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First report of the entomopathogenic nematode, *Steinernema carpocapsae*, from Moghan region of Iran and its efficacy against the turnip moth, *Agrotis segetum* Denis and Schiffermuller (Lepidoptera: Noctuidae), larvae

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Abstract

During a survey of entomopathogenic nematodes (EPNs) in Moghan region of Iran in 2015, a steinernematid species was isolated, using the *Galleria*-baiting method. Based on its morphological and phylogenetic analysis of molecular data, the isolate was identified as *Steinernema carpocapsae*. The ITS rDNA sequence was deposited in NCBI (National Center for Biotechnology Information) with accession number of MF187616.1. Nucleotide row data was edited, using MEGA 6.0 software, and homologous sequences were involved in analysis, using Blast software. Sequences were aligned using Clustal W. Bootstrap analysis. The phylogenetic tree was constructed by the maximum likelihood method, using MEGA 6.0 software, and *Steinernema feltiae* was used as out-group. The turnip moth, *Agrotis segetum* Denis and Schiffermuller (Lepidoptera: Noctuidae), is one of the most important and destructive cutworm pests in Moghan. Therefore, the lethal effect of *S. carpocapsae* isolate Moghan (IRMoghan) was evaluated in a soil assay against the last instar larvae of *A. segetum* under laboratory conditions. The bioassay results showed high susceptibility of the larvae to *S. carpocapsae*. The LC_{10}, LC_{50}, and LC_{90} values were 9.9, 54.13, and 246.2 IJs (infective juveniles) per larva of the pest, respectively ($\chi^2 = 7.36$; df = 3, *P* value = 0.061). Reproduction of the EPNs within the dissected cadavers was observed. The bioassay results indicated that the new isolate is a promising biocontrol agent against *A. segetum* larvae with success recycling through them.

Keywords: *Steinernema carpocapsae*, ITS rDNA sequence, *Agrotis segetum*, LC_{50}, Virulence

Background

Entomopathogenic nematodes (EPNs) belonging to the genera *Steinernema* and *Heterorhabditis* are obligate parasites of insects, which kill their hosts through the septicemia caused by their symbiotic bacteria. EPNs have been used for several decades for biological control of many important insect pests worldwide (Georgis et al. 2006). EPNs possess many of the attributes as effective biological control agents and have been used as classical, conservational, and augmentative biological control agents (Kaya and Gaugler, 1993 and Koppenhöfer, 2007). *S. carpocapsae* is the most commonly applied species for control of foliar and other aboveground pests. This species possess a sit and wait foraging strategy (ambushers) and therefore is effective in cryptic and soil surface habitats (Laznik and Trdan, 2011 and Lacey and Georgis, 2012).

The turnip moth, *Agrotis segetum* Denis and Schiffermuller (Lepidoptera: Noctuidae), is an important pest in Europe, Asia, and parts of Africa. It generally lives in the ground. The pest feeds on seedlings of a wide range of the

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most important crops including corn, sugar beet, potato, cabbage, and many other plants (El-Salamouny et al., 2003). Due to their soil-dwelling habits, cutworms are difficult to control; however, *S. carpocapsae* as an ambusher species is a promising biological control agent against *A. segetum*. Some previous studies indicated the efficacy of this EPN against *Agrotis* species under laboratory and field conditions (Morris et al., 1990; Levine and Oloumi-Sadeghi, 1992 and Yan *et al.*, 2014).

Various genera and species of the EPNs have been isolated from Iran and investigated in different aspects such as morphology and phylogeny (Kary et al., 2009 and Nikdel et al., 2010, 2011), natural incidence in insect hosts (Karimi et al., 2009), efficacy against agricultural pests (Ebrahimi et al., 2011, 2014, 2016), interaction of entomopathogenic nematodes and chemical pesticides (Sheykhnejad et al., 2014), and their interaction with insect immune system (Ebrahimi *et al.*, 2018).

Moghan region (39° 41′ N 47° 32′ E, 40–50 m above from sea level in north-west of Iran) is one of the most important agricultural regions of Iran. Despite of specific topographic conditions of the region, survey for EPNs was not carried out there. The present study aimed to report the first record of *S. carpocapsae* from Moghan region of Iran and document its virulence against *A. segetum* under laboratory conditions.

Materials and methods

Sampling, isolation, and culturing of the nematode

Soil sampling and trapping the samples with the last instar larvae of the greater wax moth, *Galleria mellonella* (L.), was carried out according to Woodring and Kaya (1988) method. The samples were collected from Moghan region, Ardabil province, Iran, during 2015 and 2016. Infective juveniles of the nematodes cultured in the last instar larvae of *G. mellonella* were stored in 40-ml distilled water at 5 °C.

