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Isolation of Entomopathogenic Fungi From Soils and *Ixodes scapularis* (Acari: Ixodidae) Ticks: Prevalence and Methods

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Abstract

Entomopathogenic fungi are commonly found in forested soils that provide tick habitat, and many species are pathogenic to *Ixodes scapularis* Say, the blacklegged tick. As a first step to developing effective biocontrol strategies, the objective of this study was to determine the best methods to isolate entomopathogenic fungal species from field-collected samples of soils and ticks from an Eastern deciduous forest where *I. scapularis* is common. Several methods were assessed: (1) soils, leaf litter, and ticks were plated on two types of media; (2) soils were assayed for entomopathogenic fungi using the *Galleria* bait method; (3) DNA from internal transcribed spacer (ITS) regions of the nuclear ribosomal repeat was extracted from pure cultures obtained from soils, *Galleria*, and ticks and was amplified and sequenced; and (4) DNA was extracted directly from ticks, amplified, and sequenced. We conclude that (1) ticks encounter potentially entomopathogenic fungi more often in soil than in leaf litter, (2) many species of potentially entomopathogenic fungi found in the soil can readily be cultured, (3) the *Galleria* bait method is a sufficiently efficient method for isolation of these fungi from soils, and (4) although DNA extraction from ticks was not possible in this study because of small sample size, DNA extraction from fungi isolated from soils and from ticks was successful and provided clean sequences in 100 and 73% of samples, respectively. A combination of the above methods is clearly necessary for optimal characterization of entomopathogenic fungi associated with ticks in the environment.

Keywords

biological control; entomopathogen; fungi; *Ixodes scapularis*; blacklegged tick

Ixodes scapularis Say, the blacklegged tick, is an ectoparasite and a vector for several disease-causing agents. Among these are *Borrelia burgdorferi*, *Babesia microti*, and *Anaplasma phagocytophilum*, the causative agents of Lyme disease, human babesiosis, and human granulocytic anaplasmosis, respectively. Presently, pesticides are the most effective method of tick control. However, increasing resistance of ticks to pesticides (Li et al. 2007) and concern regarding negative effects of the pesticides on the environment have spurred the search for

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alternative control methods (Samish and Rehacek 1999), including landscape modification, host-targeted measures, and the use of tick parasitoids, predators, and pathogens (see Wilson and Deblinger 1993 and Ginsberg and Stafford 2005 for reviews). Of the latter biological control methods, entomopathogenic fungi seem to be the most promising (Kaaya 1992, Samish and Rehacek 1999; for review, see Chandler et al. 2000).

Entomopathogenic fungi may be widely distributed in forest soils (Brownbridge et al. 1993) and have been isolated from the interface between leaf litter (Oi horizon) and the organic (Oe + Oa horizons) layer of soil (Hajek et al. 2000). Ticks occupy this environment when they are not actively seeking hosts (Samish and Rehacek 1999). For example, larval *I. scapularis* have been found inactive in the leaf litter for several weeks before beginning to quest (Daniels et al. 1996). In addition, all stages of replete *Ixodes ricinus* ticks drop to the ground and burrow down to the deeper layers of leaf litter (Lees 1969), where they come in contact with potentially lethal spores. Conidia (asexual spores) that adhere to the surface of the tick release extracellular enzymes including lipases, proteases (Kucera and Samšiňáková 1968), and chitinases (Tanada and Kaya 1993) that help breach the tick's chitinous exoskeleton. The spores germinate and produce a germ tube that penetrates the cuticle, allowing the mycelia to grow inside the host body (Tanada and Kaya 1993, Inglis et al. 2001), eventually killing it. Therefore, application of entomopathogenic fungi to soil surfaces may be a suitable method for controlling tick populations.

