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Assessment of the Natural Presence of Entomopathogenic Nematodes in Kayseri Soils, Turkey

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ABSTRACT

Entomopathogenic nematodes (EPNs) have lots of ideal attributes as biological control agents, such as a wide host range, rapid host mortality, ease of commercial production *in vivo* or *in vitro*, active host-seeking ability, long-term efficacy, easy application, compatible with many chemical pesticides and environmental safety. In the present study, 61 EPNs isolates from 174 soil samples were collected for the districts of Kayseri Province and from 5 different habitats (Forest, pasture, field crops, vegetable, and fruit orchards). The positive soil samples ratio was (35.1%). Forty-seven of isolates were *Steinernema* with the ratio of (27%). Prevalence of *Heterorhabditis* was (8%) (14 isolates). Nematodes were identified by morphometric and molecular methods. Forty-one of these isolates were identified as *Steinernema feltiae*. Two isolates were *S. carpocapsae*, one was *S. bicornutum*, 6 were unknown *Steinernema* spp., and 14 were *Heterorhabditis bacteriophora*. Of these nematodes, *S. bicornutum* was a first record in Turkey. The soil textures of the EPN positive soils were loam, sandy loam, loamy sand and sandy clay loam with weakly basic (pH 7.57-7.91) and 1.15-3.06% organic content. The electrical conductivity of the nematode positive soils varied from 0.19 to 0.79 (none saline) dS/m.

Key words: Entomopathogenic nematodes, *Steinernema, Heterorhabditis*, natural occurrence, Turkey.

INTRODUCTION

Diseases and pests like: fungi, bacteria, viruses, insects, and weeds have a negative impact on agriculture. Controlling of them with non-toxic residues, minimal impact on non-target organisms, without leading to pest resistance has been a struggle in modern agriculture. Over years, chemical pesticides have made a great contribution to the management of pests and diseases. However, their widespread and long-term use has severely affected the abiotic and biotic environmental components. Therefore, there is an increasing social pressure to replace them gradually with eco-friendly alternative methods.

One of the effective alternatives is the use of bio-control agents such as; predators, parasitoids, microorganisms as nematodes, and pathogens including bacteria, viruses, and fungi to suppress pest populations. In terms of crop protection, although there are many different control options against pests, the biological control is highly preferred over other methods, because of its safety to human, animal and environment, maintaining the ecological balance and sustainability. Recently, as bio-control agents, entomopathogenic nematodes (EPNs) attracted attention increasingly in plant protection and have been employed to control soil-inhabiting insects in different parts of the world (Gaugler, 1981, Klein, 1990 and Smart, 1995). Above ground, insects live in cryptic habitats such as: the coddling moth, navel orange worm, carpenter worms, the artichoke plume moth and clear wing moths have also been managed successfully by using EPNs (Bedding & Miller 1981, Ralph 1981, Begley 1990, Kaya 1990, and Vreditelyami *et al.*, 1992). These nematodes have many excellent attributes such as a broad host range, rapid host mortality, simple production *in vivo* or *in vitro*, host searching ability, easy application, long-term efficacy, accordance with many chemical pesticides, being safe for the environment and no registration requirement in many countries. Bedding and Akhurst (1975) developed the *Galleria*-bait method, afterwards a number of surveys have been carried out around the world (Hominick, 2002). The habitats and soil characteristics where these nematodes were isolated vary: pastures, forests, field crops, orchards, beaches, and in calcareous, sandy loam, loam, humus and sandy, and humus and organo-mineral soils (Akhurst and Brooks 1984, Hara *et al.*, 1991 and Hominick 2002).

The present study focused on a survey of the EPNs and their presence in various soil types and habitats in different locations of Kayseri, Turkey.

MATERIALS AND METHODS

Soil sampling for entomopathogenic nematodes

One hundred and seventy-four soil samples from 5 various habitats in the districts of Kayseri, Turkey

were collected on different dates in 2011 and 2012 (Table 1). The habitats were forest, pasture, cropland (soybean/corn/cotton), vegetable, and orchard/vineyard. Five to eight random samples were picked up from a 50m^2 area at 1-3 m intervals to a depth of 15-20 cm at each site with a hand shovel or soil corer with a volume of 1500-2000 cc. The shovel was surface sterilized between sampling sites. The samples were placed in plastic bags (43 x 46 x 20 cm) and mixed well. Approximately 500 ml of soil was taken and put in plastic pots with a cover containing nine 1mm-diameter holes. Associated vegetation, date and site location were recorded on the containers. The samples were held in a cooler at 12-15ºC during transportation.