Morphological and morphometric identification of the nematode

Males and females, collected from 5-day infected *Galleria* cadavers, were dissected in distilled water. Infective juveniles (IJs) were collected after emergence from *Galleria* cadavers in White traps (Woodring and Kaya 1988). The nematodes were killed and processed to anhydrous glycerin by a slow evaporation method (Woodring and Kaya 1988), then mounted on microscopic slides. Morphological and morphometric measurements were recorded. The morphometric measurements included the following: *L* = body length, *W* = greatest body width, EP = distance from anterior end to excretory pore, NR = distance from anterior end to nerve ring, ES = esophagus length, *a* = L/W, *b* = L/ES, *c* = L/T (T = tail length), *D*% = EP/ES × 100, *E*% = EP/T × 100, ABW = anal body width, SL = spicule length, GL = gubernaculum length, IJ = infective juvenile, ratio SW = spicule length/ABW, ratio GS = gubernaculum length/spicule, MUC = mucron, A = absent, P = present.

Molecular identification of the nematode: DNA extraction, amplification, and sequencing

DNA was extracted from 10 IJs, using by modified method of Hominick *et al.* (1997). Briefly, the IJs were crashed in 8-μl deionized distilled (dd) H_2O in a sterilized 0.5-ml microcentrifuge tube. then 12-μl lysis buffer was added, and the mixture was homogenized for 1 min. After adding 2 μl proteinase K, the tube was frozen at − 20 °C for 2 h. The tube was incubated at 67 °C for 1 h, followed by a 97 °C incubation for 10 min to digest the proteins and inactivate proteinase K. Subsequently, the tube was centrifuged at 4000 rmp for 2 min. The supernatant containing the DNA was collected and used in PCR amplification. The ITS region from of the ribosomal DNA was amplified in a sterile 0.5-ml tube. The following were used to assemble the reaction: 15 μl of Master Mix, 10 μl of dd H_2O, 1 μl of 10 pM forward primer, 1 μl of 10 pM reverse primer, and 3 μl DNA extract. The primers were TW81:5′-GTTTCCGTAGGTGAACCTGC-3′ (forward) and AB28: 5′-ATATGCTTAAGTTCAGCGGGT-3′ (reverse). PCR reactions were conducted in a Thermocycler. The PCR cycles were as follows (Hominick *et al.*, 1997): 1 cycle at 95 °C for 4 min followed by 33 cycles at 95 °C for 30 s, 57 °C for 40 s, and 72 °C for 30 s. The last step was 72 °C for 5 min. PCR product was purified and sequenced by Bioneer Corporation, Korea. The obtained sequence was deposited at the NCBI (National Center for Biotechnology Information) database with the accession number of MF187616.1. Nucleotide row data was edited using MEGA X software (Kumar *et al.*, 2018), and homologous sequences were involved in analysis using Blast software. Sequences were aligned, using Clustal W. Bootstrap analysis. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The phylogenetic tree was constructed by the maximum likelihood method, using MEGA 6.0 software, and *Steinernema feltiae* was used as out-group.

Insects

Galleria mellonella

The greater wax moth, *G. mellonella*, was reared in plastic boxes with ventilated lids at 26 ± 2 °C, 50 ± 5% RH, and 16:8 (L:D) photoperiod and on an artificial diet composed of 1200-g wheat flour (44.1%), 600-g honey (22.1%), 500 ml of 99% glycerol (18.4%), 300-g yeast (11%), and 120-g bee wax (4.4%).

Agrotis segetum

Last instar larvae of *A. segetum* were collected from tomato fields in Ardebil Agricultural Research Station in Moghan region and transferred to the laboratory of Plant Protection Research Department. The larvae were reared on corn seedlings. Prepupae were placed in the egg lying dishes described by Sherlock (1979). The eggs were collected, and the developed last instar larvae were treated based on Sherlock (1979) method, with exception of using fresh corn seedlings as feeding source for the larvae. Rearing conditions were 25 ± 1 °C and of 70 ± 10% RH and photoperiod 16:8 (L:D). In 24-h time intervals, last instar larvae were collected from rearing dishes and were used in the bioassay experiments.

Nematode

Infective juveniles of *S. carpocapsae* IRMoghan were cultured on the last instar larvae of *G. mellonella* (Woodring and Kaya, 1988). The IJs were stored in distilled water at 5 °C and used in all experiments within 30 days of emerging from the host. Before starting the experiments, nematodes were kept at 25 °C for 20–30 min.

