Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Isolation, identification, and pathogenicity of entomopathogenic nematodes occurring in Cappadocia Region, Central Turkey

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Abstract

A survey for naturally occurring entomopathogenic nematodes (EPNs) was conducted in various agricultural fields in central Turkey, Nevsehir, between 2015 and 2016 years. EPNs were recovered from 20 of 112 soil samples (17.9%). Seventeen isolates were identified as *Steinernema feltiae* (Rhabditida: Steinernematidae). One was unknown *Steinernema* sp. (Rhabditida: Steinernematidae), and two were *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae). The most common species was *S. feltiae*, which was recovered from 5 out of 8 sites. The pathogenicity of these isolates was evaluated on the last larval instar of *Galleria mellonella* L. (Lepidoptera: Pyralidae) at different concentrations (25, 50, 100 IJs) in the laboratory at 25 ± 1 °C. Maximum mortality rate (87%) was achieved from the *H. bacteriophora* AVB-15 isolate at the lowest concentration after 48 h post-inoculation, while the only isolate that caused maximum mortality of (100%) at the concentration of 50 IJs/ml, 24 h post-inoculation was *S. feltiae* DDKB-17 isolate. All isolates showed high pathogenicity on *G. mellonella* last instar ranging from 63 to 100% at the concentration of 100 IJs/ml, 48 h post-inoculation. The present survey revealed that these EPNs are commonly present at Nevsehir, and they might have a good potential in biological control of insect pests.

Keywords: Entomopathogenic nematodes, *Heterorhabditis bacteriophora*, Soil nematodes, *Steinernema feltiae*, Survey

Background

Long-standing improper use of pesticides has led to an increased risk of contamination of the environment and harmful effects on food security. Concerns about the destructive effects of chemical pesticides have prompted a growing number of researchers across the world to find more sustainable and environmentally friendly novel control methods that offer the production of safer foods to consumers in the management of pests (Canhilal et al. 2016). Entomopathogenic nematodes (EPNs) from the families Heterorhabditidae and Steinernematidae possess many features that distinguish them from other EPNs and enable them to provide a more successful control against insect pests that live in both soil and cryptic habitats (Hazir et al. 2004; Kaya et al. 2006 and Lacey and Georgis 2012). Recently, EPNs as biological control agents proved to be successful in the management of major insect pests in agriculture (Yuksel et al. 2018; Yuksel and Canhilal 2018 and Majić et al. 2019). Therefore, there is a great scientific interest in both obtaining EPNs and determining their efficacy against the most damaging species (Canhilal et al. 2016 and Kepenekci et al. 2018).

The occurrence and distribution of EPN have been studied in some parts of Turkey, but no study has been conducted at Nevsehir (Kepenekci and Susurluk 2000; Canhilal et al. 2016 and Canhilal et al. 2017).

The objective of the present study was to determine the distribution and species composition of EPNs at Nevsehir Province, and to evaluate their pathogenicity on *Galleria mellonella* L.

Materials and methods

A survey took place between 2015 and 2016 years, and soil samples were collected randomly with a hand shovel from different habitats such as forest, pasture, field crops, vegetable, and fruit orchards following rainy days in spring (March–June) and autumn (September–October) (Majić et al. 2018). A total of 112 soil samples were collected. Each one (approximately 1 kg) consisted of 6–8 subsamples

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taken at a depth of 10–15 cm over an area of 30–40 m^2. Samples were placed in polyethylene bags to avoid dehydration, kept in a cool box (approximately at 15 °C) and transported to the laboratory. The hand shovel was sterilized with 70% ethanol after each sampling.

EPN were recovered from the soil samples using *Galleria* traps method described by Bedding and Akhurst (1975). Subsamples, taken from each soil sample, were placed into a clean plastic box with 8 individuals of *G. mellonella* last instar. The boxes were covered by lids allowing air flow, turned upside down every 24 h to allow up and down free movement of the wax moth larvae inside the plastic boxes and raise their chance to meet nematode infective juveniles (IJs). The boxes were kept in the dark at 25 °C for 1 week. The *Galleria* larvae were checked at 3-day intervals for the presence of the dead larvae, and insect cadavers were placed individually to modified White traps (White 1927) for the emergence of the infective-stage juveniles. Emerging IJs within the first week were cleaned by distilled water and each nematode isolate was tested against 10 *G. mellonella* larvae to confirm Koch's postulates for pathogenicity (Kaya and Stock 1997). The juveniles emerging from newly inoculated larvae were maintained on *G. mellonella* larvae, with regular inoculation each month and stored at 11 °C after rinsed three times with sterile distilled water.