Isolation of entomopathogenic nematodes

Larvae of greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) reared in the laboratory, and then used to bioassay the soil samples within 24 h after sampling (Bedding and Akhurst, 1975). Five to seven larvae were buried in each sample and the containers were held at 25±2ºC. Checks for mortality were made 3-6 days after the larvae were buried. Dead larvae from each soil sample, after rinsing in sterile distilled water, were placed individually in a White-trap to collect infective juveniles (IJs) that migrated into the water (White, 1927). The IJs at the doses of (20-25) IJs per larva were exposed to 5-8 *G. mellonella* larvae placed on a filter paper in a (100 x 15mm) Petri dish . The IJs were cultured in the laboratory (Kaya and Stock, 1997). Positive soil samples for nematodes were analyzed for pH, electrical conductivity, organic matter and soil texture in the Soil Science Department, Faculty of Agriculture, Erciyes University, Turkey.

Morphological identification of entomopathogenic nematodes

One to seven days old 3rd stage nematodes (IJs) were used for morphological studies. Hermaphrodites of heterorhabditids were gained from the cadavers 6-7 days after the IJs infected *Galleria* larvae at 25ºC. Second generation females and males were similarly obtained 8-9 days after infection. All nematodes, used for observations, were not older than a week. Nematodes in Ringer´s solution were fixed on slides and insulated with paraffin. Observations were made on 15 males, 5 females, 5 hermaphrodites and 30 IJs for each nematode sample. Then, they were drawn and measured using a drawing camera. The standards were proposed by Nguyen and Smart (1996), Hominick (2002), and Nguyen and Hunt (2007), such as body length, maximum body diameter, anal body diameter, excretory pore position, distance from anterior end to the base of pharynx, distance from anterior end to nerve ring and tail length of IJs were used for morphological diagnosis.

Molecular identification and sequence analyses of entomopathogenic nematodes

For each population, DNA was attained from 5 IJs of each population, following the protocol of Holterman *et al.* (2006). The ITS region was amplified by a PCR by adding 1 µl DNA into a 49 µl reaction volume, including 23 µl ddH2O, 25 µl of Dream Taq PCR Master Mix (2×) (Fermentas Life Sciences, Germany), 1 µM of forward primer5'-CGTAACAAGGTAGCTGTAG-3' and 1 µM of the 5'-TCCTCCGCTAAATGATATG-3' (Ferris *et al.*, 1993). The PCR products were sequenced in a sequencing facility (Refgen, Ankara, Turkey) in both directions to obtain matching sequences of both DNA strands. The sequences were formatted and analyzed. Mega5 (Tamura *et al.*, 2013) was employed for phylogenetic analysis of ranged sequences. A maximum likelihood method based on the Hasegawa-Kishino-Yano model was employed to conclude the evolutionary history (Hasegawa *et al.*, 1985). Branches corresponding to fractions proliferated in less than 60% bootstrap replicates were taken out. To represent the evolutionary history of the taxa analyzed, the bootstrap consensus tree concluded from 200 replicates was taken (Kumar *et al.*, 2015). Initial tree(s) for the heuristic search were acquired spontaneously by implementing Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and later choosing the topology with superior log likelihood value. The Basic Local Alignment Search Tool, or BLAST (http://blat.ncbi.nlm.nih.gov/Blast.cgi) was used to compare sequences to known sequences in the public database. The ITS-r DNA PCR products from 61 new samples represented different species and populations. The sequences from this research were deposited in GenBank with the accession numbers KX462906 - KX462966.

