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

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ARTICLE INFO

Article history:
Received 28 September 2006
Received in revised form
1 December 2006
Accepted 7 January 2007
Published online 26 January 2007
Corresponding Editor:
Richard A. Humber

Keywords:
Azygospores
DNA extraction
Entomophthoraceae
Entomophthorales
Forest soil
Zygomycetes
Zygomycota

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.

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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0953-7562/$ – see front matter
doi:10.1016/j.mycres.2007.01.010

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

Table 2 – Sampling locations and properties of the soils used in this study

Sampling location	Texture	Composition (%)			Moisture (%)	Organic matter (%)	pH
		Sand	Silt	Clay			
Site 1 (Potomac Road), Finger Lakes National Forest, Hector, NY (FLNF1)	Silty loam	17	68	15	5.2	77.9	4.6
Site 2 (Ravine Loop Trail) Finger Lakes National Forest, Hector, NY (FLNF2)	Silty loam	20	56	24	2.6	15.8	5.6
McGowan Woods, Ithaca, NY (MG)	Silty loam	29	63	8	3	22.7	4.5
Plot 22N, Michigan (MI)	Sandy loam	85	12	3	1	8.4	4.2

from each site was submitted to the Cornell Nutrient Analysis Laboratory to determine soil texture, organic matter, moisture and pH (Table 2).

DNA extraction from resting spores in soil

The initial step was to break the thick-walled resting spores (21.5–40 μm in diameter with 0.5–4 μm thick cell wall) (Soper *et al.* 1988). Preliminary studies were conducted using beads of different types (glass, zirconia/silica, and zirconia; BioSpec Products Bartlesville, OK) and sizes (0.1, 0.5, 1, 2.4 and 2.5 mm) at different bead:soil ratios and at different homogenization times (10, 20, 30, 60, and 120 s) at 5000 rev min^{-1} to achieve over 95 % lysis of resting spores in soil for DNA extraction. Lysis of resting spores was evaluated by microscopic examination.

Trials were also conducted to compare use of the PowerSoil DNA kit (MoBio Laboratories, Carlsbad, CA) *versus* the method developed by Kuske *et al.* (1998) for extraction of genomic DNA from soil. The latter was found to yield at least ten times more DNA than the former and was adapted, with modifications, for this study. Genomic DNA from soil was extracted using a detergent and bead mill homogenization treatment followed by purification through Sephadex–polyvinylpolypyrrolidone (PVPP) microcolumns to remove co-extracted contaminants (Kuske *et al.* 1998), in particular humic acids, which are common contaminants of DNA extracted from soils. Purification microcolumns were prepared as follows: TE buffer (pH 7.6) was added to hydrated Sephadex G50 fine resin (Sigma-Aldrich, St Louis, MO) (9 ml deionized water to 1 g resin incubated at 4 °C overnight, plus another 2 ml water added the next day) at a resin:buffer ratio (v/v) of 2:1. Granular PVPP (20 mg/ml; Sigma-Aldrich, St Louis, MO) was added to the resin–buffer suspension, vortexed, and the mixture allowed to equilibrate for several hours. Five-hundred microlitres of the suspension was pipetted into each microcolumn (Ultrafree-MC centrifugal filters, Millipore, Billerica, MA), and the columns were centrifuged in a swinging rotor at 500 ×*g* for 5 min, rotated 180°, and spun another 10 min at 750 ×*g* at room temperature. This procedure resulted in an even packing to 400 μl of each microcolumn. Microcolumns were prepared daily before use, but could also be stored before use at 4 °C in small batches in plastic bags to prevent drying.