The morphometric assessments of IJs and adults were made using a drawing camera (camera lucida) (Axioplan Axiophoto) attached to a ZEISS (MC100 SPOT) light microscope based on the criteria suggested by Stock and Kaya (1996), Hominick et al. (1997), and Nguyen and Hunt (2007). For morphometric characterization of each isolate, 20 first-generation hermaphrodites of *Heterorhabditis* species and 20 IJs and 20-s-generation males of *Steinernema* species were selected at random by dissecting different *G. mellonella* cadavers after 2–4 and 5–7 days post-infection, respectively. Selected specimens of each isolate were killed by heat, fixed (Seinhorst 1959) in TAF solution (triethanolamine formalin), and transferred to glycerin to mount on slides (Kaya and Stock 1997).

The following taxonomic characters were measured: (L) total body length; (W) maximum body diameter, (ABD) anal body diameter, (EP) excretory pore position, (ES) distance from anterior end to base of pharynx, (NR) distance from anterior end to nerve ring position, (T) tail length, a (total body length divided by maximum body diameter); b (total body length divided by distance from anterior end to base of pharynx); c (body length divided by tail length); (SL) the length of the spicules, (GU) length of the gubernaculum, D% (excretory pore position divided by distance from anterior end to base of pharynx); E% (excretory pore position divided by tail length). After morphological characteristics were made, isolates were placed into related species-groups.

Molecular characterization of each isolate was performed by ITS rDNA (internal transcribed spacer) sequences. DNA extraction was performed, following the protocol of Waeyenberge et al. (2000). Newly harvested live 5–10 IJs of each population were used for DNA extraction. The ITS region of the isolates rDNA was amplified by a total of 50 μl PCR reaction mixture containing of 2 μl DNA, 32 μl ddH2O, 15 μl of Dream Taq PCR Master Mix (Fermentas Life Sciences, Germany), 0.5 μM of forward primer 5′-CGTAACAAGGTAGCTGTAG-3′ and 0.5 μl of the 5′-TCCTCCGCTAAATGATATG-3′ (Joyce et al. 1994). The PCR products were sequenced in a sequencing facility in both directions at Refgen, (Ankara, Turkey) and the amplified fragments of *Heterorhabditis* and *Steinernema* isolates (Rhabditida: Heterorhabditidae and Steinernematidae) were compared to the sequences of the *Heterorhabditis* and *Steinernema* species available in GenBank (NCBI).

A dose-response bioassay was performed by the isolates obtained against last instar larvae of *G. mellonella* to evaluate their virulence in a Petri dish arena. Ten *G. mellonella* larvae were placed in sterile 9-cm Petri dishes including 2 moist filter papers (100 × 15 mm) and exposed to IJs suspended in 1 ml of sterile water containing concentrations of 25, 50, and 100 IJs. The Petri dishes applied at different concentrations of IJs were put in a dark incubator at 25 ± 1 °C, R.H. 60%, and only sterile water was applied to the Petri dishes used for control treatments. The bioassay was replicated 3 times and mortality rates were recorded first and second-day post-treatment. Dead larvae were removed from the Petri dishes, incubated individually on modified White Traps at 25 ± 1 °C and dissected to confirm the presence of nematodes.

The data obtained were analyzed by factorial analysis of variance (ANOVA) using SPSS (Version 11.0) statistical software package and Tukey's multiple range test ($P < 0.05$) was used to separate mean values.

Results and discussion

EPNs were isolated from 20 of the 112 soil samples collected from the districts of Hacibektas, Urgup, Avanos, Kozakli, Gulsehir, Acigol, Derinkuyu, and Nevsehir Center with a (17.9%) recovery rate. No nematodes were isolated from the soil samples collected from Gulsehir and Hacibektas districts. Urgup District had the highest proportion of positive soil samples (35%) among others. Vegetable habitats demonstrated a higher recovery frequency (35%) than other habitats and 12 soil samples collected from the forest habitats yielded no nematodes (Table 1).

