

## A new entomopathogenic nematode species for Turkey, *Heterorhabditis megidis* Poinar, Jackson & Klein 1987 (Rhabditida: Heterorhabditidae)

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**Abstract:** During a survey on the occurrence of entomopathogenic nematodes (EPNs) in the Eastern Black Sea region of Turkey, a heterorhabditid species was isolated using the Galleria-baiting technique. Based on morphology and morphometrics, the isolate was identified as *Heterorhabditis megidis*. Sequences of the ITS region of its rDNA confirmed this identification. The species is recorded for the first time from Turkey. A more intensive survey to determine the distribution of this species, covering all parts of the Black Sea region of Turkey, is currently underway.

**Key words:** Entomopathogenic nematode, *Heterorhabditis megidis*, ITS, Turkey

### Türkiye için yeni bir entomopatojenik nematod türü *Heterorhabditis megidis* Poinar, Jackson & Klein 1987 (Rhabditida: Heterorhabditidae)

**Özet:** Türkiye'nin Doğu Karadeniz Bölgesi'nde entomopatojenik nematodların varlığını belirlemek için yapılan bir survey çalışması sırasında, Galleria tuzak böcek yöntemi kullanılarak bir heterorhabditid türü tespit edildi. Yapılan morfolojik ve morfometrik incelemeler sonucunda izole edilen türün *Heterorhabditis megidis* olduğu belirlendi. Bu identifikasyon, rRNA'nın ITS bölgesi sekansı ile doğrulandı. Bu çalışma ile *Heterorhabditis megidis* Türkiye'den ilk kez izole edilmiştir. Karadeniz Bölgesi'nin tamamını kapsayan detaylı survey çalışması halen yürütülmektedir.

**Anahtar sözcükler:** Entomopatojenik nematod, *Heterorhabditis megidis*, ITS, Türkiye

### Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* Travassos and *Heterorhabditis* Poinar are obligate pathogens that infect a wide range

of insects (Kaya and Gaugler, 1993). These nematodes are symbiotically associated with bacteria from the family Enterobacteriaceae that are pathogenic to insects; steinernematids are associated with

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*Xenorhabdus* spp. and heterorhabditids with *Photorhabdus* spp. (Boemare, 2002). The third-stage infective juvenile (IJ) is the only free-living stage that persists in the soil in search of a susceptible arthropod host. Following entry through the cuticle and/or natural openings (i.e. the mouth, anus, and spiracles), IJs release the symbiotic bacteria into the insect hemocoel, multiply, and kill the host, usually within 24-48 h (Ciche and Ensign, 2003). Nematodes feed on the symbiotic bacteria and complete 1-3 generations in the host cadaver. When food resources become depleted, new IJs either remain at the site where they were produced or disperse in search of new hosts.

When screening EPN species or strains for controlling a specific insect pest, the better adapted an EPN is to local environmental conditions and the targeted pest, the higher the level of efficacy likely to be achieved (Bedding, 1990). Therefore, it is important to survey and preserve indigenous EPN species or strains. Different nematode species and strains exhibit differences in survival, search behavior, and infectivity, which make them more or less suitable for particular insect pest control programs (Del Pino and Palomo, 1996). As such, there is great interest in finding wild populations to obtain new species and strains for possible use in biological control. At the time of the writing this paper, there were about 76 valid EPN species that belong to the Steinernematidae (60) or Heterorhabditidae (16).

Turkey, a country of 814,578 km<sup>2</sup>, is located between Europe and Asia. The country has diverse climates, suggesting the possibility of large EPN diversity. A few EPN surveys reported the presence of *S. affine*, *S. carpocapsae*, *S. feltiae*, *S. weiseri*, *S. anatoliense*, *H. bacteriophora*, and *H. marelata* (Özer et al., 1995; Kepenekçi et al., 1999; Kepenekçi and Susurluk, 2000, 2003; Susurluk et al., 2001, 2003; Kepenekçi, 2002; Hazır et al., 2003a, 2003b; Ünlü et al., 2007). Herein we report the occurrence of *H. megidis* in Turkey for the first time.