The developed protocol is as follows, 0.5 g soil samples were transferred to 2 ml bead-beater tubes with 3 g of 2.4 mm zirconia beads and 1 ml 2× TENS buffer [100 mM Tris–HCl (ph 8), 40 mM EDTA, 200 mM NaCl, 2 % (w/v) sodium dodecyl sulphate] was added to each tube and the mixture was briefly vortexed. Samples were homogenized for 1 min at 5000 rev min^{-1} in a Mini Bead Beater (BioSpec Products) to disrupt soil material and break fungal resting spores without shearing genomic DNA. Tubes were then centrifuged at 12,000 ×*g* for 10 min at room temperature and ~800 μl of the supernatant collected. The soil–bead pellet was washed once with 1 ml of 2× TENS buffer and the second supernatant was pooled with the first. Nucleic acids were precipitated by adding 1/10 volume of 3 M sodium acetate and 0.54 volume of room temperature isopropanol. Precipitates were washed with 70 % ethanol, air-dried, resuspended in 100–200 μl TE (pH 8). Fifty microlitre aliquots (maximum volume per microcolumn) of the suspension were pipetted onto Sephadex–PVPP microcolumns, spun in a fixed angle rotor at 750 ×*g* for 10 min, and eluates from the same extraction sample pooled together. DNA was precipitated as above, resuspended in 100 μl TE (pH 8), and analysed using a spectrophotometer (readings at 230, 260 and 280 nm) (BioPhotometer, Eppendorf, Westbury, NY) and by gel electrophoresis. All DNA samples were stored at −20 °C until use.

Soil seeding experiments

To determine efficacy of the DNA extraction method and sensitivity of the real-time PCR assays on different forest soils, subsamples from each sampling site were seeded with suspensions of *Entomophaga maimaiga* 7123 resting spores in deionized water. Five gram soil samples were seeded with different concentrations of resting spores delivered in volumes of 100 μl g^{-1} of soil to obtain titres of 4×10^1, 2×10^2, 1×10^3, 5×10^3, and 2.5×10^4 resting spores g^{-1} of soil, representative of the range of resting spore titres detected in the field (Hajek *et al.* 2004a, 2004b). Seeded soil was mixed using a sterile spatula and three subsamples of 0.5 g were obtained for separate DNA extractions. DNA samples were pooled together for each soil sample-resting spore titre combination and stored at −20 °C until use. Genomic DNA was also extracted from unseeded soil samples to determine amount of background DNA present. Genomic DNA from pure resting spores of different concentrations, as used for soil seeding, was extracted using the same method used for seeded soils.

Primers and TaqMan probe

Real-time PCR primers and probe were designed using the Primer Express software, version 2 (Applied Biosystems, Foster, CA), based on the nuclear ribosomal ITS sequence of

Entomophaga maimaiga strains 1400, 3828, 5568, 6053, 6162, 7127, and 7139 (GenBank accession numbers DQ534745, DQ534747, DQ534748, DQ534749, DQ534750, DQ534751, and DQ534752, respectively) and *E. aulicae* strains 3039 and 7142 (GenBank accession numbers DQ534746 and DQ534753, respectively). *E. maimaiga* is a member of the *E. aulicae* species complex (Soper *et al.* 1988), the members of which infect forest lepidopterans (Walsh 1996), and thus, other species of this closely related group could co-occur with *E. maimaiga* resting spores in forest soils. Therefore, numerous isolates of *E. maimaiga* from Asia and North America, as well as three isolates of *E. aulicae* from forest lepidopterans were included in primer selection. Primers EmTqF1 (5′ CTCTTTGTTTATTCTTTGCTATTGATTGAG) and EmTqR1 (5′ GCACAAAAAGTACCTCCACTGATG) amplified a 91 bp region of the ITS region. The probe EmTqP1 (5′ TTAAATT TGATGGATTTAGGTCTGGCGTAAAGTGA) was labelled at the 5′-end with fluorescent FAM reporter dye and the 3′-end was labelled with TAMRA quencher dye (Applied Biosystems).