Most of the nematode isolates belonged to the genus *Steinernema* spp., which were isolated from 18 (90%) out of 20 positive soil samples. The most common species was *Steinernema feltiae*, which was recovered from 17 soil samples (85%), followed by *Heterorhabditis bacteriophora*,

Table 1 Habitat types and percentage of sites in which entomopathogenic nematode isolates were recovered at Nevsehir in 2015 and 2016

Habitats	No. of positive sites/no. sampling sites	No. and % of *Steinernema feltiae*	No. and % of *Heterorhabditis bacteriophora*	No. and % of *Steinernema* sp.	Total %
Vegetable	7/29	7–24.1	0–00.0	0–00.0	24.1
Field crops	4/19	3–15.8	0–00.0	1–5.3	21.1
Pasture	4/20	3–15.0	1–5.0	0–00.0	20.0
Fruit orchards	5/32	4–12.5	1–3.1	0–00.0	15.6
Forest	0/12	0–0.0	0–00.0	0–00.0	0.0

found only in 2 soil samples (10%) and unidentified *Steinernema* sp. found only in one soil sample (5%) according to the morphometrical and molecular examination of the isolates (Tables 2 and 3).

The virulence of the obtained isolates was evaluated on *G. mellonella* in Petri dish experiments. The results indicated that all isolates were effective and caused significant mortality rates ranging between 13 and 100% on the first day and 30 and 100% on the second day post-treatment at 25 °C. Larval mortality increased as the concentration of the isolates tested gradually increased and no mortality occurred in control treatments. The maximum mortality rate (100%) on *G. mellonella* larvae was achieved by *S. feltiae* DDKB-17 isolate at all concentrations and exposure times, except on the first day at the concentration of 25 IJs. Four of the isolates (UTP-5, UMK-7, AVB-15, and DDKB-17) generally caused higher mortality rates than others at all concentrations and exposure times (Figs. 1 and 2).

The present study is the first comprehensive survey that indicates the common presence and wide distribution of EPNs in the Cappadocia Region, Nevsehir. During the survey, 20 EPN isolates were obtained by a recovery rate of (17.9%) from 112 soil samples collected from 8 districts throughout the province. The recovery rate of EPN isolates (17.9%) was similar to that reported at Karaman province (19.2%) (Yavuzaslanoglu et al. 2016) but remarkably higher than other surveys having recovery rates of (4.71%) in Rize (Ozer et al., 1995), 9% at Adana and Kahramanmaras provinces (Canhilal et al. 2016),

Table 2 Entomopathogenic nematode isolates with habitat characteristics of positive sampling sites and ITS sequence information

Isolate code	Species	Location	Elevation (m)	Habitat	District	Accession numbers
UKK-1	*Steinernema feltiae*	38°33′13 N 34°35′4 E	1309	Vegetable	Urgup	MG462715
MKB-2	*S.feltiae*	38°30′0 N 34°36′3 E	1382	Fruit orchards	Nevsehir center	MG462716
UCK-3	*S.feltiae*	38°32′48 N 34°46′2 E	1372	Fruit orchards	Urgup	MG602329
KBC-4	*S.feltiae*	39° 6′46 N 34°45′3 E	1119	Pasture	Kozakli	MG602330
UTP-5	*S.feltiae*	38°30′8 N 34°56′5 E	1585	Vegetable	Urgup	MG602331
DTK-6	*S.feltiae*	38°29′6 N 34°45′9 E	1428	Vegetable	Derinkuyu	MG602332
UMK-7	*Heterorhabditis bacteriophora*	38°33′50 N 34°55′1 E	1191	Pasture	Urgup	MG602333
MCB-8	*S.feltiae*	38°40′40 N 34°29′5 E	1007	Vegetable	Nevsehir center	MG602334
UCY-9	*S.feltiae*	38°32′42 N 34°45′5 E	1393	Pasture	Urgup	MG602335
DTF-10	*S.feltiae*	38°26′59 N 34°44′5 E	1412	Vegetable	Derinkuyu	MG602336
DDKY-11	*S.feltiae*	38°24′19 N 34°47′4 E	1446	Fruit orchards	Derinkuyu	MG602337
MAY-12	*S.feltiae*	38°38′26 N 34°35′2 E	1210	Pasture	Nevsehir center	MG602338
ATB-13	*S.feltiae*	38°33′20 N 34°34′2 E	1307	Field crops	Acigol	MG602339
MBB-14	*Steinernema* sp.	38°37′7 N 34°37′5 E	1245	Field crops	Nevsehir center	MG602340
AVB-15	*H.bacteriophora*	38°43′43 N 34°52′1 E	977	Fruit orchards	Avanos	MG602341
ÜCB-16	*S.feltiae*	38°31′4 N 34°56′3 E	1353	Field crops	Urgup	MG602342
DDKB-17	*S.feltiae*	38°23′9 N 34°45′3 E	1374	Vegetable	Derinkuyu	MG602343
DME-18	*S.feltiae*	38°29′23 N 34°45′1 E	1436	Fruit orchards	Derinkuyu	MG602344
UIP-19	*S.feltiae*	38°36′11 N 34°50′1 E	1340	Vegetable	Urgup	MG602345
DMB-20	*S.feltiae*	38°28′35 N 34°46′1E	1459	Field crops	Derinkuyu	MG602346

