Insect pathogens and entomopathogenic nematodes
IOBC/wprs Bulletin Vol. 66, 2011
pp. 113-115

Molecular diversity of the *Metarhizium anisopliae* lineage in an agricultural field

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Abstract: Entomopathogenic fungal isolates identified by morphology as *Metarhizium anisopliae* may belong to different species when identified by molecular characters. We isolated *Metarhizium* spp. from a Danish agricultural field using *Tenebrio molitor* as bait insect to assess the molecular diversity within the soil of a single field. Isolates were analyzed using DNA sequencing and applying SSR markers. Within the former *M. anisopliae* lineage, we found *M. brunneum* (86.3%), *M. robertsii* (11.3%) and *M. majus* (3.4%) in the soil samples. Several genotypes of each species were identified based on SSR markers. Differences in abundance of the species and their genotypes suggest different adaptations to the soil environment of the agricultural field.

Key words: entomopathogenic fungi, cryptic species, SSR makers, Elongation Factor-1 alpha

Introduction

The fungal genus *Metarhizium* (Hypocreales; Ascomycota) has a worldwide distribution infecting insects of several orders (Zimmermann 2007). Fungal propagules of *Metarhizium* spp. also occur widely in the soil environment. Surveys of soils from different sites suggest that *Metarhizium* spp. are the most abundant fungal entomopathogens in the soil of agricultural fields in temperate regions (Bidochka et al. 1998). Isolates mentioned in the literature are mostly identified as *M. anisopliae* based on morphological characters and little is known about the genetic diversity of these fungi. Recently, Bischoff et al. (2009) redescribed the *M. anisopliae* lineage based on multilocus phylogenetic analyses. The analysis allowed to recognize nine terminal taxa within the *M. anisopliae* species complex, i.e., *M. acridum, M. anisopliae, M. brunneum, M. globosum, M. guizhouense, M. lepidiote, M. majus, M. pingshaene* and *M. robertsii.* Similar conidial morphology can be observed for five of these species, *M. brunneum*, *M. lepidiotae*, *M. pingshaense*, *M. robertsii* and *M. anisopliae*. Thus studies reporting on occurrence of the morphospecies *M. anisopliae* may include any of these newly erected species as well as potentially other species showing very similar or overlapping morphological characters, including *M. guizhouense* and *M. majus* (Bischoff et al. 2009). It is currently unknown which of these newly described species that are included in naturally occurring *Metarhizium* communities appear in the soil environment of agricultural fields.

In this study we wished to uncover the molecular diversity of the *M. anisopliae* complex in the soil of a single agricultural field in Denmark using DNA sequencing and SSR marker analysis. Previous investigations have shown that *M. anisopliae* was the dominating fungal taxon in the soil of this particular agricultural field when fungal identities were based solely on morphological characters (N. V. Meyling unpublished data).

Material and methods

The soil sampling was done in a cropping system in Årslev, Denmark (10°27'E, 55°18'N). A single field (60m x 130m) was divided into 32 plots (12.5m x 10m). One mixed soil sample was taken from each plot by combining 12 evenly distributed soil cores (Ø 6cm, depth 10cm). In the laboratory, the samples were homogenized, sieved (2mm mesh) and stored at 5°C until further processing. Entomopathogenic fungi were isolated using *Tenebrio molitor* as bait insects (modified after Zimmermann (1986); 10 insects in a 155ml cup filled with 120ml soil). Dead larvae were incubated in moist chambers and emerging fungi were isolated and identified morphologically. 116 of the isolates morphologically characterized as *M. anisopliae* were selected for further molecular analysis.

To identify the species of the isolates, the 5'- intron region of Elongation Factor 1-alpha (EF1-α intron region) (Bischoff et al., 2009) was sequenced. PCR amplifications were performed with primers EF2F (5'-GGAGGACAAGACTCACATCAACG-3') and EFjR (5'-TGYTCNCGRGTYTGNCCRTCYTT-3'). PCR products were purified using the GFX PCR DNA and Gel band purification kit (GE Healthcare, UK) and sequenced with the primers mentioned above. Sequencing was performed by MWG (Ebersberg, Germany). The sequences were aligned using BioEdit and compared with reference sequences obtained from GenBank (Bischoff et al. 2009).

To determine the different genotypes within the *Metarhizium* species, 16 SSR markers, i.e., Ma145, Ma325, Ma2049, Ma2054, Ma2056, Ma2057, Ma2060, Ma2063, Ma2069, Ma2070, Ma2077, Ma2089, Ma2283, Ma2287, Ma2292, Ma2296 (Oulevey et al. 2009), were applied to each of the 116 isolates. PCR amplifications were performed as described by Oulevey et al. (2009). PCR product sizes (SSR allele sizes) were determined on an ABI Prism 3130xl genetic analyzer equipped with 36-cm capillaries and POP-7 polymer (Applied Biosystems, Foster City, CA). Fragment sizes were determined using GenMarker v1.51 (SoftGenetics LLC, State College, PA) software and GeneScan ROX400 (Applied Biosystems) as internal size standard.

Results and discussion

Three *Metarhizium* species as defined by Bischoff et al. (2009) were identified among the 116 isolates obtained from the agricultural field: *M. brunneum*, *M. robertsii* and *M. majus* (Tab. 1). This is the first time that these species are documented from a single agricultural field. Until recently, all isolates would have been identified (based on morphology) as *M. anisopliae*.

Within the field, *M. brunneum* was most abundant (86.3%), followed by *M. robertsii* (11.3%) and *M. majus* (3.4%). Application of the 16 SSR markers allowed to further discriminate 5 genotypes (strains) for *M. brunneum* and *M. robertsii* each and 2 genotypes for *M. majus*. The 16 SSR loci were successfully amplified from all the three species except for Ma325 and Ma2060, which could not be amplified from *M. majus*. The genotype "H" from *M. brunneum* was by far the most abundant genotype in the investigated field. All other genotypes had much lower occurrence often represented by only a single isolate. These findings suggest that *M. brunneum* and especially strain "H" seems to be favored by some factors (biotic and/or abiotic) occurring in the investigated agricultural field.

In a next step the following questions will be addressed: Which characteristics make *M. brunneum* and specifically genotype "H" so successful in the soil environment? Is the

observed pattern just a local phenomenon or can the dominance of *M. brunneum* be found in a larger area of agricultural fields?

Table 1. *Metarhizium* species (n=116), frequency and number of genotypes identified from the soil of a single agricultural field.

***Metarhizium* species**	**Frequency**	**Genotypes (n)**
M. brunneum	86.3%	H (93) J (3) G (1) K (1) L (1)
M. robertsii	11.3%	F (7) C (2) M (2) D (1) E (1)
M. majus	3.4%	A (2) B (2)

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