RESULTS AND DISCUSSION

Occurrence of entomopathogenic nematodes

Prevalence of *Heterorhabditis* was (8%) of the total soil samples. The only heterorhabditid obtained was *H. bacteriophora* in 14 samples. Six, 3, 2, 2 and one isolates were found in vineyards, cropland, forest, vegetable and pasture, respectively. No nematode was found in Develi district and the most ones were obtained from Ozvatan district (55.6% of the samples) (Table 1). Nematodes were obtained from all tested habitats and isolated more from the vineyard with (54.4%), followed by pasture, vegetable, cropland and forest with (31.0, 25.9, 24.4 and 12.5%), respectively (Table 2).

Most of the obtained *Steinernema* and *Heterorhabditis* were from sandy soil, followed by

Table (1): Habitats, no. samples/habitat, no. positive samples, % of habitats where *Heterorhabditis* (H) and *Steinernema* (S) detected and total %, at Kayseri, Turkey in 2011 and 2012

Location	Habitats (No. positive samples* / no. samples tested)							
	Forest	Pasture	Orchard/ vineyard	Cropland	Vegetable	% of H	% of S	Total %
Melikgazi	1(H)/5	3(S)/9	1(H),7(S)/16	2(S)/4	1(S)/3	5.4	32.4	40.5
Talas	0/2	1(S)/4	6(S)/7	0/2	0/3	0.0	33.3	38.9
İncesu	0/1	0/2	1(S)/2	1(S)/3	1(H)/2	10.0	20.0	30.0
Yeşilhisar	0/1	0/1	1(H),1(S)/3	2(S)/3	0/2	10.0	30.0	40.0
Kocasinan	0/1	1(S)/2	1(H),4(S)/5	0/4	1(H)/2	14.3	35.7	50.0
Bünyan	1(H)/3	1(H)/3	1(H)/6	1(H),1(S)/5	0/1	22.2	5.6	27.8
Pınarbaşı	0/1	1(S)/3	0/1	1(H)/4	0/1	10.0	10.0	20.0
Sarıoğlan	0/1	1(S)/1	2(S)/2	0/2	1(S)/3	0.0	44,4	44,4
Akkışla	-	0/1	2(S)/4	0/2	0/4	0.0	18.2	18.2
Özvatan	-	0/1	1(S)/3	1(S)/2	3(S)/3	0.0	55.6	55.6
Felahiye	0/1	0/1	1(H)/2	1(H)/4	1(S)/3	18.2	9.1	27.3
Tomarza	-	1(S)/1	-	1(S)/4	-	0.0	40.0	40.0
Yahyalı	-	-	1(H),1(S)/3	0/2	-	20.0	20.0	40.0
Develi	-	-	0/3	0/4	-	0.0	0.0	0.0

Table (2): Location, habitat types and percentage of sites in which nematodes were detected at Kayseri in 2011 and 2012

Habitats	No. positive sites / no. sampling sites	No. and % of heterorhabditids	No. and % of steinernematids	Total %
Pasture	9/29	1-3.5	8-25.6	31.0
Vineyard	31/57	6-10.5	27-43.9	54.4
Forest	2/16	2-12.5	0-0	12.5
Cropland	11/45	3-6.7	8-17.8	24.4
Vegetable	7/27	2-7.4	5-18.5	25.9

Table (3): Mean (SE) of characterizations of soil containing *Heterorhabditis* spp. and *Steinernema* spp., no. and % of positive samples at Kayseri in 2011 and 2012

Soil texture	(No. and %) Positive samples		% Organic matter content	Electrical conductivity (dS/m)	pH
	Total	Species			
Heterorhabditis spp.					
Loam	1-1.2	1-6.67	2.43	0.3	7.68
Sandy loam	10-15.4	10-66.7	2.96	0.34±012	7.57
Loamy sand	1-1.3	1-6.67	1.5	0.19	7.91
Sandy clay loam	3-4.7	3-20	2.31	0.38±0.23	7.66
Overall mean			2.3	0.43±0.26	7.71
Steinernema spp.					
Loam	12-19.7	12-25.5	3.06	0.61±0.59	7.68
Sandy loam	25-37.9	25-49.01	3.03	0.47±0.53	7.68
Loamy sand	7-10.6	7-13.72	1.15	0.33±0.16	7.71
Sandy clay loam	7-9.2	7-11.8	2.95	0.39±0.16	7.68
Overall mean			2.55	0.45±0.12	7.69

loam, sandy loam, loamy sand and sandy clay loam soils. Electrical conductivity averaged 0.43 dS/m for all *Heterorhabditis*-positive soils and 0.45 dS/m for *Steinernema*-positive soil samples. Soil pH ranged from 7.57 to 7.91 for *Heterorhabditis* and *Steinernema*-positive soil samples (Table 3).

