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Detection and quantification of *Entomophaga maimaiga* resting spores in forest soil using real-time PCR

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ABSTRACT

Environmental sampling to monitor entomopathogen titre in forest soil, a known reservoir of insect pathogens such as fungi and viruses, is important in the evaluation of conditions that could trigger epizootics and in the development of strategies for insect pest management. Molecular or PCR-based analysis of environmental samples provides a sensitive method for strain- or species-based detection, and real-time PCR, in particular, allows quantification of the organism of interest. In this study we developed a DNA extraction method and a real-time PCR assay for detection and quantification of *Entomophaga maimaiga* (*Zygomycetes*: *Entomophthorales*), a fungal pathogen of the gypsy moth, in the organic layer of forest soil. DNA from fungal resting spores (azygospores) in soil was extracted using a detergent and bead mill homogenization treatment followed by purification of the crude DNA extract using Sephadex–polyvinylpolypyrrolidone microcolumns. The purification step eliminated most of the environmental contaminants commonly co-extracted with genomic DNA from soil samples but detection assays still required the addition of bovine serum albumin to relieve PCR inhibition. The real-time PCR assay used primers and probe based on sequence analysis of the nuclear ribosomal ITS region of several *E. maimaiga* and two *E. aulicae* strains. Comparison of threshold cycle values from different soil samples spiked with *E. maimaiga* DNA showed that soil background DNA and remaining co-extracted contaminants are critical factors determining detection sensitivity. Based on our results from comparisons of resting spore titres among different forest soils, estimates were best for organic soils with comparatively high densities of resting spores.

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Introduction

Entomopathogenic fungi can have a significant impact on arthropod population densities, both through continuously present low levels of infection or through development of epizootics. These fungi persist through time in reservoirs in the soil. Thus, studying entomopathogenic fungi in the soil is important to understanding the epizootiology of diseases caused by these pathogens. Some hypocrealean fungi infecting arthropods are culturable and selective media are available for their extraction and quantification from soil (Hajek *et al.* in press). However, an important fungal group, the order *Entomophthorales*, is more fastidious and cannot be cultured from soil. An alternative method of detection uses standard, or end-point, PCR with species- or strain-specific markers, as has been developed for soil-borne stages of a number of anamorphic entomopathogens in the *Hypocreales* (e.g., Castrillo *et al.* 2003; Entz *et al.* 2005). Although these methods can be

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sensitive, they do not adequately quantify fungi in soil samples. Regardless, such methods have not been developed for species in the order *Entomophthorales*.

The fungal pathogen *Entomophaga maimaiga* (*Zygomycetes*: *Entomophthorales*) has been of particular interest based on the recurrence of epizootics that have maintained populations of the gypsy moth (*Lymantria dispar*), a forest defoliator capable of devastating outbreaks, under control in many areas of the USA. This host-specific fungal pathogen is native to Asia (Nielsen *et al.* 2005) and was first found in the USA in 1989 (Andreadis & Weseloh 1990; Hajek *et al.* 1990b), after which time it spread across the contiguous distribution of the gypsy moth in northeastern USA. *E. maimaiga* infects gypsy moth larvae during the approximately two-month period they are present in spring (Hajek 1999). The fungus actively ejects conidia from infected larval cadavers, but once late instar larvae are present, the fungus usually produces thick-walled azygospores (resting spores) within cadavers that are left hanging on tree trunks. These cadavers fall from tree trunks, and resting spores are leached into the organic layer of soil.

At present, one method has been developed for quantifying *E. maimaiga* resting spores in soil based on visual counts after wet sieving followed by density gradient centrifugation (Hajek & Wheeler 1994). However, this method is very time-consuming. Alternative methods that are less time-consuming rely on visual counts of resting spores, but these are also less accurate (Weseloh & Andreadis 2002). Most species of *Entomophthorales* produce resting spores, which have few distinctive morphological features to allow discrimination among different species.

Real-time PCR, which allows quantification of starting nucleic acid of target organisms in a reaction using fluorescent detection techniques, has been developed for detection and quantification of several species of fungi in soil (Lees *et al.* 2002; Atkins *et al.* 2003; Kabir *et al.* 2003). This method offers greater sensitivity and precision than conventional end-point PCR and yields accurate quantification of target organisms.

The objectives of the present study were to develop efficient DNA extraction and purification methods and a real-time PCR-based assay for detection and quantification of *E. maimaiga* resting spores in forest soil. We quickly learned that the organic-layer soil, in which most resting spores are found, can interfere with DNA extraction (Tsai & Olson 1992; Tebbe & Vahjen 1993) so we evaluated the effects of soil type on DNA extraction. We also assessed the effects of soil background DNA on the efficacy of the real-time PCR detection assay for the target fungus.

Materials and methods

Fungal strains and resting spore production

The *Entomophaga maimaiga* and *E. aulicae* strains used in this study are listed in Table 1. All strains are stored and maintained at the USDA, ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY). DNA samples for primer development and PCR specificity assays were isolated from liquid cultures using the DNeasy Tissue Kit (Qiagen, Valencia, CA) (Nielsen *et al.* 2005). Resting spores of *E. maimaiga* strain 7123 were obtained from laboratory-infected gypsy moth larvae following the protocols reported by Hajek *et al.* (1990a).

Soil samples

Forest soil samples were collected from four sites (Table 2) with no known history of *Entomophaga maimaiga* according to the method reported by Hajek *et al.* (1998). Briefly, soil was collected from around the bases of three large red (*Quercus rubra*) or white oak (*Q. alba*) trees per sampling site. Samples were taken from the soil surface, no more than 3 cm deep and 10 cm away from the trunk of each tree, where *E. maimaiga* resting spores are most likely to be found. Three samples were collected per tree, pooled together, and stored at 4 °C in a plastic bag until use. A subsample (*ca* 100–230 g)

Table 1 – List of *Entomophaga maimaiga* and *E. aulicae* strains used in this study

Species/strain (ARSEF No.)	Insect host	Collection site	Year
Entomophaga maimaiga			
1400	*Lymantria dispar* (*Lepidoptera*: *Lymantriidae*)	Ishikawa, Japan	1984
3828	"	New York, USA	1996
5384	"	Maryland, USA	1996
5568	"	Virginia, USA	1997
6053	"	Michigan, USA	1998
6162	"	Chiba, Japan	1998
7104	"	Iwate, Japan	2001
7123	"	Massachusetts, USA	2003
7124	"	Massachusetts, USA	2003
7127	"	Khabarowsk, Russia	1999
7139	"	Heilongjiang, China	2002
7353	"	Pennsylvania, USA	2003
E. aulicae			
2898	*Choristoneura fumiferana* (*Lepidoptera*: *Tortricidae*)	Newfoundland, Canada	1978
3039	*Heterocampa guttivitta* (*Lepidoptera*: *Notodontidae*)	New York, USA	1990
7142	*Euproctis chrysorrhoea* (*Lepidoptera*: *Lymantriidae*)	Maine, USA	2003

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