Entomopathogenic fungi have been suggested as promising biological control agents for managing *I. scapularis* populations (Benjamin et al. 2002, Kirkland et al. 2004) and those of other Ixodid species (Gindin et al. 2002, Gomathinayagam et al. 2002, Yoder et al. 2003). A number of laboratory bioassays have been conducted with suspected fungal entomopathogens (Zhioua et al. 1997, Monteiro et al. 1998, Samish et al. 2001, Gindin et al. 2002), and several of these studies have identified *Metarhizium anisopliae* (Metschnikoff) Sorokin as the most pathogenic fungus among species tested (Zhioua et al. 1997, Monteiro et al. 1998, Samish et al. 2001). Although field application of *M. anisopliae* does reduce tick numbers, its virulence relative to other entomopathogenic fungi is unknown because comparison studies have not been reported (Benjamin et al. 2002). Furthermore, there is some evidence that *M. anisopliae* is less virulent in the field than in the laboratory (Benjamin et al. 2002), suggesting that environmental conditions may limit the ability of *M. anisopliae* to reduce tick numbers. If so, other entomopathogenic fungi that occur naturally in Eastern deciduous forests and that might be better adapted to local conditions may be important in regulating tick populations. However, there is little information relating the frequency and distribution of fungal species present in blacklegged tick habitat to the virulence of those fungi being developed as biocontrol agents. To our knowledge, only two surveys have attempted to identify fungi associated with field-collected *I. scapularis* (Zhioua et al. 1999, Benoit et al. 2005), and only a few have looked at fungi associated with other ticks (Kalsbeek et al. 1995, Yoder et al. 2003). To help clarify the role that these fungi may have in the life cycle of *I. scapularis*, optimization of methods to survey and isolate fungi occurring in situ is needed. The objective of this study was to develop and evaluate methods to isolate and identify entomopathogenic fungal species from field-collected samples of soils and ticks from eastern deciduous forests. Data derived from such surveys can provide the foundation for future biological control strategies that more effectively use naturally abundant entomopathogenic fungi to reduce tick populations.

Materials and Methods

Site Descriptions

The study was conducted at the Louis Calder Center, the biological field station of Fordham University located in Armonk, Westchester Co., NY (41°8′ N, 73°48′ W), which is characterized by a mixed hardwood forest with a canopy dominated by oak (*Quercus* spp.),

maple (*Acer* spp.), birch (*Betula* spp.), and hickory (*Carya* spp.) trees. Understory vegetation is sparse, comprised of several shrubs (e.g., barberry [*Berberis thunbergii*], mountain laurel [*Kalmia latifolia*], spice bush [*Lindera benzoin*]), and grasses, and a variable layer of leaf litter that typically covers the ground. The Calder Center is within the New England Uplands physiographic region overlying Precambrian gneiss, schists, and limestones (Schuberth 1968). Soils are well-drained, moderate to shallow sandy loam soils, mesic Inceptisols, in the Charlton and Hollis series (Hill et al. 1980). Blacklegged tick populations are established at this site and have been the focus of an ongoing tick research program since the mid-1980s (Daniels et al. 2000). Ticks were also obtained from a 176-ha privately owned, largely mixed deciduous tract in Bridgeport, CT. The site is characterized by a canopy comprised mostly of oak (*Quercus* spp.) and hickory (*Carya* spp.) species and a dense understory dominated by barberry (*Berberis thunbergii*) and Greenbriar (*Smilax* spp.) (Stafford et al. 2003). In addition, adult ticks were collected from Lake Gaillard in North Branford, New Haven County, CT (41° 20′ N, 72°46′ W). Forest at this site is generally dominated by oak (*Quercus* spp.) and hickory (*Carya* spp.), with dogwood (*Cornus* spp.), arrow-wood (*Viburnum dentatum*), and laurels (*Kalmia* spp.) constituting much of the understory.

Isolating and Identifying Entomopathogenic Fungi

Several methods were used to collect and identify potential entomopathogenic fungi. These involved culturing fungi from samples of soil, leaves, bait insects, and host-seeking ticks and identifying common entomopathogenic fungi, as described below. Additionally, the use of molecular techniques was used because it allows a broader range of fungal species to be identified quickly and accurately compared with identification of fungi through morphological characteristics alone (Bruns et al. 1991).

Culturing Entomopathogenic Fungi From Soil, Leaf Litter, and Ticks

Soil (Oe and Oa horizons) and leaf litter (Oi horizon) were sampled from nine randomly chosen 10 by 10-m quadrats on a 60 by 60-m permanent study grid that was established in 1987 to monitor tick populations (Daniels et al. 2000). Samples were obtained from 28 May to 6 June 2003 in a typical season for the site, when the mean temperature was 17.07°C and mean precipitation was 12.7 cm (unpublished data). From each quadrat, 10 soil cores, 5 cm in diameter and 5 cm deep, were removed and placed together as subsamples in one plastic bag and homogenized. Five leaf litter subsamples were obtained from each quadrat by placing a coffee can ≈15.24 cm (6 in) in diameter on the ground and collecting litter within it. All subsamples from the same plot were placed together in a plastic bag and homogenized. Samples were returned to the laboratory and stored at 4°C until processing.