## Materials and methods

### Origin, nematode isolation, and morphological observation

An entomopathogenic nematode isolate named P69 was obtained in July 2006 from a soil sample

collected from the bank of a brook close to a tea plantation in Pazar (41°07'N, 40°45'E; 5 m asl; city of Rize, Eastern Black Sea region of Turkey). The soil sample, a composite of 5 random sub-samples (in total ca. 1 kg), was taken from an area of 100 m<sup>2</sup> and from a depth of 20 cm. Soil type was light yellow sandy loam. A subsample of 200 g was baited with 10 last instar *Galleria mellonella* (L.) larvae (Bedding and Akhurst, 1975). One week later, dead larvae were placed onto White traps (White, 1927) and emerging IJs were collected and stored at 15 °C in aerated water (Kaya and Stock, 1997). To confirm their pathogenicity to insects, the IJs were transferred onto moist filter paper in petri dishes to which living *G. mellonella* larvae were added.

First and second generation adult nematodes were obtained by dissecting infected last instar *G. mellonella* larvae 5-6 and 8-9 days after infection, respectively. For the identification of EPN, 20 specimens from each developmental stage (first generation hermaphroditic female, male, and IJ) were randomly selected from different *G. mellonella* cadavers. Nematodes were killed and fixed in hot 4% formalin (50-60 °C) and kept in this solution for 48 h. Fixed nematodes were transferred to anhydrous glycerin, according to Seinhorst's (1959) rapid method as modified by De Grisse (1969), and mounted on slides using cover-glass supports to avoid flattening. All measurements were made using a drawing tube attached to an Olympus BX50 light microscope. The nematodes were identified according to a selection of morphological and morphometrical criteria (Stock and Kaya, 1996).

### DNA extraction, PCR, and DNA purification and sequencing

DNA was extracted from a single hermaphroditic female using a modification of the method of Joyce et al. (1994). The specimen was cut in 8 µL of double distilled H<sub>2</sub>O. The nematode fragments were transferred into an Eppendorf tube to which 8 µL of worm lysis buffer (500 mM of KCl, 100 mM of Tris-Cl [pH 8.3], 15 mM of MgCl<sub>2</sub>, 10 mM of DTT, 4.5% Tween 20, and 0.1% gelatin) and 2 µL of proteinase K (600 µg mL<sup>-1</sup>) were added. After freezing (-70 °C for 10 min) the tubes were incubated at 65 °C for 1 h and then at 95 °C for 10 min.

After centrifugation (13,000 ×g for 1 min) of the tubes, 5 µL of the DNA suspension was added to a PCR reaction mixture that contained 5 µL of 10× PCR buffer, 2 µL of MgCl<sub>2</sub> (25 mM), 1 µL of dNTP mixture (10 mM each), 0.3 µL (500 nM) of each primer, 1.5 U of *Taq* DNA polymerase, and 36 µL of double distilled water, to a final volume of 50 µL. The forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') were used in the PCR reaction for amplification of the complete ITS region (Joyce et al., 1994). The amplification profile was carried out using a PTC-100 thermocycler, which was preheated to 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and then 72 °C for 8 min. After DNA amplification, 5 µL of the product was loaded on 1% agarose gel for DNA checking. Amplified products were purified using a Qiagen Gel Purification Kit (Qiagen Ltd, Leusden, The Netherlands).

The purified PCR product was cloned using pGEM-T vector and JM109 High Efficiency Competent Cells (Promega, Leiden, The Netherlands), according to the manufacturer's instructions. PCR products from selected clones were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems AH Nieuwerkerk a/d Ijssel, The Netherlands). The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge BioSystems Inc., Gaithersburg, MD, USA). The DNA sequence was edited with Chromas v.1.45 and aligned using Clustal W (Thomson et al., 1994) with sequences of related species and isolates available in GenBank.

## Results

The nematode population that was isolated from the sample taken at Pazar showed the typical developmental pattern of heterorhabditids in insect hosts. Morphological and morphometric investigations of the isolate were performed, and the data are given in Table 1 and Figure 1.