Specificity of the primer pair was tested against most of the sequenced fungal strains, plus *E. maimaiga* strains 5384, 7104, 7124, and 7353 and *E. aulicae* strain 2898. The *E. maimaiga* strains tested reflected the genotypic diversity of this species in the USA, along with representatives from Japan and Russia (Nielsen *et al.* 2005). Specificity was tested using standard end-point PCR assays. PCR reaction mixtures (25 μl volume) contained 1× PCR buffer with 1.5 mM $MgCl_2$; 200 μM each of dATP, dCTP, dGTP, and dTTP; 0.8 μM of each primer; 50 ng fungal DNA; and 2.5 units *Taq* PCR enzyme (Qiagen). PCR amplification was performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA) programmed for initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Reaction tubes were held at 4 °C before visualization of the PCR products in a 1 % (w/v) agarose gel stained with ethidium bromide. Assays were repeated at least twice for each strain. Additional assays were conducted using a lower primer concentration of 0.5 μM and a gradient cycle from 55 to 65 °C to eliminate non-specific bands generated in *E. aulicae* 7142.

PCR inhibition by soil components (i.e., humic acid) was tested by standard PCR assays as reported above (with 0.5 μM primer concentration and 55 °C annealing temperature) using genomic DNA extracted from McGowan Woods and Michigan soils seeded with *E. maimaiga* 7123 resting spores. Assays were conducted using undiluted and diluted (1:10 with pH 8 TE buffer) genomic DNA from soil, with bovine serum albumin (BSA; New England Biolabs, Beverly, MA) at concentrations of 0, 0.4, 0.6, 0.8 and 1 μg μl^{-1} final concentration added to relieve inhibition (Kreader 1996). Experiments were repeated at least twice, and results were visualized in 1 % agarose gels stained with ethidium bromide and examined under UV light.

Real-time PCR

Optimal primer and probe concentrations for real-time PCR were determined empirically. Combinations of different primer concentrations (forward and reverse primers at 0.05, 0.1, 0.2, 0.4, 0.5 and 0.8 μM) and probe concentrations (0.125, 0.25 and 0.5 μ M) were tested to obtain the lowest threshold cycle (Ct) values at 90 to 110 % assay efficiency, which can be directly correlated to the starting concentration of the sample target DNA. Optimization trials included a standard curve of pure *Entomophaga maimaiga* 7123 DNA from resting spores, comprised of five series of ten-fold dilutions; genomic extract from unseeded soil from the Finger Lakes National Forest (FLNF) site 1 spiked with DNA used for the standard curve (from 0.1 ng to 1 pg); and a no-template control.

Real-time PCR assays were conducted using the iCycler iQ Real-time PCR detection system (BioRad Laboratories, Foster, CA). Each reaction mixture (25 μl final volume) contained 1× TaqMan Universal Primer Mastermix (Applied Biosystems, Branchburg, NJ), 0.5 μM each primer, 0.125 μM probe, 0.8 μg μl^{-1} BSA (final concentration), and 2 μl of each of the appropriately diluted sample DNA (*E. maimaiga* standard and soil genomic DNA). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and single-step annealing and extension at 60 °C for 1 min. Each PCR assay included a standard curve (ten-fold dilutions from 100 or 10 ng to 1 pg) for determining starting DNA concentration of unknowns and a no-template control in addition to genomic DNA from soil samples (1:10 dilution). Assays were performed with duplicates of each sample. At least three independent PCR assays were conducted for each experiment.

Detection of different strains

Sensitivity of the real-time PCR assay of diverse strains of *Entomophaga maimaiga* present in the northeast USA was determined in assays against strains 3828, 5384, 6053, 7124 and 7353 at 10 ng DNA. The standard curve was composed of *E. maimaiga* 7123 DNA (ten-fold dilutions from 100 ng to 1 pg).