All samples were diluted 1:100 or 1:1000 in a potassium phosphate buffer (232 mM) containing 0.2 M monobasic potassium phosphate and 0.4 M dibasic potassium phosphate and were plated on potato dextrose agar (PDA) media to assess the frequency of entomopathogenic fungi found in samples. Half-strength PDA plates were prepared with a 10-mg/liter penicillin-streptomycin solution (1,663 U Penicillin G/mg, 748 U streptomycin sulfate/mg). From 90 soil and 45 leaf litter samples, 145 and 140 plates were prepared, respectively. The macroscopic and microscopic characteristics of any resultant fungal growth were examined using reference texts (Barron 1968, Hazard et al. 1981, King and Humber 1981, Samson 1981, Domsch et al. 1993) to determine whether the fungal colonies were one of the four focal taxa known to be entomopathogenic (*M. anisopliae*, *Beauveria bassiana*, *Paecilomyces* spp., *Lecanicillium lecanii*). Fungal colonies that were not identified as one the four taxa of interest were classified as “other fungi,” which may or may not have been entomopathogenic.

Nymphal ticks were collected from 28 May to 30 June 2003 by drag sampling in which a 1-m^2 white corduroy cloth was pulled along the ground and over vegetation (Daniels et al.

2000). Host-seeking ticks grabbing onto the cloth were removed and brought to the laboratory for subsequent work. Ticks were either streaked across the plate, plated whole, or had visible fungal growth removed and plated on rabbit food agar (RFA) media containing a 10-mg/liter tetracycline-streptomycin solution (900 μg/mg tetracycline/mg, 748 U streptomycin sulfate/mg) to assay for the presence of entomopathogenic fungi. In all, 64 plates were prepared and microscopically screened for morphological structures that would indicate the taxa of interest, as described above.

Isolating Entomopathogenic Fungi Using the *Galleria* Bait Method

Entomopathogenic fungi were isolated from soil using *Galleria mellonella* L. (greater wax moth) larvae (Zimmermann 1986). The same soils used above were maintained at 4°C for ≈4 wk until they were used in this assay. Four *G. mellonella* larvae (Grubco, Fairfield, OH) were placed in a plastic container with 275.3 ml (0.5 pint) of each of the 90 soil samples; containers were sealed with perforated lids and held at room temperature. In addition, three to five *G. mellonella* larvae were placed in containers with sterile soil (negative control), no soil (negative control), or sterilized soil to which fungi obtained from one plate (approximate colony surface area of 28.3 cm^2) of each of three known entomopathogenic fungal cultures were added (positive controls). The known entomopathogenic fungi, obtained from the American Type Culture Collection (ATCC), were *M. anisopliae* (ATCC 16085), *B. bassiana* (ATCC 24318), and *P. fumosoroseus* (ATCC 16312). Containers were examined every other day, and dead larvae were collected. Carcasses were surface-sterilized for 3 min in a 1% sodium hypochlorite solution, rinsed in sterile diH_2O, plated, and incubated at 27°C in a humidity chamber at 100% RH to permit growth of fungi (Brownbridge et al. 1993). In total, 204 larvae were dissected and plated on half-strength PDA with apenicillin-streptomycin solution as described above. The number of plates infected with fungus was recorded, the number of entomopathogenic fungal colonies was determined, and the number of fungal colonies suspected to be entomopathogenic was also noted.

Identifying Fungal Infections in Ticks

Throughout the summer and fall of 2003, questing nymphal *I. scapularis* ticks were collected by drag sampling from the Louis Calder Center; adult ticks were collected from the Louis Calder Center, from Bridgeport, CT, and from deer at Lake Gaillard in Branford, CT. In February 2004, a total of 488 nymphal and adult ticks was surface-sterilized for 3 min in 1% sodium hypochlorite and rinsed in sterile diH_2O, with the exception of 38 adult ticks that were surface-sterilized with a solution containing 1% sodium hypochlorite and a small amount of detergent. Each tick was cut in half with a sterile scalpel, and the interior dorsal and ventral viscera were plated on half-strength PDA containing a penicillin-streptomycin solution, as described above. Plates were incubated at 27°C until the presence of fungi was observed. The fungi were sub-cultured to obtain pure cultures and identified by microscopic characteristics as described above.

Extraction of DNA From Fungi Grown in Culture and Directly From Ticks

In addition to the cultures obtained from ATCC, fungal cultures isolated in the previous assays were maintained until DNA was extracted in spring 2005. To culture fungi from ticks, ticks were collected in the summer of 2005 and 2006 by drag sampling, as described above. Within 48 h of collection, ticks, tick eggs, or fungus removed from ticks were plated. All fungal cultures were grown at 27°C on half-strength PDA containing a penicillin-streptomycin solution, as described. As soon as pure fungal cultures were obtained, DNA was extracted from all morphologically distinct cultures using the MoBio (Carlsbad, CA) Soil Ultra Clean DNA Extraction kit. Polymerase chain reaction (PCR; GeneAmp PCR System 2400; Applied Biosystems, Foster City, CA) was conducted in 25- and 50-μl reactions with the following