A BLAST search indicated 99% similarity between the *H. megidis* P69 sequence and that of the 2 *H.*

*megidis* isolates previously submitted to GenBank (accession numbers EF043439 and AY321480), and 97% similarity with another *H. megidis* isolate (accession number AY293284). Other comparable sequences were those from *H. downesi* (AY321482), with 93% similarity, and *H. marelatius* (EF043441), with 91% similarity. The alignment of the sequences of the above-mentioned isolates is presented in Figure 2. The sequences of all other *Heterorhabditis* species available in GenBank showed less than 90% similarity.

## Discussion

Taxonomic investigations showed that the population was conspecific with *H. megidis*. With respect to morphometrics, the main difference between the infective stages of *H. megidis* and the other species of the genus is body length. Mean length of *H. megidis* IJs, mean distance from the head to the secretory/excretory pore, and mean length of the pharynx separate this species from other described *Heterorhabditis* species. For the males, the ratio of the length of the gubernaculum to the length of the spicule distinguishes *H. megidis* (< 0.5 µm) from other species (Adams and Nguyen, 2002). The morphometrics of both the hermaphroditic female and the male of *H. megidis* P69 (Table 1) fit those of the original description of the species (Poinar et al., 1987). Characteristic data of the IJ are slightly lower than those of the original description.

*Heterorhabditis megidis* was originally isolated from the Japanese beetle, *Popillia japonica* (Coleoptera, Scarabaeidae), in Ohio (Poinar et al., 1987). The nematode is widespread in the temperate regions of Europe (Hominick, 2002). It was also isolated in Greece (Menti et al., 1997), Israel (Glazer et al., 1993), Japan (Yoshida et al., 1998), Russia (Fischer-Le Saux et al., 1998), and Canada (Mracek and Webster, 1993). This is the first record of the natural occurrence of *H. megidis* in Turkey. We isolated the nematode from sandy loam soil in Pazar, at a very low altitude. Heterorhabditids are frequently found at sites adjacent to the sea (Hara et al., 1991; Waturu, 1998; Yoshida et al., 1998; Griffin et al., 1999; Griffin et al., 2000; Rosa et al., 2000). Their

Table 1. Morphometrics (in  $\mu\text{m}$ ) of *Heterorhabditis megidis* P69. Data are expressed as mean  $\pm$  SD (range).

