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Wide interguild relationships among entomopathogenic and free-living nematodes in soil as measured by real time qPCR

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ABSTRACT

Entomopathogenic nematodes (EPNs) are promising biological control agents of soil-dwelling insect pests of many crops. These nematodes are ubiquitous in both natural and agricultural areas. Their efficacy against arthropods is affected directly and indirectly by food webs and edaphic conditions. It has long been suggested that a greater understanding of EPN ecology is needed to achieve consistent biological control by these nematodes and the development of molecular tools is helping to overcome obstacles to the study of cryptic organisms and complex interactions. Here we extend the repertoire of molecular tools to characterize soil food webs by describing primers/probe set to quantify certain free-living, bactivorous nematodes (FLBNs) that interact with EPNs in soil. Three FLBN isolates were recovered from soil baited with insect larvae. Morphological and molecular characterization confirmed their identities as *Acrobeloides maximum* (RT-1-R15C and RT-2-R25A) and *Rhabditis rainai* (PT-R14B). Laboratory experiments demonstrated the ability of these FLBNs to interfere with the development of *Steinernema diaprepesi*, *Steinernema riobrave* and *Heterorhabditis indica* parasitizing the weevil *Diaprepes abbreviatus* ($P < 0.001$), perhaps due to resource competition. A molecular probe was developed for the strongest competitor, *A. maximum*. We selected the highly conserved SSU rDNA sequence to design the primers/probe, because these sequences are more abundantly available for free-living nematodes than ITS sequences that can likely provide better taxonomic resolution. Our molecular probe can identify organisms that share $\geqslant 98\%$ similarity at this locus. The use of this molecular probe to characterize soil communities from samples of nematode DNA collected within a citrus orchard revealed positive correlations ($P < 0.01$) between *Acrobeloides*-group nematodes and total numbers of EPNs (*S. diaprepesi, H. indica* and *Heterorhabditis zealandica*) as well as a complex of nematophagous fungi comprising *Catenaria* sp. and *Monachrosporium gephyropagum* that are natural enemies of EPNs. These relationships can be broadly interpreted as supporting Linford's hypothesis, i.e., decomposition of organic matter (here, insect cadavers) greatly increases bactivorous nematodes and their natural enemies.

1. Introduction

Entomopathogenic nematodes (EPNs) are a group of species in the order Rhabditida that have been studied for decades because of their potential as biological control agents (Georgis et al., 2006; Kaya et al., 2006). More recently these nematodes are receiving special attention as models to investigate more general biological, ecological and evolutionary questions (Denno et al., 2008; Goodrich-Blair and Clarke, 2007). EPNs also require symbiotic bacteria in their life cycles (*Xenorhabdus* spp. in the case of the nematodes in the genus *Steinernema* and *Photorhabdus* spp. for those in the genus *Heterorhabditis*), making them ideal to study symbiosis and co-evolution (Stock and Goodrich-Blair, 2008). Recent, rapid advances in knowledge of fundamental biology and ecology derived from these nematodes have resulted from the development of new techniques to study cryptic organisms, the availability of public platforms to share information and advances in techniques to analyse complex sets of environmental data (Campos-Herrera et al., 2012).

EPNs are reported to have worldwide occurrence, with the exception of Antarctica (Adams et al., 2006; Hominick, 2002). The third-stage, infective juvenile (IJ) is the only stage that can reside in soil and locate and infect a host. In the host hemocoel, IJs liberate their symbiotic bacteria which kill the insect and reproduce by digesting its tissues. The IJs complete their development through several generations by feeding on the bacteria and the insect tissues. As resources are depleted, nematode development ar-

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rests at the IJ stage which retains bacteria in the gut and emerges into the environment (Adams and Nguyen, 2002).

The duality of the relationship between this nematode and their mutualistic bacteria, both associated with the food resource of the arthropod host, suggests an adaptation from ancestral trophic behaviour by free-living bacterivorous nematodes (FLBNs) and this is supported by phylogenetic studies (Blaxter et al., 1998; De la Ley and Blaxter, 2002; Poinar, 1993). Field evidence suggests that competition occurs between some FLBNs and EPNs, although the extent to which FLBNs might affect EPN population dynamics is unknown. FLBNs emerged from cadavers of sentinel insects buried in Florida citrus orchards at significantly higher rates when EPNs were added to soil than when soil remained untreated (Duncan et al., 2003, 2007). When the FLBN *Pellioditis* sp. was combined with *Steinernema riobrave* in laboratory trials, numbers of EPNs emerging from insect cadavers declined, whereas numbers of *Pellioditis* sp. increased, compared to treatments with just a single species (Duncan et al., 2003). Other studies have reported similar responses by FLBNs to augmentation of EPNs (Ishibashi and Kondo, 1986, 1987), although there are also reports of no interactions (García et al., 2011; Grewal et al., 1997; Somasekhar et al., 2002).

