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Characterization of biocontrol traits in the entomopathogenic nematode *Heterorhabditis georgiana* (Kesha strain), and phylogenetic analysis of the nematode's symbiotic bacteria

David I. Shapiro-Ilan [a,*], George N. Mbata [b], Khuong B. Nguyen [c], Scott M. Peat [d], Dana Blackburn [d,e], Byron J. Adams [d]

[a] USDA-ARS, Southeastern Fruit and Tree Nut Research Lab., Byron, GA 31008, USA
[b] Department of Biology, Fort Valley State University, Fort Valley, GA 31030, USA
[c] Entomology & Nematology Department, University of Florida, Gainesville, FL 32601, USA
[d] Department of Biology, and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT 84602, USA
[e] Molecular Genetics and Microbiology Department, University of Florida, Gainesville, FL 32610, USA

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ABSTRACT

Our objective was to estimate the biocontrol potential of the recently discovered entomopathogenic nematode species *Heterorhabditis georgiana* (Kesha strain). Additionally, we conducted a phylogenetic characterization of the nematode's symbiotic bacterium. In laboratory experiments, we compared *H. georgiana* to other entomopathogenic nematodes for virulence, environmental tolerance (to heat, desiccation, and cold), and host seeking ability. Virulence assays targeted *Acheta domesticus*, *Agrotis ipsilon, Diaprepes abbreviatus, Musca domestica*, *Plodia interpunctella, Solenopsis invicta*, and *Tenebrio molitor*. Each assay included *H. georgiana* and five or six of the following species: *Heterorhabditis floridensis, Heterorhabditis indica*, *Heterorhabditis mexicana*, *Steinernema carpocapsae*, *Steinernema feltiae, Steinernema rarum*, and *Steinernema riobrave*. Environmental tolerance assays included *Heterorhabditis bacteriophora, H. georgiana, H. indica*, *S. carpocapsae, S. feltiae*, and *S. riobrave* (except cold tolerance did not include *S. carpocapsae* or *S. riobrave*). Host seeking ability was assessed in *H. bacteriophora, H. georgiana*, *S. carpocapsae*, and *Steinernema glaseri*, all of which showed positive orientation to the host with *S. glaseri* having greater movement toward the host than *S. carpocapsae* (and the heterorhabditids being intermediate). Temperature range data (tested at 10, 13, 17, 25, 30 and 35 °C) indicated that *H. georgiana* can infect *Galleria mellonella* between 13 and 35 °C (with higher infection at 17–30 °C), and could reproduce between 17 and 30 °C (with higher nematode yields at 25 °C). Compared with other nematode species, *H. georgiana* expressed low or intermediate capabilities in all virulence and environmental tolerance assays indicating a relatively low biocontrol potential. Some novel observations resulted from comparisons among other species tested. In virulence assays, *H. indica* caused the highest mortality in *P. interpunctella* followed by *S. riobrave; S. carpocapsae* caused the highest mortality in *A. domesticus* followed by *H. indica;* and *S. riobrave* was the most virulent nematode to *S. invicta.* In cold tolerance, *S. feltiae* exhibited superior ability to cause mortality in *G. mellonella* (100%) at 10 °C, yet *H. bacteriophora* and *H. georgiana* exhibited the ability to produce attenuated infections at 10 °C, i.e., the infections resumed and produced mortality at 25 °C. In contrast, *H. indica* did not show an ability to cause attenuated infections. Based on the phylogenetic analysis, the bacterium associated with *H. georgiana* was identified as *Photorhabdus luminescens akhurstii*.

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1. Introduction

Entomopathogenic nematodes (genera *Steinernema* and *Heterorhabditis*) are biological control agents that are used to control a variety of economically important insect pests (Shapiro-Ilan et al., 2002; Grewal et al., 2005). These nematodes have a mutualistic symbiosis with a bacterium (*Xenorhabdus* spp. and *Photorhabdus* spp. for steinernematids and heterorhabditids, respectively) (Poinar, 1990). Infective juveniles (IJs), the only free-living stage, enter hosts through natural openings (mouth, anus, and spiracles), or in some cases, through the cuticle. After entering the host's hemocoel, nematodes release their bacterial symbionts, which are primarily responsible for killing the host within 24–48 h, defending against secondary invaders, and providing the

* Corresponding author. Address: USDA-ARS, Southeastern Fruit and Tree Nut Research Lab., 21 Dunbar Road, Byron, GA 31008, USA. Fax: +1 478 956 2929.
E-mail address: David.Shapiro@ars.usda.gov (D.I. Shapiro-Ilan).

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nematodes with nutrition (Dowds and Peters, 2002). The nematodes molt and complete up to three generations within the host after which IJs exit the cadaver to find new hosts (Kaya and Gaugler, 1993). Although entomopathogenic nematodes are (collectively) pathogenic to a wide variety of insect pests (e.g., some nematode species can infect more than 100 different hosts [Poinar, 1979]), successful commercialization has been limited to relatively few targets (Shapiro-Ilan et al., 2002; Grewal et al., 2005).

Discovery of new entomopathogenic nematode species can substantially expand or improve biological control utility. For example, discovery of *Steinernema riobrave* (Cabanillas et al., 1994) resulted in improved control of *Diaprepes abbreviatus* and pink bollworm, *Pectinophora gossypiella* (Saunders) (Duncan and McCoy, 1996; Shapiro-Ilan et al., 2002), discovery of *Steinernema scapterisci* (Nguyen and Smart, 1990) led to enhanced suppression of mole crickets, *Scapteriscus* spp. (Parkman et al., 1994), and the discovery of *Steinernema scarabaei* (Stock and Koppenhöfer, 2003) promises to expand nematode control of white grubs (Koppenhöfer and Fuzy, 2003a). Additionally, discovery of endemic entomopathogenic nematodes can be beneficial in terms of finding strains or species that are well adapted to the local environment (for targeting pests in that geographic region). Once a new species is discovered, characterization of certain biological and ecological properties may facilitate an estimation of the newly described organism's potential as a biocontrol agent (Koppenhöfer and Fuzy, 2003b; Koppenhöfer and Kaya, 1999; Koppenhöfer et al., 2000; Shapiro-Ilan et al., 2005).

In a soil survey conducted during the summer of 2006, a new entomopathogenic nematode species, *Heterorhabditis georgiana* Nguyen, Shapiro-Ilan, and Mbata, was discovered in Byron, GA (Nguyen et al., 2008). The nematode population (designated "Kesha" strain) was isolated from a single composite soil sample taken from a soil surrounding a pecan tree within a pecan orchard. The objective of this study was to estimate the relative biocontrol potential of the Kesha strain. Additionally, given that the bacterium associated with this nematode had not yet been identified, we conducted a phylogenetic analysis to characterize the nematode's symbiont.

2. Materials and methods

To estimate relative biocontrol potential, we conducted an array of laboratory bioassays. Laboratory assays that compare relative virulence to particular insect pests, and tolerance to environmental extremes, among nematode species may be used to help predict biocontrol abilities under field conditions (Shapiro-Ilan et al., 2002). We conducted a broad temperature range experiment to determine the range in which *H. georgiana* can infect and reproduce. Additionally, we measured virulence to a variety of insect pests that are commercial targets or potential targets for entomopathogenic nematode control (Poinar, 1979; Shapiro-Ilan et al., 2002, 2005; Mbata and Shapiro-Ilan, 2005; Ramos-Rodríguez et al., 2006): the black cutworm, *Agrotis ipsilon* (Hufnagel), the Diaprepes root weevil, D. abbreviatus (L.), the house fly, *Musca domestica* L., the house cricket, *Acheta domesticus* (L.), the Indianmeal moth, *Plodia interpunctella* (Hübner), and lesser mealworm, *Tenebrio molitor* L., and the red imported fire ant, *Solenopsis invicta* Buren. Furthermore, we assessed environmental tolerance (to heat, cold, and desiccation), and host seeking ability. For each trait, *H. georgiana* was compared to several other entomopathogenic nematodes species. For most comparisons, at least one nematode species was known to possess high capabilities for that trait (based on previous studies).