Bioassay

Last instar larvae of *A. segetum* were used in the bioassay experiments. The experiments were conducted in plastic cups (9 cm height and 7.5 cm diameter), filled with 250-g autoclaved moist sandy soil (85 sand, 10 silt and 5 clay and 10% moisture (w/w)). Based on preliminary experiments, a range of 10–100 IJs per insect (i.e., 10, 25, 50, 75, and 100 IJs per larva) was used. IJs were added in certain concentrations in 0.5 ml of water to the surface of the soil, separately. Finally, one larva was placed on the soil, and the cups were covered by ventilated lids to avoid desiccation. Control cups received 0.5-ml distilled water without nematodes. A piece of stem of fresh corn seedlings was used as feeding source which was renewed daily. Fifteen cups were used for each nematode concentration and the control. The dead or alive insects were counted after 7 days. The experiment was replicated 3 times. All dead insects were collected and dissected to ensure the presence of nematodes inside the cadavers.

Statistical analysis

LC_{20}, LC_{50}, and LC_{80} values were obtained by Probit analysis, using SAS software (SAS Institute 2004). Analysis of variance was done, and the means were evaluated by Duncan's multiple-range test (SAS Institute 2004). Lethal experimental data was transformed into square root of $(x + 1)$ where needed, before analysis.

Table 1 Morphometric of infective juveniles of *Steinernema carpocapsae* IRMoghan. All measurements are in micrometers (mean ± SE)

Character	Measurement	Character	Measurement
L	538.50 ± 35.5 (505–620)	*a*	23.20 ± 2.52 (19.81–28.25)
W	23.35 ± 1.80 (22–27)	*b*	4.77 ± 0.43 (4.08–5.49)
EP	30.90 ± 3.51 (25–34)	*c*	11.24 ± 1.38 (9.71–13.29)
NR	77.90 ± 5.69 (73–85)	*D*%	27.31 ± 2.99 (22.94–32.69)
ES	113.20 ± 6.23 (104–125)	*E*%	65.12 ± 13.85 (44.64–89.19)
T	48.50 ± 5.95 (37–56)	*N*	20
ABW	13.40 ± 3.50 (9–18)		

Ratios: *a* = L/W, *b* = L/ES, *c* = L/T, *D*% = EP/ES × 100, *E*% = EP/*T* × 100
L body length, *W* max. body diam, *EP* excretory pore, *NR* nerve ring, *ES* pharynx, *T* tail length, *ABD* anal body diam

Results and discussion

Morphometric and molecular identification

Morphometric data of IJs and first-generation males of the isolate are shown in Tables 1 and 2.

Infective juvenile: Body slender, enclosed in a sheath. Pharynx long and narrow, often degenerate, tail elongate and conical.

Males: Body C-shaped when heat-relaxed. Cuticle smooth. Stoma shallow. Pharynx with cylindrical procorpus, slightly enlarged metacarpus, followed by an isthmus and a basal bulb with valve. Nerve ring surrounding isthmus. Excretory pore anterior to nerve ring. Reproductive system monorchic, testis reflexed. Spicules paired, slightly curved. Gubernaculum boat-shaped in Bursa absent.

Based on its morphological and molecular properties, the isolate was identified as *Steinernema carpocapse*.

Table 2 Morphometric data of first-generation males of *Steinernema carpocapsae* IRMoghan1. All measurements are in micrometers (mean ± SE)

Character	Measurement	Character	Measurement
EP*	54.00 ± 4.54 (50–61)	ABW	37.60 ± 3.95 (30–42)
ES	163.00 ± 4.81 (142–172)	SW	1.87 ± 0.21 (1.67–2.2)
SL	69.80 ± 4.05 (65–76)	GS	0.68 ± 0.1 (0.51–0.80)
GL	47.20 ± 5.14 (38–54)	*N*	10

Ratios: SW = SL/ABD, GS% = GL/SL × 100
EP excretory pore, *ES* pharynx, *SL* spicule length, *GL* gubernaculum length, *ABD* anal body diam

