Research Article

Isolation and identification of entomopathogenic nematodes (Nematoda: Rhabditida) from the Eastern Black Sea region and their biocontrol potential against *Melolontha melolontha* (Coleoptera: Scarabaeidae) larvae

Zeynep ERBAŞ[1], Cihan GÖKÇE[1], Selçuk HAZIR[2], Zihni DEMİRBAĞ[1], İsmail DEMİR[1],*
[1]Department of Biology, Faculty of Sciences, Karadeniz Technical University, Trabzon, Turkey
[2]Department of Biology, Faculty of Science, Adnan Menderes University, Aydın, Turkey

Abstract: A survey was conducted for entomopathogenic nematodes in various agricultural fields in the Eastern Black Sea region of Turkey. A total of 77 soil samples were collected from 15 distinct geographic areas during 2010–2011. Seven entomopathogenic nematode isolates (ZET02, ZET04, ZET09, ZET28, ZET31, ZET35, and ZET76) were detected from the soil samples (9.1% positive) using the Galleria baiting technique. Morphological and molecular characterizations of the isolates were performed for species identification. Five isolates were identified as *Heterorhabditis bacteriophora* (ZET02, ZET04, ZET09, ZET28, and ZET35) and 2 isolates were identified as *Steinernema feltiae* (ZET31 and ZET76). The efficacy of all isolates was tested on *Melolontha melolontha* larvae in plastic boxes and pot experiments. Different concentrations of nematodes at 0, 500, 1000, or 2000 infective juveniles (IJs)/mL and 2 different temperature regimes (15 and 25 °C) were used. One hundred percent mortality was obtained from the ZET09 and ZET35 isolates at a concentration of 2000 IJs/mL at 25 °C. The same isolates also provided 100% protection with 100 IJs/cm² in strawberry planted pot experiments.

Key words: Entomopathogenic nematodes, biological control, *Melolontha melolontha*

1. Introduction

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae with their associated symbiotic bacteria (*Xenorhabdus* and *Photorhabdus*, respectively) are widely distributed in soils throughout the world (Hominick, 2002; Adams et al., 2006). These nematodes are parasites of insects, killing them within 48 h with the aid of their associated bacterial symbionts, and have a great importance as biological control agents of many insect pests (Hazır et al., 2003a; Laznik et al., 2011). Nematodes are commercially available in many countries for the control of soil-inhabiting insects (Grewal et al., 2005; Susurluk, 2011). Since they are now grown in large-scale liquid culture, production costs have been significantly reduced and their application in horticulture, agriculture, and forestry is increasing (Ehlers, 2001; Grewal et al., 2005). Several companies in Europe, Asia, and North America mass-produce nematodes either on a small scale in vivo or on a large scale in vitro using bioreactors (Shapiro-Ilan and Gaugler, 2002). EPNs are found in a variety of habitats, and the various species and isolates exhibit considerable variation in their host range, reproduction, infectivity, and conditions for survival (Bedding, 1990; Laznik and Trdan, 2012a).

The northeastern region of Turkey has suitable climatic conditions for cultivation of agricultural crops, resulting in a broad range of insect pests attacking these crops. This region also has a diversity of climatic conditions (Susurluk, 2007), suggesting the possibility of different EPN strains and species adapted to these climates. Research on EPNs in Turkey has only recently been initiated. A few surveys of native EPNs have been conducted and the presence of *Steinernema affine* (Hazır et al., 2003a), *S. carpocapsae* (Kepenekçi, 2002), *S. feltiae* (Özer et al., 1995; Susurluk et al., 2001; Hazır et al., 2003a), *S. weiseri* (Ünlü et al., 2007), *S. anatoliense* (Hazır et al., 2003b) from the genus *Steinernema* and *Heterorhabditis bacteriophora* (Susurluk et al., 2001; Hazır et al., 2003b; Susurluk and Toprak, 2006), and *H. megidis* (Yılmaz et al., 2009) have been isolated. However, the few surveys conducted in the Eastern Black Sea region of Turkey in undisturbed habitats have revealed few EPN isolates (Yılmaz et al., 2009), and no survey has been conducted to document the occurrence of EPNs in disturbed habitats.

In this study, an extensive survey was conducted to isolate and identify EPNs from disturbed habitats in the Eastern Black Sea region. In addition, the efficacy of the

* Correspondence: idemir@ktu.edu.tr

newly found isolates was evaluated against the white grubs of the common European cockchafer (*Melolontha melolontha* [L.], Coleoptera, Scarabaeidae), which is one of the most serious pests in agriculture and forestry in Turkey (Ministry of Agriculture of Turkey, 2008) and in Europe (Muska et al., 2012).

2. Materials and methods

2.1. Soil sampling and isolation of nematodes

Soil samples were collected from different agricultural areas (tobacco, strawberry, lettuce, tomato, potato, brassica, etc.) in the Eastern Black Sea region of Turkey during 2010–2011. Each sample contained 3–5 subsamples (10–20 cm deep), which were randomly taken in an area of 100 m². The subsamples were combined to form a single composite sample of approximately 1 kg. All soil samples were transported to the laboratory and were homogenized before extraction of EPNs (Hazır et al., 2003a).

