Trinh *et al.*
Egyptian Journal of Biological Pest Control (2021) 31:147
https://doi.org/10.1186/s41938-021-00496-1

Egyptian Journal of Biological Pest Control

RESEARCH Open Access

First report of entomopathogenic nematode *Steinernema surkhetense* and its pathogenic potential to larvae of the Greater Wax Moth (*Galleria mellonella* L.) in Vietnam

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Abstract

Background: Entomopathogenic nematodes (EPNs) are evidently a useful nematode group for the biocontrol of insect pests. It is well known that efficacy of different EPN strains, even belonging to the same species, can be significantly varied in different localities. Therefore, exploring EPNs and testing their efficacy in various ecological regions is of crucial importance to find out more efficient EPN strains. On the other hand, this practice is also needed to enhance the knowledge on diversity and distribution model of EPNs over the world.

Results: In this study, a species belonging to the genus *Steinernema*, *S. surkhetense*, has been characterized for the first time in Vietnam based on morphological and molecular characterizations. Morphological characterizations of infective juveniles, the first and second-generation adults, and molecular characterization of D2-D3 expansion segment of 28S rRNA region were given. Molecular phylogeny of the genus *Steinernema* was also provided. In addition, the study showed that the lethal efficacy of this local strain to larvae of *Galleria mellonella* L. was relatively higher than other reported EPN strains in Vietnam.

Conclusions: The Vietnamese EPN population found in this study was determined to be conspecific with *S. surkhetense*, revealed its new distribution in Vietnam. Besides, detailed morphological and molecular characterizations of it was provided with small variations compared to other populations in the world, and its relatively high lethal efficacy on larvae of *G. mellonella* implied that this strain can be potentially a useful strain for biological control of insect pests.

Keywords: Entomopathogenic nematodes, *Steinernema surkhetense*, Lethal efficacy, *Galleria mellonella*, Central Highlands, Vietnam, Biocontrol

Background

Entomopathogenic nematodes (EPNs) are obligate parasites and have significant potential in the biological control of insects. They are capable of parasitizing and causing death to hundreds of insect pests without having any clear adverse effect on humans and other vertebrate animals (Nguyen and Hunt 2007). Although most EPN species can control a wide range of insect pests, they are also known that different EPN strains belonging to the same species having different efficacy to control the same host insect (Laznik et al. 2010). Therefore, the works such as isolating, identifying, and testing the efficacy of EPNs strains from different localities are of crucial importance in providing excellent EPN strains for producing biological pesticides (Laznik and Trdan 2011). Besides, the above works also help to provide the information about diversity, distribution, and host range of EPNs over the

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world, which is necessary to better understand the evolution of life.

Currently, more than 121 valid EPNs species have been described in the world (Didiza et al. 2021). Among them, *Steinernema surkhetense* Khatri-Chhetri, Waeyenberge, Spiridonov, Manadhar and Moens 2011 belongs to *carpocapsae*-group (presence of short juveniles). This species has been reported from several regions of Nepal, India, and China (Bhat et al. 2020). In Vietnam, 13 EPN species belonging to the genera *Steinernema* and *Heterorhabditis* have been found (Phan et al. 2014). The present study provides the first morphological and molecular characterizations of the EPN *S. surkhetense* from Vietnam, and molecular phylogeny of the genus *Steinernema*. Besides, the pathogenic potential of this strain to the larvae of great wax moth, *G. mellonella,* was also tested.

Methods

Nematode isolation and rearing

Fifty soil samples were collected randomly from Lam Dong and Ninh Thuan, Vietnam. Nematodes were recovered from soil samples using the modified *Galleria* bait method (Bedding and Akhurst 1975). Positive samples were found at the following coordinates: 11°45′ 37″ N, 109°35′ 47″ E and 11°45′ 55″ N, 109°11′ 01″ E. Subsequently, they were cultured on the larvae of *G. mellonella* using the method described by Nguyen (2008). The first and second-generation adults were obtained by dissecting infected cadavers after 3 and 5 days, respectively. Infective juveniles (IJs) were obtained when they emerged from the cadavers after 8–10 days.

