogenic genera were distinct but closely related; *Mucor* was the most closely related free-living genus. More studies of this type are needed to cover a wider range of genera and isolates. The results may help to clarify, for example, the controversial distinction between *Erynia* and *Zoophthora*.

Three species which have been introduced into other countries either accidentally or deliberately are *Entomophaga maimaiga* into the USA (Andreadis and Weseloh, 1990), *Zoophthora radicans* into Australia (Milner *et al.*, 1982), and *Entomophaga grylli* into both Australia (Milner, 1985) and the USA (Carruthers and Onsager, 1993). PCR studies of all these species have been published in addition to those on the ubiquitous aphid pathogen *Erynia neoaphidis* (syn. *Pandora neoaphidis*).

The gypsy moth, *Lymantria dispar*, is an important pest of forests in the USA. In 1989, extensive mortality of caterpillars occurred in the north-east USA and the causative organism was provisionally identified as a member of the *Entomophaga aulicae* complex, an entomophthoran pathogen of a variety of species of Lepidoptera in North America. Hajek *et al*. (1990) used allozymes and RFLP to compare 20 isolates of *E. aulicae* from a variety of hosts in the USA and Canada, with six isolates of *E. maimaimga*, a morphologically similar fungus from the *L. dispar* in Japan, and three isolates of the new fungus from gypsy moth caterpillars infected in the north-east USA. Hajek *et al*. (1996b) have extended these studies using PCR products from two primers as well as RFLP used previously. rDNA primers for the 18S and 25S genes were used to amplify 200 bp of the 3′ end of the 18S gene and 1100 bp of the 5′ end of the 25S gene. The results supported the splitting of *E. aulicae* into three groups based on RFLP analysis (Walsh *et al*., 1990) and showed that a new isolate obtained from *Orygia vetusta*, a lymantriid closely related to the gypsy moth, belonged to group 2 *E. aulicae* and was distinct from *E. maimaiga* from the gypsy moth in the USA and Japan. Unfortunately the original host of the group 2 *E. aulicae* reference strain is not known for certain but is thought to be a *Heliothis* sp. These data provide strong evidence that there are four cryptic species within the *E. aulicae* complex in the USA which are ecologically separated by attacking different hosts. Only one of these species, *E. maimaiga*, has been characterized and named.

Recent PCR studies on two other apparently broad host-range entomophthoran fungi, *Zoophthora radicans* and *Erynia neoaphidis*, have also demonstrated that these are species complexes. Hodge *et al*. (1995) used RAPDs to compare 38 isolates from a variety of hosts around the world. The aim of the study was to determine whether or not seven isolates of this fungus, originally from Serbia and field-released near Ithaca, New York (for biological control of the potato leafhopper, *Empoasca fabae*) had become established. The results gave a high level of correlation between isolates from Serbia and those subsequently isolated from field-release sites at Ithaca. More interesting was the finding that aphid-derived isolates were distinct from both the ciccadelid isolates and from isolates derived from Lepidoptera. In all some ten groups could be distinguished on the basis of RAPD patterns. Further work is needed to determine the taxonomic implications of these results. They support previous studies which proposed host-specific groups within *Z. radicans* (Milner and Mahon, 1985; Glare *et al*., 1987). The successful introduction of a strain of *Z. radicans* into Australia from Israel for the control of the spotted alfalfa aphid, *Therioaphis trifollii* f. *maculata*, was undertaken on the understanding that isolates already present in Australia on Lepidoptera, Diptera and Hymenoptera, were unable to attack aphids (Milner *et al*., 1982).

The relationships within *Zoophthora* spp. were further investigated by Hajek *et al*. (1996a) who investigated the origin of an outbreak of *Zoophthora*

phytonomi in North American alfalfa weevils, *Hypera punctata*. Similar methods, using RAPDs, were used to those of Hodge *et al*. (1995). The results clearly delineated two distinct groups within *Z. phytonomi*, both of which were distinct from two isolates of *Z. radicans* from a lepidopteran and a homopteran which gave identical patterns. The authors postulated that one of the two genotypes may be 'new' to North America, either because of importation from Europe or through genomic change (e.g. by mutation). They found that spore size, traditionally a key taxonomic character, was too variable to be of any taxonomic value.

In a similar study, Rohel *et al*. (1997) compared RAPD patterns for 31 isolates of the *Erynia neoaphidis* complex. As with the *E. aulicae* complex, only a single species *Er. kondoiensis* had been formally recognized and described as a distinct species. *Er. kondoiensis* was distinguished from *Er. neoaphidis* on the basis of allozymes, spore size and ecological factors (Milner *et al*., 1983). Rohel *et al*. (1997) showed that the *Er. neoaphidis* complex clearly displays size polymorphism in the internally transcribed spacer (ITS) region. RAPD data showed that there were four major groups within the 31 isolates studied. The majority of isolates, which were from aphid hosts in France and other countries, were placed in group 1100, two isolates from non-aphid hosts in Mexico were group 575, the *Er. kondoiensis* isolate from *Acyrthosiphon kondoi* in Australia plus two other isolates from *Aphis* sp. in Mexico and *Acyrthosiphon pisum* in Brazil constituted group 1000, and seven isolates all from aphids in France constituted group 1450. Both groups 1000 and 1450 had smaller conidia than the (probably 'typical') *Er. neoaphidis* group 1100, and they grew more rapidly on artificial media. Further ecological and molecular studies are needed to determine the differences between these groups, especially groups 1100 and 1450, both of which occur on a range of aphid species in France. The two isolates from non-aphid hosts are likely to constitute a distinct species.

8.3 Recent Studies on PCR of Ascomycota

A large number of species of fungi from the *Ascomycota* have been described as pathogens of insects including the genera *Cordyceps*, *Torrubiella* and *Nectria*. Recent molecular research has focused on the *Ascosphaera* spp. which cause the disease known as chalkbrood in bees (Bissett *et al*., 1996). Lu *et al*. (1996) found that RAPD patterns could be used to detect the infection in leafcutter bees and to distinguish between bees infected with *A. aggregata* and those infected with *A. larvis*. They reported that a quick 'vortexing' method for extracting DNA was effective for use in RAPD analysis. All the primers except one gave clear banding patterns and these were consistent between infected cadavers and those from pure cultures; while uninfected insects gave quite different patterns. There were obvious differences in banding patterns between insects infected with either species of *Ascosphaera*

and non-sporulating cadavers gave banding patterns similar to those from sporulating cadavers. Lu *et al*. (1996) concluded that this method provides a rapid and accurate identification of the infection in leafcutter bees and that species-specific ITS primers would provide a more reliable way of recognizing species.

Recently five new species of *Ascosphaera* have been described from bee material in Australia, using traditional taxonomic methods (Anderson and Gibson, 1997), taking the total of known species to 25. They also compared 20 of these species using sequence analysis of the ITS1 and ITS2 regions (Anderson *et al*., 1997). The methodology used was essentially that adopted for *Metarhizium* by Curran *et al*. (1994). Sequences were found to be remarkably uniform within species and small differences between species were therefore considered important and diagnostic. The ITS1 region was found to be more variable than the ITS2 region. Four distinct groups were detected which correlate with morphological data. For example, the eight species clustered with *A. apis* all have large, blackish spore cysts, with ellipsoidal to subcylindrical ascospores that are small or of average size. Because of the limited differences in morphology, the molecular data were used as the primary character for separating some species, e.g. *A. subcuticulata* from *A. aggregata*. Interestingly these clusters did not correlate with the host taxonomy, though there was some evidence that the saprophytic/parasitic life cycle correlated with these clusters. The results of Anderson *et al*. (1997) also support the conclusions of Berbee and Taylor (1992, 1995) who found, using sequence data from the nuclear 18S rDNA gene, that *Ascosphaera* and *Eremascus* are closely related genera.

