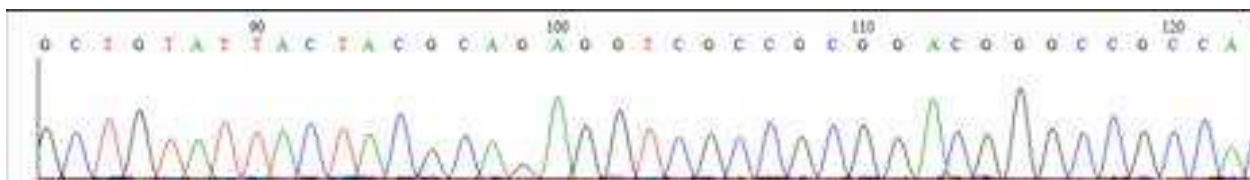
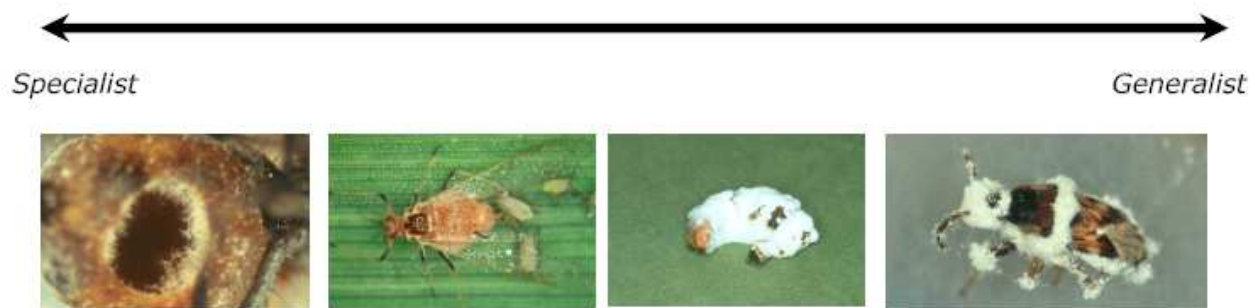


PCR-based characterisation of entomopathogenic fungi for ecological studies

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Preface

This text is Deliverable 5.2 from workpackage 5 in the ICROFS (the former DARCOF III) project VegQure. The present text is meant to provide a review of some of the PCR-based methods used in the scientific literature to characterise entomopathogenic fungi from the Hypocreales (Ascomycota). It is not meant to be a laboratory protocol, but to provide an overview as well as a list of relevant references that others can use for inspiration on how to characterise fungi. Furthermore, I wish to provide some thoughts of how to design sampling schemes for the investigation of ecological aspects of entomopathogenic fungi in the environment. Sampling schemes are rarely considered in published studies. However, well designed sampling is essential for generation of ecological data.

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Summary

The implementation of PCR-based tools for characterisation of organisms has greatly advanced our understanding of the phylogenies and species boundaries in entomopathogenic fungi, especially the widespread taxa *Beauveria bassiana* and *Metarhizium anisopliae*. These fungi have received a lot of interest due to their potential as biocontrol agents of pests. However, there is still a lack of knowledge about the fundamental ecology of these fungi in both managed and natural ecosystems, but such information is necessary both for risk assessments prior to release of biocontrol agents in the environment as well as if we wish to understand the distribution of the fungi and their impact on host populations. This latter focus is essential if the fungi are to be included in pest management strategies based on conservation biological control.

A series of unspecific PCR methods have been used to characterise isolates of *B. bassiana* and *M. anisopliae*, and many studies have concluded that the fungi contain a lot of genetic diversity. The problem with these methods is that they provide little phylogenetic information of the fungi and the characters can not be used to explicitly compare data between studies. Sequences from specific targets in the DNA provide tools for explicit comparison between isolates across studies. There is now a range of primer sets published along with reference sequences in GenBank to make these comparisons possible. Multi-gene phylogenies have revealed that both *B. bassiana* and *M. anisopliae* are complexes of species that contain cryptic taxa or clades. Using merely morphological characteristics will not result in the same degree of taxa identification as will the use of DNA sequence data. Similarly, when using microsatellite markers for population genetic studies of fungal communities, it is necessary to first separate the populations in clades as allele sizes can convert across populations. In the near future, USDA-ARS in Beltsville will launch a web-based platform (MBID, *Metarhizium-Beauveria* ID) including lists of primers, databases of reference sequences and type isolates of new described species for the reliable identification of taxa for the scientific community. This tool should provide a common framework and nomenclature for scientists and create basis for making comparisons among studies. There should be great potential to learn more about the ecology of the entomopathogenic fungi with the application of these molecular markers. However, designing an appropriate sampling scheme in the environment is absolutely necessary to conduct these ecological studies. The use of isolates from culture collections should be avoided.