Morphological characterization

Heat-killed nematodes were fixed using TAF (Formalin 40%: 7 ml, Triethanolamine: 2 ml, distilled-water: 91 ml) for 3–4 days and subsequently transferred to glycerin to make permanent slides, following Seinhorst (1959). Measurements and pictures of all nematode stages were taken from permanent slides using Carl Zeiss Axio Lab. A1 light microscope equipped with a Zeiss Axiocam ERc5s digital camera.

Molecular characterization

The D2-D3 region of 28S rRNA was amplified using D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (3′-TCCTCGGAAGGAACCAGCTACTA-5′) primers (De Ley et al. 1999). The obtained sequence was analyzed following Nguyen et al. (2020). Blast search was used to search for closely related species on GenBank (Altschul et al. 1997). Forward and reverse sequences were assembled and analysed using Geneious R11. The best fit model was chosen using Mega 7, following Nguyen et al. (2020). Phylogenetic trees were created using MrBayes 3.2.6 Add-in in Geneious R11 under GTR + G + I model (Markov chains were set with 1×106 generations, four runs, 20% burn-in, and subsampling frequency of 500 generations) following Nguyen et al. (2020).

Virulence of S. surkhetens from Vietnam

In this study, an experiment on the lethal efficacy of *S. surkhetense* to larvae of *G. mellonella* was done, following Nguyen (2008). There were 10 treatments with the concentration of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 IJs per larva of *G. mellonella*. Each treatment was repeated 3 times, 15 larvae were used for a replication resulted in a total of 45 larvae per treatment. In the control treatment, IJs were replaced by distilled-water. The experiment was checked after 48 h. at 28 °C. Cadavers of larvae were used for a white trap to confirm the infection of IJs (Nguyen 2008).

Statistical analysis

Results were analyzed using SPSS version 25 to determine LC_{50}, to detect significant differences and correlation (IBM Corp 2017).

Results

Measurements

Data of different measurements are presented in Table 1.

Morphological characterization

Female (first generation)

Body was spiral-shaped. Cuticle was smooth under LM (Fig. 1A). Lip region was rounded and slightly offset to body contour. Mouth was funnel-shaped. Cheilorhabdions and cardia were prominent. Secretory-excretory pores were prominent and located anterior to nerve ring (Fig. 1B). Vulva was slit-like with protruding lips, located at mid-body with short epiptygmata (Fig. 1C). Genital tract was amphidelphic-didelphic with reflexed ovaries. Tail was conical with short mucron (Fig. 1D).

Female (second generation)

Similar to the first-generation females. Body size was smaller and slightly curved ventrally. Tail was elongate and tapering to a pointed tail end. (Fig. 1E and F).

Male (first generation)

Body was J-shaped. Head region was similar to female. Testis was reflexed ventrally (Fig. 1H). Spicules were paired, symmetrical, and moderately curved. Gubernaculum was boat-shaped (Fig. 1I). A single precloacal papilla and 11 pairs of genital papillae were arranged in normal position for *Steinernema* (*i.e.* 6 or 7 pairs precloacal subventral, one pair adcloacal, one pair lateral, 2 pairs subterminal, and one pair subdorsal).

Table 1 Morphometric data of *Steinernema surkhetense* from different localities. All measurements are in µm (except for ratio)