Effect of soil background DNA on sensitivity of real-time PCR

The effect of soil background DNA on the detection limit of *Entomophaga maimaiga* resting spores in the soil was tested by spiking 1:10 dilutions of genomic DNA from unseeded subsamples from each sampling site with various concentrations of *E. maimaiga* 7123 pure DNA (0.1 pg to 1 ng). This method was used to reduce the effect of possible interactions between soil sample and the DNA extraction process, which could affect the concentration of resting spore DNA in background soil DNA. Real-time PCR assays also included genomic soil DNA with 10 ng *E. aulicae* 7142 DNA added.

Detection of resting spore titres in different soil samples

Comparative sensitivity of real-time PCR on various *Entomophaga maimaiga* titres in different forest soils was evaluated in assays of genomic DNA from seeded soil samples. Detectable *E. maimaiga* DNA concentrations, based on Ct values obtained for each soil sample-resting spore titre mix, were used to calculate predicted resting spore number using regression analysis. Correlation of pure resting spore number and DNA concentration was calculated based on quantity of DNA extracted from resting spore suspensions of known concentrations (4×10^1, 2×10^2, 1×10^3, 5×10^3, and 2.5×10^4 resting

spores), as were used for the soil seeding study. Predicted resting spore numbers were then compared with the actual spore titre in each soil sample–resting spore mix.

Data analysis

Analysis of variance and Tukey *post hoc* tests of real-time PCR data and regression analysis of *Entomophaga maimaiga* DNA extracted from known resting spore concentrations were conducted using StatView (SAS Institute Inc, Cary, NC).

Results

DNA extraction from soil

A mix of 3 g of 2.4 mm zirconia beads and 0.5 g soil in 2 ml bead-beater tubes homogenized at 5000 rev min^{-1} for 1 min was found to be optimal, by producing ~100 % lysis of resting spores (by microscopic inspection) in the maximum possible soil weight of 0.5 g without shearing genomic DNA. A longer homogenization time of 2 min was not necessary, even though it still did not result in shearing, as DNA yields were found to be comparable at both processing times (data not shown).

Crude DNA extracts from the different sampling sites contained co-extracted contaminants, visible as brown colouration. The darkest extracts came from Michigan soil, while FLNF site 2 samples had the lightest colour. Purification though microcolumns with Sephadex and PVPP resins resulted in the visible loss of most of the brown colouration in the genomic DNA. However, spectrophotometer readings at 230 and 260 nm, for determining the level of humic acid contamination, showed that this contaminant was still present. The highest level of contamination was observed in the Michigan samples with an A260/A230 of 1.16. A A260:A230 ratio below 2 is indicative of humic acid contamination, and the Michigan samples had the lowest ratio observed. Absorbance ratios from the other samples were near 2, indicating cleaner DNA extracts. DNA yield from the different unseeded forest soils varied. Mean background genomic DNA from McGowan Woods, Michigan Plot 22N, FLNF sites 1 and 2 were 23.4, 35.2, 15.3 and 14.2 $\mu g\, g^{-1}$ of soil, respectively.

Primer specificity

End-point or standard PCR assays to test primers EmTqF1 and EmTqR1 showed that similar sized PCR products of ~91 bp were generated from the different *Entomophaga maimaiga* strains tested (data not shown). No PCR product was generated from *E. aulicae* strains 2898 or 3039 and two non-specific amplicons, ~3000 and <90 bp, were generated from *E. aulicae* strain 7142 (data not shown). Specificity was improved when primer concentration was reduced to 0.5 μM and annealing temperature was raised above 61 °C; these changes resulted in the loss of non-specific products. No changes were made on the primer design as the real-time PCR assay included a probe based on *E. maimaiga* ITS sequence, which would further improve assay specificity, and had an annealing temperature of 60 °C.