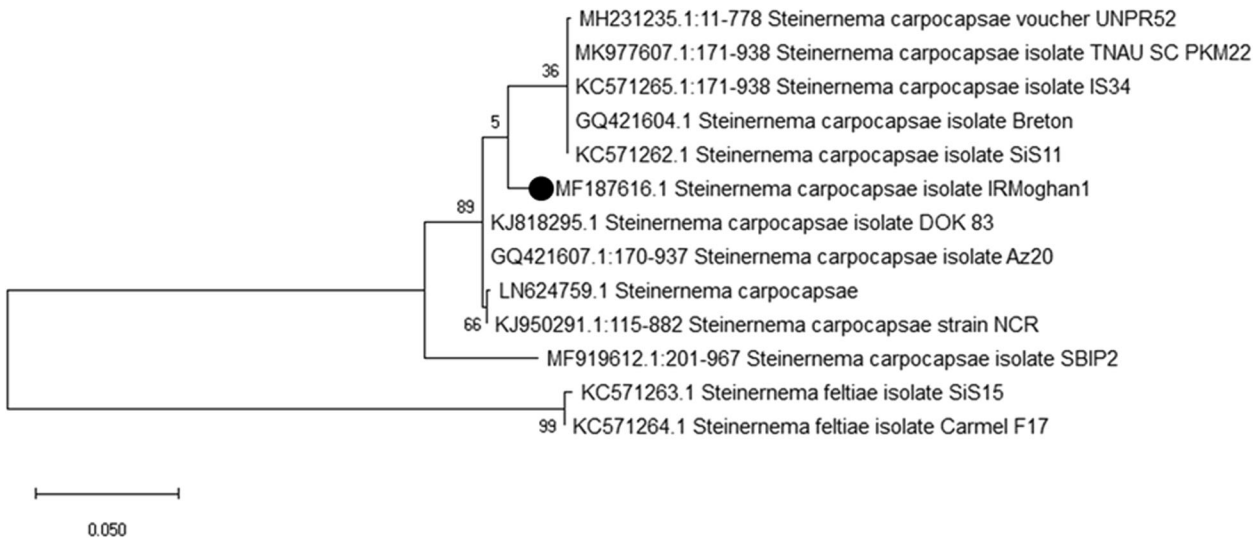

Fig. 1 Evolutionary relationships of new EPN isolate (IRMoghan1). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site

The analysis of the ITS rDNA sequence confirmed the species identification.

The phylogenetic tree was constructed, and the tree with the highest log likelihood (– 2815.96) was shown. The relative phylogenetic position of the new isolate, *S. carpocapsae* MoghanIR1, was determined according to neighbor joining based on the 28S rDNA sequence (Fig. 1). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated, using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

Table 3 Analysis of variance of mortality percentage of *Agrotis segetum* in different concentrations of *Steinernema carpocapsae* IRMoghan

Source of variation	df	Mean square	*F* value
R	2	6.02	9.04
Treatment	5	26.34**	39.54
Error	10	0.67	

**Statistically significant ($P < 0.01$)

Bioassay

Last instar larvae of *A. segetum* were found susceptible to infection with *S. carpocapsae* IRMoghan. Analysis of variance revealed significant differences among varied concentrations of *S. carpocapsae* IRMoghan on *A. segetum* mortality ($F = 39.52$; df = 5; $P < 0.0001$) (Table 3). There were no dead insects in control cups during experimental period. At the highest concentration, the nematode caused greater mortality than all other concentrations. All the treated nematode concentrations caused greater mortality than the control and those of 10 IJs/larvae (Table 4).

The LC_{10}, LC_{50}, and LC_{90} values for *S. carpocapsae* IRMoghan1 on *A. segetum* were 9.9, 54.13, and 246.2 IJs per larva of the pest, respectively ($\chi^2 = 7.36$; df = 3, *P* value = 0.061) (Table 5). Regression analysis revealed a significant correlation between log concentration

Table 4 Mean comparison of *Agrotis segetum* mortality in different concentrations of *Steinernema carpocapsae* IRMoghan

Nematode concentrations (IJs per insect)	Mortality %
100	83.33^{a}
75	56.67^{b}
50	30^{c}
25	30^{c}
10	10^{d}
0	0^{d}

Insignificant differences are present between the values marked with similar superscripts ($P < 0.01$); $N = 45$ insects for each concentration

Table 5 LC_{10}, LC_{50}, and LC_{90} values for *Steinernema carpocapsae* IRMoghan against *Agrotis segetum*

Chi-square (df, *P* value)	LC_{10}[a] (95% CL)	LC_{50} (95% CL)	LC_{90} (95% CL)	R^2	N
7.36 (3, 0.061)	11.9 (5.28–18.1)	54.13 (41.69–73.9)	246.2 (149.7–662.9)	0.91	150

CL confidence limits
[a]LC values are based on IJ per insect larva

and insect mortality (Fig. 2). Because goodness-of-fit Chi-Square value was insignificant for Probit analysis; no heterogeneity factor was used in the calculation of confidence limits and LC_{10}, LC_{50}, and LC_{90} values. Reproduction of the EPNs within the dissected cadavers was observed.