Characters	*S. surkhetense* from Vietnam					*S. surkhetense* from Nepal (Khatri-Chhetri et al., 2011)				
	Females		Males		Infective juveniles	Females		Males		Infective juveniles
	1st generation	2nd generation	1st generation	2nd generation		1st generation	2nd generation	1st generation	2nd generation	
n	10	10	10	10	10	20	20	20	20	20
L	7405 ± 1103 (5962–9990)	1632 ± 108 (1400–1768)	1664 ± 91 (1549–1828)	879 ± 65 (802–993)	474 ± 21 (437–500)	2970 ± 922 (1894–5692)	1072 ± 101 (875–1290)	1224 ± 194 (887–1576)	824 ± 89 (621–945)	415 ± 15 (393–450)
a	28 ± 3 (24–34)	13 ± 0.7 (12–14)	10.6 ± 0.8 (9–11)	15 ± 1 (13–16)	20 ± 1 (19–22)	19 ± 2.7 (13–25)	13 ± 1.4 (11–16)	12 ± 1.3 (10–14)	12 ± 1.9 (8.8–15)	19 ± 1.8 (16–24)
b	33 ± 4 (26–41)	10.5 ± 1 (9.0–12)	11.2 ± 1 (10–12.7)	7.2 ± 0.2 (6.9–7.5)	5 ± 0.2 (4.7–5.2)	24 ± 8.8 (14–49)	5.6 ± 0.5 (4.6–6.7)	11 ± 1.6 (8.0–15)	6.8 ± 0.8 (5.2–8.0)	5 ± 0.2 (4.3–4.8)
c	201 ± 37 (152–283)	40 ± 4 (36–48)	58 ± 6 (49–67)	44 ± 4.8 (35–50)	10.5 ± 0.3 (10–10.8)	161 ± 54 (91–309)	11 ± 1.6 (8.7–15)	65 ± 12 (43–85)	44 ± 6.9 (31–59)	9 ± 0.5 (8.3–10)
c′	0.4 ± 0.1 (0.3–0.6)	1 ± 0.1 (0.9–1.1)	0.7 ± 0.0 (0.6–0.8)	0.8 ± 0.1 (0.7–0.9)	3.9 ± 0.1 (3.8–4.1)	–	–	–	–	–
V	53 ± 2.3 (48–54)	57 ± 2.6 (53–62)	–	–	–	54 ± 3.4 45–59	50 ± 2.7 (42–54)	–	–	–
W	268 ± 28 (225–318)	123 ± 11 (103–142)	163 ± 15 (145–186)	60 ± 5.2 (51–69)	23 ± 0.8 (22–25)	154 ± 34 (102–236)	80 ± 10 (64–97)	105 ± 19 (75–132)	73 ± 13 (56–96)	21 ± 1.8 (18–25)
EP	92 ± 16 (56–107)	76 ± 5 (69–83)	73 ± 6 (62–84)	61 ± 4 (55–66)	34 ± 1 (32–35)	53 ± 13 (33–86)	137 ± 21 (96–195)	55 ± 10 (43–78)	44 ± 4 (35–50)	32 ± 1.5 (28–34)
NR	154 ± 14 (134–179)	108 ± 6 (98–118)	106 ± 11 (82–117)	90 ± 5.4 (82–100)	67 ± 3.4 (61–70)	93 ± 17 (65–114)	159 ± 13 (142–198)	92 ± 19 (59–140)	90 ± 10 (73–109)	63 ± 3.1 (57–70)
ES	226 ± 12 (207–244)	157 ± 11 (139–180)	149 ± 10 (124–160)	122 ± 7 (114–133)	95 ± 3 (90–99)	132 ± 31 (52–190)	193 ± 16 (173–236)	115 ± 15 (86–146)	122 ± 11 (106–141)	92 ± 4.1 (84–101)
ABD	92 ± 8.8 (80–110)	41 ± 3.8 (33–46)	41 ± 1.4 (40–44)	26 ± 1.4 (24–28)	11.6 ± 0.5 (10.8–12)	47 ± 10 (25–68)	28 ± 3.2 (21–34)	31 ± 3.8 (23–38)	27 ± 2.3 (23–32)	12 ± 0.8 (11–14)
T	37 ± 4.4 (31–46)	41 ± 5 (31–47)	29 ± 1.7 (25–32)	20 ± 1.4 (18–23)	45 ± 1.5 (43–47)	19 ± 5.0 (12–30)	103 ± 15 (65–135)	19 ± 2.1 (16–23)	19 ± 3.6 (15–27)	45 ± 3.2 (38–53)
HT	–	–	–	–	19.6 ± 1 (18.4–22)	–	–	–	–	26 ± 3.9 (20–32)
SPL	–	–	74 ± 6 (62–81)	60 ± 3.6 (57–68)	–	–	–	70 ± 5.2 (58–78)	64 ± 5.4 (55–76)	–
SPW	–	–	11.6 ± 1 (10.2–13)	10 ± 0.7 (8.6–10.8)	–	–	–	–	–	–
GUL	–	–	53 ± 4 (48–58)	43 ± 4.2 (38–50)	–	–	–	52 ± 5.1 (42–63)	44 ± 5.6 (35–53)	–
GUW	–	–	7 ± 0.6 (6.3–7.8)	6 ± 0.8 (4.7–7.6)	–	–	–	–	–	–
H%	–	–	–	–	44 ± 2.5 (40–47)	–	–	–	–	57 ± 7.4 (44–73)
D%	41 ± 7 (24–46)	49 ± 3.7 (43–56)	49 ± 4.8 (40–55)	50 ± 2.5 (46–55)	35 ± 1 (34.7–37)	44 ± 20 (28–117)	71 ± 10 (50–87)	48 ± 8.7 (37–64)	36 ± 3.3 (31–42)	35 ± 2.2 (31–40)
E%	247 ± 42 (160–302)	193 ± 26 (162–250)	256 ± 29 (203–296)	301 ± 34 (239–340)	75 ± 2 (72–78)	292 ± 84 (156–453)	137 ± 28(85–189)	295 ± 66 (205–429)	237 ± 45 (167–313)	72 ± 6.4 (54–84)

