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Molecular diversity of the entomopathogenic fungal *Metarhizium* community within an agroecosystem

Bernhardt M. Steinwender [a,*], Jürg Enkerli [b], Franco Widmer [b], Jørgen Eilenberg [a], Kristian Thorup-Kristensen [c,d], Nicolai V. Meyling [a,*]

[a] Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
[b] Molecular Ecology, Institute for Sustainability Sciences, Agroscope Reckenholzstrasse 191, 8046 Zürich, Switzerland
[c] Department of Plant and Environmental Sciences, University of Copenhagen, Højbakkegård Allé 13, 2630 Taastrup, Denmark
[d] Department of Horticulture, Aarhus University, Kirstinebjergvej 10, 5792 Aarslev, Denmark

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ABSTRACT

The entomopathogenic fungal *Metarhizium anisopliae* lineage harbors cryptic diversity and was recently split into several species. *Metarhizium* spp. are frequently isolated from soil environments, but the abundance and distribution of the separate species in local communities is still largely unknown. Entomopathogenic isolates of *Metarhizium* spp. were obtained from 32 bulked soil samples of a single agroecosystem in Denmark using *Tenebrio molitor* as bait insect. To assess the *Metarhizium* community in soil from the agricultural field and surrounding hedgerow, 123 isolates were identified by sequence analysis of 5′ end of elongation factor 1-α and their genotypic diversity characterized by multilocus simple sequence repeat (SSR) typing. *Metarhizium brunneum* was most frequent (78.8%) followed by *M. robertsii* (14.6%), while *M. majus* and *M. flavoviride* were infrequent (3.3% each) revealing co-occurrence of at least four *Metarhizium* species in the soil of the same agroecosystem. Based on SSR fragment length analysis five genotypes of *M. brunneum* and six genotypes of *M. robertsii* were identified among the isolates. A single genotype within *M. brunneum* predominated (72.3% of all genotypes) while the remaining genotypes of *M. brunneum* and *M. robertsii* were found at low frequencies throughout the investigated area indicating a diverse *Metarhizium* community. The results may indicate potentially favorable adaptations of the predominant *M. brunneum* genotype to the agricultural soil environment.

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1. Introduction

Metarhizium Sorokin is a globally distributed genus of soil borne entomopathogenic fungi (Ascomycota: Hypocreales) that infect a broad spectrum of insects from which several strains have been developed as biological control agents (Roberts and Leger, 2004; Zimmermann, 2007). However, profound knowledge of natural occurrence and distribution, genetic diversity and community structure of the species within *Metarhizium* in managed habitats is required to evaluate consequences of biocontrol initiatives including evaluation of the potential to develop strategies for conservation biological control (Eilenberg et al., 2001; Meyling and Eilenberg, 2007). Recently, Bischoff et al. (2009) provided a multilocus phylogeny of the *Metarhizium anisopliae* (Metschn.) Sorokin lineage and revised the taxonomy of *Metarhizium* accordingly recognizing nine species within the *M. anisopliae* lineage, including several cryptic species (Bischoff et al., 2009). Fungal isolates which formerly have been identified as *M. anisopliae* could potentially belong to any of the nine taxa and implementation of the revised taxonomy will reveal new aspects of *Metarhizium* diversity and ecology.

Given the cryptic diversity within the *M. anisopliae* lineage, discrimination of species cannot solely be based on morphology but requires the use of molecular methods for accurate identification. Although Bischoff et al. (2009) used five gene regions for their phylogeny, the 5′ end of the elongation factor 1-α (5′ EF1-α) was highlighted as a reliable marker for species discrimination. However, this region does not provide sufficient resolution for identification of genotypes within species. Simple sequence repeat (SSR) markers, also known as microsatellites, are currently among the most suitable markers for genotyping and assessing within species diversity (Enkerli and Widmer, 2010). Enkerli et al. (2005) and

* Corresponding authors at: Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark (B.M. Steinwender).

E-mail addresses: bmsw@plen.ku.dk (B.M. Steinwender), nvm@plen.ku.dk (N.V. Meyling).

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Oulevey et al. (2009) have developed SSR markers for strain-level genotyping within the *M. anisopliae* lineage, which have successfully been used to investigate molecular diversity of isolate collections (Oulevey et al., 2009; Velasquez et al., 2007).

Soils of agricultural fields have been reported to harbor high abundances of *Metarhizium* spp. compared to different ecosystems within the same region, such as forests and grasslands (Bidochka et al., 1998; Keller et al., 2003; Vänninen, 1996). Even within a limited geographical area a considerable genetic variability of *Metarhizium* spp. can be found (Bidochka et al., 2001; Inglis et al., 2008; Wyrebek et al., 2011). However, within species genotypic diversity in particular habitats in the context of the revised taxonomy is largely unknown. Bidochka et al. (2001, 2005) reported the occurrence of two cryptic groups of the *M. anisopliae* lineage in Ontario, Canada, which were genetically distinctive, non-recombining and strongly associated with soil environments of two habitat types, agricultural fields and forests. The two groups were later identified as *M. robertsii* and *M. brunneum*, respectively (Bischoff et al., 2009), representing an important emphasis on factors governing community and population structure of entomopathogenic fungi, i.e., habitat association and not host insect association, which is the traditional paradigm of insect pathology (St. Leger et al., 1992; Fegan et al., 1993; Bridge et al., 1997; Bidochka et al., 2001).