2.1. Nematodes, insects, and experimental conditions

Nematodes used in all experiments were cultured in parallel in last instar *Galleria mellonella* (L.) (obtained from Webster's Waxie Ranch, Webster, WI) according to Kaya and Stock (1997); IJs were stored at 13 °C for less than 3 weeks prior to use in experiments. Nematodes included among the various experiments were: *Heterorhabditis bacteriophora* Poinar (Hb1 strain), *Heterorhabditis floridensis* Nguyen, Gozel, Koppenhöfer & Adams, *H. georgiana, Heterorhabditis indica* Poinar, Karunakar & David (Hom1 strain), *Heterorhabditis mexicana* Nguyen et al. (MX4 strain), *Steinernema carpocapsae* (Weiser) (All strain), *Steinernema feltiae* (Filipjev) (SN strain), *Steinernema glaseri* (Steiner) (NJ43 strain), *Steinernema rarum* (Doucet) (17C&E strain), and *S. riobrave* (355 strain).

Adult *A. domesticus* were obtained from Russell's Premium Crickets (Thomson, GA), and *M. domestica* maggots from Carolina Biological (Charlotte, NC). Third instar *A. ipsilon,* reared on artificial diet, were obtained from the USDA-ARS Corn Insects Research unit (Ames, IA). *D. abbreviatus* (7–10th instar) were supplied by the Florida Department of Agriculture, Division of Plant Industry (Gainesville, FL), *S. invicta* workers were provided by the University of Georgia Experiment Station (Griffin, GA), and *T. molitor* (ca. 0.8 ± 0.05 mg per insect) were obtained from Southeastern Insectary (Perry, GA). *P. interpunctella* was reared at the Department of Biology, Fort Valley State University on a diet of cornmeal, chick laying mash, chick starter mash, oats and glycerin (volumetric mixture at 4:2:2:2:1) (Mbata, 1985); third instars were used in experiments. All bioassays (virulence, environmental tolerance, and host-finding experiments) were conducted in the laboratory at approximately 25 °C, arranged as completely randomized designs, and each experiment was repeated once in time (in entirety) unless otherwise stated.

2.2. Virulence assays

Parameters for virulence assays (substrate, rate of application, time of assessment) were based on previously published studies, and/or preliminary experiments (unpublished data). Except for the assessments with *A. ipsilon* and *D. abbreviatus,* virulence assays were conducted in Petri dishes lined with filter paper (Whatman No. 1) based on Kaya and Stock (1997), Mbata and Shapiro-Ilan (2005), and Shapiro-Ilan et al. (2005). Virulence assessments for each insect were conducted separately; all experiments contained an untreated (water only) control.

Virulence assays for *A. domesticus, M. domestica*, and *S. invicta* were conducted in 100 mm Petri dishes. Each dish received a total of 1 ml water or nematode suspension, and contained 5 *A. domesticus*, 10 *M. domestica*, or 10 *S. invicta* (only 5 *A. domesticus* were used because of their greater size and potential crowding in the dishes). The rate of application was 200 IJs per insect for *A. domesticus* and *M. domestica*, and 6000 IJs per insect for *S. invicta*. Insect mortality was assessed 1 and 2 d (24 and 48 h) after application for *A. domesticus* and *M. domestica*, and after 1 and 4 d for *S. invicta*. There were four replicates of each dish. Treatments included *H. floridensis*, *H. georgiana, H. indica*, *S. carpocapsae*, *S. feltiae*, *S. rarum*, and *S. riobrave*. To help in determining which nematode species to include, we noted that prior studies had demonstrated consistent pathogenicity or high relative virulence of *S. carpocapsae* to *A. domesticus* (Poinar, 1979) and *S. invicta* (Drees et al., 1992), and *S. feltiae* to *M. domestica* (Taylor et al., 1998).

Plodia interpunctella and *T. molitor* assays were conducted in 60 mm Petri dishes. In separate trials (conducted one time each), approximately 100 or 500 IJs were applied in 350 µl water to dishes containing a single larva each. Larval mortality was evaluated 1 and 2 d after application. Treatments were the same as those indicated above (e.g., for *A. domesticus*) except *H. mexicana* was included whereas *S. carpocapsae* and *S. feltiae* were not. There were four replicates of 10 dishes for each treatment. Treatment choices for the *P. interpunctella* assay included *H. indica* and *S. riobrave* based on relatively high virulence to this insect observed in prior

studies (Mbata and Shapiro-Ilan, 2005; Ramos-Rodríguez et al., 2006).

Virulence assays for *A. ipsilon* and *D. abbreviatus* were conducted in a soil substrate based on procedures described by Shapiro and McCoy (2000) and Shapiro-Ilan et al. (2005). For *A. ipsilon*, experiments were conducted in plastic cups (Bioserv Inc., Frenchtown, NJ) (3–4 cm in diam, 3.5 cm deep) filled with 27 g of (oven-dried) soil from the USDA-ARS pecan orchard (Byron, GA), and contained one larva each. The soil was a loamy sand with the percentage sand:silt:clay = 84:10:6, pH 6.1, and organic matter = 2.8% by weight. Nematodes were pipetted onto the soil surface of each cup in 0.5 ml of water so that the final moisture was standardized at field capacity (14%). Each experiment contained four replicates of 10 cups per treatment and a water only control. Approximately 10 or 50 IJs were applied to each cup and larval mortality was assessed after 1 d (24 h); treatments included the same nematodes as those listed for *A. domesticus* except *S. feltiae* was not included. The nematode, *S. carpocapsae* was chosen in particular because relatively high levels of virulence were previously observed against *A. ipsilon* (Capinera et al., 1988; Levine and Oloumi-Sadeghi, 1992).

Virulence assays with *D. abbreviatus* were conducted in a similar manner. Experimental units consisted of 30 ml cups filled with sand, which was standardized at approximately 8% moisture by weight. A single *D. abbreviatus* larva was placed on the bottom of each container prior to filling with soil, and 500 or 1000 IJs were applied to the soil surface in 0.5 ml water. Larval mortality was determined 7 d post-inoculation. Treatments and replicates were the same as those for the *A. ipsilon* assays except *H. mexicana* was included instead of *S. carpocapsae*. The experiment included *S. riobrave* and *H. indica* based on high virulence observed in previous comparisons (Shapiro et al., 1999; Shapiro and McCoy, 2000).

2.3. Environmental tolerance

An experiment was conducted to estimate the range of temperatures (and optima) at which *H. georgiana* can infect and reproduce. The host insect, last instar *G. mellonella*, was exposed to nematodes in 100 mm Petri dishes lined with filter paper (Whatman No. 1). Each dish contained 10 *G. mellonella* and received 2000 *H. georgiana* IJs in 1 ml tap water. The dishes were immediately placed in incubators at 10, 13, 17, 25, 30, or 35 °C. Insect mortality was checked daily and the total number of dead *G. mellonella* was recorded after 7 d. There were four replicate dishes in each temperature and an equal set of no-nematode control dishes (water only) was also included at each temperature. Cadavers showing signs of nematode infection were placed individually on White traps (Kaya and Stock, 1997), and the IJ yield per insect was determined 30 d post-inoculation. There were 10 replicate White traps for each temperature.

In addition to the temperature range experiment focusing on *H. georgiana*, the environmental tolerance of *H. georgiana* to desiccation, heat, and cold relative to other nematode species. All assays included the following treatments: *H. bacteriophora*, *H. georgiana, H. indica*, *S. carpocapsae*, *S. feltiae*, and *S. riobrave*, except the cold tolerance assays did not include *S. carpocapsae* or *S. riobrave*. The heat and desiccation tolerance assays contained three replicates of each treatment, and cold stress contained four replicates.

Desiccation tolerance was compared among nematode strains based on procedures described by Solomon et al. (1999). Approximately 2000 IJs were pipetted onto filter paper (Whatman No. 1). Excess moisture was removed from the filter paper through vacuum filtration. The filter paper containing nematodes was then placed into the open lid of a 60 mm Petri dish, and then into a plastic desiccator (23 cm maximum diam × 24 cm height, Nalgene®, Rochester, NY) that was set to 85% RH based on a saturated solution of KCl. After 24 and 48 h of incubation at 25 °C, the filter paper was removed and placed in approximately 5 ml tap water for an additional 1 d at which time percentage nematode mortality was determined based on lack of movement response when probed with a dissecting needle. Each treatment (strain) contained four replicates in a randomized block design (blocked by desiccator). To assist in making treatment choices, we considered that *S. carpocapsae* and *S. feltiae* have been reported to be more desiccation tolerant than several other entomopathogenic nematode species (Glazer, 2002).