Nematodes were recovered from the soil samples by using the insect-baiting technique (Bedding and Akhurst, 1975). Each soil sample was divided into 4 subsamples, placed into a clean 250-mL plastic box and baited with 8 last instar larvae of *Galleria mellonella* L. The boxes were inverted and kept in the dark at 20–25 °C. During a 7-day holding period, the samples were periodically checked for the presence of dead insects. The dead larvae were transferred individually to modified White traps (Kaya and Stock, 1997).

Nematodes were harvested within the first week of emergence and used to inoculate *G. mellonella* larvae, to confirm Koch's postulates for pathogenicity as described by Kaya and Stock (1997). To confirm their pathogenicity to insects, the infective juveniles (IJs) were transferred onto moist filter paper in petri dishes to which living *G. mellonella* larvae were added. The new generation IJs were collected in a beaker and rinsed 3 times with sterile distilled water and stored at 11 °C. Each nematode isolate was passed through *G. mellonella* every 3 months for *Heterorhabditis* and 6 months for *Steinernema* (Rosa et al., 2000).

2.2. Morphometric characterization of isolates

The newly obtained isolates, reared in vivo on *G. mellonella* larvae, were used for the morphometric characterization studies. For morphometric analysis of each isolate, 20 IJs and 20 first-generation males for *Steinernema* or males from the first generation hermaphrodite for *Heterorhabditis* were randomly selected from different infected *G. mellonella* larvae. IJs were collected for 1 week after they first appeared on cadavers (Nguyen and Smart, 1995). The males and IJs were killed and fixed using hot 4% formalin (60 °C) for 2 min and kept in this solution for 12 h at room temperature. Fixed nematodes were transferred to anhydrous glycerin and mounted on slides using cover-

glass supports to avoid flattening them. Morphological observations were made following the taxonomic criteria of Hominick et al. (1997). Measurements were taken using a Leica IM50 microscope equipped with differential interference contrast optics. Microsoft Excel was used for analysis of the morphometric variables of the males and IJs.

2.3. Molecular characterization of the isolates

Molecular characterization of the isolates was performed by analysis of the ITS rDNA sequences. DNA was extracted from 1 first-generation female for steinernematids or 1 hermaphrodite for heterorhabditids using a modified method published by Joyce et al. (1994). The specimen was cut in 8 µL of double-distilled H₂O. The nematode fragments were transferred into a microcentrifuge tube to which 8 µL of worm lysis buffer (500 mM of KCl, 100 mM of Tris-HCl (pH 8.3), 15 mM of MgCl₂, 10 mM of DTT, 4.5% Tween-20, and 0.1% gelatin) and 2 µL of proteinase K (600 µg/mL) were added. The tubes were frozen at -70 °C for 10 min and incubated at 65 °C for 1 h and then at 95 °C for 10 min. After centrifugation of the mixture at 12,000 rpm for 2 min, the top 20 µL containing nematode DNA was transferred to a clean 0.5-µL Eppendorf tube and kept at -20 °C until used.

The ITS region of the nematode rDNA was amplified by PCR in a 50 µL reaction mix containing 5 µL of the DNA suspension, 5 µL of 10X PCR buffer, 2 µL of MgCl₂ (25 mM), 1 µL of dNTP mixture (10 mM of each dNTP), 1.5 U of *Taq* DNA polymerase and 36 µL of double-distilled water, 1 µL of the forward primer TW81: 5'–GTTTCCGTAGGTGAACCTGC–3', and 1 µL of the reverse primer AB28: 5'–ATATGCTTAAGTTCAGCGGGT–3' and ddH₂O to final volume (Joyce et al., 1994). Subsequently, 5 µL of the product was loaded on a 1% agarose gel, and a target fragment was purified using a Qiagen Gel Purification Kit (Qiagen Ltd, Netherlands).

The purified PCR product was cloned into a pGEM-T easy vector and transferred to DH10β high efficiency competent cells (Promega, Netherlands), according to the manufacturer's instructions. After selection of transformed colonies, plasmid isolation was performed and digested by restriction enzymes to confirm whether the gene was successfully cloned into the vector or not. Plasmid DNA samples that had the right clone were sequenced (Macrogen, Korea). The obtained sequence of *Heterorhabditis* and *Steinernema* isolates were compared with sequences of the *Heterorhabditis* and *Steinernema* species available in GenBank (NCBI). The DNA sequences were edited using BioEdit (Hall, 1999) with sequences of related species and new isolates available in GenBank. The evolutionary relationship of the isolates with 16 species for *Heterorhabditis* and 14 species for *Steinernema* were evaluated (Swofford, 2002). Phylogenetic (neighbor

joining) analyses the ITS sequence data were done using MEGA (Tamura et al., 2007). *Caenorhabditis elegans* obtained from GenBank (EU131007) was used as the outgroup taxon.