In a recent study in Denmark, Meyling et al. (2011) reported high abundances of *Metarhizium* spp. in the soil environment within a single agroecosystem. The fungi were identified morphologically as belonging to the *M. anisopliae* lineage, and it was further revealed that these isolates were exclusively confined to the soil environment and did not occur as infections in hosts above ground (Meyling et al., 2011). However, knowledge of the community structure of *Metarhizium* spp. within the agroecosystem based on molecular diversity is important for potential inclusion of these entomopathogens in conservation biological control strategies (Meyling and Eilenberg, 2007).

The aim of this study was to investigate the molecular diversity and community structure of *Metarhizium* spp. in the soil of the agroecosystem previously surveyed for entomopathogenic fungi by Meyling et al. (2011). To achieve this objective we first analyzed the genotypic diversity within a collection of 123 *Metarhizium* isolates obtained with bait insects of soil samples by using multilocus SSR markers and then assigning resulting genotypes to species by DNA sequencing.

2. Material and methods

2.1. Soil sampling

Soil sampling was performed in an agricultural field and adjacent hedgerow at the Research Center Aarslev, Denmark (10°27′E, 55°18′N) on July 20th–21st, 2009. The field was rectangular (40 × 130 m) and divided into 32 plots (12.5 × 10 m) (see Thorup-Kristensen et al., 2012). Twelve evenly distributed soil cores (∅6 cm, depth 10 cm) were taken per plot using a Gardena (Ulm, Germany) onion planter and the 12 cores were pooled creating a bulked sample for each of the 32 plots. The soil of the adjacent hedgerow, which surrounded two thirds of the field, was sampled by collecting soil cores within 21 plots of 2 × 4.5 m evenly distributed along the hedgerow. In each of the 21 hedgerow plots, six single soil cores as above were collected and pooled into a single hedgerow bulked sample. The sampling tools were rinsed thoroughly after collecting the cores of each plot using water, 70% ethanol and again water. The 53 soil samples were stored in black plastic bags, protected from direct sunlight and transferred to the laboratory within 12 h. The samples were homogenized by hand in the plastic bag and sieved (2 mm mesh). The sieved soil samples were stored in sealed plastic bags in the dark at 5 °C for up to three months before further processing.

2.2. Fungus isolation

Entomopathogenic fungi were isolated from mixed soil samples using the insect bait method described by Zimmermann (1986). Plastic cups (155 mL) were filled with 120 mL soil and up to 3 mL tap water was added to obtain sufficient moisture levels of the samples before adding 10 *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae to each cup. Cups were sealed with perforated lids and incubated in the dark in closed boxes at 20 °C. A moist paper towel (34 × 23 cm) was added to each box to maintain humidity. The cups were inverted daily over a period of five weeks and assessed for presence of dead larvae every fifth day. Cadavers were washed three times with demineralized water and remaining water was absorbed with a piece of filter paper. Subsequently, cadavers were incubated at 100% relative humidity in 30 ml medicine cups at 20 °C in darkness and checked for emerging fungi after 5–7 days. Fungal taxa were morphologically identified under a dissection microscope and by light microscopy (400× magnification) according to Humber (2012). Tools used during handling of cadavers were sterilized with 70% ethanol followed by flaming. Unintentional transfer of conidia was checked by imprinting the tools in Sabouraud Dextrose Agar (SDA; Merck, Darmstadt, Germany), and placing SDA plates open for 10 min in the room during handling. No colony forming units (CFUs) of entomopathogenic fungi were observed in any of these tests. Conidia from all sporulating *T. molitor* cadavers were transferred to SDA plates and incubated in the dark at 23 °C.

2.3. Molecular characterization

For the molecular characterization, 108 *Metarhizium* isolates were selected as representatives of the agricultural field by including isolates from each of the 32 plots and a maximum of five isolates per plot. If a plot yielded *Metarhizium* isolates of different colony color or morphology, all separate morphologies were included. Additionally, all *Metarhizium* isolates obtained from the hedgerow ($n = 15$) were included resulting in a total of 123 isolates. Four of the 123 isolates were morphologically identified as *M. flavoviride* based on their bright green colony color and conidia dimensions that are characteristic for this species. In addition, a reference isolate of *M. brunneum* originally isolated in Switzerland (ARSEF 7524) was included in the molecular characterization. Each of the selected isolates were stored in a 1:1 mixture of skimmed milk and 100% glycerol at −70 °C in the culture collection at University of Copenhagen. Conidia from each isolate were inoculated into 15 ml liquid medium (2% peptone, 3% sucrose and 0.2% yeast extract) in sterile Erlenmeyer flasks and incubated at room temperature on a rotary shaker at 170 rpm for 3–4 days. The resulting fungal material was filtered through filter paper (Munktel, Grade 3 W, Grycksbo, Sweden) under suction and lyophilized (HETOSICC CD 53-1, HETO Lab Equipment, Birkerød, Denmark) for 12–16 h.

DNA was extracted from the lyophilized mycelia using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. To determine the different genotypes represented by the *Metarhizium* isolates, 18 SSR markers, i.e., Ma145, Ma325, Ma307, Ma2049, Ma2054, Ma2055, Ma2056, Ma2057, Ma2060, Ma2063, Ma2069, Ma2070, Ma2077, Ma2089, Ma2283, Ma2287, Ma2292, Ma2296 (Oulevey et al., 2009), were PCR amplified from each of the 124 isolates. Maximal resolution of the SSR analysis was tested by applying the 23 remaining published SSR markers (Enkerli et al., 2005; Oulevey et al., 2009) to 29 isolates representing the most frequent genotype (Suppl. Table 4). No further resolution was achieved. PCR amplifications

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