Characters	<i>H. megidis</i> P69			<i>H. megidis</i> (after Poinar et al., 1987)		
	Hermaphroditic female	Male	Infective juvenile	Hermaphroditic female	Male	Infective juvenile
<b>n</b>	20	20	20	15	15	15
<b>L</b>	3889.2 $\pm$ 606.0 (3058.2-4447.9)	1009.7 $\pm$ 59.7 (769.0-1071.7)	730.0 $\pm$ 29.4 (665.3-752.2)	3600 (2400-4900)	1000 (800-1100)	768 (736-800)
<b>EP</b>	224.7 $\pm$ 27.3 (196.2-259.8)	173.2 $\pm$ 14.6 (144.2-192.6)	111.7 $\pm$ 4.9 (102.8-117.9)	209 (193-270)	156 (139-176)	131 (123-142)
<b>NR</b>	180.3 $\pm$ 25.0 (149.2-207.3)	114.6 $\pm$ 10.1 (99.7-131.9)	95.9 $\pm$ 2.6 (93.4-99.6)	162 (139-178)	104 (96-112)	109 (104-115)
<b>ES</b>	248.4 $\pm$ 26.5 (218.7-281.7)	135.9 $\pm$ 4.3 (130.1-142.2)	138.6 $\pm$ 4.5 (130.9-145.5)	229 (206-269)	128 (122-134)	155 (147-160)
<b>T</b>	109.5 $\pm$ 18.9 (97.6-137.4)	41.5 $\pm$ 5.2 (34.6-47.9)	93.0 $\pm$ 7.0 (80.3-103.8)	105 (95-124)	39 (35-43)	119 (112-128)
<b>ABW</b>	66.5 $\pm$ 14.7 (48.8-84.0)	30.3 $\pm$ 4.0 (23.1-36.3)	13.7 $\pm$ 1.9 (11.2-16.8)	63 (38-86)	26 (22-31)	NA
<b>W</b>	219.3 $\pm$ 32.7 (175.9-255.0)	51.1 $\pm$ 2.3 (47.3-54.8)	26.2 $\pm$ 1.3 (23.8-27.9)	209 (120-333)	47 (44-50)	29 (27-32)
<b>SL</b>	NA	54.1 $\pm$ 2.8 (48.5-58.0)	NA	NA	49 (46-54)	NA
<b>SW</b>	NA	6.8 $\pm$ 1.5 (4.3-8.2)	NA	NA	6 (5-8)	NA
<b>GL</b>	NA	22.3 $\pm$ 1.8 (19.9-26.3)	NA	NA	21 (17-24)	NA
<b>GW</b>	NA	1.4 $\pm$ 0.6 (0.4-2.1)	NA	NA	1.1 (0.3-1.6)	NA
<b>TR</b>	NA	197.8 $\pm$ 53.5 (112.0-295.7)	NA	NA	138 (117-230)	NA
<b>V%</b>	46.0 $\pm$ 2.5 (43.5-48.9)	NA	NA	48 (45-50)	NA	NA
<b>a</b>	18.0 $\pm$ 3.5 (13.9-21.9)	20.9 $\pm$ 1.4 (17.2-22.4)	27.9 $\pm$ 1.9 (25.5-31.2)	17 (14-24)	19 (18-22)	26.5 (23-28)
<b>b</b>	15.6 $\pm$ 1.5 (14.0-17.5)	7.9 $\pm$ 0.5 (6.5-8.3)	5.3 $\pm$ 0.1 (5.1-5.4)	15 (12-21)	8 (7-9)	5 (4.6-5.9)
<b>c</b>	35.9 $\pm$ 5.9 (30.6-42.7)	26.8 $\pm$ 1.9 (23.3-29.1)	7.9 $\pm$ 0.4 (7.0-8.3)	34 (23-49)	26 (23-31)	6.5 (6.1-6.9)
<b>D%</b>	90.4 $\pm$ 1.4 (88.9-92.2)	126.4 $\pm$ 10.0 (109.3-142.7)	80.6 $\pm$ 2.9 (76.1-84.2)	91.3	122	84.5 (81-91)
<b>E%</b>	206.7 $\pm$ 14.5 (189.0-221.4)	426.2 $\pm$ 30.5 (389.0-480.3)	120.5 $\pm$ 6.7 (110.8-128.9)	199	400	110.1 (103-120)
<b>f</b>	2.0 $\pm$ 0.2 (1.8-2.2)	1.3 $\pm$ 0.1 (1.2-1.4)	0.3 $\pm$ 0.0 (0.3-0.3)	2	1.2	0.2

NA: Not available; n: number of species; L: total body length EP: distance from the anterior end to the excretory pore; NR: distance from the anterior end to the nerve ring; ES: distance from the anterior end base of the basal bulb; T: tail length; ABW: anal body width; W: maximum body width; SL: spicule length; SW: spicule width; GL: gubernaculum length; GW: gubernaculum length; TR: testis reflection; V%: distance from the anterior end to vulva/total body length  $\times$  100; a: L/W; b: L/ES; c: L/T; D%: (EP/ES)  $\times$  100; E%: (EP/T)  $\times$  100; f: W/T