2.4. Pathogenicity of the isolates against *Melolontha melolontha* larvae

Larvae of *Melolontha melolontha* were collected from various agricultural fields (strawberry, tomato, and potato) in the vicinity of Trabzon, Turkey, in autumn 2011. They were obtained by digging out the soil and placing them into plastic boxes (17 × 11 × 7 cm) with some soil and roots, covered with perforated lids to permit airflow, and transported to the laboratory.

2.5. Pathogenicity tests

Experiments were conducted for all nematode isolates to determine their pathogenicity on *M. melolontha*. Healthy third instar *M. melolontha* larvae were used to evaluate the efficacy of the nematodes with tests carried out in plastic cups (3.5 × 6 × 7 cm). Ten grams of sterilized sand was adjusted to 7% (w/v) moisture by adding distilled water. Nematode concentrations were calculated according to the technique of Smith (2000). In dose response experiments, 0, 500, 1000, or 2000 IJs per larva were inoculated onto the sand in each cup. The final moisture content for the sand was 10%. The treated cups were kept at room temperature for 1 h, at which point 1 third instar larva was placed on the sand surface of each cup and the cups were capped with a lid. Control cups were prepared as above except that they were only treated with distilled water. Experimental cups were incubated at 2 different temperatures (15 °C and 25 °C) in the dark. Five days later, each cup was checked and the number of dead larvae was recorded. The infection of the insects with nematodes was confirmed by dissection with the aid of a stereomicroscope. All dead larvae were placed individually onto White traps and the emergence of IJs from the cadavers was recorded. There were 10 replicates for each nematode isolate and the control experiment was conducted 3 times.

2.6. Pot experiments

After the sand experiments in plastic cups, pot experiments were conducted to determine the susceptibility of *M. melolontha* grubs to 3 nematode isolates (ZET09, ZET31, and ZET35) using strawberry plants in pots. The experiments were established according to the technique of Ansari et al. (2003). Each plastic pot (6 cm deep, 3.4 cm in diameter, and a surface area of 9 cm²) was filled with about 80 g of autoclaved soil, planted with a strawberry plant, and watered every 2–3 days until the end of the experiment. The strawberry was allowed to grow for 1 week before 5 third instar larvae were placed on the soil surface in each pot. After 2 days, IJs suspended in water suspension were applied at the rate of 100 IJs/cm² to

each pot. The controls were treated with water only. The air temperature in the laboratory was about 25 °C. There were 5 replicates for each nematode isolate and the control experiment was conducted twice.

2.7. Statistical analysis

One-way analysis of variance was used to compare the mortality of *M. melolontha*. Means were compared at the P = 0.05 level, and Duncan's test was used to separate means. Arcsine transformation was carried out on mortality (%) before analyses.

3. Results

A total of 77 soil samples from the Eastern Black Sea region of Turkey were surveyed for EPNs, of which 7 (9.1%) resulted in positive nematode isolates (Table 1). Two of the samples were positive for *S. feltiae* and 5 were positive for *H. bacteriophora*.

3.1. Morphometric and morphological identification

The morphometric and morphological examination of IJs and males of all nematode isolates matched the original descriptions of the respective species (Tables 2 and 3).

3.2. Molecular characterization of nematodes

Based on the ITS rDNA sequences, 5 of the 7 isolates were identified as *H. bacteriophora* and 2 of the isolates were *S. feltiae*, confirming our morphometric and morphological data. The evolutionary relationship of the isolates and other closely related species were evaluated. Approximately 700–850 bp segments of ITS rDNA were used for the phylogenetic analysis by MEGA. The phylogenetic analysis of the ITS rDNA sequence data placed *Heterorhabditis* and *Steinernema* isolates in a clade with other isolates of *Heterorhabditis* and *Steinernema* species (Figures 1 and 2).

3.3. Pathogenicity of the isolates against *Melolontha melolontha* larvae

In the plastic box experiments, all isolates caused mortality to *M. melolontha* larvae ranging between 33% and 100% at 25 °C (Figures 3 and 4). However, species/strain of nematode, application rate of IJs, and ambient temperature affected the pathogenicity of the isolates. Increasing the concentration of nematodes and temperature gradually increased the larval mortality. The mortality rate at 25 °C was greater than at 15 °C for all tests. *H. bacteriophora* was more effective than *S. feltiae* strains. The highest and fastest mortality (100%) on *M. melolontha* larvae was obtained with *H. bacteriophora* strains (ZET09 and ZET35) at 2000 IJs at 7 days postinfection in the sand experiments (F = 155.336; df = 6, 20; P < 0.05). After 7 days, applications of 500 IJs/mL (F = 164.708; df = 6, 20; P < 0.05) and 1000 IJs/mL (F = 175.433; df = 6, 20; P < 0.05) of *H. bacteriophora* isolate ZET09 resulted in 66% and 94% larval mortality, respectively.   Analysis of the pooled results showed that larval mortality of *M. melolontha* was significantly

Table 1. Characteristics of positive localities for entomopathogenic nematodes.