Heat tolerance was measured using procedures described by Shapiro et al. (1996) and Shapiro-Ilan et al. (2005). Approximately 2000 IJs in 0.2 ml were pipetted into 5 ml of tap water in a 20-ml glass scintillation vial. The vial had already been equilibrated to 37 °C prior to addition of nematodes. After incubation for 3 and 4 h in a water bath shaker (rpm 70) at 37 °C, 0.2 ml of the suspension was transferred to a 60-mm Petri dish containing 9 ml tap water. The dishes were incubated at 25 °C for 1 d at which time the percentage nematode mortality was determined as described above. To assist in making treatment choices, we considered that prior studies indicated relatively high levels of heat tolerance in *S. riobrave* (Grewal et al., 1994a) and *H. indica* (Shapiro and McCoy, 2000).

Cold tolerance was measured by evaluating the ability of nematodes to infect the host and cause mortality at 10 °C. Experimental arenas consisted of 100 mm Petri dishes containing sand (standardized at 8% moisture) and 10 last instar *G. mellonella*. Approximately, 500 IJs were added to each dish, which was then placed in an incubator at 10 °C. After 5 d, dishes were removed from the incubator, and the number of live and dead *G. mellonella* was assessed. Immediately after the mortality assessment, the larvae from each dish were rinsed thoroughly, and placed in a new Petri dish lined with filter paper (Whatman No. 1); these dishes were kept in an incubator at 25 °C for 3 d (8 d after treatment), at which time larval mortality was assessed a second time. A set of control dishes containing *G. mellonella* but without nematodes were treated in an identical manner. Additionally, a set of positive control plates containing both nematodes and *G. mellonella* were kept at 25 °C.

2.4. Host seeking

Host seeking ability was measured on agar plate assays (Schmidt and All, 1978; Barbercheck and Kaya, 1991; Shapiro-Ilan et al., 2005). Plastic Petri dishes (100 mm) were filled to approximately 2/3 depth with 2% agar. A pipette tip (1000 µl capacity) was inserted into a hole in the lid on opposite ends of the plate (approximately 4 mm from the edge of the plate), and a nematode inoculation port (1.0 cm diameter) was created in the center of the lid and sealed with duct tape. On treatment plates, one *G. mellonella* larva was placed in one pipette tip and the other tip was left empty. In control plates both tips were left empty; all tips were sealed with parafilm. During the 1.5 h that followed placement of the insect in the pipette tip, a gradient of volatile host cues was created. Subsequently, approximately 2000 IJs in 0.35 ml water were applied to a 60 mm filter paper disc (Whatman No. 1), excess water was removed by vacuum filtration, and the disc was inserted into the center of the agar dish, which was then resealed with duct tape. After 3 h, the number of IJs found in a 1 cm diameter circle under each pipette was recorded. Host seeking ability (% attraction) was estimated by the percentage of IJs found under the circle with the host relative to the total under the host plus those under the empty pipette tip. In control plates one side was randomly designated as the host side for calculation purposes. Treatments included *H. bacteriophora*, *H. georgiana, S. carpocapsae*, and *S. glaseri* (NJ43). The first trial contained eight replicates per

treatment and the second trial contained 10 replicates per treatment. Both *S. glaseri* and *H. bacteriophora* are known to migrate toward host volatile cues (Lewis et al., 1992; Grewal et al., 1994b).

2.5. Phylogenetic characterization of H. georgiana's bacterial symbiont

2.5.1. Bacterial isolation

Bacteria were extracted from IJs of *H. georgiana.* Additionally, the symbiont of *H. mexicana*, which had not been characterized previously, was also extracted and included in the analysis for comparison with known species and strains. Five *G. mellonella* larvae were placed on filter paper covering the bottom of a 60 mm Petri dish. Approximately 300 IJs suspended in 200 ml of solution were evenly dispersed on the filter paper. After 72 h insect cadavers were sterilized with a 70% ethyl alcohol solution. Dried sterilized cadavers were torn open with flame-sterilized tweezers and the viscera were spread across lipid agar plates. Plates were incubated at 30 °C. After 48 h lawns of bacteria had grown on each of the five plates. Cells obtained for DNA extraction were collected directly from plate cultures.

2.5.2. Sequence generation

DNA was extracted using a DNeasy Tissue kit (Qiagen Inc., Valencia, CA). The 16S rRNA gene was PCR amplified using forward primer 5′-GAAGAGTTTGATCATGGCTC-3′ and reverse primer 5′-AAGGAGGTGATCCAGCCGCA-3′ (Fischer-Le Saux et al., 1999). The gyrase B (gyrB) gene was PCR amplified using forward primer 5′-TAARTTYGAYGAYAACTCYTAYAAAGT-3′ and reverse primer 5′-CMCCYTCCACCARGTAMAGTTC-3′ (Dauga, 2002). Initial sequencing was performed using the same primer sets. 16S sequencing yielded approximately 350 and 780 bp sequences for *H. georgiana* and *H. mexicana*, respectively. Initial gyrase B sequencing yielded 320 and 680 bp sequences for *H. georgiana* and *H. mexicana*, respectively. To obtain longer sequences internal primers were designed to extend in opposite directions. 16S primers for both *H. georgiana* and *H. mexicana* were forward primer 5′-AAGGAGGTGATCCAACCG CAG-3′ and reverse primer 5′-GGAACGCTGAGACAGGTG-3′. Gyrase B primers for *H. georgiana* were forward primer 5′-GACTGGTGAT GATGCTCGTG-3′ and reverse primer 5′-CGTCGTTTGGATTTTC CAGT-3′. Gyrase B primers for *H. mexicana* were forward primer 5′-CGATGGCTTCCAGGAAAATA-3′ and reverse primer 5′-CTAAA CCTTCCCGAGCATCA-3′. All sequencing was conducted at the Brigham Young University DNA sequencing center using the Applied Biosystems Model 3730xl DNA analyzer (Applied Biosystems, CA). Contiguous sequences were assembled using Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, MI) with final 16S sequence lengths for *H. georgiana* and *H. mexicana* of 939 and 1267 bp, respectively, and final gyrase B sequence lengths for *H. georgiana* and *H. mexicana* of 924 and 956 bp, respectively.

2.5.3. Phylogenetic analysis

Sequence data for 16S ribosomal DNA (rDNA) and the gyrase B gene (*gyr*B) were obtained from GenBank (at the National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html) for all of the taxa except for the *Photorhabdus* isolates from *H. georgiana* (Kesha) and *H. mexicana* (MX4). Twenty three ingroup taxa and two outgroup taxa were included in the 16S analyses, while eighteen ingroup taxa and three outgroup taxa were included in the *gyr*B analyses. Each molecular data set (1455 bp for 16S and 1155 bp for *gyr*B) was aligned separately using the default parameters in Muscle (Edgar, 2004). Following alignment, sequences were visually inspected using MacClade 4.05 (Maddison and Maddison, 2002).

ModelTest 3.7 (Posada and Crandall, 1998) was utilized to calculate the best fit model of evolution for each dataset using the Akaike Information Criterion (AIC). Maximum Likelihood analyses were conducted on each dataset in PHYML (Guindon and Gascuel, 2003). The 16S likelihood analysis was conducted using the TrN + *I* + *G* model of evolution with six substitution types and substitution rate matrix and nucleotide frequencies estimated from the nucleotide alignment. The *gyr*B likelihood analysis was conducted using the TIMef + *I* + *G* model of evolution with six substitution types and substitution rate matrix and nucleotide frequencies estimated from the nucleotide alignment. Bootstrap values for the likelihood tree were calculated in PHYML using 1000 bootstrap replicates.

Bayesian analyses were conducted in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003), using the same models that were used in the ML analyses. Two runs were conducted for each dataset using 20,000,000 generations and sampling every 1000 generations. Stationarity was estimated using Tracer v1.4 (Rambaut and Drummond, 2007), with the 16S analysis having a burn-in value of 50,000 and the *gyr*B dataset having a burn-in of 30,000. Bremer support values were calculated by constructing a 50% majority rule consensus tree of the remaining trees (19,950 for the 16S and 19,970 for the *gyr*B) using PAUP*4.0b10 (Swofford, 2002). Log likelihood values for each run were compared to ensure that each Bayesian run converged on similar log likelihood mean values for each of the two independent runs for each gene.

2.6. Statistical analyses

Treatment effects in all bioassay experiments were analyzed with ANOVA. If the ANOVA detected a significant difference ($P \leqslant 0.05$) then treatment differences were elucidated through LSD (SAS, 2001). For each experiment, data from both trials (repeated in time) were combined, and variation among trials was accounted for as a block effect. In the *A. ipsilon* and *D. abbreviatus* virulence experiments, data from both nematode rates were combined because the interactions between rate and treatment effects were not significant ($P > 0.05$). Data were not combined across rates in the *P. interpunctella* and *T. molitor* experiments because the rates were not applied and evaluated simultaneously. Percentage data (mortality and host-seeking) were arcsine transformed and numerical data (nematode yield) were square-root transformed prior to analysis (Southwood, 1978; Steel and Torrie, 1980; SAS, 2001); non-transformed means are presented in figures.