Effect of BSA on PCR inhibition

PCR assays on genomic DNA from seeded soils showed that remaining humic acid contaminants after purification through Sephadex–PVPP microcolumns were sufficient to inhibit amplification. The addition of BSA relieved inhibition in assays with diluted (1:10) genomic DNA but not with undiluted samples for the different soils tested. Levels of co-extracted contaminants were still enough in the undiluted samples to inhibit reactions even in the presence of BSA. Among the different BSA concentrations tested, 0.8 $\mu g\, \mu l^{-1}$ final concentration was found optimal, resulting in a distinct PCR product from each of the seeded soil tested (Fig 1). At a concentration of 1 $\mu g\, \mu l^{-1}$, BSA itself was found to be inhibitory to amplification and resulted in the loss of PCR products.

Detection of different strains

Real-time PCR assays of *Entomophaga maimaiga* strains 3828, 5384, 6053, 7104, 7124 and 7353 at 10 ng DNA per reaction

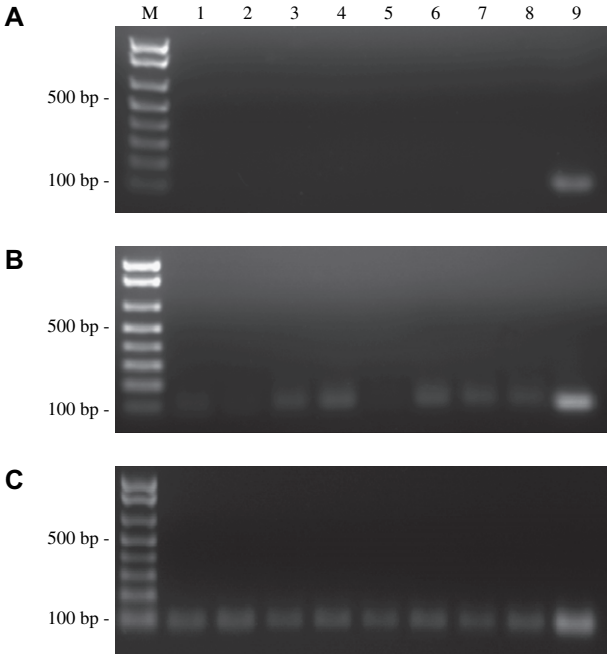

Fig 1 – The effect of bovine serum albumin (BSA) concentration on the amplification of *Entomophaga maimaiga* extracted directly from soil samples. BSA at 0.2–1 mg ml^{-1} final concentration was tested to relieve PCR inhibition from contaminants co-extracted with *E. maimaiga* DNA from soil. No amplification products were generated in the absence of BSA (A). A concentration of 0.8 mg/ml BSA (C) was found optimal, generating consistent results and distinct PCR products compared with 0.4 mg ml^{-1} (B). Lanes 1–4 and 5–8 represent soil samples from McGowan woods and Michigan, respectively, seeded with *E. maimaiga* resting spores. Lane 9 represents a positive control of 20 ng pure *E. maimaiga* DNA. The marker is a 1 kb Plus DNA ladder (Invitrogen, Valencia, CA).

resulted in Ct values ranging from 20.81 ± 1.58 to 22.52 ± 1.56. Mean Ct value of standard strain 7123 was 21.58 ± 1.56 at the same concentration. No significant differences were observed among the different strains ($F = 0.15$, $df = 5$, $P = 0.97$), thus indicating that the assay is robust to genetic differences between strains of this fungus.

Effect of soil background DNA on sensitivity of real-time PCR

Analysis of Ct values of different concentrations of *Entomophaga maimaiga* 7123 DNA added to genomic DNA from unseeded soils showed that sensitivity of real-time PCR assays was affected by interactions between soil sample and *E. maimaiga* DNA concentration ($F = 529.16$, $df = 12$, $P = <0.001$). Although no significant differences were observed among the Ct values for DNA from the four soil samples at high concentrations of 1000, 100 or 10 pg *E. maimaiga* DNA per reaction, significant differences were observed among Ct values at low concentrations of 1 and 0.1 pg *E. maimaiga* DNA (Fig 2). At 1 pg *E. maimaiga* DNA, the Ct value for the Michigan soil sample was the highest and the Ct for FLNF site 1 was the lowest, while FLNF site 2 and McGowan Woods had comparable results (Tukey test, $P < 0.001$). At 0.1 pg *E. maimaiga* DNA, the fungus was no longer detectable in Michigan soil. Among the three other soil samples, each had a significantly different Ct value at 0.1 pg, with the highest for FLNF site 2 and the lowest for FLNF site 1 (Tukey test, $P < 0.001$).