Isolates (GenBank accession numbers)	Sampling time	Coordinate	Altitude (m)	Species
ZET02 (JX544063)	3 April 2010	N 40°45′ E 39°41′	89	*H. bacteriophora*
ZET04 (JX544064)	3 April 2010	N 40°50′ E 39°41′	156	*H. bacteriophora*
ZET09 (JX544065)	15 April 2010	N 40°40′ E 39°39′	364	*H. bacteriophora*
ZET28 (JX544066)	30 May 2010	N 40°39′ E 39°40′	404	*H. bacteriophora*
ZET31 (JX544067)	30 May 2010	N 40°39′ E 39°40′	384	*S. feltiae*
ZET35 (JX544068)	22 June 2010	N 40°37′ E 39°41′	365	*H. bacteriophora*
ZET76 (JX544069)	28 August 2010	N 40°38′ E 39°39′	1134	*S. feltiae*

influenced by temperature at 15 °C (F = 155.336; df = 6, 20; P < 0.05) and 25 °C (F = 145.592; df = 6, 20; P < 0.05). The highest mortality obtained with the *Steinernema* isolates was 83% from *S. feltiae* ZET31 at 2000 IJs/mL at 7 days postinfection.

3.4. Pot experiments
We observed significant differences among the *H. bacteriophora* strains (ZET09 and ZET35) and the *S. feltiae* (ZET31) in the pot experiments (F = 3828.500; df = 2, 8; P < 0.05). The nematodes protected the strawberry plants from insect damage and allowed them to survive in the pots when the ZET09 and ZET35 isolates were applied. Strawberries applied with ZET31 were observed to be stunted and dying, whereas all strawberries in the control were dead at the end of day 15.

M. melolontha larvae were highly susceptible to ZET09 and ZET35 isolates in the pot experiment (Figure 5). Larval mortality reached 100% with these isolates after 15 days of treatment, and we confirmed microscopically that death had been due to nematodes. Larval mortality caused by *S. feltiae* was 42% (Figure 5).

5. Discussion
The current study represents the first survey of disturbed habitats and the second systematic survey of indigenous EPN species in the Eastern Black Sea region of Turkey, which has a variety of different climatic regions with high temperatures and humidity. With large agricultural areas and a mountainous region with different continental climate and altitudes that also result in a high diversity of insects, it is reasonable to suppose that the Eastern Black Sea region is an undisturbed niche for a number of different species and strains of EPNs (Özer et al., 1995; Hazır et al., 2003a; Yılmaz, 2009).

During the survey of agricultural fields, 7 positive EPN isolates were recovered from 77 soil samples collected from 9 towns throughout the province. Five isolates of *Heterorhabditis* and 2 isolates of *Steinernema* were identified and characterized using a combination of morphological and molecular techniques. Molecular identifications based on sequences of the ITS region were supported by the morphometric and morphological observations. According to the results obtained, the heterorhabditids and steinernematids were *H. bacteriophora* and *S. feltiae*, respectively.

The occurrence of EPNs in the study area of our survey was relatively low (9.1%). We hypothesize that the low recovery rate for EPNs for this region may be due to several factors. First, only *Galleria mellonella* was used as a trap insect and it may not be an appropriate host for all EPNs (Spiridonow and Moens, 1999). Second, only room temperature was used for baiting the soil samples during EPN isolation. Third, a high rate of chemical pesticides is being used to control pests in agricultural areas (Kary et al., 2009). Another reason may be the sample size. A larger sample size covering more areas may increase the species diversity and the number of positive samples. As is known, chemical pesticides can dramatically affect EPNs both directly and indirectly by reducing host and nematode abundance (Laznik et al., 2012b). However, we should

Table 2. Comparative morphometrics of the IJs of *Heterorhabditis* and *Steinernema* found during the survey. Measurements are in mm, and data are expressed in the form of mean ± SD (range).