This is the first record of *S. carpocapsae* form Moghan region of Iran. Two surveys have been conducted in Iran and several species of EPNs including *S. carpocapsae* were reported (Kary *et al.,* 2009 and Nikdel *et al.,* 2010). Both surveys were carried out in mountainous and cold climatic region of Iran, while the Moghan Plain has a completely different climate. The Moghan Plain due to the special topographic conditions basically has a contrary climate in contrast to other parts of Azerbaijan and even its southern regions. Based on the weather data of the Pars-Abad synoptic meteorological station, Moghan region has a mild semi-desert climate with mild winters and warm and humid summers (Shiri *et al.,* 2015).

Various studies have investigated the effect of EPNs on different species of *Agrotis* in various methods (Unlu *et al.,* 2007; Gokce *et al.,* 2013; Yan *et al.,* 2014 and Yuksel and Canhilal 2018). Obtained results are consistent with other researches concerning the effect of *S. carpocapsae* on *A. segetum*. Unlu *et al.* (2007) evaluated the effect of the Turkish isolate of *S. feltiae*, *S. weiseri*, and *S. carpocapsae* (10 to 100 IJs per insect) against *A. segetum* and reported a higher efficacy of *S. carpocapsae* than the other species. One hundred IJs per insect concentration of *S. carpocapsae* Turkish isolate caused (75%) mortality of *A. segetum*, while the same concentration of *S. carpocapsae* IRMoghan isolate showed (83.83%) mortality of the insect. Despite of a high mortality due to Moghan isolate in the highest concentration of nematodes, 50 IJs of Moghan isolate caused 30% mortality of the insect, while it was 60% for the Turkish isolate. Gokce *et al.* (2013) reported a high susceptibility of *A. segetum* larvae to *S. websteri*, as 100 IJs g^{-1} of dry sand caused about 84% mortality rate of the insect.

The results of the bioassay revealed a high susceptibility of *A. segetum* to *S. carpocapsae* IRMoghan isolate. Overall, most of the lepidopteran insects are susceptible to EPNs due to their defense mechanisms (Lacey and Georgis, 2012). On the other hand, *S. carpocapsae* has a wide host of insect range (Labaude and Griffin, 2018). However, other factors like virulence of the nematodes due to the symbiotic bacteria against *A. segetum* and probable compatibility of the native isolate of *S. carpocapsae* with the pest could be effective in susceptibility of *A. segetum* larvae. Native species are often expected to be adapted to local conditions, and their use is preferred (Labaude and Griffin, 2018).

Conclusion

The results of this study indicated that *S. carpocapsae* IRMoghan isolate is a promising biocontrol agent against *A. segetum* with success recycling through the pest. Isolating *S. carpocapsae* from Moghan region and its compatibility with the insect pests of the region could incite more investigations on applicable use of this nematode species in integrated pest management programs in Moghan.

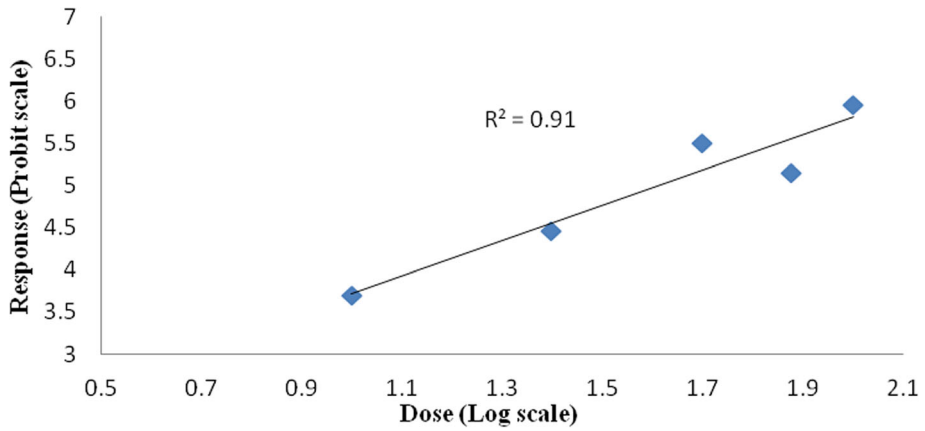

Fig. 2 Mortality (Probit-transformed) of *Agrotis segetum* treated with different concentrations of *Steinernema carpocapsae* IRMoghan

Abbreviations
EPNs: Entomopathogenic nematodes,; IJs: Infective juveniles; NCBI: National Center for Biotechnology Information

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Authors' contributions
LE carried out nematode isolation and identification and the experiments and was the major contributor in writing the manuscript. ZT participated in the morphological and morphometric and molecular identification of the nematode. PSH participated in nematode sampling and insect rearing. All authors read and approved the final manuscript.

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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
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests

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