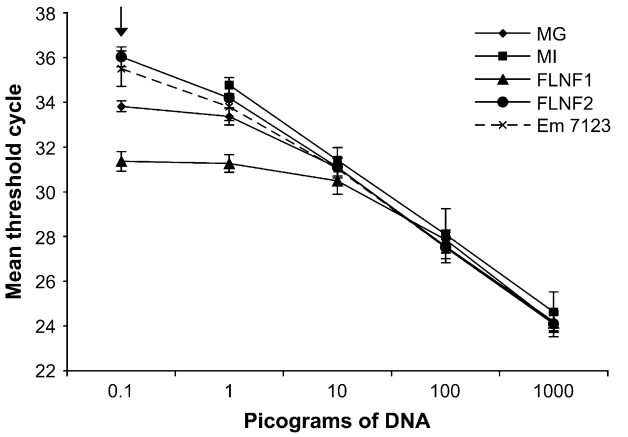

Fig 2 – The effect of the soil sample on the detection sensitivity of the real-time PCR assay. Genomic DNA from unseeded soil samples was spiked with pure *E. maimaiga* 7123 DNA, from 1 ng to 0.1 pg, to test the effect of the soil sample on the threshold of detection. The results showed significant interactions between soil samples and DNA concentrations ($P < 0.001$, Tukey test). The sensitivity of the assay was lowest in soil samples from Michigan (MI) at 1 pg *E. maimaiga* DNA, and at 0.1 pg (↓) of DNA, the fungus was not detected in the same soil sample. At higher *E. maimaiga* DNA concentrations, Ct values among the four soil samples, McGowan Woods (MG), Finger Lakes National Forest sites 1 and 2 (FLNF1 and FLNF2, respectively), and MI, were comparable. A five series 10-fold dilution of pure *E. maimaiga* 7123 DNA (Em 7123) was used as the standard. Three independent assays were conducted, with duplicates of each sample per assay.

Detection of various resting spore titres

Entomophaga maimaiga DNA was not detected in any of the unseeded soil samples. Among the seeded samples detectable *E. maimaga* DNA in 0.5 g of soils with titres of 20–12,500 resting spores ranged from 0.01–1.89, 0.13–2.87, 0.35–3.75 and 0.02–3.77 ng for Michigan, McGowan, FLNF site 1, and FNLF site 2, respectively (Table 3). Analysis of data on detectable *E. maimaiga* DNA in various soil sample–resting spore titre mixes showed no significant interaction between soil sample and resting spore titre ($F = 1.49$, $df = 12$, $P = 0.17$) but significant differences among soil samples ($F = 10.11$, $df = 3,4$, $P < 0.0001$). At either 4×10^1 or 2×10^2 resting spores g^{-1} forest soil, FLNF site 2 sample has significantly higher detectable *E. maimaiga* DNA than the other three soil samples. At higher concentrations of 1×10^3, 5×10^3 and 2.5×10^4 resting spores g^{-1} soil, no significant differences were observed among the four soil samples.