| Isolates Species	H. bacteriophora (ZET02)	H. bacteriophora (ZET04)	H. bacteriophora (ZET09)	H. bacteriophora (ZET28)	S. feltiae (ZET31)	H. bacteriophora (ZET35)	S. feltiae (ZET76)	H. bacteriophora[1]	S. feltiae[2]
n	20	20	20	20	20	20	20	15	20
L	590 (587–621)	627 (601–643)	610 (599–621)	568 (540–599)	897 (847–944)	575 (568–601)	800 (685–868)	588 (512–671)	849 (736–950)
W	24 (21–26)	22 (20–25)	25 (23–27)	22 (20–24)	30 (25–36)	23 (21–25)	28 (20–74)	23 (18–31)	26 (22–29)
EP	97 (90–107)	96 (89–105)	99 (89–114)	99 (90–108)	62 (49–112)	98 (88–107)	56 (47–64)	103 (87–110)	62 (53–67)
NR	83 (74–87)	85 (78–90)	88 (82–93)	87 (81–92)	104 (68–118)	88 (82–93)	100 (89–117)	85 (72–93)	99 (88–112)
ES	127 (125–130)	126 (116–130)	127 (125–133)	122 (116–129)	134 (117–148)	127 (125–133)	126 (108–138)	125 (100–138)	136 (115–150)
T	87 (80–94)	86 (81–95)	89 (84–98)	86 (82–92)	55 (50–62)	87 (80–94)	71 (50–83)	98 (83–112)	81 (70–92)
a	21 (21–25)	23 (22–26)	25 (21–28)	25 (22–27)	29 (25–34)	27 (24–30)	30 (9–37)	25 (17–30)	31 (29–33)
b	4.7 (4.5–5.3)	4.9 (4.2–5.7)	4.7 (4.2–5.1)	4.6 (4.3–5.0)	6.7 (6.0–7.8)	4.3 (4.5–5.4)	6.4 (5.0–7.2)	4.5 (4.0–5.1)	6.0 (5.3–6.4)
c	6.8 (6.5–7.6)	6.6 (6.0–7.4)	6.7 (5.5–7.8)	6.5 (5.9–7.1)	16.2 (13.8–18.7)	6.8 (6.5–7.6)	11.4 (9.8–15.9)	6.2 (5.5–7.0)	10.4 (9.2–12.6)
D%	83 (73–89)	84 (74–90)	84 (72–88)	81 (74–89)	46 (38–78)	83 (75–90)	44 (37–51)	84 (76–92)	45 (42–51)
E%	–	–	–	114 (109–116)	112 (94–199)	–	80 (62–115)	112 (103–130)	78 (69–86)

NA = not available, n = number of species, L = total body length, EP = distance from anterior end to excretory pore, NR = distance from anterior end to nerve ring, ES = distance from anterior end and base of basal bulb, T = tail length, W = maximum body width, a = L/W, b = L/ES, c = L/T, D% = (EP/ES) × 100, and E% = (EP/T) × 100 ([1]Poinar, 1976; [2]Poinar, 1990).

Table 3. Comparative morphometrics of the males of *Heterorhabditis* and *Steinernema* found during the survey. Measurements are in µm, and data are expressed in the form of mean ± SD (range).

| Isolates / Species	H. bacteriophora (ZET02)	H. bacteriophora (ZET04)	H. bacteriophora (ZET09)	H. bacteriophora (ZET28)	S. feltiae (ZET31)	H. bacteriophora (ZET35)	S. feltiae (ZET76)	H. bacteriophora[1]	S. feltiae[2]
n	20	20	20	20	20	20	20	15	–
L	603 (564–657)	591 (534–647)	623 (571–667)	603 (564–657)	1431 (1208–1740)	591 (534–647)	1405 (1280–1547)	820 (780–960)	–
W	25	26	26	25	96	26	98	43 (38–46)	75 (60–90)
EP	116 (99–126)	114 (91–121)	113 (94–119)	116 (99–126)	87 (84–92)	114 (91–121)	98 (89–105)	121 (114–130)	–
NR	91 (87–98)	89 (84–96)	92 (85–97)	91 (87–98)	130 (118–138)	89 (84–96)	127 (111–137)	72 (65–81)	–
ES	130 (117–147)	129 (112–146)	132 (121–148)	130 (117–147)	155 (151–160)	129 (112–145)	152 (132–165)	103 (99–105)	–
T	–	–	–	–	31 (24–38)	–	30 (26–37)	28 –	–
ABD	30 (28–32)	31 (29–33)	31 (28–33)	30 (28–32)	41 (36–48)	31 (29–33)	37 (31–43)	23 (22–25)	–
SP	39 (37–44)	40 (36–45)	39 (37–43)	39 (37–44)	71 (65–78)	40 (36–45)	76 (71–81)	40 (36–44)	70 (65–77)
GL	22 (18–26)	21 (17–27)	21 (17–25)	22 (18–26)	49 (46–54)	21 (17–28)	47 (44–52)	20 (18–25)	41 (34–47)
D%	85 (75–92)	82 (76–91)	84 (75–91)	85 (75–92)	56 (53–59)	82 (76–91)	64 (60–71)	117	60 (51–64)
E%	–	–	–	–	–	–	–	–	–