Morphological identification of entomopathogenic nematodes

Sixty-one EPNs isolates from 174 soil samples with a ratio of (35.1%) were collected from the tested habitats in the districts of Kayseri Province (Table 1). They were identified by morphometric and molecular methods. Forty-one of these isolates were identified as *Steinernema feltiae*. Two isolates were *S. carpocapsae*, one was *S. bicornutum*, 3 were *Steinernema* spp., and 14 were *Heterorhabditis bacteriophora*. *S. bicornutum* was the first record in Turkey. Morphometrics of obtained IJs of nematode species are given in table (4). Fifty isolates were

Table (4): Mean (SD) morphometrics of the infective juveniles of *Heterorhabditis* and *Steinernema* species obtained

Character	*Steinernema feltiae*	*Steinernema carpocapsae*	*Steinernema bicornutum*	*Steinernema* spp.	*Heterorhabditis bacteriophora*
Total body length (L)	909 ± 38 (792-918)	572 ± 41 (503-645)	776 ± 23 (744-817)	916±43.1 (847-968)	599 ± 21 (567-628)
Body length divided (/) by body width (a)	30.3 ±1.6 (28.2-33.5)	21.2 ±1.7 (19-24.3)	25.9 ± 1.33 (26-30)	29.5 ± 0.8 (27-31)	25 ± 2.6 (17-28)
Body length (/) oesophageal length (b)	6.7 ± 0.2 (6.1-7)	4.7 ± 0.4 (4.3-5.1)	6 ± 0.3 (5.5-6.7)	7.4 ± 0.3 (6.6-7.8)	4.8 ± 0.3 (4.2-5.3)
Body length (/) tail length (c)	10.6 ± 0.4 (10-11.4)	10 ± 0.6 (9-11)	10.6 ± 0.8 (11-13.4)	11 ± 0.5 (10-12)	6.2 ± 0.4 (5.5-6.9)
%D [(EP/ES)X100]	47 ± 1.7 (46-52)	32 ± 1.5 (29-34)	47 ± 2.4 (45-54)	50±2.5 (46-56)	85 ± 1.6 (82-88)
%E [(EP/TL)X100]	76 ± 2.2 (72 – 78)	68 ± 2.3 (64-70)	86 ± 4.1 (85-101)	76 ± 3.3 (74-82)	109 ± 3.9 (102-116)
Nerve ring (NR)	116 ± 2.5 (111-120)	88 ± 2.7 (83-92)	93 ± 4.1 (85-100)	96 ± 4.2 (90-105)	88 ± 3.2 (83-94)
Excretory pore (EP)	65 ± 2.2 (60-69)	39 ± 2.5 (34-44)	61 ± 2.6 (55-66)	62 ± 3.1 (55-69)	105 ± 3.3 (100-113)
Pharynx (ES)	139 ± 3.2 (133-145)	123 ± 3.7 (118-133)	130 ± 5.4 (114-136)	123 ± 3.4 (115-129)	124 ± 3.6 (118-131)
Body diameter (W)	30 ± 1.6 (27-32)	27 ± 2.7 (22-29)	30 ± 1.4 (26-31)	31 ± 1.4 (28-33)	24 ± 3.1 (21-31)
Tail length (TL)	86 ± 2.9 (75-85)	57 ± 3.1 (49-59)	71 ± 4 (61-74)	82 ± 4.3 (66-85)	96 ± 5.3 (84-106)

N= 25

Steinernema with the ratio of (28.7%) and *Steinernema feltiae* was obtained from 41 samples, followed by *S. carpocapsae*, *S. bicornutum* and *Steinernema* spp. from 2, 1 and 6 samples, respectively (Table 1).