Table 3 – Prediction of resting spore titre in seeded soil samples based on real-time PCR data

Resting spore g^{-1} soil	Detectable *Entomophaga maimaiga* DNA (ng)[a] in different soil samples (Predicted resting spore number g^{-1} soil)[b]			
	McGowan Woods, NY	Plot 22N, MI	Finger Lakes National Forest site1	Finger Lakes National Forest site 2
25,000	5.74 ± 1.08a (5603.28 ± 89.26)	3.78 ± 0.56a (982.4 ± 56.24)	7.50 ± 1.24a (26757 ± 102.88)	7.54 ± 0.94a (27725.14 ± 78.82)
5000	2.46 ± 0.89a (304.12 ± 75.66)	1.14 ± 0.28a (94.14 ± 43.86)	2.70 ± 0.33a (376.38 ± 45.68)	2.38 ± 0.28a (288 ± 43.86)
1000	0.71 ± 0.16a (64.02 ± 39.42)	0.54 ± 0.3a (55.24 ± 44.64)	2.34 ± 0.44a (273.36 ± 50.56)	2 ± 0.1a (195 ± 37.88)
200	0.33 ± 0.08a (45.68 ± 36.72)	0.04 ± 0.02a (35.44 ± 34.82)	2.20 ± 0.82b (241.40 + 71.36)	1.92 ± 0.96b (188.24 ± 80.3)
40	0.26 ± 0.06a (43.24 ± 36.06)	0.02 ± 0.01a (34.8 ± 34.4)	0.7 ± 0.16ab (63.68 ± 39.42)	0.06 ± 0.01a (18.03 ± 17.22)

a Detectable DNA concentrations were based on Ct values from real-time PCR assays. The standard used was a five series ten-fold dilution of *E. maimaiga* DNA. Values with the same letters within a row are not significant ($P = 0.05$, Tukey test).

b Predicted resting spore number were calculated using regression analysis using the equation: resting spore number = ê(2.839 + 1.777* DNA concentration in micrograms) derived from correlation of *E. maimaiga* DNA concentration with resting spore concentration (five-fold series from 2.5×10^4 to 4×10^1 resting spores).

Detectable *E. maimaiga* DNA data were converted to predicted resting spore titre in various soil mixes using the equation: resting spore number = ê(2.839 + 1.777* DNA concentration in μg), based on regression analysis of pure *E. maimaiga* DNA extracted from various concentrations of resting spores. Comparison of predicted resting spore numbers showed that, although there is no significant difference among detectable DNA at the three highest titres, soil samples from FLNF sites 1 and 2 gave predictions closer to actual spore titre of 2.5×10^4 spores g^{-1} soil (Table 3). At titres of 1×10^3 and 5×10^3 spores g^{-1} soil, the four soil samples had predicted values that are only 5.5–27.3 % and 2–7.5 % of actual counts, respectively. At the lower titre of 2×10^2 spores g^{-1} soil, FLNF sites 1 and 2 soils gave values closer to the actual spore number than the McGowan Woods and Michigan samples.

Discussion

Environmental sampling to monitor entomopathogen titres in forest soil, a known reservoir of insect pathogens such as fungi and viruses (Hajek 1999; Thompson *et al.* 1981), 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, also allows quantification of the organism of interest. However, PCR-based sampling of microorganisms in the soil is dependent upon the efficient lysis of these organisms and the removal of contaminants co-extracted with microbial DNA. Humic acids, in particular, are known to inhibit *Taq* polymerase in PCR and to interfere with detection assays (Steffan *et al.* 1988; Tebbe & Vahjen 1993; Tsai & Olson 1992). In this study, we achieved effective lysis of *E. maimaiga* resting spores in various types of forest soil using detergent and bead mill homogenization and adequate purification using Sephadex–PVPP spin columns for PCR amplification. As resting spores are often found in the upper few centimetres of the soil surface, where most of the organic matter is found, it is critical that efficient spore lysis is combined with a purification method that does not result in significant loss of DNA yield. Soil genomic DNA yields obtained in this study were within the range of values obtained by other researchers using bead mill homogenization and similar purification methods (Kuske *et al.* 1998; Yeates *et al.* 1998; Miller *et al.* 1999). Background DNA yield obtained for McGowan Woods was even comparable with a yield of 26.4 μg DNA g^{-1} soil obtained by Miller *et al.* (1999) from the same forest using an SDS–bead mill extraction protocol.