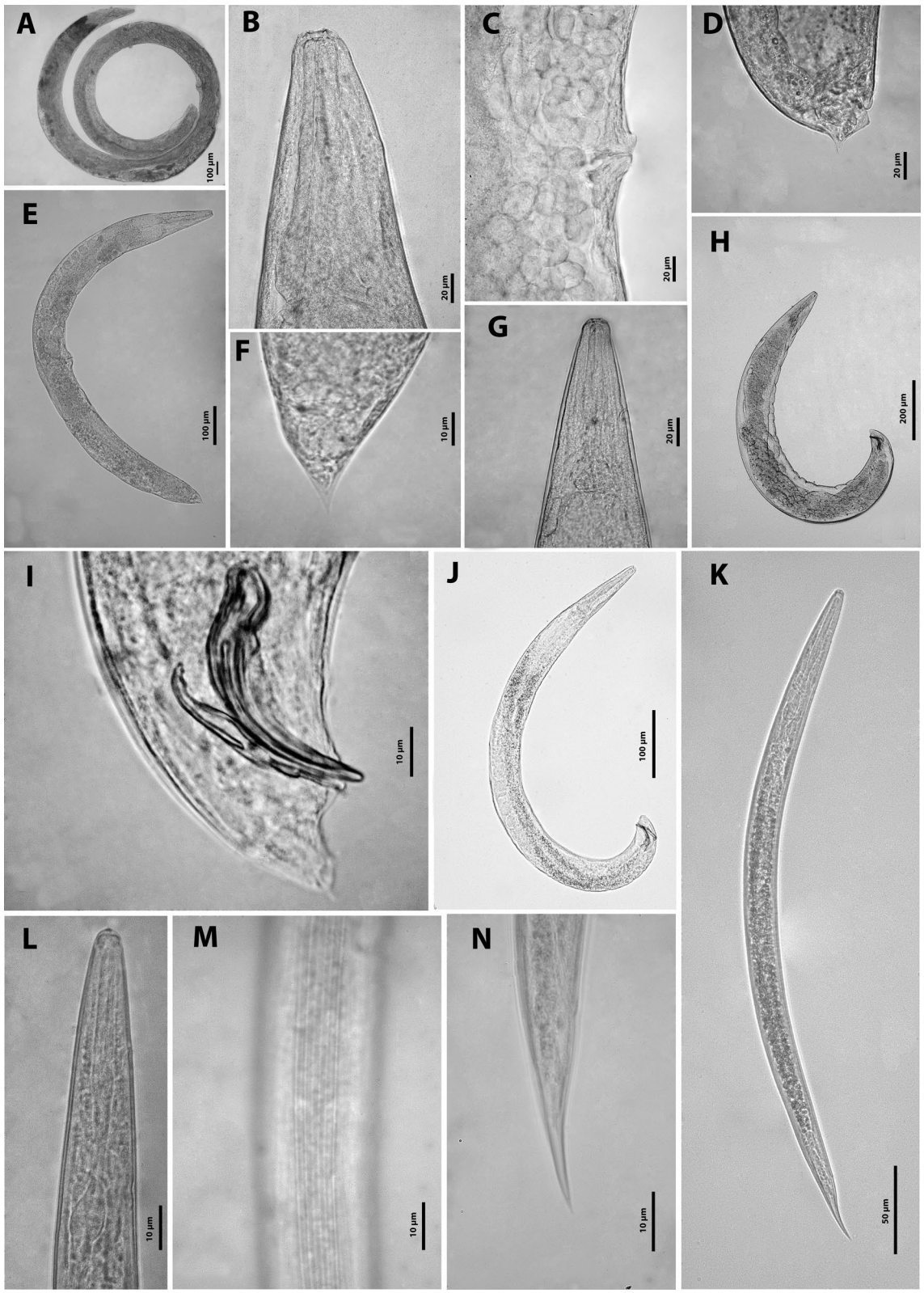

Fig. 1 LM pics of *Steinernema surkhetense* from Vietnam. **A**–**D** first-generation female. **A** entire body; **B** pharyngeal region; **C** vulva region; **D** tail region. **E**–**F** second-generation female. **E** entire body; **F** tail region. **G**–**I** first-generation male. **G** pharyngeal region; **H** entire body; **I** tail region. **J** entire body of second-generation male. **K**–**N** juvenile. **K** entire body; **L** pharyngeal region; **M** lateral field; **N** tail region

Fig. 2 BI Phylogenetic tree generated from D2-D3 of 28S rRNA sequences under GTR + G + I. Bayesian posterior probabilities (in percentage) are given next to each node. Sequence of *Steinernema surkhetense* from Vietnam is in red and bold

Table 2 Lethal efficacy of *Steinernema surkhetense* to larvae of *Galleria mellonella*

Treatments	No. of innoculation concentration (IJs/larva)	Average mortality rate (%)
Control	0	0^{a}
1	10	60^{b}
2	20	73.33^{c}
3	30	86.67^{de}
4	40	93.33^{ef}
5	50	100^{f}
6	60	100^{f}
7	70	100^{f}
8	80	100^{f}
9	90	100^{f}
10	100	100^{f}

* Note: different superscript letters in the third column indicate significant difference

Male (second generation)

Similar to first-generation of males, but body size was smaller (Fig. 1J).

Infective juvenile

Body was short, slender, slightly curved ventrally, and tapering to both two ends (Fig. 1K). Lip region was rounded and continuous to body contour (Fig. 1L). Lateral field was with 9 lines at mid-body (Fig. 1M). Amphids was slit-like. Hemizonid was distinct and located at beginning of basal bulb. Secretory-excretory pore was prominent and located at anterior third of pharyngeal region. Hyaline region was occupying half of tail length. Tail was tapering to pointed tail end (Fig. 1N). Phasmid was pore-like and located at mid-tail.

Molecular characterization

Obtained D2-D3 region of 28S rRNA sequence of *S. surkhetense* from Vietnam was 695 bp long (assession number: MW703809). It was 100% similar to other sequences of *S. surkhetense* from GenBank (accession number: MF621001, HQ190043). The phylogenetic tree showed that the D2-D3 region of 28S rRNA sequence of *S. surkhetense* from Vietnam had a sister relationship to the sequences of *S. surkhetense*, *S. nepalense* Khatri-Chhetri et al. (2011), *S. siamkayai*, *S. huense*, and *S. carpocapsae* with a maximal support (1 PP). (Fig. 2).