concentrations: 2.5 U of Qiagen (Valencia, CA) *Taq*DNA polymerase, 1 × Qiagen Buffer, 200 μM of each dNTP, 2 mM Mg^{2+}, and 0.24 μM of each primer. The primers used were the fungus-specific forward primer ITS-1 F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns 1993) and the reverse primer ITS-4 (TCCTCCGCTTATTGATATGC) (White et al. 1990), manufactured by Invitrogen Custom Primers (Carlsbad, CA). PCR was carried out using the following protocol, modified from Gardes and Bruns (1993): initial denaturation at 94°C for 85 s, followed by 37 cycles of denaturation at 95°C for 35 s, annealing at 55°C for 55 s, and extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The reaction was held at 4°C All PCR reactions had one or two positive controls, either *B. bassiana* isolated from soil at the Louis Calder Center alone or in addition to *B. bassiana* isolated from the Eastern tent caterpillar from Massachusetts (ATCC 24318), and one negative control, milli-Q water. The presence of PCR products was confirmed by gel electrophoresis on agarose gel with a concentration ranging from 0.08 to 1.5% in 0.5 × TBE buffer.

Fungal DNA was extracted from ticks collected in the summer of 2005 by drag sampling as described above using the Qiagen DNeasy Blood and Tissue kit. The manufacturer's protocol was modified as follows: ticks were incubated for 3.5 h at 56°C, and the necessary reagents were reduced to one half of the recommended volume because the starting material used was less than what the protocol described. The primers and PCR protocol are described above.

Purification, Sequencing, and Identification of Fungal DNA

Purification of PCR products was conducted using MoBio UltraClean PCR product purification kit. To estimate the concentration of DNA needed for sequencing, purified PCR products were compared with Invitrogen's Low DNA Mass Ladder. All samples were sequenced at the Cornell BioResource Center DNA Sequencing Lab (Ithaca, NY) using an Applied Biosystems Automated 3730 DNA Analyzer with Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase to sequence each PCR fragment with one sequencing reaction. Each PCR product was sequenced in the forward and reverse directions, and consensus sequences were created in Sequencher 4.8 (Gene Codes, Ann Arbor, MI). In April 2008, consensus sequences were compared against all GenBank database sequences using BLAST (blastn), and genusor species-level identifications were determined based on a minimum of 97% sequence similarity (Parrent et al. 2006). When consensus sequence comparisons produced results including more than one species or genus at the same level of sequence similarity, all sequences were aligned in ClustalX (Heidelberg, Germany), and neighbor-joining trees were constructed using PAUP 4.0 (Sinauer Associates, Sunderland, MA). In all cases in which phylogenetic analyses were necessary to resolve identification, the analyses provided strong evidence for inclusion within a single genus or species.

Results

Culturing Entomopathogenic Fungi From Soil, Leaf Litter, and Ticks

Fungi were not uniformly distributed between the soil and leaf litter samples ($\chi^2 = 45.2$, $P < 0.0001$). Fungi were grown on 51 (35.2%) of the 145 plates prepared from soil samples, whereas only 8 (5.7%) of the 140 plates prepared from leaf litter samples developed fungal growth (Table 1). Of the 51 fungal colonies isolated from soil, 35 (68.6%) were confirmed microscopically as species in one of the focal genera that include known entomopathogenic species: *Metarhizium*, *Beauveria*, and *Paecilomyces*. None of the eight fungal colonies isolated from leaf litter were identified as species in the genera of interest in this study. Additionally, fungi were cultured from soil collected in eight of the nine quadrats sampled but from leaf litter in only three of the nine quadrats (Table 1).

Fungi were cultured from 21 (33%) of the 64 plates prepared from nymphal ticks. Of the 21 colonies growing from the plated ticks, 16 were confirmed through microscopic examination to represent one of the four focal genera: *Metarhizium*, *Beauveria*, *Paecilomyces*, and *Lecanicillium*.