8.4 Recent Studies on PCR of Mitosporic Fungi

8.4.1 Verticillium *spp.*

One of the first fungi to be commercialized as a mycoinsecticide was *Verticillium lecanii* for control of aphids (trade-named Vertalec) and for whiteflies (trade-named Mycotal). Various species of insect pathogenic fungi were synomized under the name *V. lecanii* by Gams (1971). Jun *et al*. (1991) tested the validity of this revision using a range of morphological, physiological and biochemical characters to establish the taxonomic relationships between 64 isolates of *Verticillium* spp. They found that isolates of *V. lecanii* clustered according to their source and suggested that further work using enzyme patterns and DNA composition might support the case for separating entomopathogenic, fungicolous and plant-pathogenic isolates into distinct taxa. Typas *et al*. (1992) used RFLPs and found that three isolates of *V. lecanii* from aphids (two species) and whitefly (one species) clustered together and were distinct from six plant pathogenic species. Roberts *et al*. (1995) used RAPDs to compare *V. lecanii* with plant pathogenic species *V.*

albo-atrum and *V. dahliae* and concluded that *V. lecanii* was highly variable and quite distinct from the other two *Verticillium* species. Other workers such as Nazir *et al*. (1991) have used sequence data of the ITS region to differentiate between species and strains within plant-pathogenic members of the genus. These data suggest that *V. lecanii* could be incorrectly named and that it could be separated into a number of varieties/species which were ecologically distinct.

Mor *et al*. (1996) analysed 36 isolates, mostly from insects and identified as *V. lecanii*, using RAPDs. Unlike Jun *et al*. (1991) the isolates did not cluster according to source, nor was there any correlation with geographical location or virulence for the whitefly, *Bemisia tabaci*. They conclude 'it appears that virulence determinants of *V. lecanii* are also unrelated to polymorphic DNA pattern as expressed by RAPDs'.

8.4.2 Paecilomyces *spp.*

Two species of *Paecilomyces*, *P. farinosus* and *P. fumosoroseus*, are important as natural control agents of insects and are being evaluated as potential mycoinsecticides. A third species, *P. lilacinus*, is used as a biological control agent for plant parasitic nematodes in the Philippines.

Tigano-Milani *et al*. (1995b, 1995c) have been investigating variation within these species using RAPDs. The first of these studies (Tigano-Milani *et al*., 1995a) was concerned with *P. lilacinus* and compared the banding patterns of 28 isolates identified as *P. lilacinus*, mainly from soil and nematode material in Brazil, with the banding patterns of *P. fumosoroseus* (two isolates), *P. farinosus* (three isolates) and *P. amoenoroseus* (one isolate). The polyacrylamide gels showed considerable variation within *P. lilacinus* and enabled a large number of bands to be scored resulting in 293 scorable characters. Cluster analysis with *P. fumosoroeus* as the outgroup gave a large number of clusters with two isolates, code-named CG301 and CG190, being particularly divergent. The tRNA phylogenic tree gave a smaller number of clusters based on the 112 scorable characters. Interestingly, one isolate of *P. farinosus* clustered in the middle of the *P. lilacinus* isolates while CG190 was clustered with an isolate of *P. amoenoroseus* and one of *P. farinosus*. It was concluded that *P. farinosus* was not monophyletic. However apparently no attempt was made to determine whether or not CG190, isolated from the soil, was pathogenic for insects. While these results clearly indicate a high degree of variability within *P. lilacinus* and suggest that polyacrylamide gels are potentially useful for genetic fingerprinting of strains, the phylogenetic conclusions are open to question. Do the tRNA genes have adequate information content to allow the drawing of phylogenetic conclusions? Were the closely related strains of *P. fumoroseus* suitable as outgroups?

Identical methods were applied by Tigano-Milani *et al*. (1995b) to study 27 *P. fumosoroeus* isolates, of which 15 came from *B. tabaci*, in comparison with one strain of *P. lilacinus* and nine strains of *Paecilomyces* not assigned

to species. As with *P. lilacinus*, the RAPD patterns revealed a high degree of genetic divergence between strains. Cluster analysis placed the *P. fumosoroseus* isolates into three groups. Group 1 isolates were morphologically similar and resembled the typical *P. fumosoroeus*, and group 2 isolates were more polymorphic and this group contained most of the *B. tabaci* isolates. Group 1 included isolate CG170 derived from the PFR-97 pilot product being produced by ECO-tek in the USA. The group 3 isolates were highly divergent from what can be regarded as normal. This is similar to the situation in *Metarhzium* where it is also a species aggregate (see case study below). The phylogenetic analysis based on the tRNA primers did not clarify the situation because group 1 and group 2 isolates often clustered together, again suggesting that the data may be unsuitable for this type of analysis.

8.4.3 Beauveria *spp.*

Agostino Bassi is credited with being the first scientist to demonstrate that a disease was caused by a microorganism, when in 1835 he infected silkworms with *Beauveria bassiana*. Since that time, members of the genus *Beauveria* have been intensively studied all over the world. A diverse range of isolates sharing the essential features of *Beauveria* have been described and some six species are generally recognized at present: *B. bassiana*, *B. brongniartii*, *B. amorpha*, *B. vermiconia*, *B. velata* and *B. caledonica* (Glare and Inwood, 1997). Distinguishing features of *Beauveria* include conidiophores which produce one-celled conidia in whorls or dense clusters of sympodial, short or globose or flask-shaped conidial cells with apical denticulate rachi. Generally, isolates are insect pathogens which produce cadavers covered in white mycelium and conidia sometimes forming pronounced coremia. *B. calendonica*, described from moorland soil in Scotland (Bissett and Widden, 1988), is the only species not known to be pathogenic to insects and may be entirely saprophytic.

Prior to the development of PCR, Rakotonirainy *et al.* (1991) sequenced a 500 bp segment of the 28S RNA gene of nine isolates of *B. bassiana* and compared them with *Fusarium* and *Tolypocladium*. The data confirmed that these *Tolypocladia* should not be synomized with *Beauveria*. Kosir *et al.* (1991) used RFLP banding patterns to differentiate two isolates of *B. bassiana*, one a virulent isolate and the other a derived mutant of much lower virulence. Pfeifer *et al.* (1993) analysed a mitochondrial gene of *B. bassiana* isolate GK2016 using restriction enzymes, gene probe hybridization and DNA sequence comparisons. They found the mtDNA to be circular and 28.5 kbp in length. By using probes from other organisms, they were able to produce a restriction enzyme map. They were unable to find any introns and concluded that the sequence was more similar to that of *Aspergillus nidulans* than to two other species, *Podospora anserina* and *Neurospora crassa*.

The first application of PCR was by Bidochka *et al.* (1993) who compared RAPD patterns of 24 isolates of *Metarhizium* and *Beauveria* from

grasshoppers. Using three primers and analysing the data by means of the method of Nei and Li (1979), they found considerable variability within *M. anisopliae* but 12 'acridid' isolates clustered together and showed much less variability. These were referred to as *M. flavoviride* because they included ARSEF 2023, an isolate described as *M. flavoviride* var. *minus* by Rombach *et al*. (1986) (see below). Isolates of the two *Metarhizium* species were found to be more similar to each other than to *B. bassiana*. This study clearly showed the potential of RAPDs for identifying species and strains of entomopathogenic fungi.

Contrasting approaches to using RAPDs for studying variation within *Beauveria* are shown in recent papers: Maurer *et al*. (1997) have confirmed the existence of host-dependent clusters while Glare and Inwood (1997) have found evidence of a distinct New Zealand group of *B. bassiana* isolates. The 36 isolates studied by Maurer *et al*. (1997) were all classified as *B. bassiana* on the basis of conidial morphology and were derived from pyralids (mostly the European corn borer, *Ostrinia nubilalis*), curculionids (mostly *Sitona* spp.) and four isolates from chrysomelids. Isolates were from Europe, Latin America, South America and Africa. RAPD and RFLP analyses were used and the isolates were bioassayed against European corn borer and the weevil *S. lineatus*. The results for the European corn borer isolates were particularly convincing. Twelve of the 13 most virulent isolates originated from this insect, and when analysed they clustered together with the one exception: isolate Bb231 from *Sitona*, which clustered closer to the corn borer isolates and was only slightly pathogenic for *S. lineatus*. The curculionid isolates were more variable but also tended to cluster together. This study shows that genetic fingerprinting, using RAPDs or a similar method, can be a powerful tool for screening isolates to detect those highly virulent for a particular pest. Maurer *et al*. (1997) also compare two dendograms produced for the RAPD and RFLP data using an unweighted pair-group algorithm and found that these dendograms showed a remarkable degree of congruity. However, the authors correctly point out problems of using a hierarchical method for placing isolates in groups with implied relatedness.