Comparisons among unseeded samples spiked with *E. maimaiga* DNA showed that background DNA and, likely, remaining humic acid contaminants affect the threshold limit of detection of *E. maimaiga* at 1 pg DNA and below. Michigan soil, which had the highest amount of background DNA, 35 μg g^{-1} soil, and highest level of remaining co-extracted contaminants, had the highest Ct value and thus the lowest detectable DNA concentration at 1 pg of *E. maimaiga* DNA. At 0.1 pg *E. maimaiga* DNA, the fungus was not detected, thus indicating poor detection sensitivity of real-time PCR assay on the Michigan samples with low amounts of fungal DNA. In contrast, FLNF site 1, which had only 15.3 μg background DNA g^{-1} soil and had cleaner extracted DNA, had the lowest Ct values at the same two *E. maimaiga* DNA concentrations, indicating higher sensitivity of detection in this soil. These results concur with other studies showing that PCR detection of target organisms from environmental samples is dependent on the amount of background DNA and remaining co-extracted contaminants that can affect assay sensitivity (Picard *et al.* 1992; Kuske *et al.* 1998). However, the two lowest *E. maimaiga* DNA concentrations tested were well below the range of detectable *E. maimaiga* DNA extracted from 40–25,000 resting spores g^{-1} soil, which is representative of spore titres detected in the field. It is also worth noting that the soil at FLNF site 1 sample is more typical of the soil type found to harbour *E. maimaiga* resting spores (Hajek *et al.* 1998), indicating that our protocols are adequate for field studies of this fungus.

The effect of background DNA and co-extracted contaminants on the variability of detection sensitivity is probably compounded by the variability of fungal DNA extraction from small subsamples, as is evident with the large standard errors in predicted resting spore numbers. Even with attempts to mix soil samples thoroughly, it is difficult to obtain subsamples of equal consistency because of plant debris or organic matter present in the upper soil layer. This problem is more evident in soil samples with low resting spore titres. Although small subsamples allow rapid analysis of multiple sites, the small sample size may provide inaccurate data except for soil with high titres of the fungus. As the reliability of spore titre predictions from molecular detection assays is dependent upon the representative field sample, variability in detection due to sampling is also critical when comparing data among sampling sites and even over time for the same site. However, these sampling problems may be remedied by increasing the number of subsamples per site and by increasing the number of real-time PCR assays with a single extract (Dionisi *et al.* 2003).

In summary, we developed a DNA extraction method and a real-time PCR assay for the detection and quantification of *E. maimaiga* in forest soil. We showed that soil type and sample size are critical in achieving sensitive and reliable real-time PCR detection and quantification assays of *E. maimaiga* resting spores in forest soil. Based on our results from comparisons of resting spore titres among different forest soils, estimates were best for organic soils with higher densities of resting spores. Although soil factors may be difficult to eliminate and to control, knowledge of their effect in predicting resting spore titres is critical in interpreting data, especially when sampling from different forest sites.

Acknowledgements

We are indebted to Charlotte Nielsen (Department of Entomology, Cornell University, Ithaca, NY) for providing DNA samples of most of the *Entomophaga maimaiga* and *E. aulicae* strains used for primer development and specificity assays, to Joshua Hannam and Victoria Miranda (Department of Entomology, Cornell University, Ithaca, NY) for technical assistance, to Janice Thies and Chris Jones for advice and help with DNA extraction from soil samples and to Mary Burrows (USDA ARS, US Plant Soil & Nutrition Laboratory Ithaca, NY)

for advice on real-time PCR. We are also very grateful to John D. Vandenberg, Stewart Gray, and Donna M. Gibson (USDA ARS, US Plant Soil & Nutrition Laboratory, Ithaca, NY) for use of their laboratory equipment while conducting this study.

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