Isolating Entomopathogenic Fungi Using the *Galleria* Bait Method

In all, 204 experimental greater wax moth larvae were dissected and plated on PDA, 55 (27%) of which exhibited growth of fungi (Table 2). Of the 62 resulting fungal isolates, 26 (42%) were identified as species from genera known to include entomopathogenic species, such as *Metarhizium*, *Beauveria*, or *Paecilomyces*; the remaining 29 plates grew unidentified fungal colonies. Control plates in which *Galleria* were placed on either sterile soil (negative control), no soil (negative control), or on sterilized soil to which known entomopathogenic fungi were added (positive controls) resulted in fungal growth on 14 ($n = 7$), 10 ($n = 10$), and 44% ($n = 43$) of the plates, respectively. Entomopathogenic fungi were found significantly more often in the experimental soil samples than in the negative controls where larvae were placed in sterile soil and on sterile plates without soil ($\chi^2 = 18.2$, $P < 0.0001$ and $\chi^2 = 41.6$, $P < 0.0001$, respectively). Of the seven colonies that grew on plates to which *M. anisopliae* was added ($n = 15$), five resulted in positive *Metarhizium* growth and two grew fungal colonies that were potentially entomopathogenic. Thirteen plates were prepared from the positive control containing *B. bassiana* and seven (54%) resulted in fungal growth; one of these colonies was identified as *Beauveria* and six were suspected to be other entomopathogenic fungi. Four of the six fungal colonies that grew on the 15 plates prepared from the positive control containing *P. fumosoroseus* were identified as *Paecilomyces*, and two were suspected to be one of the four other entomopathogenic taxa of interest for this study.

Identifying Fungal Infections in Ticks

Plating of field-collected, surface-sterilized adult ticks on PDA yielded fungal growth from 74 (18.5%) of the 400 specimens (Table 3), although only 4 of these (1%) were positively identified as known entomopathogens. Of the 90 adults collected from the Louis Calder Center, 38 were surface-sterilized with the addition of detergent. No fungi were isolated from these ticks, and they either died or appeared less healthy (little response to chemical or mechanical stimulation) after being retrieved from the solution. For nymphs ($n = 88$), fungal growth occurred on seven (8%) plates, only four of these (4.6%) represented entomopathogenic species, and all were ticks collected at the Calder Center (Table 3).

Positive identification of three fungal colonies as members of genera including known entomopathogenic fungal species were made from ticks plated in this study (Table 3). *Paecilomyces* sp., and *Lecanicillium* sp., two known entomopathogens, were isolated from ticks ($n = 336$) collected in Bridgeport, CT, in addition to a suspected entomopathogenic *Penicillium* sp., which was cultured from ticks collected at Bridgeport ($n = 336$) and the Louis Calder Center ($n = 122$).

Extraction, Amplification, and Sequencing of Fungal DNA From Cultures

DNA extracted from pure cultures isolated from soils provided 100% ($n = 15$) clean sequence results, compared with 73% ($n = 40$) for fungal DNA extracted from cultures grown from ticks and 0% ($n = 2$) for sequence results from DNA extracted by lysing whole ticks. Fungal DNA was sequenced and identified from five cultures isolated from soils and *Galleria* at the Louis Calder Center (Table 4). Of those cultures isolated, three of the morphological identifications were identical to the BLAST identities for sequences from *M. anisopliae*, *B. bassiana*, and *P. fumosoroseus*. However, there were two cultures isolated from the Louis Calder Center, which, when sequenced and compared with those in GenBank, were identified differently from the morphological identification. The culture considered to be *Lecanicillium lecanii* was identified

as *Simplicillium lamellicola*, and the culture considered to be *Paecilomyces farinosus* was identified as *P. fumosoroseus* (Table 4). Likewise, sequences from ATCC (16085) cultures of *M. anisopliae* were identified as *M. flavoviride*. In all, DNA sequenced from 24 samples obtained from fungi cultured from ticks yielded 14 different identifications, 67% of which are known to be entomopathogenic (Table 4). The pathogenicity of other species identified from DNA sequences is unknown.

Discussion

This study indicates that a range of methods is needed for characterization of entomopathogenic fungi occurring naturally in tick habitat or associated directly with ticks. Overall, the methods used to isolate fungi by (1) plating soils, (2) using bait insects (*G. mellonella*), and (3) plating ticks were equivalent, averaging 35, 27, and 33% success, respectively, in growing potentially entomopathogenic fungi on half-strength PDA plates. In contrast, attempts to isolate these fungi from leaf litter were less successful; only ≈6% of the samples yielded potentially entomopathogenic fungal colonies. Thus, data suggest that entomopathogenic fungi are not distributed equally in each of the three "habitats"—soil, leaf litter, and ticks—examined and may reflect important deficiencies in environmental conditions needed to maintain these entomopathogenic fungi in the more exposed, generally dry leaf litter.

The *Galleria* bait method is relatively simple to use and resulted in isolation of entomopathogenic fungi from 27% of samples. This success rate was similar to that reported for a survey of Vermont soils that found entomopathogenic fungi in 32.1% of samples baited with *Galleria* (Brownbridge et al. 1993). However, the results of this study actually may be slightly lower than reported because of fungal growth that occurred in the negative control plate, even though growth occurred on only one plate in each control group and the fungus grown was not one of the known entomopathogens identified in the soil samples. Additional care to prevent contamination should resolve this problem in the future.