The insecticidal effectiveness of EPNs is mediated by biotic and abiotic factors (Stuart et al., 2006). Although many studies have focused on the abiotic environment (i.e. temperature, humidity, pH, etc.), interactions between EPNs and their natural enemies and competitors remain poorly understood (Ram et al., 2008; Strong, 2002; Stuart et al., 2008). Especially rare are field studies of guilds encompassing EPN antagonists such as nematophagous fungi (NF) (Duncan et al., 2007; Jaffee and Strong, 2005), microarthropod predators (Greenwood et al., 2011; Jabbour and Barbercheck, 2011) or nematode competitors of EPNs (Duncan et al., 2003, 2007; Hoy et al., 2008). These studies are constrained by the time, cost, and taxonomic expertise required to study cryptic organisms at the community level. The development of quantitative molecular probes is helping to resolve some of these difficulties and a number of these tools are now available to study species of EPNs, NF and even bacteria that are phoretically associated with EPNs (Atkins et al., 2005; Campos-Herrera et al., 2011a,b; Pathak et al., 2012; Torr et al., 2007; Zhang et al., 2006). In this report we describe (1) experiments to identify FLBN species that compete with indigenous EPN species in Florida, (2) molecular primers and probe that discriminate closely related FLBNs, and (3) spatial relationships between three guilds of soil organisms in a Florida citrus orchard: EPNs, NF, and FLBNs that can comprise species which compete with EPNs.

2. Materials and methods

2.1. Isolation and molecular characterization of FLBNs

In August 2010, 10 composite soil samples (10 cores, 30 cm deep × 5 cm diam.) from a citrus orchard near Land O' Lakes, FL (82 28 28.42 W and 28 15 9.69 N) were mixed and subsamples of 500 g soil from each sample were baited with five *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae in 3 × 10 cm diam. Petri dishes. The samples were incubated at room temperature (22–25 °C) for 4 days, after which dead larvae were individually placed in White traps and incubated at 25 °C. Emerging EPNs were identified based on morphology, morphometrics and species-specific molecular probes (Campos-Herrera et al., 2011a; Nguyen, 2007), but some cadavers produced large numbers of rhabditid nematodes that might have exploited insects killed by EPNs (Duncan et al., 2003). Three such isolates, two with round tails (RT-1 and RT-2) and one with pointed tails (PT), were maintained for further study. Isolates were rinsed repeatedly on a 650 mesh sieve (opening 20 μm), after which 10–15 nematodes were handpicked and placed on 1.5% nutrient agar (NA, Difco, MD, USA) to develop with the bacteria associated with their gut or cuticle. Isolates were transferred every 10–15 days for several rounds without evidence of any change in morphology.

For DNA extraction, suspensions of nematodes from three culture plates of each isolate were washed with deionized water as previously described. Nematodes were concentrated and aliquots of 300 nematodes were stored in 1.5 mL Eppendorf tubes at −20 °C until use. DNA from each of four tubes per isolate was extracted following the protocol for maximum yield of the Ultra Clean Soil™ DNA Kit (MoBio). The DNA samples were resuspended in 50 μL elution buffer and quality and quantity were determined using 1 μL per duplicate in a Nanodrop 1000 (ND-1000 v3.3.0, Thermo Scientific, Wilmington, DE). Afterwards, DNA from each tube per isolate was combined to avoid the slight differences in quantity that occurs during extraction (Campos-Herrera et al., 2011a) and DNA quantity was adjusted to 1 ng/μL for use in conventional and real time qPCR experiments.

The SSU rDNA of the FLBNs was sequenced in order to confirm morphological identification using the primers and protocols of Holterman et al. (2006). Additional partial sequences of the ITS-1 region were determined using primers and protocols described by Jones et al. (2006a). PCR amplifications were conducted in a 25 μL final volume (MJ Research PTC 200 Peltier Thermal Cycler) with 1 μL of DNA template and sterile de-ionized water for negative controls (Campos-Herrera et al., 2011a). Before initiating sequencing protocols, the PCR product sizes were checked with a TAE 2% agarose gel and visualized (UVP BioDoc-it™ System). PCR products were sequenced according to Campos-Herrera et al. (2011a) at the Interdisciplinary Center for Biotechnology Research (ICBR) of the University of Florida. These sequences were compared to reported sequences using Blast (http://www.blast.ncbi.nlm.nih.gov).

2.2. Competition between EPNs and FLBNs

To evaluate the competitive abilities of the FLBN isolates, laboratory experiments were performed following protocols described by Duncan et al. (2003). Three EPN species, *Steinernema diaprepesi* HK31, *S. riobrave* Btw1 and *Heterorhabditis indica* Kerr1, were used in the experiments. *S diaprepesi* and *S. riobrave* are reported to have foraging strategies intermediate between cruising and ambushing (Campbell et al., 2003), whereas *H. indica* behaves as a cruiser, as do many *Heterorhabditis* spp. (Campbell and Lewis, 2002). All EPNs were isolated in Florida citrus groves during a survey conducted in 2008 and identified based on morphology/morphometrics and molecular characterization (Campos-Herrera et al., 2011a; Nguyen, 2007). *S. diaprepesi* and *H. indica* are naturally occurring in Florida, whereas *S. riobrave* are descendents of commercially formulated nematodes applied in orchards to control *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae) (Duncan et al., 2003, 2007). The EPN species were cultured in *G. mellonella* larvae and stored in distilled water at 15 °C for 1 week until use. The laboratory cultures were maintained by infecting 18–20 *G. mellonella* larvae each 2 months and combining the emerging IJs, which were stored as described previously. The FLBN suspensions for the experiments were obtained from 10-day-old cultures, cleaned and prepared as previously described. The FLBN were maintained in laboratory cultures at room temperature by adding 50 μL of the nematode suspension to 4 NA Petri dishes every 2–3 weeks. Nematodes counts (EPN and FLBN) were adjusted to 600 nematodes in 200 μL by volumentric estimation following the procedure described by Glazer and Lewis (2000).

The experiments were conducted as described by Duncan et al. (2003) and repeated once for each nematode species, Briefly, soil