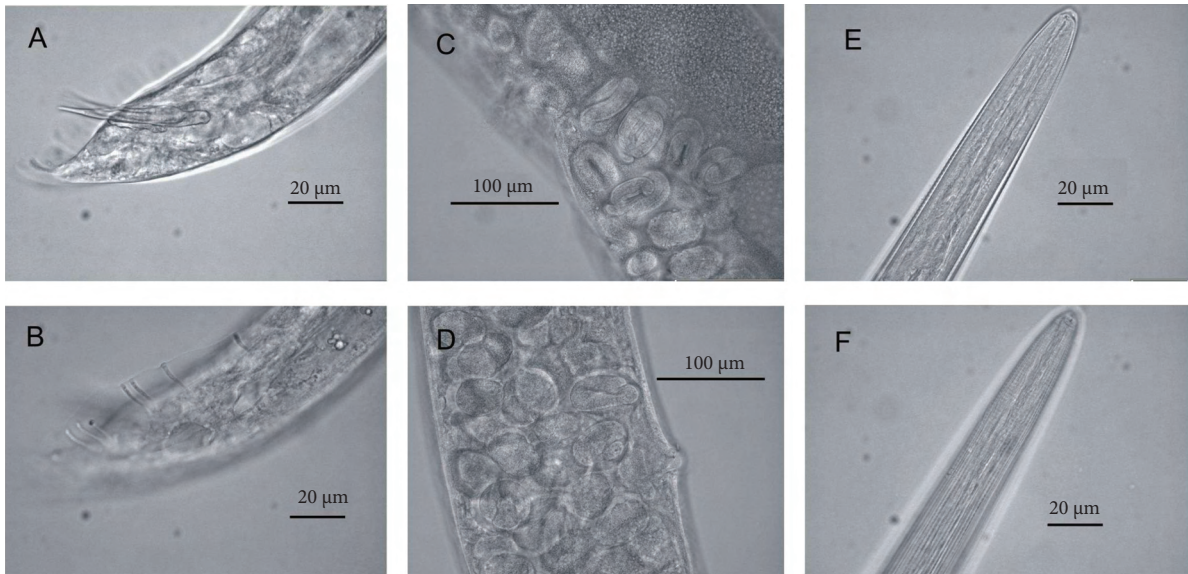


Figure 1. Light microphotographs of *Heterorhabditis megidis* P69. A: Spicule and gubernaculum. B: Bursa of a male. C: Eggs inside a female. D: Vulva of a hermaphroditic female. E: Head of an infective juvenile. F: Annulations of an infective juvenile.