Conversely, the positive controls, in which soils were inoculated with known entomopathogens, exhibited less fungal growth than expected (44% versus an expected 100%). Furthermore, entomopathogenic species other than those used in the inoculations of the positive controls were found in 23% of the plates. Possible explanations for this include an inadequate volume of known fungi plated and contamination of plates with unknown fungi that either grew unrestricted or, in the case of plates receiving an adequate inoculum of known entomopathogens, outcompeted the known species. The likelihood that the *Galleria* larvae used in this study were contaminated must also be considered. To avoid contamination in future studies using *Galleria* larvae, a clean laboratory culture could be maintained, following Brownbridge et al. (1993). In the case of controls using *B. bassiana*, it is possible that growing conditions were not optimal because this species has been shown to grow more efficiently when incubated at lower temperatures (Bidochka et al. 1998) than those used in this study. Future work would involve attention to specific growing conditions for each species.

In this study, plating nymphal ticks to assess external fungal growth resulted in 25% of nymphs with confirmed entomopathogenic fungal growth. An earlier study of *I. ricinus* found the prevalence of entomopathogenic fungi on larvae, nymphs, and females to be 0.2, 0.3, and 10.3%, respectively (Kalsbeek et al. 1995). Subsequent work by Zhioua et al. (1999), in which spores were removed from field-collected *I. scapularis* and plated on Sabouraud's dextrose agar with yeast (SDAY) medium, found 0% prevalence of entomopathogenic fungi on larvae, nymphs, and males, and only 4.3% prevalence on females. In addition, they found just two entomopathogenic species on the 947 ticks examined (Zhioua et al. 1999). The methods used in this study enabled identification of four taxa known to be entomopathogenic in addition to five unidentified species that were suspected entomopathogens from a total of 64 nymphs

plated. Similarly, a survey of the external mycoflora of nonfed female *I. scapularis* resulted in the isolation of one entomopathogenic fungus, but 14 additional fungi were isolated from the 25 ticks plated (Benoit et al. 2005). Likewise, Yoder et al. (2003) used methods similar to those in this study, plating whole female American dog ticks (*Dermacentor variabilis*), and isolated three genera from the external surfaces of only 12 ticks. In a survey assessing fungi associated with three tick species (*Ixodes ricinus, Dermacentor marginatus*, and *D. reticulatus*) found in southern Moravia, Czech Republic, 552 female *I. ricinus* ticks were placed in cages in meadow, woodland, and ecotone habitats and covered with leaf litter or grass. Of these, 329 (59.6%) developed fungal infections, and fungi were successfully cultured from the external surfaces of 68 individuals. Five known entomopathogens were identified from these specimens, and Samšiňáková et al. (1974) concluded that the presence of entomopathogenic species on the cuticle of ticks may be sufficient to influence tick population dynamics. The results from this study, combined with those reported by Benoit et al. (2005), Yoder et al. (2003), and Samšiňáková et al. (1974), show that directly plating ticks is an effective method for finding entomopathogenic fungi on tick surfaces.

Morphological identification of fungi isolated from the viscera of ticks can provide important information about internal infection rates for known entomopathogens and for other potentially entomopathogenic species. In this study, 1% of adult *I. scapularis* and 4.6% of nymphs were infected internally with one of three entomopathogenic fungus species. These internal infection rates are relatively low compared with those reported in previous studies. For example, 6 of 25 (24%) surface-sterilized, nonfed female *I. scapularis* were found to harbor the entomopathogenic fungus, *Metarhizium anisopliae*, and 25% of nonfed female *Rhicephalus sanguineus* contained fungus (Benoit et al. 2005). In addition, Yoder et al. (2003) surface-sterilized, dissected, and plated female *D. variabilis* to observe internal fungal growth and found that one fungal species, identified as *Scopulariopsis brevicaulis*, was present in 100% of their 25 specimens. The fungus was applied topically to ticks and mortality increased significantly compared with controls (Yoder et al. 2003). However, the authors did not dissect the treated ticks to determine internal infection rates; therefore, tick mortality may have resulted from factors other than the internal growth of *S. brevicaulis* alone.

Identification of fungal cultures using DNA extraction, amplification, and sequencing is a useful complement to morphological identification of fungi. From sequencing fungal DNA isolated from the exterior of 10 ticks, 14 fungal taxa were identified, which is very similar to the 15 fungal taxa that were morphologically identified from 25 *I. scapularis* ticks in a study conducted by Benoit et al. (2005). Furthermore, the protocol described herein was shown to be 100% effective in producing clean sequences from pure cultures of entomopathogenic fungi that were isolated from soils and morphologically identified. There was, however, a discrepancy in the sequence identification of 25% of the pure cultures previously identified using morphological characteristics. This incongruity may be because of the limitations of identifying species through DNA sequence comparison in GenBank, where 27% of the species identification of fungal sequences may be inaccurate (Nilsson et al. 2006). Despite the relatively limited amount of reference data available for comparison (Bridge and Spooner 2001), this method was also useful for the identification of 73% of fungi cultured from ticks that could potentially be pathogenic to *I. scapularis*. Unfortunately, identification of fungal species by sequencing DNA extracted from whole tick lysates did not prove successful in this study, most likely because of competition among several species of fungi in low or equal concentration in the PCR reaction (Bridge and Spooner 2001). Fungal DNA was successfully extracted and amplified, but the sequences were not sufficiently clean to identify the species. Cloning may be a more useful method for obtaining clean sequences from environmental samples.