3. Results

3.1. Virulence assays

At 1 d post-inoculation, mortality in *A. domesticus* was higher in the *S. carpocapsae* treatment than all other treatments and the control; no other differences were detected ($F = 5.6$, df = 7.119, $P < 0.001$) (Fig. 1). At 2 d post-inoculation, *A. domesticus* mortality was higher in the *H. indica* and *S. carpocapsae* treatments than the control and all other treatments; the other nematode treatments did not exhibit higher mortality than the control ($F = 11.29$, df = 7.119, $P < 0.001$) (Fig. 1).

All treatments caused higher mortality than the control in *A. ipsilon* ($F = 8.29$, df = 6.104, $P < 0.001$) (Fig. 2). *Agrotis ipsilon* mortality was lower in *S. rarum* than all other treatments except *H. georgiana*. In the assessment of virulence to *D. abbreviatus*, all nematodes caused higher mortality than the control, the highest mortality was observed in the *H. indica* and *S. riobrave* treatments (which were not different from each other), and the lowest was observed in *S. rarum* ($F = 45.27$, df = 6.104, $P < 0.001$) (Fig. 3).

At 1 d post-inoculation, *M. domestica* mortality was higher than the control in the *S. feltiae* treatment, followed by the *H. indica*, and *S. carpocapsae* treatments ($F = 9.26$, df = 7.55, $P < 0.001$) (Fig. 4). At 2 d post-inoculation, all nematode treatments caused higher *M.*

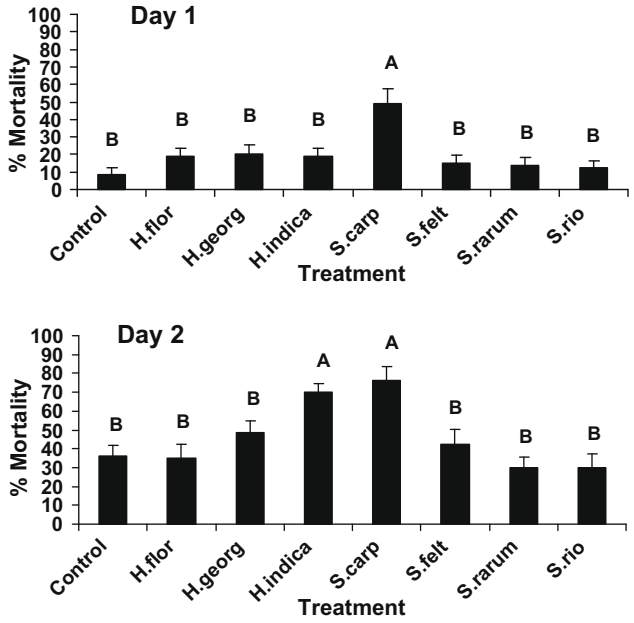

Fig. 1. Mean percentage mortality (±SE) of *Acheta domesticus* adults 1 or 2 d after treatment with 200 infective juvenile nematodes. Treatments included *Heterorhabditis floridensis* (=H.flor), *H. georgiana* (=H.georg), *H. indica*, *Steinernema carpocapsae* (=S.carp), *S. feltiae* (S.felt), *S. rarum*, and *S. riobrave* (=S.rio), and a water only control. Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

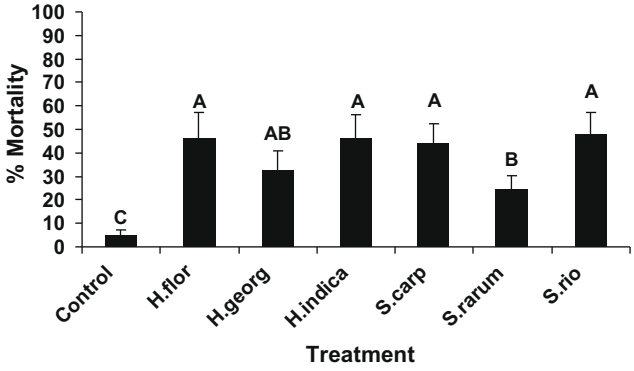

Fig. 2. Mean percentage mortality (±SE) of *Agrotis ipsilon* larvae 1 d after treatment with 10 or 50 infective juvenile nematodes (data were combined across rates). Treatments included *Heterorhabditis floridensis* (=H.flor), *H. georgiana* (=H.georg), *H. indica*, *Steinernema carpocapsae* (=S.carp), *S. rarum*, and *S. riobrave* (=S.rio), and a water only control. Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

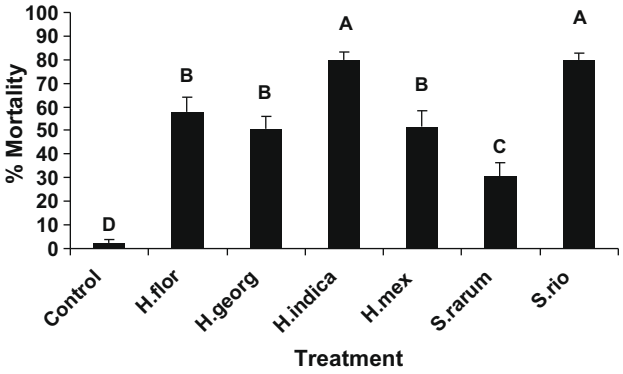

Fig. 3. Mean percentage mortality (±SE) of *Diaprepes abbreviatus* larvae 7 d after treatment with 500 or 1000 infective juvenile nematodes (data were combined across rates). Treatments included *Heterorhabditis floridensis* (=H.flor), *H. georgiana* (=H.georg), *H. indica*, *H. mexicana*, *Steinernema rarum*, and *S. riobrave* (=S.rio), and a water only control. Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

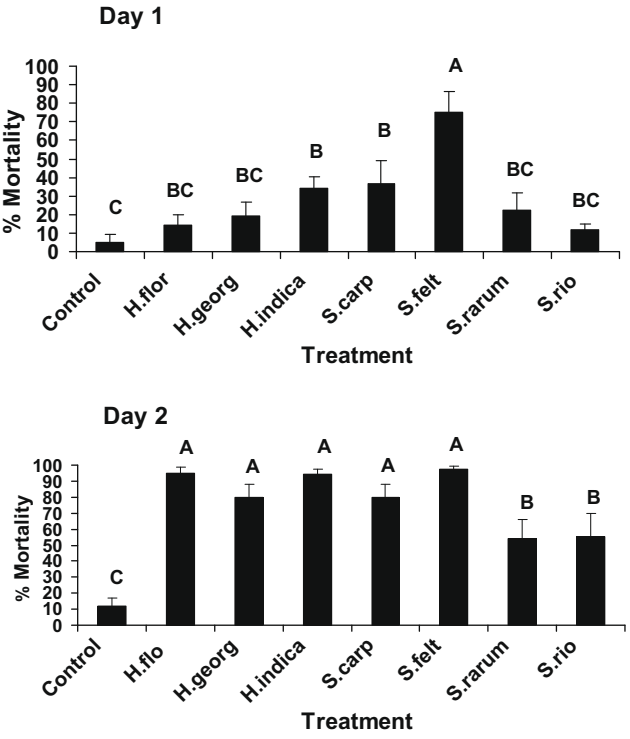

Fig. 4. Mean percentage mortality (±SE) of *Musca domestica* larvae 1 or 2 d after treatment with 200 infective juvenile nematodes. Treatments included *Heterorhabditis floridensis* (=H.flor), *H. georgiana* (=H.georg), *H. indica*, *Steinernema carpocapsae* (=S.carp), *S. feltiae* (S.felt), *S. rarum*, and *S. riobrave* (=S.rio), and a water only control. Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

domestica mortality than the control; mortality in the *S. rarum* and *S. riobrave* treatments was lower than in other treatments ($F = 19.63$, df = 7.54, $P < 0.001$) (Fig. 4).

