Egyptian Journal of Biological Pest Control

RESEARCH

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Heterorhabditis alii n. sp. (Nematoda: Heterorhabditidae), a novel entomopathogenic nematode from Egypt used against the fall armyworm, *Spodoptera frugiperda* (Smith 1797) (Lepidoptera: Noctuidae)

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Abstract

Background Isolation of novel species of entomopathogenic nematodes (EPNs) with biocontrol potential against important insect pests is very important for the sustainable management of economic pests damaging food crops and providing protection to the agricultural environment. This study was aimed to new indigenous EPN isolates from Egyptian agricultural soils and studies its biocontrol potential for further use in the biological control programs. Five out of 15 soil samples obtained from a farm located at the Cairo–Alexandria desert highway was positive for the presence of EPN, using the greater wax moth baiting method.

Results Sequencing of the internal transcribed spacer (ITS) region of 4 of the nematode isolates suggested that they belong to the species *Heterorhabditis indica*. However, one isolate does not show a high similarity to any of the *H. indica* previously recorded in the database of the Gen Bank and hence was identified as a new *Heterorhabditis* species and was deposited at the National Center for Biotechnology Information (NCBI) and registered under accession no. (OP555450) under the name of *Heterorhabditis alii*. This new species was also registered in the ZooBank under the registration link of: LSID urn: lsid: zoobank.org: act: 306F9D57-CC30-4B8E-8B19-4F0E42B08F34. No males were found in this species. Morphological characterization using the light microscope (LM) and scanning electron microscope (SEM) confirmed the identification of this nematode as a new species of the genus *Heterorhabditis*. Moreover, virulence of this new species against the fall armyworm (FAW), *Spodoptera frugiperda* (Smith 1797) (Lepidoptera: Noctuidae) was tested in comparison with the foreign EPN species, *Heterorhabditis bacteriophora* (HP88) and the local *Heterorhabditis indica* (Mango 2 isolate) and proved to be more effective against this devastative insect pest than the two compared species.

Conclusions The present study found out a new species of the EPN genus, *Heterorhabditis* in Egypt. Our results were confirmed by both morphological and molecular analyses. The efficacy of this new species against the FAW proved to be a potent and safe biocontrol agent that can be used in biological control programs against this invasive insect pest of corn in Egypt and other global countries.

Keywords Entomopathogenic nematodes, *Heterorhabditis* sp., DNA sequencing, *Spodoptera frugiperda*

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Background

Entomopathogenic nematodes (EPNs) are very important biocontrol agents that are characterized by the ability to search and find their insect hosts, they are specific pathogens of insects and safe on nontarget insects and beneficial organisms (Gozel and Gozel 2016). EPNs of the family Heterorhabditidae are obligate insect parasites that spend part of their life cycle inside their hosts. The third juvenile stage in the cuticle of the second stage is the infective juvenile (IJ). Once the IJs penetrate the host, they release the bacteria that live in symbiosis within the EPN gut and multiply fast and under optimal conditions causing host mortality within 24–48 h (Bhat et al. 2020). Isolation of novel species and/or isolates of the EPNs with biocontrol potential against economic insect pests is very promising for the sustainable management of those pests on economic crops in addition to providing protection to the environment (Tarasco et al. 2023). Globally, about 22 species of *Heterorhabditis* and 102 species *Steinernema* have been reported before (Hazir et al. 2022). However, new EPN species still being found and described from different parts of the world (Půža et al. 2020). In Egypt, many surveys of different soil types were conducted and a lot of nematode isolates and different species were found from all over the country (Khashaba and Abd El Azim 2021).

In the study, the isolation of a new species of *Heterorhabditis* from Egypt was reported by morphological and molecular identification. In addition, the efficacy of the new species against the FAW compared to two local EPN isolates of *Heterorhabditis indica* was determined.