NA = not available, n = number of species, L = total body length, EP = distance from anterior end to excretory pore, NR = distance from anterior end to nerve ring, ES = distance from anterior end to base of basal bulb, T = tail length, W = maximum body width, SL = spicule length, GL = gubernaculum length, a = L/W, b = L/ES, c = L/T, D% = (EP/ES) × 100, E% = (EP/T) × 100, and f = W/T ([1]Poinar, 1976; [2]Poinar, 1990).
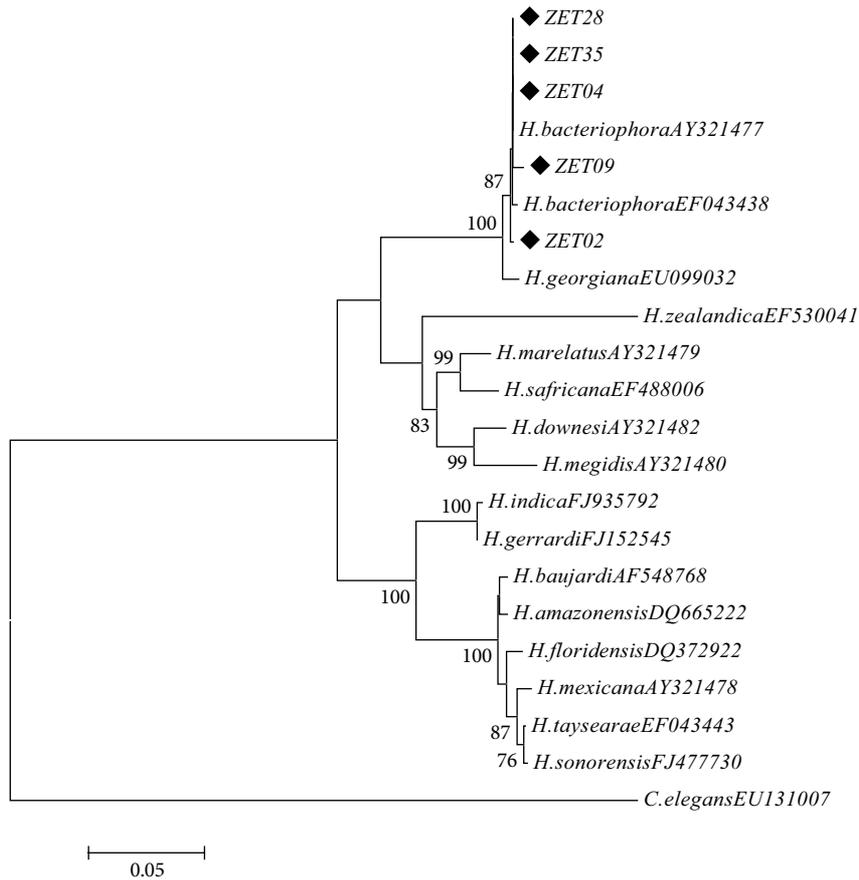
Figure 1. Phylogenetic relationships of the *Heterorhabditis* species based on analysis of ITS rDNA regions using the neighbor joining method. Numbers higher than 70% on branches indicate the percentage of 1000 bootstrap replicates. *Caenorhabditis elegans* (EU131007) was used as the out group.
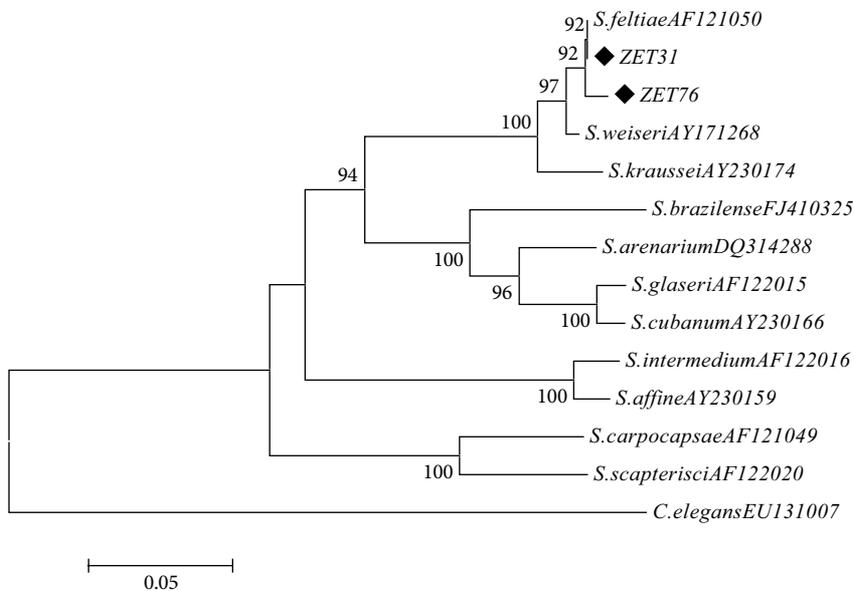