Differences among nematode species in virulence to *P. interpunctella* were observed. One d after nematode application, only *H. indica* and *S. riobrave* caused higher mortality than the control ($F = 5.88$, df = 6.21, $P = 0.001$ for rate of 100 IJs per insect, and $F = 6.64$, df = 6.21, $P = 0.0005$ for the 500 IJ rate) (Fig. 5). At 2 d post-inoculation and 100 IJs per larva, all nematodes caused greater mortality than the control, and *H. indica* caused greater mortality than *H. mexicana*, *S. rarum*, and *S. riobrave* ($F = 8.19$, df = 6.21, $P = 0.0001$) (Fig. 5). At 2 d post-inoculation and 500 IJs per larva, all nematodes caused greater mortality than the control, and mortality caused by *S. riobrave* was higher than mortality caused by *H. georgiana* but not different from *H. indica* or *S. rarum*; *H. mexicana* and *H. floridensis* caused the lowest mortality ($F = 24.62$, df = 6.21, $P < 0.0001$) (Fig. 5).

At 1 d post-inoculation, *S. invicta* mortality was higher in the *S. riobrave* treatment than all other treatments except *S. feltiae*; four treatments (*H. indica, S. carpocapsae, S. feltiae*, and *S. riobrave*) caused higher mortality than the control, whereas three did not (*H. floridensis, H. georgiana*, and *S. rarum*) ($F = 9.49$, df = 7.55, $P < 0.0001$) (Fig. 6). At 4 d post-inoculation, all nematode treatments caused higher mortality than the control, and *S. riobrave*

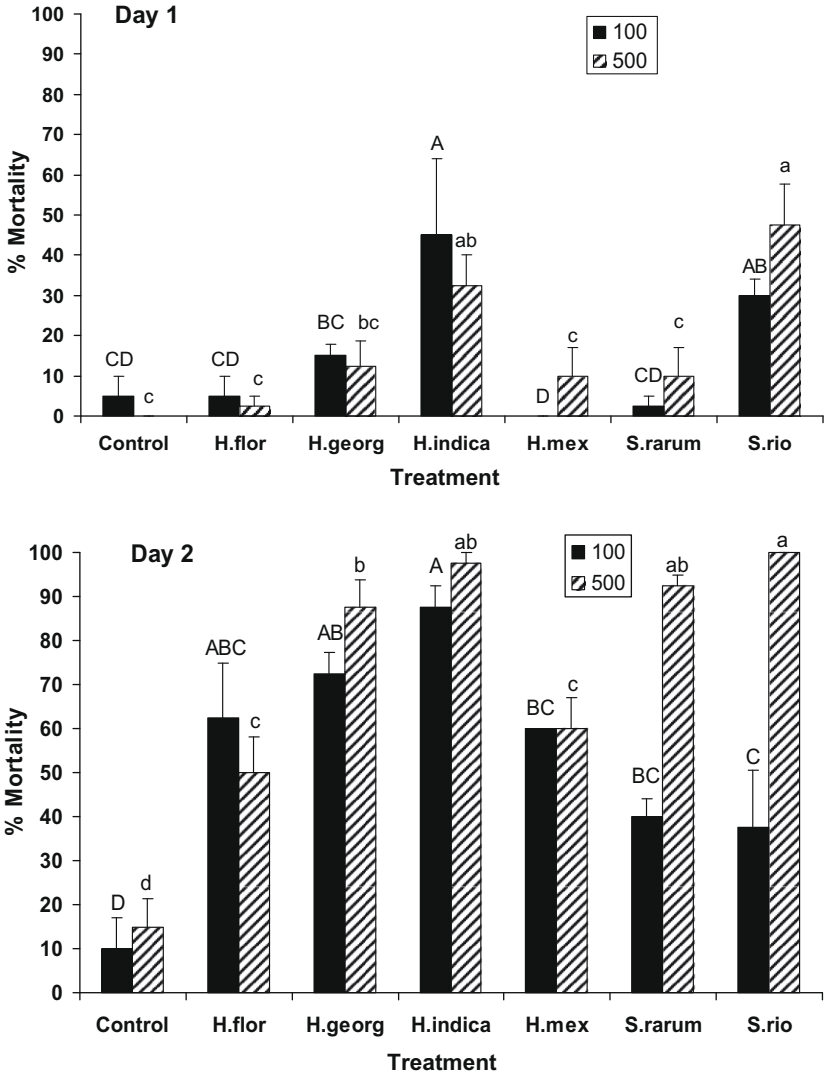

Fig. 5. Mean percentage mortality (±SE) of *Plodia interpunctella* larvae 1 or 2 d after treatment with 100 or 500 infective juvenile nematodes. Treatments included *Heterorhabditis floridensis* (=H.flor), *H. georgiana* (=H.georg), *H. indica*, *H. mexicana*, *Steinernema rarum*, and *S. riobrave* (=S.rio), and a water only control. Different upper and lower case letters above bars indicate statistical differences at the 100 and 500 nematode application rates, respectively ($P \leqslant 0.05$, LSD).

caused higher mortality than all other treatments ($F = 5.41$, df = 7.55, $P < 0.0001$) (Fig. 6).

When exposed to 100 IJs per larva for 1 d, *T. molitor* mortality was higher in all treatments relative to the control except in the *H. mexicana* and *H. floridensis* treatments ($F = 5.59$, df = 6.21, $P = 0.0013$) (Fig. 7). When exposed to 500 IJs per larva for 1 d, *T. molitor* mortality was higher in all treatments relative to the control except in the *H. mexicana* treatment ($F = 19.99$, df = 6.21, $P < 0.0001$) (Fig. 7). At 2 d post-inoculation and 100 IJs per insect, all nematodes caused higher *T. molitor* mortality than the control, and *H. georgiana* and *H. mexicana* treatments resulted in lower mortality than the other nematodes ($F = 23.46$, df = 6.21, $P < 0.0001$) (Fig. 7). At 2 d post-inoculation with 500 IJs, mortality was higher in all nematodes relative to the control and no differences among the nematode species were detected ($F = 39.66$, df = 6.21, $P < 0.0001$) (Fig. 7).

3.2. Environmental tolerance

When determining the range of temperatures at which *H. georgiana* could infect, nematodes caused significant *G. mellonella* mortality relative to the non-treated control at all temperatures ($P \leqslant 0.05$) except 10 °C ($P > 0.05$); control mortality ranged from 0% at 10 °C and 13 °C to 20.2% at 35 °C (not shown). The following levels of *G. mellonella* mortality (±SE) were observed after correcting for control mortality with Abbott's formula (Abbott, 1925): 3.2 ± 2.3% for 10 °C, 7.5 ± 4.1% for 13 °C, 33.1 ± 10.8% for 35 °C, and 100% for 17, 25, and 30 °C. The corrected percentage mortalities at 17, 25, and 30 °C were higher than at 35 °C, which in turn was higher than 10 or 13 °C (10 and 13 °C were not different from each other) ($F = 93.92$, df = 5.41, $P < 0.0001$; LSD test). In terms of nematode yield, *H. georgiana* only produced progeny IJs on White traps in three temperatures: 17, 25, and 30 °C. Average (±SE) numbers of IJs produced per cadaver were 47436.1 ± 12404.4 for 17 °C, 247705.0 ± 28448.7 for 25 °C, and 31190.8 ± 8938.7 for 30 °C. The number of IJs produced at 25 °C was greater than the number produced at either of the other temperatures (which were not different from each other) ($F = 54.98$, df = 2.55, $P < 0.0001$; LSD test).

In general, greater desiccation and heat tolerance was observed in the steinernematids compared with the heterorhabditids (Figs. 8 and 9). After 1 d of desiccation, tolerance (indicated by lower nematode mortality) was greatest in *S. carpocapsae*, followed by *S. feltiae*,

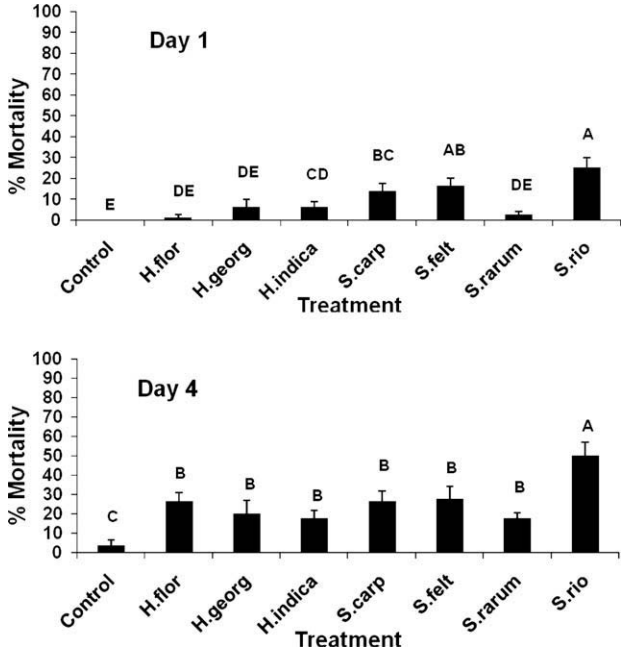

Fig. 6. Mean percentage mortality (±SE) of *Solenopsis invicta* larvae 1 or 4 d after treatment with 6000 infective juvenile nematodes. Treatments included *Heterorhabditis floridensis* (=H.flor), *H. georgiana* (=H.georg), *H. indica*, *Steinernema carpocapsae* (=S.carp), *S. feltiae* (S.felt), *S. rarum*, and *S. riobrave* (=S.rio), and a water only control. Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