Applying molecular markers in ecological studies of entomopathogenic fungi

Researchers studying entomopathogenic fungi have traditionally focused on the development of biocontrol agents that can be applied to a cropping system to control a target pest. This approach has led many researchers to investigate both the populations of the target pests as well as population of other arthropod species for the prevalence of natural infections of fungal pathogens; the ecological host range. These pathogens may hold potential as biocontrol agents, also for target pests that they do not naturally infect; the physiological host range.

However, there has been limited focus on understanding the ecological role of entomopathogenic fungi in both managed and natural ecosystems. Examples of natural infections in different host species have often been mostly lists of species names, and qualitative data exist mostly for pest species. There is increasing interest in understanding the relevance of pathogens in populations of non-pest hosts, both from the viewpoint of pure ecology as well as from applied ecology. The latter is receiving more focus since the non-pest populations may function as a reservoir of natural enemies that may be recruited to suppress the pest populations in an adjacent crop. This principle is contained within the biocontrol strategy of *conservation biological control* (Eilenberg *et al.*, 2001). In order to apply this strategy in practice, knowledge of the ecological host range of the pathogen, the interactions between the hosts and other organisms, and effects of biotic and abiotic factors on the distribution of the pathogen is essential (Meyling & Eilenberg, 2007).

Studying natural populations require tools for reliable identifications of the species involved. In the entomopathogenic fungi, researchers have traditionally used morphological characters based on general appearance of macro- and microscopical structures. Especially, the shapes and sizes of conidia are used as diagnostic criteria for separating species in the genera *Beauveria* and *Metarhizium*. However, recent evidence suggests that these features are often ambiguous and that the genera contain cryptic species, both in *Beauveria* (Rehner, 2005; Rehner & Buckley, 2005) and *Metarhizium* (J.F. Bishoff and S.A. Rehner, pers. comm.). The placement of the fungi in separate phylogenetic clades or species based on molecular markers will provide much more information to their ecology in both managed and natural ecosystems than will traditional morphological characterisation. The aim of this report is to provide a review of which markers are currently available in the published literature. It is also an aim to emphasise the importance of sampling strategies in order to yield more information of the ecology of the fungi when using molecular tools.

Unspecific PCR-based methods

A number of unspecific DNA based methods have been used in published studies of especially fungi from *Beauveria* (Glare, 2004). The widespread method of random amplified polymorphic DNA (RAPD) has been used in many studies. It is based on the use of short general primers that anneal to unspecified regions in the template DNA. It is not necessary to have any prior knowledge about the DNA of the organism, but pure cultures are needed as all DNA is targeted. The method produces numerous bands and the banding patterns are used to separate genetic groups ("fingerprint"). However, the method suffers from lack of reproducibility between laboratories (Glare, 2004) and it is not possible to compare fingerprints between studies. Another comparable method, universally primed (UP) PCR is based on longer general primers and a higher annealing temperature which makes it more robust in terms of reproducibility (Bulat *et al.*, 1998; Bulat *et al.*, 2000; Lübeck *et al.*, 1999). The limited use of this method may be that it was developed in Russia while RAPD was developed in the US (M. Lübeck, pers. comm.). UP-PCR has been used to separate sympatric isolates of *Beauveria* in Denmark and was used to place isolates in genetic groups (Meyling & Eilenberg, 2006).

Both of these multi-band producing PCR methods suffer from the issue of the scoring of band which tends to be quite subjective and dependent on the person doing the scoring. One way to reduce the effect of variability in banding pattern is to repeat the PCR and only score bands that are consistently produced (Nielsen *et al.*, 2001). This should be a standard approach when applying these methods. The advantage of using these methods is that no knowledge of the structure of the genome is necessary. However, the methods tend to be subjective, can be unreliable and require pure cultures of the fungus for DNA extraction since the methods by nature are unspecific. The methods also suffer from the fact that they are not comparable between studies.