Figure 2. Phylogenetic relationships of the *Steinernema* species based on analysis of ITS rDNA regions using the neighbor joining method. Numbers higher than 70% on branches indicate the percentage of 1000 bootstrap replicates. *Caenorhabditis elegans* (EU131007) was used as the out group.
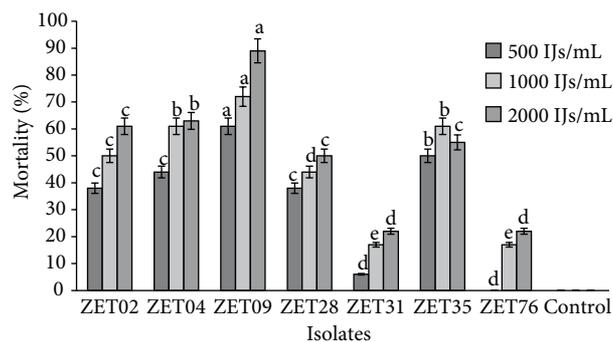
Figure 3. Dose-mortality response of third instar *Melolontha melolontha* larvae at different concentrations of nematode isolates within 7 days at 15 °C (means ± SE; columns with the same letter do not differ significantly at P = 0.05).
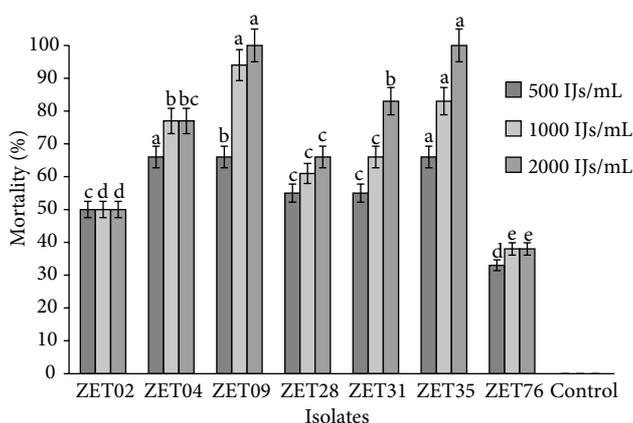
Figure 4. Dose-mortality response of third instar *Melolontha melolontha* larvae at different concentrations of isolates within 7 days at 25 °C (means ± SE; columns with the same letter do not differ significantly at P = 0.05).
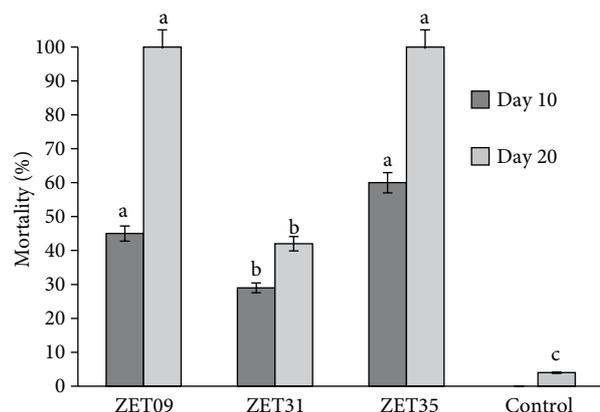
Figure 5. Percent mortality of third instar *Melolontha melolontha* larvae in pot experiments 8 and 15 days after treatment with *Heterorhabditis bacteriophora* ZET09, *Steinernema feltiae* ZET31, and *H. bacteriophora* ZET35 (means ± SE; columns with the same letter do not differ significantly at P = 0.05).

point out that the low recovery rate is not unusual, as other surveys have also recorded similar low rates (Hazır et al., 2001). For instance, similar results were obtained in the research of Laznik et al. (2009a), where they confirmed the occurrence of EPNs in Slovenian soils in only 2.5% of soil samples.

The occurrence of steinernematids and heterorhabditids in most of the surveyed areas indicates the potential role of nematodes in natural regulation of insect populations. This shows the need for further research on host ranges and characterization of these nematodes in view of their possible use for biological control, which could help to minimize the use of chemical pesticides in the Eastern Black Sea region of Turkey.

The morphometric and morphological characters of the IJs and males of isolates belonging to *Heterorhabditis* resemble the original description of *H. bacteriophora* by Poinar (1976). The IJs of the original description of *H. bacteriophora* are characterized by body length and E% (Table 2). A body length of 588 μm (512–671) in the original description of *H. bacteriophora* is longer than for isolate ZET28, which has a body length of 568 μm (540–599). However, the E% (112) of *H. bacteriophora* and that of ZET28 are (114) very similar. The spicule and gubernaculum length of the male also falls within the range of the original description, as well as the D% (Table 3). The comparisons indicated that the ZET28 isolates correspond to the original morphological descriptions of *Heterorhabditis*.

The steinernematid isolates ZET31 and ZET76 were compared with the original description of *S. feltiae* (Poinar, 1990). In comparing the IJs, we found that the body length, EP, tail length, D%, and E% fell in the range of the original description for *S. feltiae* (Table 2). As the length of the ZET31 IJ was generally longer, some of the other features were also longer than in the original description (Table 2), although most fell in the range of the original description. In the first-generation male, the lengths of the spicule and gubernaculums were longer (Table 3). Thus, the current steinernematid isolates were similar to the original description.

In Turkey, the most common EPN species isolated has been *S. feltiae*, followed by *H. bacteriophora*. Both of these EPNs are also widely distributed throughout the world (Hominick et al., 1996; Adams et al., 2006). According to Hominick (2002), steinernematids are generally recovered more often than heterorhabditids during nontargeted surveys. However, in the current survey, *H. bacteriophora* was found to be the dominant nematode species in the agricultural fields.