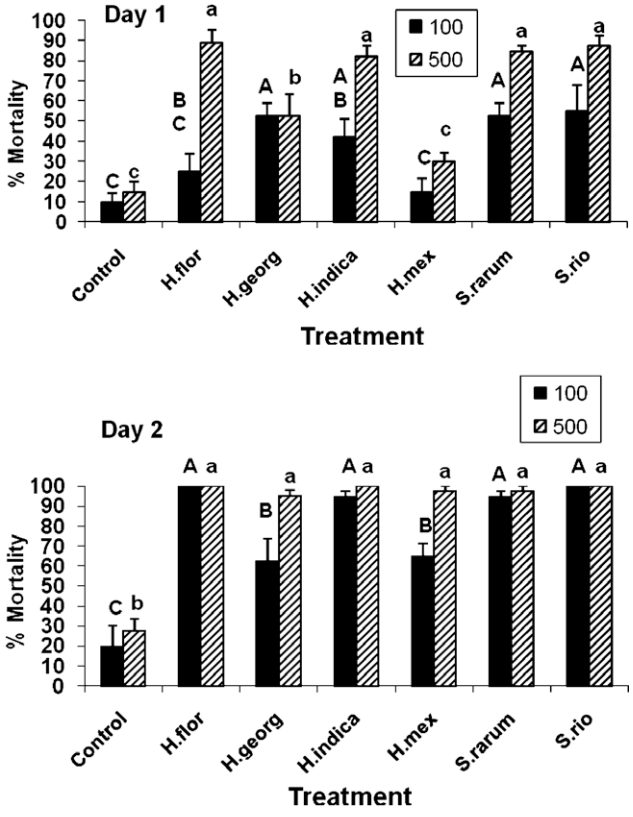

Fig. 7. Mean percentage mortality (±SE) of *Tenebrio molitor* larvae 1 or 2 d after treatment with 100 or 500 infective juvenile nematodes. Treatments included *Heterorhabditis floridensis* (=H.flor), *H. georgiana* (=H.georg), *H. indica*, *H. mexicana, Steinernema rarum*, and *S. riobrave* (=S.rio), and a water only control. Different upper and lower case letters above bars indicate statistical differences at the 100 and 500 nematode application rates, respectively ($P \leqslant 0.05$, LSD).

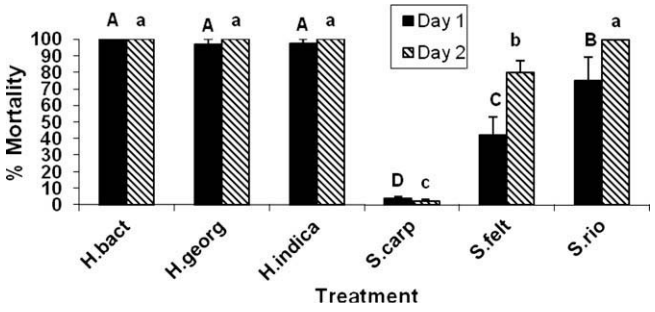

Fig. 8. Mean percentage mortality (±SE) of infective juvenile nematodes following exposure to 85% RH for 1 or 2 d. Treatments included *Heterorhabditis bacteriophora* (=H.bact), *H. georgiana* (=H.georg), *H. indica*, *Steinernema carpocapsae* (=S.carp), *S. feltiae* (S.felt), and *S. riobrave* (=S.rio). Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

S. riobrave, and the three heterorhabditid species (which were not different from each other) ($F = 31.57$, df = 5.29, $P < 0.0001$) (Fig. 8). The desiccation tolerance results at 2 d were identical to those at 1 d except that mortality in the *S. riobrave* treatment was not different from the heterorhabditid treatments ($F = 139.62$, df = 5.29, $P < 0.0001$) (Fig. 8). Heat tolerance (based on nematode mortality at 37 °C) at 3 h of exposure, was highest in *S. carpocapsae* and *S. riobrave* followed by *H. bacteriophora* and *H. indica*, and with the lowest tolerance observed in *H. georgiana* and *S. feltiae* ($F = 86.62$, df = 5.29, $P < 0.0001$) (Fig. 9); heat tolerance at 4 h of exposure was similar except that *S. riobrave* showed greater heat tolerance than *S. carpocapsae* ($F = 196.46$, df = 5.29, $P < 0.0001$) (Fig. 9).

Several of the nematodes tested were able to invade and or cause mortality in *G. mellonella* at 10 °C (Fig. 10). Only *S. feltiae* and *H. indica* caused greater mortality than the control in *G. mellonella* after 5 d at 10 °C, with *S. feltiae* causing >8-fold higher mortality than *H. indica* ($F = 254.65$, df = 4.34, $P < 0.0001$) (Fig. 10). However, when infections initiated at 10 °C resumed at 25 °C (for 3 d longer), *H. bacteriophora*, *H. georgiana*, and *S. feltiae* caused higher mortality than the control with *H. bacteriophora* and *S. feltiae* causing higher mortality than *H. georgiana* ($F = 63.03$, df = 4.34, $P < 0.0001$) (Fig. 10). In contrast, *G. mellonella* mortality in the *H. indica* treatment did not increase when the insects were moved from 10 to 25 °C, and given the increased mortality that developed in the control at 25 °C relative to 10 °C (11.25% and 3.75%, respectively), mortality in the *H. indica* treatment and the control were not statistically separated at 25 °C (Fig. 10). In all positive controls (nematodes exposed to *G. mellonella* at 25 °C) 100% insect mortality was observed (data not shown).

3.3. Host seeking

All nematode species tested exhibited positive attraction to the host based on significant differences in percentage movement between respective control and treatment plates ($F = 11.03$, df = 7.135, $P < 0.0001$) (Fig. 11). The level of attraction to the host was higher in *S. glaseri* than *S. carpocapsae* with *H. bacteriophora* and *H. georgiana* being intermediate (Fig. 11).

3.4. Phylogenetic characterization of H. georgiana's bacterial symbiont

All analyses (Figs. 12 and 13) show the Kesha strain (isolated from *H. georgiana*) forming a monophyletic group with strains previously identified as *Photorhabdus luminescens akhurstii*. The MX4 strain (isolated from *H. mexicana*), which also had not been characterized, consistently forms a monophyletic group with strains previously identified as *Photorhabdus luminescens luminescens*.

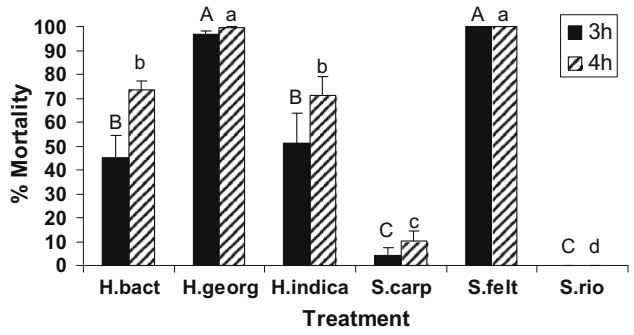

Fig. 9. Mean percentage mortality (±SE) of infective juvenile nematodes following exposure to 37 °C for 3 or 4 h. Treatments included *Heterorhabditis bacteriophora* (=H.bact), *H. georgiana* (=H.georg), *H. indica*, *Steinernema carpocapsae* (=S.carp), *S. feltiae* (S.felt), and *S. riobrave* (=S.rio). Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