For ecological studies, RAPD was used in combination with specific methods to separate isolate genotypes of *Metarhizium anisopliae* from Canadian soil (Bidochka *et al.*, 2001) and relate the genotypes to the origin of isolation.

Specific PCR-based methods

Specific methods aim to characterise selected targets in the DNA of the organism. One commonly used method is the digestion of PCR products of specific DNA regions, such as genes or ITS, with restriction enzymes, yielding fragments of variable sizes which can then be separated on an agarose gel. These PCR-RFLPs (Restriction Fragment Length Polymorphism) have been used for the characterisation of both *Beauveria* and *Metarhizium* species (Bidochka *et al.*, 2001). Although the method is specific and should in principle be explicit and reproducible, the scoring of fragment size is mostly done from gel photos and this has some subjectivity to it. Furthermore, only few variables are obtained from a single restriction enzyme, thus several of these must be used for each target region to increase variability.

More variables can be obtained with the method of AFLP (Amplified Fragment Length Polymorphism). This method relies on the initial digestion of the entire genomic DNA with restriction enzymes into fragments of variable size. Specific adaptors are then attached to the sticky ends, and the fragments are amplified by PCR with primer pairs that anneal to the adaptors. The fragment sizes can be scored on polyamide gels or if they are labelled with fluorescent tags the fragments can be sized more objectively on a capillary sequencer. This fingerprinting method is more reproducible and has recently been used to characterise genotypes of *B. bassiana* and *M. anisopliae* (de Muro *et al.*, 2003; Inglis *et al.*, 2008; de Muro *et al.*, 2005).

Multiple band profiles are also possible to obtain with the method inter-simple-sequence-repeats (ISSR) PCR. This method targets specific regions, the simple sequence repeats (SSRs or microsatellites, see below), and primer pairs amplify the regions of variable lengths between these repeats. Fingerprinting *Beauveria* isolates with this method has recently been applied to collections from Japan (Takatsuka, 2007), China (Wang *et al.*, 2005) and the Middle East (de Muro *et al.*, 2005), but apparently not on *Metarhizium* isolates.

DNA sequence data: a common framework

For most organisms, sequencing of regions in the DNA are increasingly used to separate species based on construction of phylogenetic data sets. Sequence data sets are explicit and can be deposited in on-line databases such as [GenBank](#). This information is available to everyone and provides reference sequences that can be used to compare sequences that are produced from one own's

data. One problem with the sequences in GenBank is that the deposited information is not peer reviewed thus one should trust the depositor that the identification of the organism is reliable.

For entomopathogenic fungi, the first attempts were made to sequence the ITS region using general fungal primer sets (Driver *et al.*, 2000; Glare, 2004; de Muro *et al.*, 2003), but this region does not contain enough variability for interspecific or inter-genus delineation that many studies are focussing on (S.A. Rehner, pers. comm.). Primer sets that amplify selected regions in the genomes of *Beauveria* and *Metarhizium* have now been developed and create useful tools for an explicit characterisation of isolates. These primers are either published (Rehner & Buckley, 2005; Rehner *et al.*, 2006) or in process of being published (S.A. Rehner, pers. comm.), and they will be valuable tools for future studies. Regions for which primer sequences are published include EF1-alpha (Rehner & Buckley, 2005) and Bloc (Rehner *et al.*, 2006) which are regions that contain much more variability and thus information than ITS. The regions are beginning to be applied in studies of *Beauveria* spp. (Glare *et al.*, 2008; Reay *et al.*, 2008).

In the study by Rehner *et al.* (2006) the first attempts were made to explicitly name the clades of *B. bassiana* according to geographic region, e.g. NA_1 and Eu_1 for North America 1 and Europe 1, respectively. These explicit names should be a good reference terminology for future studies, and this approach is currently used for data of *Beauveria* spp. collected in Denmark (N.V. Meyling, in prep.; see also Fig. 1).

In order to use the same terminology (speak the same language), the establishment of a web-based database for sequence data and taxonomic revisions of the genera *Beauveria* and *Metarhizium* is ongoing at the USDA-ARS in Beltsville, MD, USA. The outline of the platform was presented at a workshop at the annual meeting of the Society of Invertebrate Pathology in Warwick, UK, in August, 2008 by Stephen Rehner. It is the intension that the database, MBID (*Metarhizium-Beauveria* ID), will create basis for a common reference platform for all scientists working with these fungi. The database will hold information about primer sequences, reference sequences of identified isolates that can be used for comparisons as well as the newest taxonomy of the fungi. This tool will hopefully be quickly adopted by the scientific community once it has been launched.