The difficulties posed by identifying isolates of *Beauveria* based on morphological characters led Glare and Inwood (1997) to use molecular tools to compare isolates from New Zealand with those from other countries. They compared 26 isolates, most of which were classified as *B. bassiana* or *B. brongniartii*, on the basis of conidial morphology. Other isolates studied were single isolates of *B. amorpha*, *B. caledonica* and *B. velata* of the ARSEF collection, one isolate with curved conidia from Chile referred to as *B. vermiconia* and an isolate of *Akanthomyces* sp. from Australia which was used as an outgroup in the analysis. DNA was extracted and analysed by RAPDs and by RFLPs of the ITS region. Neither RAPD patterns nor RFLP banding from the ITS region separated out *B. bassiana* and *B. brongniartii*. The RAPD analysis provided many more differences in scorable bands and using these data three clusters were apparent: *B. bassiana* from New Zealand

and other countries plus two overseas *B. brongniartii*, a second cluster containing only New Zealand isolates of *B. bassiana*, and a final cluster containing only *B. brongniartii* from New Zealand and other countries. The other four species clustered together with the *Akanthomyces* sp. outgroup. However, as discussed by Maurer *et al*. (1997), it is difficult to draw phylogenetic conclusions from this type of analysis. The RFLP patterns gave less variation but supported the conclusion that there was a 'New Zealand' genotype of *B. bassiana* which clustered with *B. amorpha*, *B. caledonica* and *B. vermiconia*. The second major cluster contained all the other *B. bassiana* plus *B. brongniartii* isolates. An interesting speculation in this paper is that the two genetically distinct groups of *B. bassiana* found in New Zealand represent an indigenous ('New Zealand') form and a post-European settlement group. It is also interesting that *B. brongniartii* might have been successfully introduced from France in the 1890s.

Hegedus and Khachatourians (1993) used the restriction enzyme *Sau* 3A to generate a number of small randomized DNA fragments and then used dot-blots to test the specificity of these fragments for *B. bassiana*. Several fragments, ranging in size from 1180 to 2700 bp were found to hybridize to DNA from *B. bassiana* but not *B. brongniatii*, *B. caledonica*, *B. densa*, *M. anisopliae*, and several other species of entomopathogenic and non-entomopathogenic fungi. These results were extended in a later paper (Hegedus and Khachatourians, 1996) in which the use of combinations of three primers (P1, P3 and P5) as species-specific probes was described. The PCR products derived from the P1–P3 primer set were then studied using single-strand conformation analysis. This enabled identification of a specific strain of *B. bassiana*.

Polymorphism of the ITS region as detected using RFLPs was also used by Neuvéglise *et al*. (1994) to compare isolates of *B. brongniartii* from *Haplochelus marginalis*. This study, while confirming the high degreee of variability within the ITS region of *B. brongniartii*, found a remarkable degree of congruence with isolates from *H. marginalis* having similar RFLP patterns and being the only isolates to be virulent for this scarab host. An unrooted tree computed by the PAUP program (Swofford, 1993) shows that this group of *B. brongniartii* from *H. marginalis* forms a distinct cluster, closely related to other *B. brongniartii*, but more distantly related from clusters containing isolates of *B. brongniartii* from *Melolontha melolontha* and isolates of *B. bassiana*. A complete sequence for the ITS and 5.8S RNA coding gene for two isolates of *B. bassiana* has recently been published by Shih *et al*. (1995).

The first report of a group 1 intron in an entomopathogenic fungus was by Neuvéglise and Brygoo (1994) when they found three insertion elements each 350–450 bp in the 28S ribosomal RNA gene of *B. brongniartii*. In a further study of 37 isolates of *B. brongniartii*, two isolates of *B. bassiana* and one isolate of *Metarhizium anisopliae*, Neuvéglise *et al*. (1997) reported 14 variant forms of introns in four different positions. Isolates from *H.*

marginalis all showed one of three patterns, another pattern was unique to isolates from *M. melolontha* and eight further patterns were from isolates from other hosts including the *M. anisopliae* isolate and the two *B. bassiana* isolates. Interestingly with plant parasitic fungi, similar introns have been found in the host and the fungus (Nishida and Sugiyama, 1995) suggesting that there may have been intron transfer during evolution from host to pathogen in the *Haplochelus–B. brongniartii* model.

Another promising method for genetic fingerprinting of strains is the use of teleomeric polymorphisms (Viaud *et al*., 1996). The teleometric sequence was cloned from another fungus, *Botrytis cinerea* and used to analyse nine isolates of *B. bassiana*. RFLP banding patterns of the amplified DNA showed that most French isolates from the European corn borer were similar, and those from other insects gave distinct fingerprints. Glare has confirmed that teleomeric fingerprinting is a useful tool for comparing isolates of *Beauveria* spp (T.R. Glare, Christchurch, 1997, personal communication). Viaud *et al*. (1996) found that chromosome number varied between seven and eight among these isolates and that there were significant chromosomal length polymorphisms within *B. bassiana*.

In conclusion, recent PCR studies have shown:

1. That there is considerable genetic diversity within the two main species, *B. bassiana* and *B. brongniartii* and that various methods such as RAPD (Bidochka *et al*., 1993; Glare and Inwood, 1997; Maurer *et al*., 1997), teleomeric fingerprinting (Viande *et al*., 1996) and RFLPs (Neuvéglise *et al*., 1994; Glare and Inwood, 1997) are useful for distinguishing between isolates;
2. That the morphological characters are unreliable (St Leger *et al*., 1992a) and in particular the distinction between *B. bassiana* and *B. brongniartii* is not supported. However, it could be that a distinction should be made between 'true' *B. brongniatii* from strains of *B. bassiana* which tended to form more cylindrical conidia (T.R. Glare, Christchurch, 1997, personal communication);
3. That phylogenetic relationships are most reliably determined by RFLP studies using the ITS region (Neuvéglise *et al*., 1994; Glare and Inwood, 1997). However, this work now needs to be extended to sequence data from this region. Limited results to date suggest that *B. bassiana* and *B. brongniartii* are closely related and that four other species are distinct but correctly placed within the *Beauveria* genus. *B. bassiana*-specific gene probes have been developed (Hegedus and Khatchatourians, 1996) but have not yet been tested by other workers. Another species, *Microhilum oncoperae* is very closely related to *B. bassiana* and its taxonomic position needs to be reassessed (Riba *et al*., 1994);
4. That there is no geographical clustering within the *B. bassiana–B. brongniartii* complex other than suggestion of a distinct genotype of *B. bassiana* found only in New Zealand (Glare and Inwood, 1997);

5. That there is evidence of co-evolved host-associated genotypes, shown previously for isolates from coffee berry borer (Bridge *et al*., 1990), with the scarabs *Haplochelus marginalis* (Neuvéglise *et al*., 1994), and *Melolontha melolontha*, the *Sitona* weevils and the pyralid, *Ostrinia nubilalis* (Maurer *et al*., 1997);
6. That isolates of *Beauveria* may contain group 1 introns which can be used to distinguish between isolates (Neuvéglise *et al*., 1994, 1997).

8.5 A Case Study: Variation in *Metarhizium* spp.

8.5.1 Introduction

'Green muscardine disease' is one of the most frequently found fungal diseases of insects and occurs worldwide. The causative agent is a Hyphomycete fungus which produces uninuclear, haploid, phialidic conidia in chains. These conidia are usually green and cover the cadaver, hence the 'green muscardine' appearance. In nature, the fungus is found only on infected insects or as dormant conidia in the soil but, in the laboratory, it is easily grown on a wide range of media. No sexual stage is known, however some genetic exchange is possible by means of a parasexual cycle (Al Aidroos, 1980).