Table 3 Morphometrics of some of the isolates of *Heterorhabditis* and *Steinernema* species obtained (μm)

Character	*Steinernema feltiae* (MKB-2) *n* = 20, mean ± Sd (min-max)		*Steinernema* sp. (MBB-14) *n* = 20, mean ± Sd (min-max)		*Heterorhabditis bacteriophora* (AVB-15) *n* = 20, mean ± Sd, (min-max)	
	IJ	Male	IJ	Male	IJ	Male
Total body Length (L)	815 ± 20 (778–842)	1558 ± 48 (1491–1651)	600 ± 44 (517–670)	1667 ± 72 (1555–1796)	583 ± 31 (529–632)	805 ± 22 (756–842)
Body length/body width (a)	28.9 ± 1.9 (25.3–34.4)	11.7 ± 1.5 (9.5–16.1)	25 ± 3.2 (19.5–31.1)	11 ± 1 (10–16)	24 ± 3 (17–28)	19 ± 1 (16–21)
Body length/esophageal length (b)	5.9 ± 0.2 (5.7–6.2)	9.7 ± 0.8 (8.4–12.2)	4.7 ± 0.3 (3.9–5.6)	10 ± 1 (8–13)	4.8 ± 0.5 (4.0–5.8)	8 ± 1 (6–9)
Body length/tail length (c)	9.9 ± 0.3 (9.4–10.4)	36.6 ± 3.4 (30.4–47.4)	6.5 ± 0.5 (5.4–7.4)	39 ± 2 (31–42)	6 ± 1 (5.2–7.0)	33 ± 2 (26–38)
%D[(EP/ES)X100]	45.8 ± 1.8 (42–50)	74 ± 6 (65–96)	76 ± 6.1 (69–92)	76 ± 10 (60–103)	80 ± 11 (64–99)	116 ± 12 (92–137)
%E[(EP/TL)X100]	77 ± 3.7 (69–86)	282 ± 22 (246–336)	103 ± 7.4 (93–122)	292 ± 24 (240–332)	102 ± 12 (79–122)	478 ± 49 (415–600)
Nerve ring (NR)	113 ± 4 (105–119)	117 ± 10 (95–141)	83 ± 3.1 (75–88)	110 ± 6 (99–125)	80 ± 4 (74–89)	75 ± 3 (69–81)
Excretory pore (EP)	62 ± 2.9 (55–69)	119 ± 5 (111–131)	96 ± 4.9 (91–110)	124 ± 6 (108–329	97 ± 7 (88–111)	116 ± 8 (103–135)
Pharynx (ES)	136 ± 2.4 (131–141)	161 ± 12 (151–192)	126 ± 6.7 (111–136)	164 ± 20 (126–201)	121 ± 10 (104–139)	100 ± 7 (91–117)
Maximum body width (W)	28 ± 2.1 (24–33)	134 ± 15 (102–161)	24 ± 2.5 (20–28)	142 ± 14 (102 + 171)	25.3 (19–31)	41 ± 2 (36–49)
Tail length(TL)	81.6 ± 1.5(79–85)	42 ± 3 (33–49)	92 ± 5 (82–101)	42 ± 3 (40–55)	95 ± 7 (82–112)	24 ± 2 (21–31)
Spicule length (SL)	–	67 ± 4 (60–74)	–	68 ± 4 (62–76)	–	42 ± 3 (33–48)
Gubernaculum Length (GL)	–	47 ± 6 (32–57)	–	56 ± 10 (45–77)	–	21 ± 2 (18–26)