Microsatellite markers

Another specific method that has been developed over the past decades for population studies of organisms is microsatellite markers or simple sequence repeats (SSR). There are primer set

sequences available for *B. brongniartii* (Enkerli *et al.*, 2001), *B. bassiana* (Rehner & Buckley, 2003), *M. anisopliae* (Enkerli *et al.*, 2005) as well as for the entomopathogenic fungus *Isaria fumosorosea* (formerly known as *Paecilomyces fumosoroseus*) (Gauthier *et al.*, 2007; Dalleau-Clouet *et al.*, 2005). The main usage of microsatellite markers is to focus on relatedness of individuals in populations and to which degree genetic mixing occurs in and between populations, principally in diploid organisms. For *B. bassiana* and *M. anisopliae*, evaluation of relatedness and degree of genetic mixing is not as straightforward since the fungi represent the anamorphic (asexual) state and are thus haploid. The teleomorph (sexual) states of these anamorphs are currently only known from East Asia. However, the study of allele frequencies may provide data whether sexual recombination occurs in regions where only the anamorphs are known. Furthermore, allele frequencies can be used to evaluate the degree of clonality and to assess how widespread specific clones are in ecosystems. In addition, the identification of mating types can reveal whether sexual reproduction has the potential to occur in a population, since both mating types must be present within a clade for mixing to occur. It is not advantageous to use microsatellite markers alone to characterise isolates (McGuire *et al.*, 2005) since allele sizes can converge although the fungi are not related. Thus it is best to first identify which populations are present in the isolate collection and then characterise each population with microsatellite markers. Defining a population is also an important aspect to consider, see below.

Microsatellite markers can also be used to track the fate of a biocontrol agent. This has been done for *B. brongniartii* in Switzerland where treated areas were sampled several years after application to evaluate whether the biocontrol agent had established (Enkerli *et al.*, 2004). This is possible if a unique allele profile can be established for the isolate. Furthermore, the markers can also be used to specifically target a fungus, e.g. *M. anisopliae*, in DNA extracted from bulk soil and thereby establish whether the fungus is present in the soil sample and how many alleles are present (J. Enkerli, pers. comm.).

Microsatellite markers hold good potential to learn more about reproduction in ecosystems and the tracking of isolates in the environment. However, it is important to keep in mind how the isolates are collected to make ecological sense.

Sampling strategies for ecological studies

Most published studies on genetic diversity of *Beauveria* and *Metarhizium* species are based on isolates from culture collections or regional samples (e.g. relatively large geographical areas within a large country), isolates from specific pests or from specific environmental compartments, e.g. soil. It is important to keep in mind when collecting isolates what the specific research question is and whether this can be answered based on the compiled isolates. Culture collections are valuable for compiling isolates for the generation of phylogenetic data, since they contain isolates from broad geographical regions and hosts, as well as they have explicit reference isolates that scientists can use mutually. However, ecological studies can not be based on isolates from culture collections.

For ecological studies, it is essential to consider the context in which the isolates are collected and which interactions they may have with each other, their host organisms and the environment. For *Beauveria* and *Metarhizium* species, the fungi can be isolated from soil and insects, and *B. bassiana* can also be isolated from plant parts and from the air (Meyling and Eilenberg, 2007). All these environmental compartments should be considered when collection schemes are planned. If collecting only specific pests that are infected, then the collection will not give information of how the pathogens interact with the surroundings, including other host species. Furthermore, in the basic ecological sciences there are increasing focus on indirect interactions between organisms in communities, as it is increasingly recognised that these interactions are important as structuring forces of e.g. insect communities (Bukovinszky *et al.*, 2008; van Veen *et al.*, 2008; van Veen *et al.*, 2006). The pathogens may also be involved in indirect interactions and may therefore also have relevance to basic ecological studies of insect communities. However, if such studies are to be conducted it is important to select appropriate sampling strategies.