The genus *Metarhizium* was erected to cover all isolates of green muscardine fungus and the first species was *M. anisopliae* from Russia. It was named after its scarab host – *Anisoplia austriaca* (Zimmermann, 1993). Since then various new names have been erected for other strains which differed from *M. anisopliae* often in minor ways such as colour of the conidia. The currently accepted taxonomy owes much to papers by Tulloch (1976) and Rombach *et al*. (1987). Three species are recognized, distinguished by the morphology of the conidia, the conidiophores and the growth characteristics. *M. anisopliae* has green, elongate and often slightly waisted conidia produced on parallel-sided conidiophores. There are two varieties, var. *anisopliae* with smaller conidia 5–7 × 2–3 μm, and var. *majus* with conidia approximately twice that size (it has been suggested that these conidia are diploids; Samuels *et al*., 1989). *M. flavoviride* is slower-growing, lighter green in colour and has swollen conidia borne on swollen conidiophores. Again two varieties are recognized, var. *flavoviride* with larger conidia 7–9 × 4.5–5.5 μm, and var. *minus* with conidia 4.5–7.0 × 2–3 μm. The third species, *M. album*, has almost white conidia and grows on plates on a subhymenial zone of inflated hyphal bodies. The conidia are swollen and measure 3–6 × 1.5–2.5 μm.

Besides these three species, isolates of which are available from many culture collections, four additional species of *Metarhizium* have been described from China: *M. pingshaeme* Chen and Guo, *M. cylindrosporae* Chen and Guo, *M. guizhousense* Chen and Guo (Guo *et al*., 1986), and *M. taii* Liang and Liu, and its teleomorph *Cordeyceps taii* (Liang *et al*., 1991).

Since the publication of the Rombach *et al*. (1987) key, our knowledge of diversity within *Metarhizium* has increased greatly due to new strains being found and the use of biochemical and molecular techniques to provide new taxonomic characters. Morphological studies have shown that the distinction between *M. anisopliae* with cylindrical phialides and conidia and *M. flavoviride* with its swollen phialides and ovoid conidia is difficult to apply, and some isolates can display both forms depending on factors such as cultural conditions and age of culture (Glare *et al*., 1996). In addition, detailed measurements of conidia have failed to show a distinction between these two species (T.R. Glare, Christchurch, 1997, personal communication). Various authors have used RAPDs to establish differences between strains from sugar cane insects (Fegan *et al*., 1993) from cercopids and the soil in Brazil (Tigano-Milani *et al*., 1995c; Fungaro *et al*., 1996), from different countries (Leal *et al*., 1994a), from acridid compared with other hosts (Cobb and Clarkson, 1993; Bridge *et al*., 1993; Bidochka *et al*., 1993), and from New Zealand (Glare *et al*., 1997). Similar studies by RFLPs on rDNA have also shown high levels of diversity between isolates (Pipe *et al*., 1995). Using allozymes combined with a number of other characters, St Leger *et al*. (1992b) detected 'cryptic' species within *M. anisopliae*. Rath *et al*. (1995) using germination temperature profiles (McCammon and Rath, 1994) and carbohydrate utilization patterns described a new cold-temperature variety, *M. anisopliae* var. *frigidum*. A powerful method for identifying strains, useful for direct determination in infected insects, is the use of primers which detect polymorphisms in the *Pr1* gene and RFLP to produce scorable bands (Leal *et al*., 1996). Recently Bailey *et al*. (1996) have described introns in the large subunit of nuclear ribosomal RNA genes from *M. anisopliae*.

The present case study aims to extend that of Curran *et al*. (1994) to help resolve some of the problems with the current taxonomy of *Metarhizium*. These problems include:

1. Should the three specific names, *M. anisopliae*, *M. flavoviride* and *M. album* be retained and if so, how are they to be defined given that the morphological basis has been shown to be invalid? A radical solution, already proposed, is that *M. flavoviride* and *M. album* should be synomized with *M. anisopliae* which would then be subdivided into a number of varieties (Milner *et al*., 1994);
2. What is the correct name for the genetically distinct group of isolates from acridid hosts now known as *M. flavoviride* group 3 (Bridge *et al*., 1997)? The first of these isolates (ARSEF 2023) was isolated from an acridid in the Galapagos Islands and described primarily on the basis of conidial morphology as *M. flavoviride* var. *minus* (Rombach *et al*., 1986). Another isolate (ARSEF 324) was isolated from *Austracris guttulosa* from Australia and named *M. anisopliae* (St Leger *et al*., 1992b), again on the basis of conidial morphology. It has since been shown to be almost identical genetically to

ARSEF 2023 and a number of other so-called acridid isolates from many countries in Africa, as well as Madagascar, Brazil and Australia (Bidochka *et al*., 1994). Bidochka *et al*. (1994) revised their opinion of ARSEF 324, and on the basis of PCR-amplified banding patterns described it as *M. flavoviride*. These isolates are all very similar when studied using RAPDs, isozymes and protease production, yet are quite distinct from isolates of *M. flavoviride* from the soil or from leafhoppers in the Philippines and the Solomon Islands (Bridge *et al*., 1997);

3. What is the taxonomic validity of *M. anisopliae* var. *frigidum* (Rath *et al*., 1995)?

4. Another group of isolates, like the acridid group, which are genetically distinct from all others studied have been identified from New Zealand (Curran *et al*., 1994; Rakotonirany *et al*., 1994; Glare *et al*., 1997). What is the correct taxonomic placement for these isolates?

Therefore in this study, we attempt to redefine the phylogenetic, and by inference, the taxonomic relationships of the major morphological species clusters in *Metarhizium*. We correlated RAPD banding patterns with sequence data from the ITS and 5.8S rDNA. Here we report on phylogenetic estimates made by parsimony and distance using the ITS and the 5.8S region of the rDNA. The following is a summary of this work which is published in detail elsewhere (Driver *et al.*, 1998).

8.5.2 Methods

Isolates

The 26 representative isolates used in this study are given in Table 8.1. Another 95 isolates have also been sequenced but did not provide significant additional data. The isolates were chosen either because they are the subject of on-going CSIRO research to develop a range of mycoinsecticides based on *Metarhizium* spp. (Milner and Jenkins, 1996), or because they are important (such as isolates derived from type material) for a comprehensive understanding of genetic diversity within *Metarhizium* spp.

ITS amplification and sequencing

PCR (Saiki *et al*., 1988) was 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, spanning the ITS1, the 5.8S rDNA and the ITS2. Primer sequences and reaction conditions were those described by Curran *et al*. (1994). Sequencing reactions were done using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing mix from Applied Biosystems Inc. (ABI) Australia.

RAPD amplifications and analysis

The presence or absence of RAPD band patterns was scored visually in order to correlate any relationships with ITS sequence data without any attempt to produce an hierarchic arrangement of clusters. RAPD groups

Table 8.1. List of representative isolates used for case study on *Metarhizium*.

Clade	Morphological species	FI number	Other designation	Host	Geographical origin	Reference
1	*M. album*	FI-MaF	ARSEF 1941	*Nephotetti viresens* (Homoptera)	Philippines	Rombach *et al.* (1997)
	M. album	FI-1165	ARSEF 1942	*Nephotetti viresens* (Homoptera)	Philippines	Rombach *et al.* (1997)
2	*M. flavoviride* var. *minus*	FI-1173	ARSEF 2948	Unknown (Homoptera)	Brazil	Humber (1992)
	M. anisopliae var. *anisopliae*	FI-152		*Lepidiota consobrina* (Coleoptera)	Australia	
3	*M. anisopliae* var. *anisopliae*	FI-698	F10	Unknown (Lepidoptera)	New Zealand	Glare *et al.* (in press)
	M. anisopliae 'var. *frigidum*'	FI-1124	DAT-F220	Soil	Australia	Rath *et al.* (1995)
	M. anisopliae 'var. *frigidum*'	FI-1125	DAT-F368	Soil	Australia	Rath *et al.* (1995)
4	*M. anisopliae* var. *anisopliae*	FI-72	IMI 177416	*Pemphigus treherni* (Homoptera)	Britain	IMI (1982)
5	*M. flavoviride* var. *minus*	FI-1172	ARSEF 1764	*Nilaparvata lugens* (Homoptera)	Philippines	Rombach *et al.* (1986)
	M. flavoviride var. *minus*	FI-403	ARSEF 2037	*Nilaparvata lugens* (Homoptera)	Philippines	Rombach *et al.* (1986)
6	*M. flavoviride* var. *flavoviride*	FI-1170	ARSEF 2025	Soil	Germany	Gams and Roszypal (1973)
	M. flavoviride var. *flavoviride*	FI-405	ARSEF 1184	*Otiorrhynchus sulcatus* (Coleoptera)	France	Rombach *et al.* (1986)
	M. flavoviride var. *flavoviride*	FI-402	ARSEF 2024	*Otiorrhynchus sulcatus* (Coleoptera)	France	Rombach *et al.* (1986)
	M. anisopliae 'var. *frigidum*'	FI-38	DAT-F001	*Adoryphorus couloni* (Coleoptera)	Australia	Yip *et al.* (1992)