An interesting result of this study was the relatively low infection rate of questing adult ticks compared with nymphs. Reasons for this might involve the tendency of adult *I. scapularis* to quest higher on vegetation, i.e., above the forest floor, thus reducing the frequency that they encounter soil-borne entomopathogenic fungi. Another reason might be that infection and mortality occur more often in nymphs before molting occurs, leading to fewer infected adults. Consequently, the high susceptibility to infection that adults exhibit in laboratory experiments may not predict mortality in the field because of differences in habitat (Zhioua et al. 1997, Kirkland et al. 2004) and tick behavior. Further studies are needed to compare the virulence of entomopathogenic fungi under laboratory conditions to the virulence in the field. In addition, future research should include comparisons of the pathogenicity of fungi to different tick life stages to determine the most efficient strategy of using fungi as a biological control of *I. scapularis* populations.

Methodological limitations to identification of entomopathogenic species associated with ticks still remain. Isolation and culturing of fungi from soils alone may result in identification of fungi that grow rapidly under laboratory conditions or are more competitive on general media (Bridge and Spooner 2001). However, identification of fungal species by purely molecular methods does not discriminate between living and dead material or active and dormant organisms (Bridge and Spooner 2001). Given their respective limitations, we conclude that the best approach to characterizing entomopathogenic fungi that may regulate tick populations must involve a combination of methods—isolation and culturing of fungi and DNA extraction and sequencing—that are most effective when used together.

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Table 1

Field surveys of soil and leaf litter for potential entomopathogenic fungi

Sample type	Quadrat	No. samples	No. fungal colonies			
			Metarhizium	*Beauveria*	*Paecilomyces*	Other
Soil	G1	10	4	1	1	7
	G6	10	2	0	0	1
	B1	10	0	0	0	2
	B6	10	0	1	0	1
	E4	10	0	1	0	4
	B3	10	1	3	0	0
	D1	10	6	5	6	1
	G3	10	0	0	0	0
	D6	10	0	4	0	0
	Totals	90	13	15	7	16
Leaf litter	G1	5	0	0	0	0
	G6	5	0	0	0	0
	B1	5	0	0	0	3
	B6	5	0	0	0	2
	E4	5	0	0	0	0
	B3	5	0	0	0	0
	D1	5	0	0	0	3
	G3	5	0	0	0	0
	D6	5	0	0	0	0
	Totals	45	0	0	0	8

Fungi obtained by direct plating on half-strength PDA containing a penicillin-streptomycin solution.

Table 2

Galleria bioassays

Quadrat/control	No. Galleria used	No. plates prepared	No. plates with fungal growth	Percent plates with fungal growth	No. entomopathogenic fungal colonies			
					Metarhizium	*Beauveria*	*Paecilomyces*	Other
G1	40	0	0	0.00	0	0	0	0
G6	40	30	9	30.00	0	0	1	1
B1	40	28	8	28.57	0	1	3	4
B6	40	35	8	22.86	0	3	0	5
E4	40	31	11	35.48	0	4	0	7
B3	40	24	10	41.67	1	2	2	5
D1	40	15	7	46.67	0	1	3	3
G3	40	21	1	4.76	0	0	0	1
D6	40	20	8	40.00	1	2	2	3
Totals	360	204	62	27.78	2	13	11	29
M. anisopliae (control)	16	15	7	46.67	5	0	0	2
B. bassiana (control)	16	13	7	53.85	0	1	0	6
P. fumosoroseus (control)	16	15	5	33.33	0	0	4	2
Sterile soil (control)	10	7	1	14.29	0	0	0	1
No soil (control)	10	10	1	10.00	0	0	0	1

Galleria larvae were placed in each of 90 soil samples from 9 quadrats (10 per quadrat, 4 larvae per sample).