In a Danish study insect species from selected hedgerow plants were collected and incubated for the presence of latent infections of *Beauveria* spp. (Meyling, 2005). Because the insect populations are from the same community in the same habitat they may interact indirectly for example by apparent competition through their shared natural enemies (Muller & Godfray, 1997). The collection of hosts that occur in sympatry may also provide evidence for the degree of host range of specific *Beauveria* clades in this spatial area and to which extent the clades show niche partitioning. A first step in such investigations is to establish whether host species, which do not interact directly through for example competition, share natural enemies. In the investigated community, many species shared clades of *B. bassiana* across host plants thus this system hold

potential for many indirect interactions mediated through pathogens. This approach is new and shows the relevance and necessity of using molecular tools in ecological studies of insect-pathogen interactions. If only the crude identification of morphology was used to characterise the fungal isolates then the detailed foodweb in fig. 1 would not be possible.

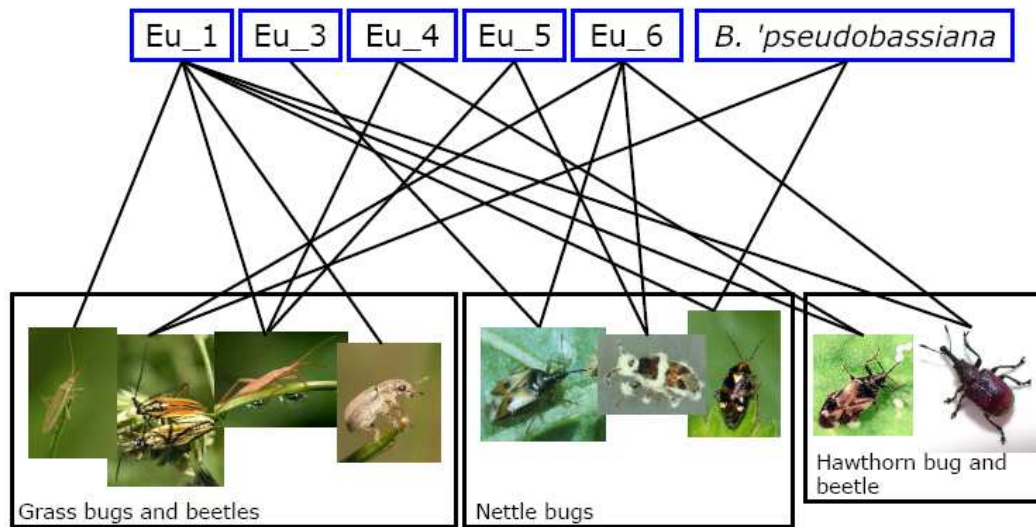


Figure 1. Example of a foodweb linking *Beauveria* spp. clades to their natural hosts in an insect community of a hedgerow in Denmark. The construction of the foodweb is only possible based on molecular characterisation of the isolated fungi as these share common morphological features. This figure was presented at the symposium *Role of disease in the regulation of non-pest insects* at the annual meeting of the Society for Invertebrate Pathology, Warwick, UK 2008.

Research questions that should be addressed in future studies of the ecology of entomopathogenic fungi could be:

- Host sharing of entomopathogenic fungi, pathogen sharing by host insects and apparent competition in insect communities
- Distribution of clades of *Beauveria* and *Metarhizium* in different ecosystems
- To which degree are the clades of *Beauveria* and *Metarhizium* clonal in agroecosystems?
- Are clades in fungal communities from pristine habitats recombining?

Besides that we need to learn more about the fundamental ecology of *Beauveria* and *Metarhizium*, we know currently nothing about the ecology of the species in *Isaria* (formerly *Paecilomyces*). The few published studies on these taxa are likewise based on material from isolate collections or relatively few isolates from a single host species (Gauthier *et al.*, 2007). Using similar approaches as mentioned above for *Beauveria* and *Metarhizium* when designing sampling schemes will also provide valuable new information about these widespread entomopathogenic fungi.

It is important to consider where isolates are collected, the timing of collection, which part of the fungal life cycle is collected, how many isolates are collected, what is an isolate, etc. All these aspects are relevant if we are to learn more of the ecology of these fungi.

For future studies, it will be most beneficial if the scientists within insect pathology speak the same language, principally when it comes to taxonomy and nomenclature. The explicit characters based on DNA sequences provide tools for this to be the case and reference sequences will create basis for a common framework to refer to. The application of sequences and microsatellite markers hold great potential to conduct exciting ecological studies of entomopathogenic fungi. If researchers consider in more detail what their sampling strategies will be, then we will learn much more of the ecology of these interesting fungi in the near future.

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