7	*M. flavoviride* var. *minus*	FI-1216	ARSEF 2023	Acridid (Orthoptera)	Galapagos Islands	Rombach *et al.* (1986)
	M. anisopliae var. *anisopliae*	FI-985	ARSEF 324	*Austracris guttulosa* (Orthoptera)	Australia	St Leger *et al.* (1992)
	M. flavoviride	FI-987	IMI 330189	*Ornithacris cavoisi* (Orthoptera)	Niger	Bridge *et al.* (1993)
	M. flavoviride	FI-1028	IMI 324673	*Zonocerus variegatus* (Orthoptera)	Tanzania	Bridge *et al.* (1993)
8	*M. anisopliae* var. *anisopliae*	FI-1042		*Dermolepida albohirtum* (Coleoptera)	Australia	
	M. anisopliae var. *anisopliae*	FI-147		*Lepidiota consobrina* (Coleoptera)	Australia	
9	*M. anisopliae* var. *anisopliae*	FI-1029	IMI 168777ii	*Schistocerca gregaria* (Orthoptera)	Eritrea	Tulloch (1976)
	M. anisopliae var. *anisopliae*	FI-1034	IIBC I91-614	*Patanga succincta* (Orthoptera)	Thailand	Bidochka *et al.* (1994)
	M. anisopliae var. *anisopliae*	FI-1099	ARSEF 445	*Teleogryllus commodus* (Orthoptera)	Australia	Bridge *et al.* (1993)
10	*M. anisopliae* var. *majus*	FI-388	ARSEF 1914	*Oryctes rhinoceros* (Coleoptera)	Philippines	Humber (1992)
	M. anisopliae var. *majus*	FI-389	ARSEF 2151	*Oryctes rhinoceros* (Coleoptera)	Indonesia	Humber (1992)
*	*Beauveria bassiana*	FI-297		Soil	Australia	
*	*Paecilomyces* sp.	FI-360		*Inopus rubriceps* (Diptera)	Australia	
*	*Paecilomyces* sp.	FI-442		*Inopus rubriceps* (Diptera)	Australia	

*Outgroups.

were assigned on the basis of perfectly identical, or nearly identical, banding patterns (Fig. 8.1).

Analysis of rDNA sequences

Sequences for complementary strands were checked and the resulting sequences aligned using CLUSTAL W (T. Gibson, D. Higgins, J. Thompson, EMBL, Heidelberg, Germany, May 1994) at default settings. The alignment was checked visually and minor adjustments made manually. The alignment

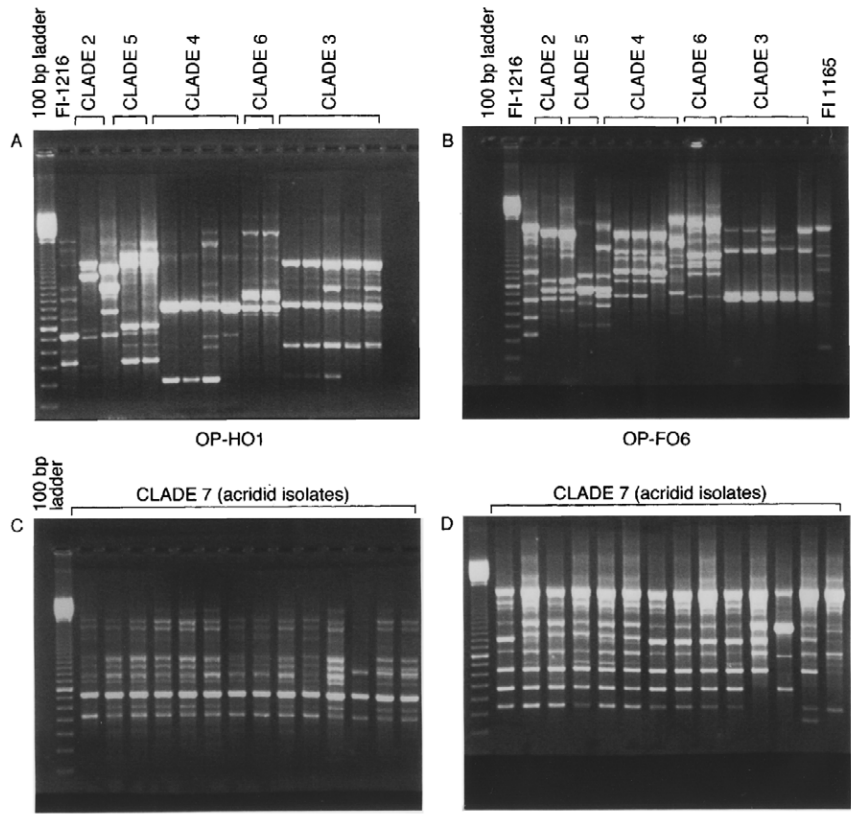

Fig. 8.1. RAPD–PCR banding patterns using two primers, OP-H01 and OP-F06. Panels A and B, lanes 1–17: 100 bp ladder; FI-1216 (ARSEF 2023, clade 7); FI-152, 1173 (clade 2); FI-403, 1172 (clade 5); FI-405, 402, 1170, 38 (clade 4); Fi-72, 1101 (clade 6); FI-698, 699, 702, 1125, 1126 (clade 2); and panel B only FI-1165 (*M. album*, clade 1). Panels B and C illustrate the genetic homogeneity of acridid strains (clade 7), lanes 1–15: 100 bp ladder; FI-1216 (Galapagos Islands); FI-1189, 1190, 1191, 1192, 1193 (Brazil); FI-1067, 983, 984, 986 (Benin); FI-987 (Niger); FI-1028 (Tanzania); and FI-985 (ARSEF 324), 1155 (Australia).

was translated into #NEXUS format for analysis using PAUP4d52-53 (beta test version of PAUP; Swofford, 1997).

The partition homogeneity test (Farris *et al*., 1995) as implemented in PAUP indicated no incongruence amongst the ITS1, 5.8S and ITS2 regions of the molecule. Subsequently the regions were analysed separately and together, but final phylogenetic results are based on the combined data. The ITS1 region (positions 14–219 in the file) contained 71 parsimony informative positions, the 5.8S region (positions 220–377) contained seven, and the ITS2 region (positions 378–604) contained 80. With alignment gaps treated as missing data and with no differential weighting of transversions against transitions, parsimony analysis of either the whole data set or the ITS1 region identified in excess of 40,000 most parsimonious (mp) trees, apparently on a single TBR-branch-swapper defined island. Separate analysis of the ITS2 region produced a lesser number of mp trees, the strict consensus of which was consistent with the strict consensus of the ITS1 or whole data set trees (Fig. 8.2). Some branches are supported by several synapomorphic changes. The large number of trees results from the lack of informative character state changes within certain clades (primarily single point deletions in a number of *M. anisopliae* isolates).

8.5.3 Results

The RAPD–PCR banding patterns showed a high level of diversity even within some clades, for example clade 9 which contains a very large number of isolates normally classified as *M. anisopliae* var. *anisopliae*. Small changes in ITS sequence, e.g. single point mutations or deletions with respect to the type-strain of *M. anisopliae* var. *anisopliae*, are often accompanied by dramatically different RAPD patterns with most primers. Low levels of sequence divergence in most other clades of isolates, including var. *majus* and the acridid isolates, were mirrored by much smaller levels of polymorphism in their RAPD banding patterns (Fig. 8.1, panels A, B, C and D).