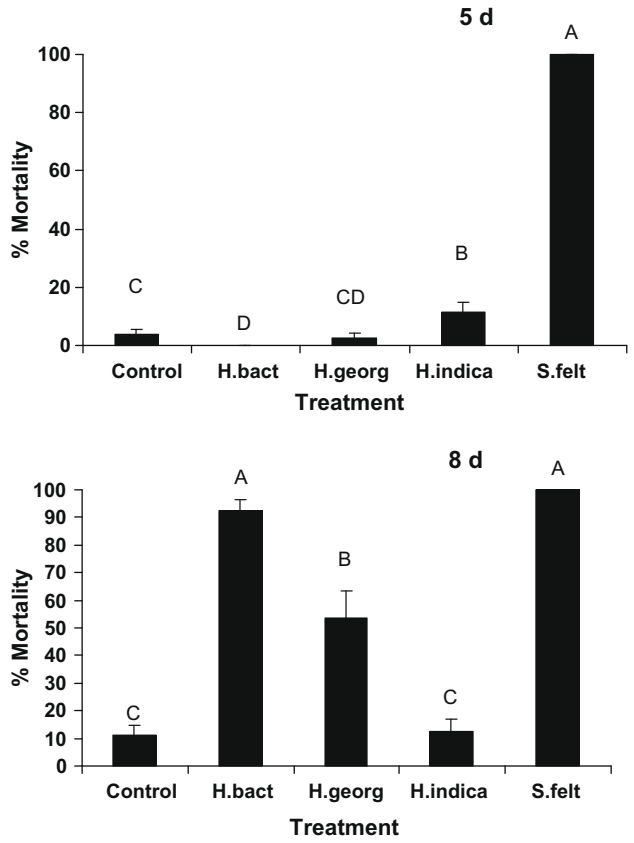

Fig. 10. Mean percentage mortality (±SE) of *Galleria mellonella* following exposure to nematodes at 10 °C for 5 d, or transfer from 10 °C (after 5 d) to 25 °C for an additional 3 d (8 d total). Treatments included *Heterorhabditis bacteriophora* (=H.bact), *H. georgiana* (=H.georg), *H. indica*, and *Steinernema feltiae* (S.felt), and a control (no nematodes). Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

4. Discussion

We have characterized *H. georgiana* (Kesha strain) for a variety of biological and ecological characteristics important to biological control. Our results indicate that *H. georgiana* generally possesses poor to moderate abilities in virulence and environmental tolerance relative to other entomopathogenic nematodes for the range of hosts and conditions tested. In each of the virulence and environmental tolerance assays (except virulence to *A. ipsilon*), at least

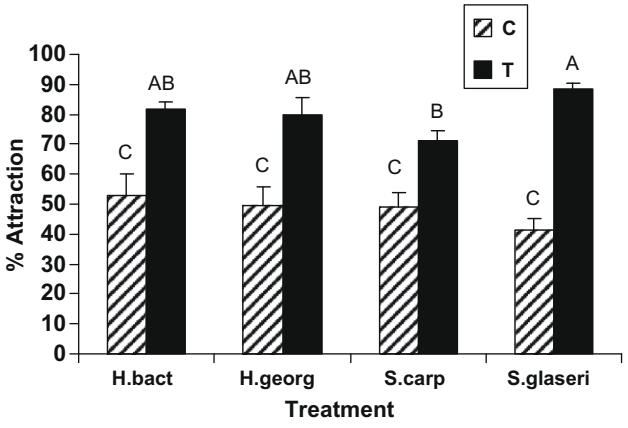

Fig. 11. Mean percentage of infective that moved on agar-filled Petri dishes toward *Galleria mellonella* host cues (T, treatment dishes), or toward a randomly selected side of a dish that did not contain any host cues (C, control dishes). Treatments included *Heterorhabditis bacteriophora* (=H.bact), *H. georgiana* (=H.georg), *Steinernema carpocapsae* (=S.carp), and *S. glaseri*. Different letters above bars indicate statistical differences ($P \leqslant 0.05$, LSD).

one other nematode species was superior to *H. georgiana,* which tended to fall into the lowest tier of nematodes tested or in some cases an intermediate tier. Host finding was the only trait in which *H. georgiana* exhibited an equal ability compared with the other nematodes tested (though was not superior to any tested). In terms of temperatures at which *H. georgiana* can infect and reproduce, the nematode appears to have a fairly wide niche breadth though perhaps not as wide as some other species such as *S. glaseri* and *S. riobrave* (Grewal et al., 1994a).

Based on the results of our experiments, we consider the biocontrol potential of *H. georgiana* to be poor to moderate. However, further tests against other target insects or under other conditions might prove otherwise. Conceivably, the relative abilities of *H. georgiana* for the traits we evaluated under laboratory conditions may differ under the complexity of factors experienced under field conditions. Further, it is conceivable that *H. georgiana*'s virulence may be exceptionally high to other pests not tested. Although our study included insects that are current or potential commercial targets (Shapiro-Ilan and Gaugler, 2002; Shapiro-Ilan et al., 2002), our experiments only included five insect orders and seven families, and susceptibility to nematodes among insects can vary greatly (Shapiro-Ilan et al., 2002; Grewal et al., 2005).

Beyond elucidating the biocontrol traits of *H. georgiana,* our assays involved comparisons among various other nematode species for a variety of traits. Relative, virulence, environmental tolerance, and host finding among nematode species agreed with prior studies in many cases though there were some exceptions; additionally, a number of novel discoveries were made in previously untested comparisons. For example, compared to the other nematodes tested, the findings of higher virulence in *S. carpocapsae* and *H. indica* to *A. domesticus*, and higher virulence of *S. riobrave* to *S. invicta,* were novel, yet the observation of higher virulence in *S. feltiae* to *M. domestica* was expected and supported by prior studies (Taylor et al., 1998). Additionally, based on prior studies it was expected *S. riobrave* would exhibit high levels of heat tolerance and *S. carpocapsae* would be intermediate (Grewal et al., 1994a). The superior virulence observed in *S. riobrave* and *H.* indica to *D. abbreviatus* also confirms findings of earlier studies (Shapiro et al., 1999; Shapiro and McCoy, 2000). In contrast, unlike some previous studies (Capinera et al., 1988; Koppenhöfer and Kaya, 1996; Shapiro-Ilan et al., 2005) we did not detect superior virulence in *S. carpocapsae* when targeting *A. ipsilon* relative to other species tested. Additionally, Ramos-Rodríguez et al. (2006) observed more

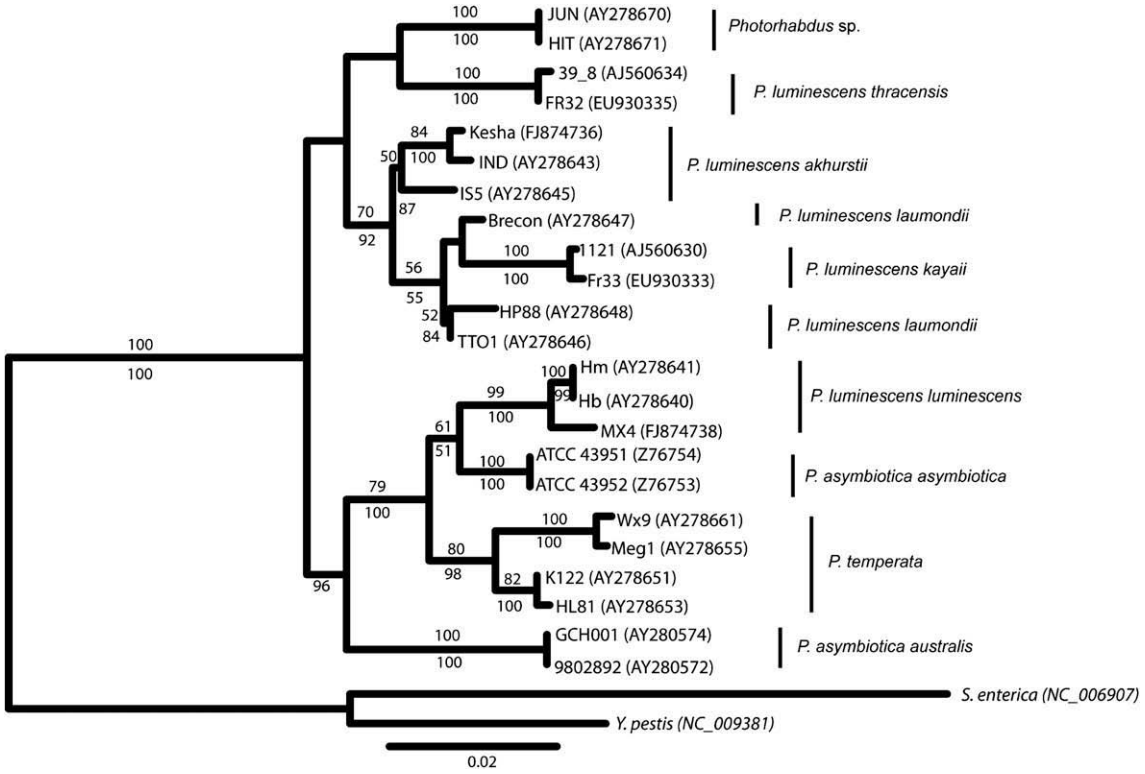

Fig. 12. 16S rDNA maximum likelihood tree as reconstructed in PHYML using the TrN + *I* + *G* model of evolution with six substitution types and substitution rate matrix and nucleotide frequencies estimated from the nucleotide alignment. Numbers above branches represent maximum likelihood bootstrap values and numbers below branches represent Bayesian posterior probability values where analyses were concordant. GenBank Accession Numbers for each strain are indicated in parentheses adjacent to each strain name.