Virulence of S. surkhetense from Vietnam

Probit analysis in SPSS version 25 showed that LC_{50} (nematode inoculation concentration kill 50% larvae of *G. mellonella*) of *S. surkhetense* was 14 IJs/larva. Besides, a highly significant correlation between nematode inoculation concentration and the mortality rate of larvae was found based on the Pearson correlation test ($R = 0.752$, $p < 0.01$). The One-way ANOVA analysis showed that the lethal efficacies of all treatments are significantly different than the control (Table 2).

Discussion

Generally, the morphology of *S. surkhetense* from Vietnam is highly in agreement with the description of Khatri-Chhetri et al. (2011). Variations in some measurements between these populations were found, including larger L, EP, ES, body diam., T, a, b, c, E% in the first-generation females; longer body length L, EP, ES, T, smaller c and E values in the first-generation males; longer body length and smaller H% value in juveniles (Table 1). However, according to Hunt and Nguyen (2016), large variations in measurements of adult *Steinernema* spp. are known to be present since their body sizes are much larger than juveniles. Besides, other populations of *S. surkhetense* from different localities in the world also showed variations in measurements.

Khatri-Chhetri et al. (2011) differentiated *S. surkhetense* from *S. nepalense* using a combination of morphological and molecular characterizations. Especially, ITS rDNA sequence of *S. surkhetense* was significantly different from that of *S. nepalense*. However, obtained result showed that D2-D3 expansion segment of 28S rRNA sequence of *S. surkhetense* in this study was very similar (only 4 nucleotides difference) to that of *S. nepalense* (accession number: HQ190045). On the other hand, Nguyen and Hunt (2007) reported that cross-hybridization study can be applied to separate closely related species of *Steinernema* spp., therefore, such studies can be done in the laboratories where populations of both *S. surkhetense* and *S. nepalense* present to support species status of these species in future.

The study of De Doucet et al. (1999) suggested that larvae of *G. mellonella* can be used as a standard host for testing and comparing virulence (based on LC_{50}) of EPNs. Their experiments on larvae of *G. mellonella* showed that LC_{50} of *Steinernema rarum* = 6 IJs/larva, *Steinernema feltiae* = 9 IJs/larva, and *Heterorhabditis bacteriophora* = 3 IJs/larva. In Vietnam, Nguyen (2008) reported that LC_{50} of 2 potential strains of EPNs (*Steinernema* S-TK10 and *Heterorhabditis* H-TN3) on larvae of *Spodoptera litura* (Fab.), *Omiodes indicate* (Fab.), *Agrotis ipsilon* (Huf.), *Spodoptera exigua* (Hub.), *Pieris rapae* (L.), and *Plutella xylostella* (Linn.) were 13–95 IJs/larva. In this study, LC_{50} of *S. surkhetense* from Vietnam was 14 IJs/larva, indicating that this is a relatively potential strain of EPNs to be used in the biocontrol of insect pests. However, it is also important to test the virulence

of this strain on other insect pests to evaluate its potential in biocontrol exactly.

Conclusions

Results conclude that *S. surkhetense* was present in Vietnam and this is a new distribution of the species. There exist small variations in measurements of *S. surkhetense* from different population, for example, variations in L, EP, ES, body diam., T, a, b, c, and E% in the first-generation females; body length, EP value, ES value, T value, c value, and E% value in the first-generation males; body length and H% value in juveniles. The new strain of *S. surkhetense* found in this study can be a potential strain for biological control.

Abbreviations

EPNs: Entomopathogenic nematodes.

Acknowledgements

Not applicable.

Authors' contributions

PQT was the supervisor of the project. DTN prepared the data. LTML was responsible for molecular data. THN prepared the manuscript. All authors contributed to writing and editing the manuscript. All authors read and approved the final manuscript.

Funding

This research was funded by the Institute of Ecology and Biological Resources, Vietnam Academy of Sciences and Technology (project code: VAST04.04/21-22).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no conflict of interest.

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Received: 24 September 2021 Accepted: 23 November 2021
Published online: 27 November 2021

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