The size of the ITS1, 5.8S and ITS2 PCR product (excluding primer sequences) varied from 506 bp for FI-1029, the type material of *M. anisopliae* var. *anisopliae*, to 573–4 bp for the two isolates of *M. album*. The size of this region is comparable to that reported for other entomopathogenic fungi (Neuvéglise *et al*., 1994; Anderson *et al*., 1997). Size polymorphisms for this region which could be detected on agarose gels, were most evident between isolates from var. *anisopliae*, var. *majus* and B-type strains of *Metarhizium*, clades 8, 9 and 10 which ranged from 505 to 510 bp, compared with all other material examined, including the acridid isolates. These larger ITS fragments, 542–544 bp in acridid isolates, 530–551 bp for all varieties of *M. flavoviride*, 545–546 bp for New Zealand isolates and 552–558 bp for the two isolates from clade 2 in the phylogenetic tree, generally arose from longer, more variable sequences in the ITS1. Interisolate variation was very low in all recognized varieties of *Metarhizium*, and newly identified clusters with most

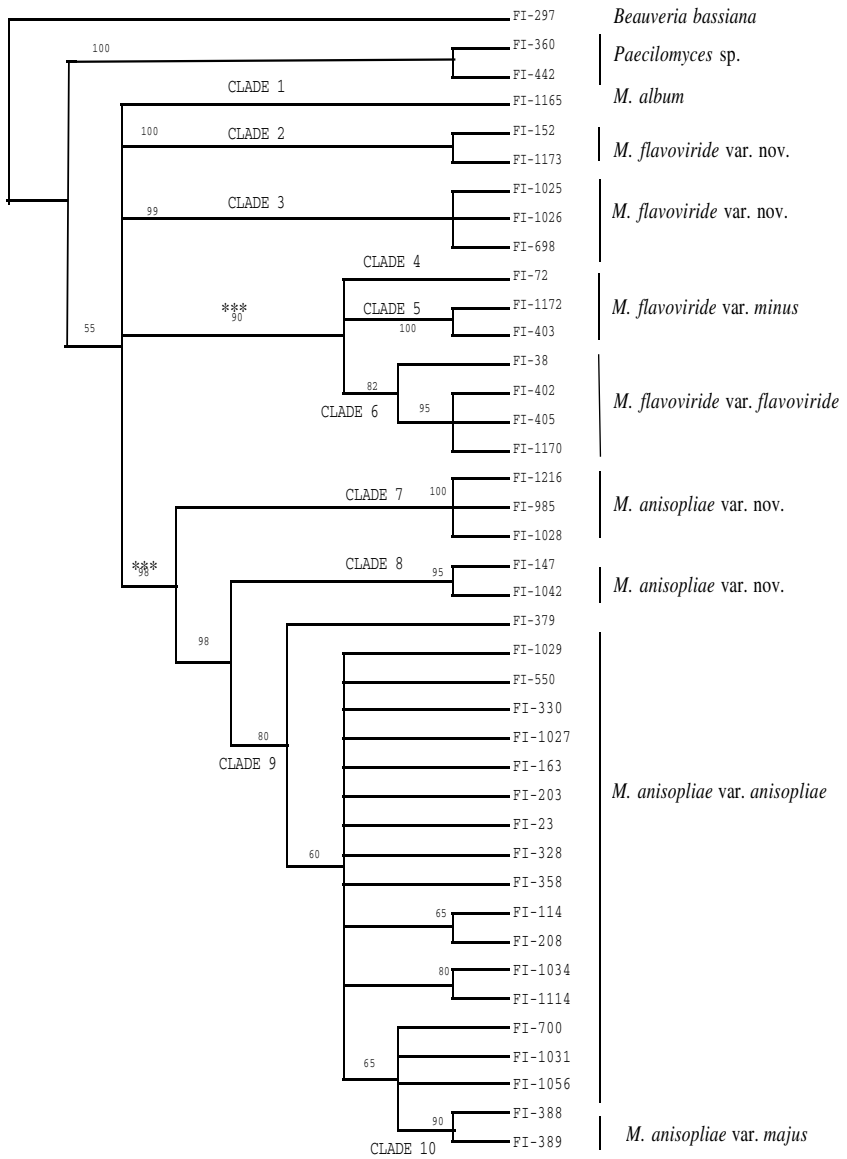

Fig. 8.2. Phylogeny of *Metarhizium* spp.: strict consensus of a maximum parsimony analysis. The numbers at branches are the bootstrap values (100 replicates), and *** indicates a T-PTP value of 0.01. Isolate designations are given in Table 8.1.

isolates exhibiting only single point mutations, or small insertions or deletions, which were generally confined to 'hot spots' of variation.

The strict consensus tree of length 394 steps from the whole data set, original alignment, with uncorrected distances, is shown as Fig. 8.2. Bootstrap tests (Felsenstein, 1985) were used to quantify the support for nodes in the consensus of mp trees. Taxon groups distinguished only by parsimony-uninformative state changes (i.e. isolates differing by only single point deletions) were combined into single nodes. Bootstrap analysis was based on 100 bootstrap replicates each with ten random-addition-sequence starting trees and keeping no more than 50 trees at each replicate, to sample the range of parsimonious trees for each resampling of the data matrix. Bootstrap proportions > 50% are shown in Fig. 8.2 where the monopoly of clades 7–10 (*M. anisopliae*) is supported.

Topologically constrained permutation tail probability (T-PTP) tests (Faith, 1991) under both equal-weighting and 2:1 transversion:transition weighting confirmed the apparent monopoly of the *M. anisopliae* clades and clades 4 and 5 (*M. flavoviride*). These clusters are not due to chance alone (PTP = 0.01). The acridoid isolates, FI-985, FI-986 and FI-1028, appear as a clade 7 closely related to clades 9 and 10 which are typical *M. anisopliae* at bootstrap support > 95%, rendering *M. flavoviride* paraphyletic. T-PTP was used to test this clustering against a conflicting hypothesis that the acridid isolates should cluster within a monophyletic clade of *M. flavoviride*. For this test, the *M. anisopliae* clades were reduced to their basal node and the group *M. anisopliae* + clade 7 isolates subjected to T-PTP tests both for monophyly and non-monophyly. A prior hypothesis of monophyly could not be falsified (PTP = 0.01). A prior hypothesis of non-monophyly could be falsified (PTP = 1.00). The data are sufficient to accept the former hypothesis and reject the latter.

Clade 6 includes isolate FI-38 which is now a commercial product in Australia (BioGreen™) under the name *M. anisopliae*, clusters within *M. flavoviride* var. *flavoviride* on all mp trees. That placement is supported by bootstrap values > 80% on the intervening branches. To confirm the prior hypothesis, a placement within the *M. anisopliae* clade could be falsified on these data, and T-PTPs for monophyly of clade 4 + *M. anisopliae* isolates were conducted. A prior hypothesis of monophyly could be falsified (PTP = 1.00). A prior hypothesis of non-monophyly could not be falsified (PTP = 0.01). These tests confirm placement of FI-38 within clade 6 (*M. flavoviride*).

The ITS region has been used extensively for fungal taxonomy (reviewed by Seifert *et al*., 1995), and it is very useful for identifying species clusters, as well as differentiating strains that are ecologically distinct (Nazir *et al*., 1991). Sequence data from this region (Driver *et al.*, 1998), have provided a sound framework for a taxonomic revision of the three accepted species of *Metarhizium*, despite the lack of resolution in the base of the tree. Processes such as gene conversion may result in the loss of clear patterns of descent in

distant relationships, and reticulation and not synapomorphies arise in the comparisons of sequences (Brower and DeSalle, 1994). The results show that the worldwide genetic diversity, as presently known, can be circumscribed by ten clades. In most cases these clades correspond to either morphologically recognized varieties, or clusters of isolates which have been identified by other molecular or biochemical markers. It is likely that most new isolates found in the future will be unequivocally placed in one of these clades. However the taxonomy is flexible and new clades may be added if the sequence data justify creation of additional clades. A key question is what level of nucleotide divergence should be attributed to define taxonomic rank at species and varietal levels? Nucleotide divergence for the ITS region between morphologically defined species in *Metarhizium* ranges between 14 and 18%, whilst divergence between recognized varieties within species does not exceed 5%. Levels of nucleotide divergence for clusters of isolates from acridoid hosts in New Zealand blur the margins between species and varietal limits. Paired clusters of isolates of clade 7/*anisopliae*, clade 7/*flavoviride* and clade 7/*album* show levels of nucleotide divergence of 9.4%, 17.2% and 16.5% respectively, whilst paired clusters of isolates from clade 3/*anisopliae*, clade 3/*flavoviride*, and clade 3/*album* are in the range 11.3%, 6.9% and 9.1% respectively. Cladistics requires that species represent monophyletic groups (Wiley *et al*., 1991) but there are no guidelines on taxonomic ranking (Seifert *et al*., 1995).