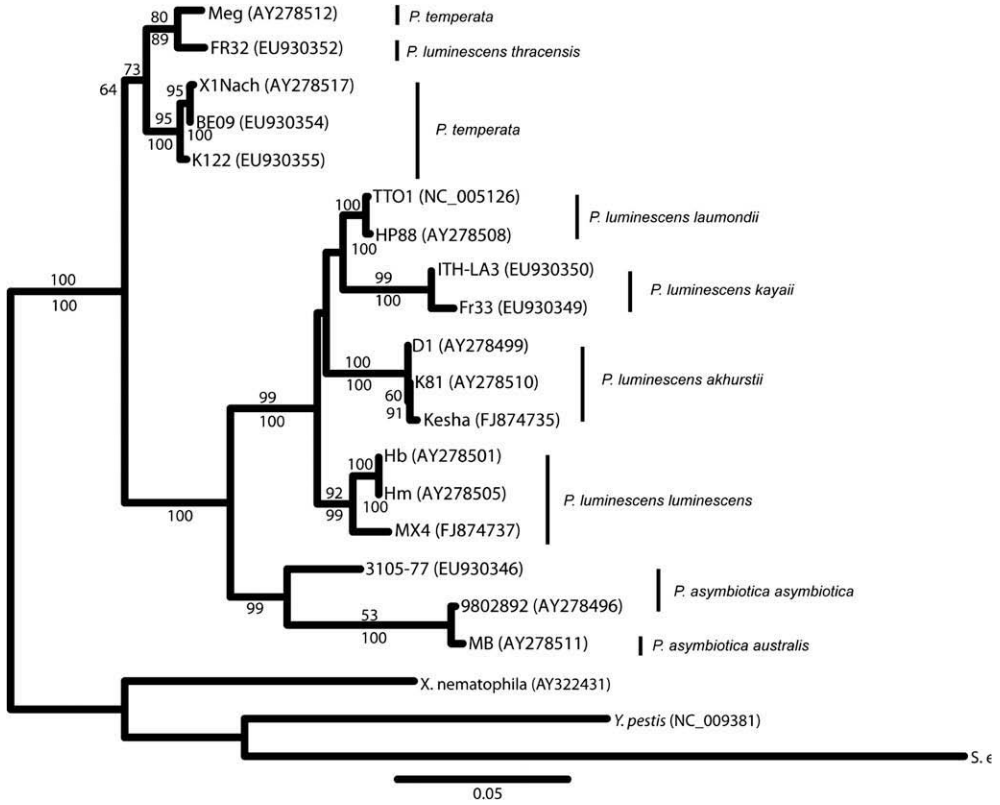

Fig. 13. Gyrase B rDNA maximum likelihood tree as reconstructed in PHYML using the TIMef + *I* + *G* model of evolution with six substitution types and substitution rate matrix and nucleotide frequencies estimated from the nucleotide alignment. Numbers above branches represent maximum likelihood bootstrap values and numbers below branches represent Bayesian posterior probability values where analyses were concordant. GenBank Accession Numbers for each strain are indicated in parentheses adjacent to each strain name.

separation in virulence to *T. molitor* among steinernematids than was observed in our study. Clearly, detection of virulence among nematode species and strains to particular target pests can vary among different studies and their associated laboratory conditions.

Regarding virulence to *P. interpunctella*, previously, both *S. riobrave* and *H. indica* were found to be virulent to this pest (Mbata and Shapiro-Ilan, 2005; Ramos-Rodríguez et al., 2006), yet the two species had not been compared with each other. In our assays; both species showed high levels of virulence and performed similarly except *H. indica* showed higher virulence at the lower rate of application (2 d post-inoculation). Although a variety of factors can affect pest suppression under field conditions, our findings regarding relative virulence among nematode species in the laboratory may be useful in predicting biocontrol efficacy.

Entomopathogenic nematode foraging strategies consist of a sit-and-wait approach (ambushers), an active search approach (cruisers), or a combination of these behaviors that are used to locate hosts (Lewis, 2002). Thus, as expected, *S. glaseri* and the heterorhabditid species, which are generally classified as cruisers, were attracted to host volatiles (Lewis et al., 1992; Grewal et al., 1994b). However, *S. carpocapsae*, an ambusher (Lewis et al., 1992; Lewis, 2002), also exhibited host attraction in our study, which is in contrast to some studies that did not detect such behavior (e.g., Grewal et al., 1994b); on the other hand, some earlier studies, in agreement with ours, have also observed response to host cues in *S. carpocapsae* (Schmidt and All, 1978; Gaugler et al., 1980). Certainly, it may be the level of response to host cues that can be considered important when considering the continuum of ambush versus cruiser foraging behavior, and in that regard our results support the model given that *S. glaseri* exhibited a higher level of directional response relative to *S. carpocapsae.*

Brown et al. (2002) observed that certain steinernematid nematodes, e.g., *S. riobrave*, can initiate an infection in *G. mellonella* at temperatures low enough to arrest the infection's progress, but once optimal temperatures are introduced pathogenesis resumes and the host dies. Similarly, we observed the same phenomenon in *H. bacteriophora* and *H. georgiana*. In fact host mortality from *H. bacteriophora* after transfer to 25 °C was similar to that caused by *S. feltiae*, which is known to be a cold tolerant nematode based on our data (having caused 100% mortality at 10 °C) as well as prior studies (Grewal et al., 1994a). Interestingly, unlike the other nematode species, *H. indica* did not produce these inapparent infections (infections without overt signs of disease) at 10 °C. The level of host mortality caused by *H. indica* did not change after transfer to a higher temperature. The ability of nematodes to invade hosts and survive in an arrested state during periods of low temperatures has adaptive value (Brown et al., 2002). We hypothesize that *H. indica* (Hom1) lacks this ability because the nematode originated in a region (Homestead, Florida) where sub-optimum temperatures are encountered less often than in the origins of other heterorhabditids tested (South Australia for *H. bacteriophora* [Poinar, 1990] and Byron, GA for *H. georgiana*). The differing abilities among nematodes to cause attenuated infections at low temperatures, and the ecological and evolutionary impact of these differences should be explored further.

While both phylogenies (16S and *gyr*B) agree on the placement of the Kesha *Photorhabdus* strain within the *P. luminescens akhurstii* clade, many aspects of the two phylogenies are incongruent. Clades resolved in the 16S rRNA phylogeny are not consistent with species-level taxonomic designations for *Photorhabdus*, with *P. luminescens luminescens* forming a monophyletic group with *Photorhabdus asymbiotica asymbiotica,* while *P. asymbiotica asymbiotica* and *P. asymbiotica australis* do not form a monophyletic group. Similar to previous studies (Akhurst et al., 2004; Hazir et al., 2004; Koppenhöfer, 2007), low or no branch support is observed at most of the deeper nodes within the 16S *Photorhabdus* tree, while robust support is present at the terminal nodes of the tree. As such, 16S rRNA, while adequate for resolving sub-specific relationships, is ineffective for resolving species level relationships within the genus *Photorhabdus* (Akhurst et al., 2004; Koppenhöfer, 2007).

With the exception of the placement of *P. luminescens thracensis* within the *Photorhabdus temperata* clade, relationships resolved using *gyr*B data are consistent with previously defined specific and sub-specific taxonomic designations. The placement of the *P. asymbiotica* clade as sister group to the *P. luminescens* clade is consistent with previous phylogenetic analyses using the *gyr*B gene (Akhurst et al., 2004; Koppenhöfer, 2007; Tailliez and Boemare, 2009) as well as an analysis that utilized a combined dataset of *gyr*B and *gln*A (Gerrard et al., 2006). More robust support at deeper nodes in the *gyr*B phylogeny suggests that *gyr*B is more capable than 16S at resolving species level relationships within the genus *Photorhabdus* (Akhurst et al., 2004; Koppenhöfer, 2007).

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