<i>H. megidis</i>	P69	MOCTGAGAT	GAATCTGCG	TCGAAACCT	ATGATATGC	TTGGACAGC	AGAGTGGT	GCACCGGAA	TCAGCTTGC	TCCGCGATT	CGATCGGAT	100
<i>H. megidis</i>	KF043439	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	100
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	100
<i>H. megidis</i>	AY293284	.....	.....	.....	.....	.....	.....	.....	.....	.....G	.....	99
<i>H. downesi</i>	AY321482	.....	.....	.....	.....	.....	.....	.....T	.....T	.....A	.....	99
<i>H. marcalatus</i>	KF043441	.....	.....	.....	.....	.....	.....	.....T	.....T	.....A	.....	99
<i>H. megidis</i>	P69	CYCCGCGCA	TCYTAGCTCT	CGGCGGCT	GTCTATGTC	ATATCGGAGT	CGCTTTGAGT	CGGCGGATG	AGGATGCGT	GTGCGATGC	CGATCGGCG	198
<i>H. megidis</i>	KF043439	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	198
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	198
<i>H. megidis</i>	AY293284	.....	.....	.....G	.....	.....G	.....	.....	.....	.....	.....	199
<i>H. downesi</i>	AY321482	.....	.....	.....	.....	.....	.....	.....A	.....T	.....T	.....G	199
<i>H. marcalatus</i>	KF043441	.....	.....	.....	.....	.....	.....	.....A	.....G	.....T	.....A	199
<i>H. megidis</i>	P69	CGGCGGCGT	AGGCGTGGG	AGCGCGGCT	GGGCGGCGC	CGGCGGCG	CGGCGGCG	TCGCGGCG	TCGCGGCG	CGGCGGCG	CGGCGGCG	292
<i>H. megidis</i>	KF043439	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	292
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	292
<i>H. megidis</i>	AY293284	.....	.....	.....G	.....	.....	.....	.....	.....	.....	.....	293
<i>H. downesi</i>	AY321482	.....	.....T	.....A	.....G	.....	.....	.....	.....	.....	.....	294
<i>H. marcalatus</i>	KF043441	.....	.....T	.....T	.....G	.....	.....	.....	.....	.....G	.....A	290
<i>H. megidis</i>	P69	CGGCGGCGA	CGGCGGCGA	CGGCGGCGT	CGGCGGCGC	CGGCGGCGC	TCGCGGCGT	TCGCGGCGA	TCGCGGCGC	CGGCGGCGT	CGGCGGCGT	392
<i>H. megidis</i>	KF043439	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	392
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	392
<i>H. megidis</i>	AY293284	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	391
<i>H. downesi</i>	AY321482	.....	.....C	.....C	.....	.....	.....	.....	.....	.....T	.....	392
<i>H. marcalatus</i>	KF043441	.....	.....T	.....G	.....C	.....	.....	.....T	.....A	.....	.....	397
<i>H. megidis</i>	P69	CGGCGGCGT	ATGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	492
<i>H. megidis</i>	KF043439	.....	.....G	.....	.....	.....	.....	.....	.....	.....	.....	492
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	492
<i>H. megidis</i>	AY293284	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	491
<i>H. downesi</i>	AY321482	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	492
<i>H. marcalatus</i>	KF043441	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	497
<i>H. megidis</i>	P69	CGGCGGCGT	TCGCGGCGA	CGGCGGCGC	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	TCGCGGCGT	TCGCGGCGT	CGGCGGCGT	CGGCGGCGT	590
<i>H. megidis</i>	KF043439	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	592
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	590
<i>H. megidis</i>	AY293284	.....	.....	.....C	.....	.....	.....	.....	.....	.....G	.....A	587
<i>H. downesi</i>	AY321482	.....	.....	.....	.....	.....	.....	.....	.....	.....T	.....A	578
<i>H. marcalatus</i>	KF043441	.....	.....	.....	.....	.....	.....	.....	.....	.....G	.....A	583
<i>H. megidis</i>	P69	ATGCGGCGT	TCGCGGCGC	TCGCGGCGC	CGGCGGCGC	CGGCGGCGT	CGGCGGCGT	CGGCGGCGC	ATGCGGCGT	CGGCGGCGC	CGGCGGCGC	690
<i>H. megidis</i>	KF043439	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	692
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....G	.....	690
<i>H. megidis</i>	AY293284	.....	.....	.....	.....	.....	.....	.....	.....	.....G	.....	687
<i>H. downesi</i>	AY321482	.....	.....C	.....	.....	.....	.....	.....	.....	.....	.....	672
<i>H. marcalatus</i>	KF043441	.....	.....C	.....	.....	.....	.....	.....	.....	.....	.....	677
<i>H. megidis</i>	P69	ATGCGGCGT	TCGCGGCGA	TCGCGGCGT	ATGCGGCGT	ATGCGGCGA	TCGCGGCGA	GGGCGGCGT	TCGCGGCGA	TCGCGGCGA	TCGCGGCGA	789
<i>H. megidis</i>	KF043439	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	790
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....G	.....	789
<i>H. megidis</i>	AY293284	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	789
<i>H. downesi</i>	AY321482	.....	.....	.....	.....	.....	.....	.....	.....	.....G	.....	770
<i>H. marcalatus</i>	KF043441	.....	.....	.....	.....	.....	.....	.....	.....	.....G	.....	775
<i>H. megidis</i>	P69	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	CGGCGGCGT	803
<i>H. megidis</i>	KF043439	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	804
<i>H. megidis</i>	AY321480	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	803
<i>H. megidis</i>	AY293284	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	799
<i>H. downesi</i>	AY321482	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	784
<i>H. marcalatus</i>	KF043441	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	789

Figure 2. Multiple sequence alignment of the ITS region of *Heterorhabditis* species.

association with sandy soils remains the most robust correlation between habitat and the presence of heterorhabditids (Hominick, 2002). *Heterorhabditis megidis* is also widely distributed in turf and weedy habitats (Stuart and Gaugler, 1994; Stock et al., 1996).

We only found one population of this species, but a more intensive survey, covering all parts of the Black Sea region of Turkey, is currently underway and may yield more populations, adding important data concerning the distribution of this nematode in Turkish soils. Further research will address the associated bacteria and insecticidal activity against economically important insect pests in this region.

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