As far as possible, it is proposed that existing species names be retained and any new names proposed will be at the variety level. Therefore the name *M. album* is retained and is now defined as applying only to isolates which fit into clade 1. At present we consider clades 2–6 to contain varieties of *M. flavoviride* while clades 7–10 contain varieties of *M. anisopliae*. Where appropriate, new varietal names have been proposed for some of these clades (Driver *et al.*, 1998). However for some clades, we have too few isolates and therefore too little data, on genetic diversity within the clade, to justify a varietal name at this time. It is likely that as our knowledge of these clades increases, especially with regard to ecological factors such as host specialization, the taxonomy will become clearer, leading to some clades being elevated to new species.

8.6 Descriptions of the Clades

Most taxonomic decisions are implicitly based on the biological species concept of actually or potentially interbreeding populations that are reproductively isolated, a view that has been widely accepted by mycologists (Seifert *et al*., 1995). It has been suggested that anamorphic species not be considered as species at all, and that the term be reserved for organisms that undergo meiosis (Perkins, 1991). In practice, morphologically defined species infer the potential to interbreed (Volger and Desalle, 1994). Such

biological species concepts of actual or inferred breeding populations to delimit taxa are not directly applicable to *Metarhizium*. The molecular data give qualified support for the existing morphological-based taxonomy, if we accept that species represent monophyletic groups. The combination of molecular, biochemical and morphological markers make a case for phylogenetically defined species or 'evolutionary significant' groups (Volger and Desalle, 1994).

Clade 1 – Metarhizium album

The only isolates identified as belonging to this clade were isolates ARSEF 1941 and ARSEF 1942, both of which were described by Rombach *et al*. (1987) from infected leafhoppers in the Philippines. Besides the apparent host specificity of this clade, other characters are the small ovoid to ellipsoid conidia measuring 4–6 × 1.5–2.5 μm, the growth of a bulging mass of hyphal bodies rather than mycelium prior to sporulation and the lack of laterally adhering conidia forming prismatic columns. The pale brown colour of the spores is also characteristic although this is of doubtful taxonomic significance.

Clade 2 – Metarhizium flavoviride

This is an unusual clade containing just two rather diverse isolates. The first, FI-1173 (ARSEF 2948) on an homopteran from Brazil was identified as *M. flavoviride* var. *minus* by Humber (1992), however all the other isolates of this variety from leafhoppers and related insects in the Philippines and Solomon Islands (Rombach *et al*., 1986) are clustered in clade 5. Morphologically these isolates are all very similar and come from similar hosts, thus it is surprising that one of the isolates should be genetically so distinct. Even more surprising is that FI-152, isolated from a scarab in Australia, and morphologically resembling *M. anisopliae* var. *anisopliae*, should also cluster into this clade. FI-152 and FI-1173 show RAPD–PCR patterns which clearly distinguish them from other isolates of *M. flavoviride* var. *minus*. More work is needed to clarify this clade and to determine if there are ecological and/or morphological characteristics which are shared by these isolates, and to collect other isolates from nature which might fit into this clade.

Clade 3 – Metarhizium flavoviride

This clade contains quite a large number of isolates, many from New Zealand, which have been recognized as distinct by several workers (Riba *et al*., 1990; Curran *et al*., 1994; Glare *et al*., 1997). Our study has shown that isolates from this clade also occur in Australia (described as Strain 2 by Yip *et al*., 1992) and that they have been included as part of a group of low-temperature isolates called *M. anisopliae* var. *frigidum* by Rath *et al*. (1995). These authors recognized that some isolates were as distinct from *M. anisopliae* var. *anisopliae* as they were from *M. flavoviride* and that they may

represent a new species. Morphologically, isolates from this clade have cylindrical conidia and correspond to the short-spored form of *M. anisopliae* described from New Zealand (Glare *et al*., 1997). All isolates in this clade grow well at low temperatures (10°C and below), infect soil insects or have been isolated from the soil and attack soil insects (both scarab larvae and lepidopterous caterpillars).

Clade 4 – Metarhizium flavoviride

This is another small clade containing just two isolates with identical collection data except the date. Both isolates were from infected root aphids in Norfolk, UK (Foster, 1975), and they have cylindrical green conidia morphologically resembling *M. anisopliae* var. *anisopliae*. However they grow well at low temperatures and cluster with *M. flavoviride*; consequently, we regard that as the correct specific name. More isolates representing this clade are needed before it can be formally given a varietal name and it is possible that a search for *Metarhizium* infection on other root aphids may reveal additional isolates.

Clade 5 – Metarhizium flavoviride *var.* minus

In our study only two isolates FI-403 and FI-1172 (ARSEF 2037 and ARSEF 1764), both morphologically identified as *Metarhizium flavoviride* var. *minus* and obtained from infected leafhoppers, were included within this clade. One of these, FI-403 (ARSEF 2037) was identified by Rombach *et al*. (1985) as the type-isolate of this variety. These isolates fit the description given by Rombach and were collected in the Philippines and the Solomon Islands suggesting a narrow host range and geographical distribution.

Clade 6 – Metarhizium flavoviride *var.* flavoviride

This clade contains FI-405 (ARSEF 2025) which was derived from the material used by Gams and Roszypal (1973) for their original description of *M. flavoviride*. It also contains other isolates described by Rombach *et al*. (1986) as *M. flavoviride* var. *flavoviride*. These isolates have large, somewhat swollen conidia which form a pale green conidial mat in culture and come from the soil or from soil-inhabiting beetles. All these isolates grow well at low temperatures. Rath *et al*. (1995) included FI-38 in *M. anisopliae* var. *frigidum* and this isolate, along with a number of other soil-derived isolates, has an ITS sequence similar to FI-405 (ARSEF 2025), unequivocally identifying these isolates as *M. flavoviride* var. *flavoviride*. RAPD–PCR banding patterns show a high degree of homogeneity and conservation with the northern European isolates suggesting that this clade probably has a very wide geographical distribution. Rath *et al*. (1995) provide a nearest-neighbour dendogram based on their carbohydrate data which showed that FI-38 (DAT F001) clustered nearer to *M. flavoviride* than to *M. anisopliae*. Our data support this finding and it is therefore proposed that the correct identity of this isolate is *M. flavoviride* var. *flavoviride*.

Clade 7 – Metarhizium anisopliae

This clade contains all of the acridoid isolates described as '*M. flavoviride* group 3' (Bridge *et al*., 1997). Our data supports and extends that of Bridge *et al*. (1993), Cobb and Clarkson (1993), Bidochka *et al*. (1993) and Bridge *et al*. (1997), in showing that these isolates are genetically quite uniform and are quite distinct from the type material of both accepted varieties of *M. flavoviride*. Bootstrapping and T-PTP tests give strong support for the phylogenetic signal that unites these isolates to the major evolutionary line that gives rise to *M. anisopliae* rather than *M. flavoviride*. Therefore, we propose that the correct identity is as a new variety of *M. anisopliae* which will be described elsewhere (F. Driver, unpublished work).

The isolates in this clade have all been isolated from acridid grasshoppers. Interestingly other orthopteran hosts such as crickets are not susceptible and in nature are attacked (exclusively) by isolates from clade 9. In our experience, grasshoppers are more often infected in nature by isolates from clade 9 but these isolates are less virulent than the rarer clade 7 isolates (Bateman *et al*., 1996).