Table 3

Total fungal growth of plated ticks collected from Bridgeport, CT, the Louis Calder Center in Armonk, NY, and from deer in Branford, CT

	Bridge port	Louis Calder Center	From deer	Total
No. adult ticks plated	280	90	30	400
No. adult ticks with fungal growth	54	12	8	74
Percent adults with fungal growth	19.3	13.3	26.7	18.5
No. nymphal ticks plated	56	32	NA	88
No. nymphal ticks with fungal growth	0	7	NA	7
Percent nymphs with fungal growth	0	22	NA	8

Fungal cultures were grown on half-strength PDA containing a penicillin-streptomycin solution.

NA, not applicable.

Table 4

Sequence identification of fungal DNA extracted from pure cultures plated from soils and ticks and directly from ticks

GenBank accession no.	DNA extraction	Culture origin	Culture source	No. BP used in BLAST	Contig length	BLAST results	Max identity (%)
EU886742	*Beauveria bassiana*	ATCC	Insect[a]	498	568	*Beauveria bassiana*	100
EU886743	*Beauveria bassiana*	LCC	Soil	498	568	*Beauveria bassiana*	100
EU886744	*Lecanicillium lecanii*	LCC	Soil	536	618	*Simplicillium lamellicola*	100
EU886745	*Metarhizium anisopliae*	ATCC	Soil—CAN	501	582	*Metarhizium flavoviride*	100
EU886746	*Metarhizium anisopliae*	LCC	Soil	393	579	*Metarhizium anisopliae*	100
EU886747	*Paecilomyces farinosus*	LCC	Soil	517	593	*Paecilomyces fumosoroseus*	100
FJ269352	*Paecilomyces fumosoroseus*	ATCC	Soil—GER	518	588	*Paecilomyces fumosoroseus*	100
EU886748	*Paecilomyces fumosoroseus*	LCC	Soil	507	598	*Paecilomyces fumosoroseus*	100
EU886749	Exterior of female tick 20a	LCC	Tick	333	592	*Paecilomyces farinosus*	100
EU886750	Exterior of female tick 21a	LCC	Tick	186	351	*Paecilomyces farinosus*	100
EU886751	Exterior of male tick 28a	LCC	Tick	430	598	*Paecilomyces farinosus*	100
EU886752	Abdomen of female tick 21a	LCC	Tick	475	588	*Paecilomyces fumosoroseus*	100
EU886753	Abdomen of female tick 21a	LCC	Tick	514	582	*Colletotrichum acutatum*	100
EU886754	Dorsal exterior of male tick 29a	LCC	Tick	506	582	*Penicillium chrysogenum*	100
EU886768	Fungus on tick adult 24b exterior	LCC	Tick	365	563	*Penicillium* sp.	98
EU886769	Fungus on tick adult 25b exterior	LCC	Tick	398	NA	*Penicillium* sp.	99
EU886755	Exterior of tick eggs	LCC	Tick	513	584	*Colletotrichum acutatum*	100
EU886756	Ventral interior of engorged female	LCC	Tick	320	558	*Colletotrichum acutatum*	100
EU886757	Ventral interior of engorged female	LCC	Tick	481	549	*Cladosporium* sp.	100
EU886758	Ventral exterior of nymphal tick 51	LCC	Tick	339	593	*Colletotrichum acutatum*	99
EU886759	Ventral exterior of nymphal tick 51	LCC	Tick	330	582	*Discostroma tricellulare*	98
EU886770	Ventral exterior of nymphal tick 51	LCC	Tick	491	632	*Colletotrichum fuscum*	100
EU886760	Dorsal exterior of nymphal tick 51	LCC	Tick	485	614	*Verticillium fungicola*	100
EU886761	Dorsal exterior of nymphal tick 51	LCC	Tick	422	619	*Cladosporium cladiosporioides*	99
EU886762	Dorsal exterior of nymphal tick A6	LCC	Tick	439	630	*Hypocrea koningii*	100
EU886763	Ventral exterior of nymphal tick A6	LCC	Tick	346	624	*Hypocrea koningii*	98
EU886764	Dorsal exterior of nymphal tick A6	LCC	Tick	304	553	*Phoma* sp.	98
EU886765	Dorsal exterior of nymphal tick A10	LCC	Tick	320	645	*Phoma* sp.	100
EU886766	Dorsal exterior of nymphal tick A10	LCC	Tick	329	598	*Myrothecium verrucaria*	97

GenBank accession no.	DNA extraction	Culture origin	Culture source	No. BP used in BLAST	Contig length	BLAST results	Max identity (%)
EU886767	Ventral exterior of nymphal tick A10	LCC	Tick	524	588	*Pestalotiopsis caudata*	99

Cultures were obtained from fungi isolated from soils and ticks sampled at the Louis Calder Center (LCC) and from the American Type Culture Collection (ATCC), where cultures were isolated from an insect ([a] *Malacosoma americanum*) and soils (sampled from Canada and Germany).