Investigations of EPNs in Turkey have recently been initiated and some surveys have been conducted. Özer

et al. (1995) initially recovered *S. feltiae* from the coast of the Black Sea region. Susurluk et al. (2001) found 2 *H. bacteriophora* isolates and 1 *S. feltiae* from natural areas in Ankara. Hazır et al. (2003a) also isolated *H. bacteriophora* and *S. feltiae* from Turkey. In these surveys, the most common species was *S. feltiae*, which was isolated from 10 sites in 6 regions, followed by *H. bacteriophora* from 7 sites in 7 regions. In the current study, we determined that *H. bacteriophora* was the most common and widely distributed EPN in the Eastern Black Sea region, followed by *S. feltiae*. Based on the previous survey results (Susurluk, 2007), *H. bacteriophora* was firstly reported from the eastern part of Turkey. In a study conducted in northwestern Iran, which shares a border region with Turkey and has similar climatic conditions, Kary et al. (2009) also found that *H. bacteriophora* was the most common species, followed by *S. feltiae*. The natural distributions of heterorhabditids and steinernematids are affected by climatic and geographical variations (Yoshida et al., 1998).

The main objective of this survey was to identify native species or strains of EPNs to use for controlling *M. melolontha*, which causes significant damage to tuber, strawberry, raspberry, and hazelnuts crops, and forest areas (Brandenburg and Villani, 1995). Using native EPNs is generally preferable to using exotic ones, because they are adapted to local conditions. Local species may have superior traits, making them suitable for direct commercial exploitation or as a source of genetic diversity for breeding improved strains (Choo et al., 1995). EPNs demonstrate great variation in their pathogenicity to insects; some species or strains are highly specific (Georgis and Manweiler, 1994). While some agents and methods have been used for the control the white grubs of the common European cockchafer, *M. melolontha*, none has been overly effective (Laznik et al., 2012c) and this insect continues to be a key pest of many agricultural crops (Sezen et al., 2007; Sevim et al., 2010). EPNs in the genera *Heterorhabditis* and *Steinernema* are already being successfully used in biological pest controls against white grubs (Scarabaeidae: Coleoptera) in Europe (Simard et al., 2001). Increasing interest in developing environmentally safe pest control methods and agents has inspired us to study the potential of EPNs for controlling *M. melolontha*.

In our current study, we demonstrated that third instar larvae *M. melolontha* were highly susceptible to indigenous strains *H. bacteriophora* ZET09 and ZET35, and *S. feltiae* ZET31. The efficiency of these isolates reached 100% with ZET09 and ZET35 within 7 days at 25 °C. In addition, ZET09 and ZET35 had the highest impact (100%) on *M. melolontha* in the sand tests and 100% bioinsecticidal activity at 100 IJs/cm^2 within 15 days in pot experiments. Other researchers have used EPNs as microbial control agents of *M. melolontha* and demonstrated that efficacy varies with species/strain. Thus, Berner and Schnetter (2001) reported that larval *M. melolontha* mortality by a *H. bacteriophora* isolate ranged from 5% to 30%. They also demonstrated that 1000 IJs of *H. megidis* against *Melolontha* larvae caused 10%–20% mortality. Lakatos and Toth (2006) indicated that *H. downesi* strain 267 was an effective biological control agent against *M. melolontha* with mortality reaching 90% at a soil concentration of 1000 IJs/g within 14 days. Several researchers demonstrated that the most effective strains for controlling other white grub species were *H. bacteriophora* GPS11 (83%–96%) (Grewal et al., 2004). Our study showed that ZET09 and ZET35 from the Eastern Black Sea region were more effective than the other *H. bacteriophora* against *M. melolontha*.

Research with *S. feltiae* also reported different mortality on *M. melolontha*. Berner and Schnetter (2001) showed 3% larval mortality with 1000 IJs/larva when the *S. feltiae* Ehlers strain was applied, but the best nematode in their study was the *S. glaseri* RS92 strain (60%). Laznik et al. (2009b) showed that the *S. feltiae* C67 strain at 20 °C at 3000 IJs/mL resulted in 53% mortality. However, our research demonstrated that the indigenous strain *S. feltiae* ZET31 attained a higher mortality rate (83%) of *M. melolontha* larvae, at 2000 IJs/larva and caused 43% mortality within 15 days with 100 IJs/cm^2 in pot tests. Our results indicated *S. feltiae* ZET31 was more effective than those tested by the other researchers on *M. melolontha*.

The effectiveness of EPNs in controlling Scarabaeidae is affected by biotic and abiotic conditions. One of the most important abiotic factors is temperature, which influenced the activity of the nematodes in our experiments. In our study, increasing temperature from 15 °C to 25 °C caused a significant increase in larval mortality of *M. melolontha* for all EPN isolates. The higher temperature regime sped mortality by *H. bacteriophora* from 10 days to 5 days, and increased the mortality from 20% to 83% for *S. feltiae*. In contrast, Laznik et al. (2009b) reported mortality caused by *S. feltiae* C67 was significantly less at 25 °C than at 20 °C. The higher mortality at 20 °C may be due to the EPNs having different optimal temperatures to control pests (Grewal et al., 2004).

As shown in our study, the most promising EPNs for controlling *M. melolontha* larvae were *H. bacteriophora* ZET09 and ZET35 (100%), and *S. feltiae* ZET31 (83%) at 25 °C. These species/strains may be good biological control agents against scarabs using an integrated pest management approach. Additional research is needed to determine the effectiveness of isolates in the field. Moreover, application methods and any long-term effects of these nematodes should be investigated.

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