Most isolates from clade 7 produce small ovoid conidia which would identify them as *M. flavoviride* var. *minus*. For example, FI-1216 (ARSEF 2023) from the Galapagos Islands described under that name by Rombach *et al*. (1985). Other isolates, though, have larger spores which can be almost cylindrical (FI-985, ARSEF 324). All isolates share some unusual characteristics such as the ability to sporulate internally and to grow well at temperatures up to 40°C (Welling *et al*., 1994).

Clade 8 – Metarhizium anisopliae

This is another clade with only a small number of known isolates. All known isolates have cylindrical spores and produce a profuse layer of green conidia in culture, and have been isolated from scarab larvae in Queensland, Australia. Using allozyme data St Leger *et al*. (1992b) were able to show considerable inter-isolate variation and the existence of cryptic 'species' or, probably more correctly, cryptic varieties within *M. anisopliae*. This cluster of isolates has previously been described as B-type isolates of *M. anisopliae* (Curran *et al*., 1994), and probably correspond to those of pathogenicity group 1 for *Lepidiota* spp; or RAPD group A, described by Fegan *et al*. (1993). More needs to be known about this clade; however, the sequence data clearly separates it from clades 7 and 9.

Clade 9 – Metarhizium anisopliae *var.* anisopliae

The type material of *Metarhizium anisopliae* var. *anisopliae* as described by Tulloch (1976) is FI-1029 (IMI168777ii) and this isolate therefore forms the basis for this clade. This clade includes the vast majority of isolates found in nature and is genetically highly diverse. St Leger *et al*. (1992b) demonstrated the existence of clonal population structures in *M. anisopliae*. Distinct groups can be identified within the clade: for example, a number

of isolates from the black field cricket, *Teleogryllus commodus*, in Australia, share identical ITS sequence data and have very similar RAPD patterns (Milner *et al*., 1996). Isolates generally have cylindrical conidia, 5–7 µm long which form green conidia in columns of chains. They normally grow poorly outside the range 15–32°C, although recently two cold temperature active isolates have been found in soil samples taken at MacQuarie Island (Roddam and Rath, 1997), confirming that cold activity is a homoplasious character.

Clade 10 – Metarhizium anisopliae *var.* majus

A typical isolate from this clade is FI-401 (ARSEF 1946) which conforms to the description by Tulloch (1976). Isolates are readily identified on the basis of the very large conidia, usually over 10 µm long, and rapidly growing colonies producing dark green conidia; they are most frequently found attacking dynastine beetles in tropical countries. Our results support those of other workers (St Leger *et al*., 1992b; Leal *et al*., 1994b) showing that genetically this clade differs less from *M. anisopliae* var. *anisopliae* isolates in clade 9 than do other isolates such as those in clade 8 (B-type), which are also currently described as var. *anisopliae*.

8.7 Discussion

Morphological characters are generally complex and may involve the expression of many genes. Traditional fungal systematics assesses the phenotype for reliable characters that should result in the recognition of homologous features with alternate character states that may be evaluated cladistically (Brower and Desalle, 1994; Seifert *et al*., 1995). The skill of the researcher resides in the ability to 'assign characters successfully to the appropriate level of analysis' (Brower and Desalle, 1994). The reproducibility and impartiality of sequence-based systematics has made an important contribution to fungal taxonomy. Although species concepts based solely on a single gene region have been considered insufficient and too trivial to be representative of all characters (Seifert *et al*., 1995), gene regions such as the ITS are being heavily relied upon to discriminate and define new species in such genera as *Ascosphera* (Anderson and Gibson, 1997), and the edible shiitake mushroom *Lentinula* (Hibbert *et al*., 1995), as well as phytopathogenic fungi such as *Colletotrichum* (Sherriff *et al*., 1994). 18S ribosomal DNA sequence data have been used to estimate ascomycete relationships, and phylogenetic trees from such data sets are highly resolved, and generally consistent with morphological evolutionary events like forcible spore discharge (Berbee and Taylor, 1995). Lutzoni and Vilgalys (1995) integrated molecular and morphological data sets to estimate fungal phylogenies in lichenized and non-lichenized *Omphalina* species. Homogeneity testing of the 28S large subunit ribosomal DNA sequences and the morphological

characters showed that the two data sets were sampling the same phylogenetic history and could be combined.

Such studies go to the heart of the matter, that is, the conflict of gene trees and the evolution of a segment of DNA and the 'true' course of evolution of the organism. Brower and Desalle (1994) consider such concerns about gene tree versus species tree problems to be overstated, and except for recently diverged or hybridizing taxa, ancestral polymorphism and lineage sorting cannot result in strong support for an incongruent topology. Congruence between cladograms derived from different gene regions or other available characters into a total evidence approach such as described above provides strong statistical support and circumvents the problem. The gene region of choice is critically important in maximizing the phylogenetic signal-to-noise ratio. Reviews by Simon *et al*. (1994) on the utility of mitochondrial gene sequences, and Brower and DeSalle (1994) on using nuclear gene regions, raise important issues about the taxonomic rank at which particular genes are useful.

8.7.1 How can the taxonomy be made more user friendly?

A major problem with a taxonomic scheme which is dependent to a large extent on molecular data is that it is difficult to provide easily applied diagnostic characters for identification. While some clades will be easy to identify others, such as clade 2, are impossible at the present time except by molecular methods. Published molecular methods for species recognition such as the probes devised by Bidochka *et al*. (1994) and Leal *et al*. (1994b) cannot be applied since they did not include all available type material, consequently there is uncertainty about the true identity of the isolates which they used as, for example, *M. flavoviride* var. *flavoviride*. Only the Gams and Roszypal (1973) strain, ARSEF 2025, can be regarded as *M. flavoviride* var. *flavoviride sensu stricto*. Consequently these methods need to be reassessed in the light of the results of our study.

While our work suggests that the most rigorous way to identify clades is by sequence determination of the ITS regions, it has also been shown that these correlate strongly with RAPD patterns, in the same manner that ITS-RFLP patterns have been shown to correlate with RAPD patterns in *Beauveria* (Maurer *et al*., 1997). It is therefore suggested that clades be identified by using either RFLP analysis of the ITS region, or RAPD–PCR patterns established by primers such as OP-H01 or OP-A03 which give rise to fewer polymorphic fragments against a set of 'standard' isolates. It is suggested that the first isolate listed under each clade in Table 8.1 be used as the standards or type material. These are mostly ARSEF isolates and it is hoped that the other isolates will be deposited prior to publication of the taxonomic revision (Driver *et al.*, 1998).

8.8 Conclusions and Future Work

Molecular methods involving PCR techniques are now accepted as being a key element in research on entomogenous fungi and some information, as summarized in this chapter, is available about most groups. However, it is still an infant technology which means that there are still substantial gaps in our knowledge: for example, clarification of strain variation in the *Entomophthora muscae* complex, a common worldwide pathogen of flies, is needed and may shed light on differences between countries in the ecology of these pathogens. In addition, there are new techniques, and improvements to existing techniques, which have yet to be applied to entomogenous fungi. Taxonomic problems, such as those highlighted in the case study on *Metarhizium* spp., could be further resolved by an analysis of mitochondrial genes (as initiated by Junior and Martinez-Rossi, 1995) or nuclear protein coding genes such as the Pr1 protease gene used by Leal *et al.* (1996) for strain identification. Other techniques which have either not been applied or only used to a limited extent include random amplified microsatellites (RAMS; Hantula and Muller, 1997) and amplified fragment length polymorphisms (AFLP; Majer *et al.*, 1996). The presence of double-stranded RNA and isometric virus-like particles in *M. anisopliae* was first announced by Leal *et al.* (1994a) and has recently been confirmed by Bogo *et al.* (1996). Similar viruses may be present also in *Beauveria bassiana* (Osborne and Rhodes, 1994). However, their possible significance in terms of virulence is unknown. In fact, very little is known of virulence genes in entomogenous fungi, though the *Pr1* gene is involved in *M. anisopliae* (St Leger *et al.*, 1996) and probably similar genes occur in some other entomogenous fungi (Leal *et al.*, 1996). With the increased use of these fungi in pest control there will be more attention given to virulence factors and the development of genetically improved, hypervirulent, isolates as reported recently by St Leger *et al.* (1995).

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