Molecular identification and sequence analyses of entomopathogenic nematodes

Sixty-one EPN populations were sequenced and identified, using their ITS-rDNA region. PCR amplification of the ITS regions of the rDNA of 47 populations belonging to the family of Steinernematidae and 14 populations belonging to the family of Heterorhabditidae, produced a single fragment which was about 800 bp. This fragment was purified and sequenced in a sequencing facility. Phylogenetic analysis grouped the obtained sequences Refgen, Ankara) with exception of 3 *Steinernema* isolates supported by bootstrap values higher or equal to (60%), together with a sequence from GenBank, representing each of the different *Steinernema* or *Heterorhabditis* species available. All populations were identified as *S. feltiae* (41 populations), *S. carpocapsae* (2 populations), *S. bicornutum* (1 population), *H. bacteriophora* (14 populations) and *Steinernema* spp. (3 populations). The results confirmed the identity obtained by morphological analysis. A discrete Gamma distribution of the family Steinernematidae to model evolutionary rate differences among sites [5 categories (+G, parameter = 0,4514)]. The analysis included 112 nucleotide sequences. All positions containing gaps and missing data were removed. There were 398 positions as total in the final data set (Fig. 1). For the family Heterorhabditidae, a discrete Gamma distribution, the same as in the family of Steinernematidae was used to model evolutionary rate differences among sites [3 categories (+G, parameter = 1,2126)]. The analysis included 30 nucleotide sequences. All positions including gaps and missing data were removed. There were 606 positions as a total in the final data set. Evolutionary analyses were carried out by MEGA7 (Kumar *et al.*, 2015) (Fig. 2).

Based on the ITS-rDNA sequences, *S. feltiae* showed intraspecific polymorphism. The *S. feltiae* populations in this study were definitively distinguished into 2 groups in phylogenetic tree grounded on the ITS-I, II, and 5.8S rDNA sequences. Two groups were composed plainly from each other in the Steinernematidae sequences which were endorsed by a moderate to high bootstrap value of (99%). These groups were added to GenBank (Fig. 1). There was no intraspecific polymorphism in *S. carpocapsae*. The *Steinernema* spp. populations also showed no intraspecific polymorphism and they were very closely related to *S. ichnusae* (EU421129), *S. litorale* (AB243441), *S. citrae* (EU740970) and *S. hebeiense* (DQ105794), endorsed by a moderate to high bootstrap value of (81%). Also, there was no intraspecific polymorphism among the