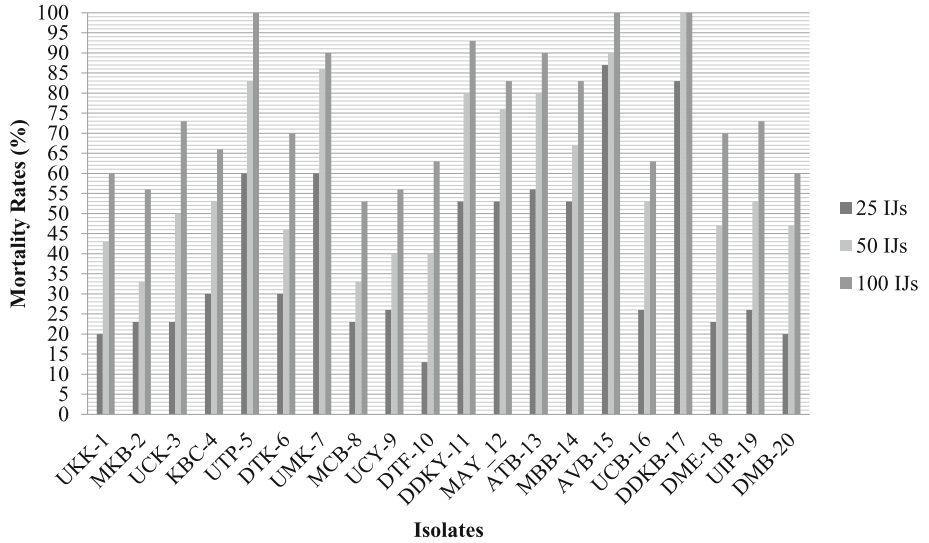

Fig. 1 The mortality rate (%) of entomopathogenic nematode isolates on *Galleria mellonella* larvae on the first day after treatment at 25 °C (25, 50, and 100 IJs). UKK-1, MKB-2, UCK-3, KBC-4, UTP-5, DTK-6, MCB-8, UCY-9, DTF-10, DDKY-11, MAY-12, ATB-13, UCB-16, DDKB-17, DME-18, UIP-19, and DMB-20: *Steinernema feltiae* isolate, UMK-7 and AVB-15: *Heterorhabditis bacteriophora* isolate, MBB-14: *Steinernema* sp

2.03% throughout Turkey (Hazır et al. 2003a). Recovery rates recorded for other European countries in sub-tropical regions were; 13.8% in Southern Italy (Tarasco and Triggiani 2016), 9.5% in Egypt (Shamseldean and Abd-Elgawad 1994) and 4.6% in Spain (Del Pino and Palomo 1996). The reason behind a high recovery rate in the present study might be due to the fact that the soil samples were collected from a narrow area of land at the most appropriate time, especially after rainfall as soil moisture and temperature are among the most crucial factors in the survival of EPNs in the soil environment (Wright 1992; Gaugler and Kaya 1990 and Ehlers and Peters 1996). Of the isolates obtained, the most prevalent ones were *S. feltiae* (Filipjev 1934) with the ratio of (85%), while the occurrence of *H. bacteriophora* (Poinar, 1985) (10%), and *Steinernema*

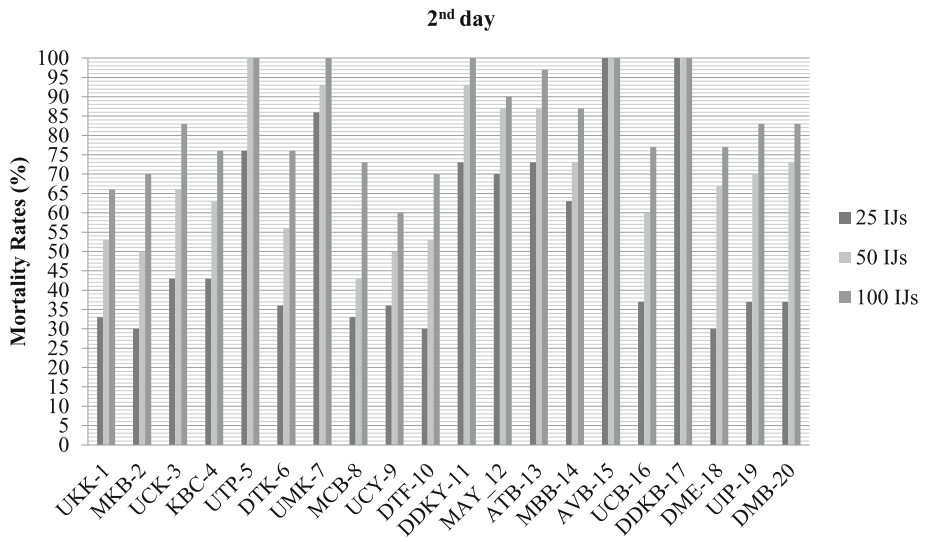

Fig. 2 The mortality rate (%) of entomopathogenic nematode isolates on *Galleria mellonella* larvae on the second day after treatment at 25 °C (25, 50, and 100 IJs). UKK-1, MKB-2, UCK-3, KBC-4, UTP-5, DTK-6, MCB-8, UCY-9, DTF-10, DDKY-11, MAY-12, ATB-13, UCB-16, DDKB-17, DME-18, UIP-19, and DMB-20: *Steinernema feltiae* isolate, UMK-7 and AVB-15: *Heterorhabditis bacteriophora* isolate, MBB-14: *Steinernema* sp