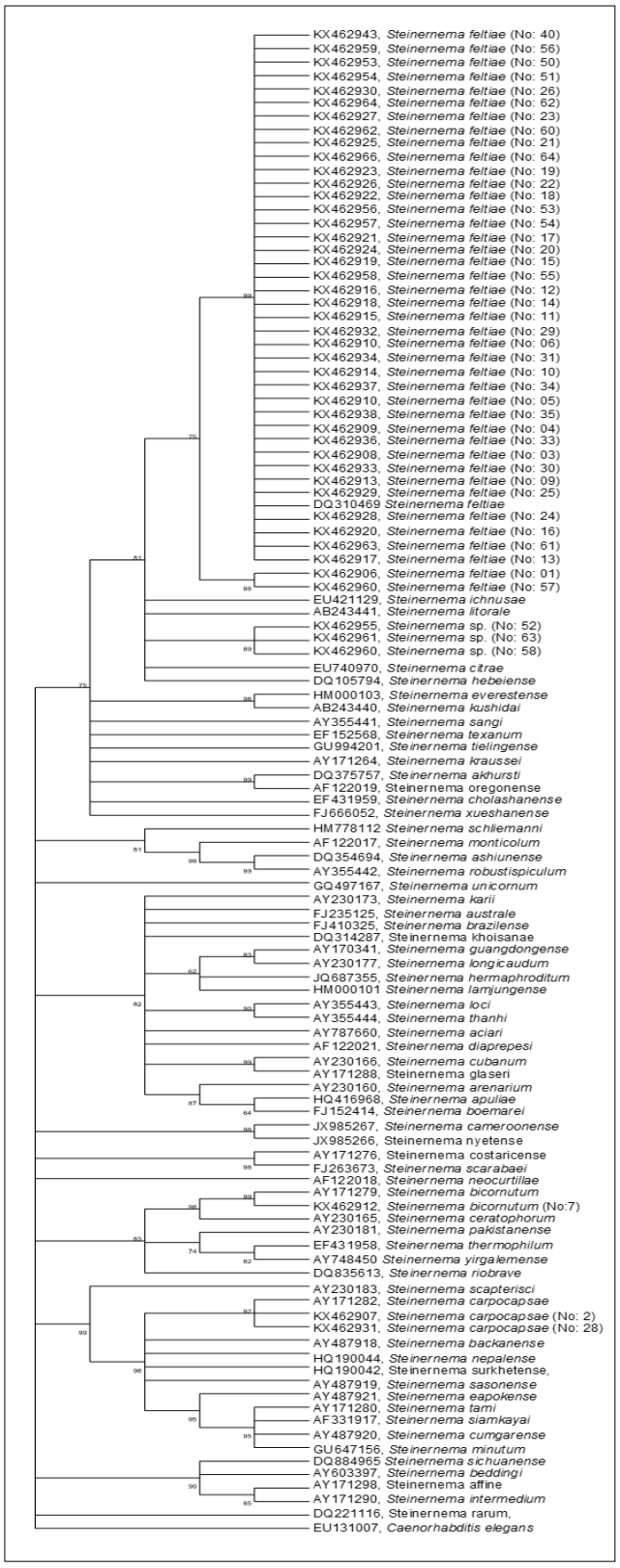


Fig. (1): Phylogenetic tree (Maximum Likelihood) constructed through the ITS sequence alignment from 47 *Steinernema* isolates of Bootstrap values (more than 60%) are given for the appropriate clades.

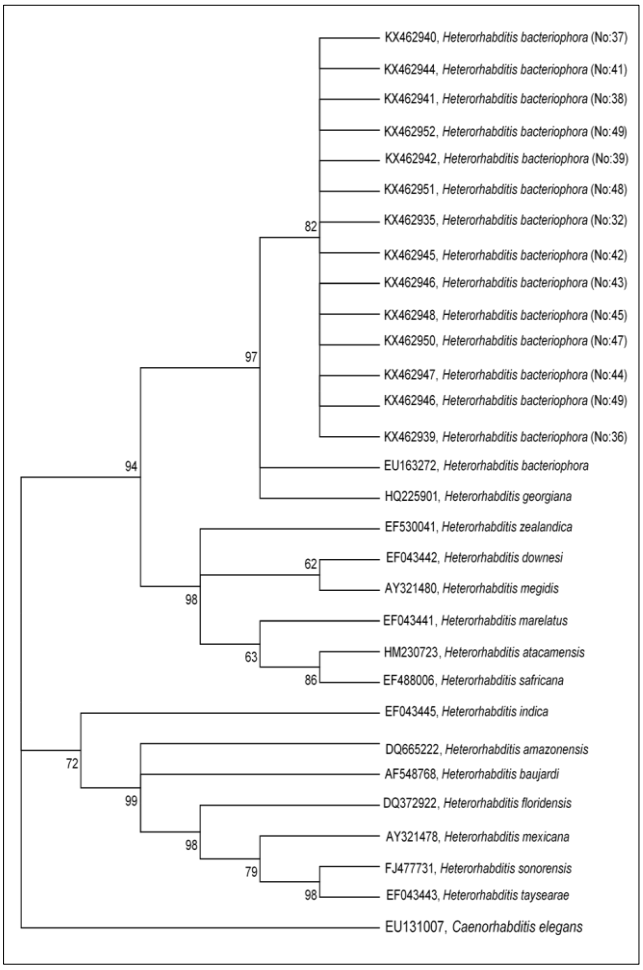


Fig. (2): Phylogenetic tree (Maximum Likelihood) constructed through the ITS sequence alignment from 14 populations of *Heterorhabditis bacteriophora* Bootstrap values (more than 60%) are given for the appropriate clades.