sp. (5%) were rare. This is in agreement with the earlier studies. *S. feltiae* has been found as the most common EPNs species isolated in the other surveys conducted in Turkey, followed by *H. bacteriophora* (Laznik et al. 2009; Canhilal et al. 2016 and Canhilal et al. 2017). Both of these EPNs are widely distributed throughout the world. Even though *S. feltiae* has been found in different climatic zones, it is one of the species that is well-adapted to cold and continental climate conditions (Hazir et al. 2001). When considering the climate of Nevsehir, as continental and its distance from coastal areas, it is an expected result for *S. feltiae* to be the most isolated EPN species. Although *H. bacteriophora* is known to be a species that is more adapted to tropical and subtropical areas (Grewal et al. 1994), it has been isolated from many areas with different climatic conditions (Griffin et al. 1999). This suggests that EPNs are distributed in varying frequencies on earth (Griffin et al. 1999).

All the isolates tested had the ability to kill *Galleria* larvae, but their pathogenicity differed remarkably among different species and/or isolates. In the present study, mortality rates varied between 63 and 100% among the isolates at the concentration of 100 IJs/ml after 48-h exposure time. Similar mortality rates ranging between 78 and 100% were found at the concentration of 100 IJ/ml after 72 h in another study (El Khoury et al. 2018). Tarasco and Triggiani (2016) reported that the mortality rates of *G. mellonella* larvae varying between 78 and 100%, when exposed to different isolates of *S. feltiae* and *H. bacteriophora,* following 72 h of exposure to 100 IJs/ml at 25 °C. In this study, the most virulent steinernematid isolate (*S. feltiae*, DDKB-17) caused (100%) mortality at the lowest concentration (25 IJ/ml) after 48 h of nematode exposure. Considering the application concentration and exposure time, *S. feltiae* DDKB-17 isolate appeared to be more virulent than other isolates in other studies. Differences in pathogenicity among the EPNs isolates belonging to the same species have been previously well documented (Canhilal 2013 and Tarasco and Triggiani 2016). These differences in pathogenicity may be due to the application techniques used in these studies or the soil habitats of these nematode species, which is one of the most important factors affecting the performance of EPNs isolates.

Conclusions

Obtained results indicate that the EPNs isolates were highly effective against the *G. mellonella* larvae where the isolates virulent behavior of *S. feltiae* UTP-5, DDKB-17 *S. feltiae*, UMK-7 *H. bacteriophora*, and AVB-15 *H. bacteriophora* performed better than the other isolates at all concentrations and exposure times. Therefore, they might have a great potential in the biological control of insect pests. The present study provides insight into the distributions of EPNs in Turkey and further studies are needed to determine the pathogenicity of these isolates against major agricultural insect pests for the selection of the appropriate EPNs isolates.

Acknowledgements
We thank Prof. Dr. Halil Elekcioğlu (Cukurova University), Assoc. Prof. Dr. Mustafa İMREN (Abant Izzat Baysal University), Abdurrahman Sami KOCA (Abant Izzat Baysal University), and Ece Bortecine KASAPOGLU (Cukurova University) for their guidance in morphometric and molecular analysis.

Authors' contributions
EY and RC conceived and designed the research. EY conducted the experiments, analyzed the data, and wrote the manuscript. Both authors read and approved the manuscript.

Funding
This study was funded by Project No. FYL-2017-7664 by the office of scientific research projects of Erciyes University. The funders had no role in the design of the study and collection, analysis, interpretation of data, and in writing the manuscript.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
All applicable institutional and national guidelines for the care and use of animals were followed.

Consent for publication
Both authors of this manuscript accepted that the paper is submitted for publication in the Journal of Pest Science, and reported that this paper has not been published or accepted for publication in another journal, and it is not under consideration at another journal.

Competing interests
The authors declare that they have no competing interests.

Received: 13 March 2019 Accepted: 20 May 2019
Published online: 19 June 2019

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