H. bacteriophora populations that was endorsed by a moderate to high bootstrap value of (82%).

Electrical conductivity for both *Heterorhabditis* and *Steinernema*-positive soil samples were very similar. It is known that EPNs are actually well adapted to different soil salinity (Thurston *et al.*, 1994). Also, it was found that survival and pathogenicity of steinernematids was reduced only a little bit as the tested soil pH dropped off from pH 8 to pH 4, but survival and pathogenicity drastically decreased at pH 10. The organic matter of *Heterorhabditis*-positive soils averaged 2.3% (1.5-2.96). For *Steinernema*-positive soils it was 2.55 (1.15-3.06) (Kung *et al.*, 1990) (Table 3).

Frequencies of EPNs varied. Steinernematids showed more than heterorhabditids in the present survey (28.7% vs. 8%). Hominick and Briscoe (1990) recovered *Steinernema* from (48.6%) of 403 sites, but only one site produced heterorhabditids in British soils. Steinernematids occurred in 33 of 35 sites but heterorhabditid nematodes were isolated from only 2 sites in Spanish soils (Gracia and Palomo, 1996). These findings are similar to the results obtained in this study. On the other hand, Akhurst and Brooks (1984) isolated heterorhabditids (*H. bacteriophora*) in (16.9%) of samples and steinernematids (*S. glaseri*, *S. feltiae* (Filipjev), and *S.* sp.) in (2.2%) of samples in North Carolina. In a survey conducted in Tennessee nursery soils, half of the positive soil samples contained heterorhabditid nematodes (Rueda *et al.*, 1993). In contrary to the present study, Hara *et al.* (1991) also obtained (6.3%) *Heterorhabditis* and (0.06%) *Steinernema*.

Morphological characters of steinernematids and heterorhabditids are indispensable and supplied base data in their identification. It is a prerequisite for proper classification, biodiversity study and their potential use in biological control programs. DNA sequence and phylogenetic analysis of EPNs provide additional criteria for identifying and delimiting species within Steinernematidae and Heterorhabditidae. DNA analysis based on PCR is a strong tool to identify the nematodes. It is well developed and broadly employed, principally in the identification of nematodes, which have similar morphology. Sequences of the internal transcribed spacer (ITS) regions of steinernematids have been employed by various authors (Nyugen *et al.*, 2001 and 2002) in taxonomic and phylogenetic studies. The recent development of PCR and DNA sequences of internal transcribed spacer (ITS) regions yielded more detailed information about variation. These spacer sequences have been used successfully to diagnose species and population of nematodes (Szalenski *et al.*,1997 and Cherry *et al.*,1997). Analyses of ITS rDNA sequences also have been used to reconstruct phylogenetic relationships and delimit nematode species (Adams *et al.*,1998 and Nadler *et al.*, 2000). Sequence analysis of ITS and 18S rRNA gene supported well the identification and redescription of isolates of *Steinernema feltiae, S. carpocapsae, S. bicornutum, Heterorhabditis bacteriophora* and *Steinernema* spp.

Intraspecific polymorphism on the basis of sequence analysis was shown among populations of *S. feltiae* in this study as in former studies (Blok *et al.*,1998 and Subbotin *et al.*, 2000). Two subgroups occurred in the obtained *S. feltiae* populations. These were distinguished from each other with (63%) rate. Because of this, they were called as Group 1 and Group 2. However, there was no intraspecific polymorphism of *S. carpocapsae*. Moreover, *Steinernema* sp. population had no intraspecific polymorphism. More genetic dissimilarities were revealed by using the same or extra chromosomal (mitochondrial) DNA (Hominick *et al.*, 1997). Obtained results showed a little intraspecific polymorphism between *H. bacteriophora* populations. Furthermore, similarly, little polymorphisms in ITS sequences were previously reported for other *H. bacteriophora* populations (Hominick *et al.*, 1997).

This study highlighted the eco-regional distribution of EPNs in the central Anatolia. Further detailed surveys and comprehensive morphological and molecular studies, especially the phylogenetic relation among the population of steinernematids and heterorhabditids using populations from different regions in Turkey are necessary to determine the distribution patterns of EPNs more accurately and to determine whether differences in an endemic population occur. These informations can be used to improve the biological control management of EPNs in Turkey.

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