Methods

Soil sampling and isolations of the entomopathogenic nematodes

In 2021, 35 soil samples were randomly collected from a farm on Alexandria Highway desert road, Cairo, Egypt, with specific coordinates of N 30° 16′ 48.7″, E 30° 41′ 50.0″. Each soil sample (1 kg) was randomly taken from a depth of 3–30 cm using a hand shovel and sealed in a plastic bag. All soil samples were kept in an icebox and transported to the laboratory located in the building of the Applied Center for Entomonematodes (ACE), Faculty of Agriculture, Cairo University, Egypt, and stored at 13 °C separately for the detection of any EPN in the isolated soils. Nematodes were recovered from the soil samples using the insect-baiting technique (Tarasco et al 2020). Each sample was baited by 10 last instar larvae of the greater wax moth, *Galleria mellonella* (Linnaeus 1758). The baited cups were inverted and kept in the dark at 25 ± 2 °C and 75 ± 5 RH. During 10 days as a holding period, the samples were checked for dead insects. Cadavers of insect larvae were transferred individually to the modified White traps (Kaya and Stock 1997). If the larvae were found still alive, the results were considered negative and the soil sample was discarded. In the positive cups, cadavers were removed, rinsed carefully with sterile distilled water, and transferred to the White trap dishes (White 1927) to harvest nematode infective juveniles (IJs). Nematodes were harvested within the first week of emergence and used to inoculate *G. mellonella* last instar larvae to confirm their pathogenicity to the insects. Infective juveniles (IJs) were transferred onto moist filter paper in Petri dishes where living *G. mellonella* larvae were added. The new generations of IJs were collected in a beaker and rinsed twice with sterile distilled water and stored at 13 °C as described by (Kaya and Stock 1997).

Molecular identification of the novel nematode species

To identify the obtained five nematode isolates with a molecular method, the amplified fragments (700–800 bp) of the 18S rRNA and ITS1 regions were sequenced. Depending on the provided sequencing charts, the quality of sequenced 18S rRNA fragments was manually assessed and the low-quality sequences were trimmed accordingly. The sequence similarity comparisons between the five isolates and those previously deposited in NCBI, using nucleotide BLAST against the whole database, were established. Molecular characterization of the isolates was performed by the analysis of the ITS rDNA sequences. Infective juveniles of each isolate were concentrated by a centrifugation at 3000 g for 10 min. in EN buffer (100 Mm NaCl, 10 mM EDTA) and then washed three times with sterile distilled water. The resulting nematode pellet was ground in liquid nitrogen and genomic DNA was extracted using the QIAamp® DNA Mini Kit (QIGEN Cat. No. 51304) according to the manufacturer's recommendations. The quality and quantity of DNA were determined using the Thermo Scientific Nanodrop 2000c. The amplification of ITS region of the nematode 18S rDNA was performed in the Veriti® 96-well Thermal Cycler (Applied biosystems) at the biotechnology laboratory of Cairo University Research Park. The 50 µl reaction mix contained 25 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific Cat. No. #K1081), 100 ng of the DNA suspension and 2 µl of each of the forward and reverse primers were observed. The PCR cycling profile included initial denaturation at 95 °C for 3 min., followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Subsequently, the product was loaded on a 1.5% agarose gel. The primer set used for the amplification was the ITS primers: 5′-TTGATTACGTCCCTG CCCTTT-3′ (forward) and 5′-TTTCAC TCGCCGTTA CTAAGG-3′ (reverse) (Subbotin et al. 2000). The PCR

products were purified using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega) following the manufacturer's instructions. PCR products were sequenced using sequence-specific primers with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem, USA) and carried out on ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, USA) in both directions by the Macrogen Inc. service, South Korea. All obtained DNA sequences were firstly edited to remove low-quality sequences, using Bioedit sequence alignment editor (Hall 1999). The identity of approximately 700–800 bp sequences was confirmed by a BLAST (Basic Local Alignment Search Tool) for sequence similarity searches at NCBI (National Center for Biotechnology Information). The obtained sequences of the heterorhabditid isolate were compared by sequences of *Heterorhabditis* spp. and submitted to the NCBI database with the accession numbers.

Identification of the isolated five heterorhabditid nematode species

All the isolated EPNs samples were identified as *Heterorhabditis* spp. due to the reddish color of their cadaver's affected by the symbiotic bacterial colonies. The isolated nematode species was cultured on the last instar larvae of *G. mellonella*. Infective juveniles (IJs) were killed in hot (55 ^{O}C) Ringer's solution (10% saline solution) or water, fixed in TAF, and processed to glycerin for measurements. Three and four days after infection, insect cadavers were dissected in Ringer's solution to recover the hermaphroditic females by dissecting the cadavers after five to six days post-infection. Further morphological identification of the obtained five heterorhabditid isolates to the species level was conducted by the use of both light and scanning electron microscope (SEM). One of the five nematode isolates was different according to the SEM examination. This isolate was identified as the new species, *Heterorhabditis alii*.

Sample preparation for scanning electron microscope (SEM)

For Scanning Electron Microscopic (SEM) examination, nematode specimens were first fixed in 3% glutaraldehyde in a 0.1 M. phosphate buffer at pH 7.2 4–8 °C for two days (Nguyen and Smart 1995). They were post-fixed overnight in 2% osmium tetroxide at 23 °C for 12 h, dehydrated in a graded ethanol series, critical point dried with liquid CO2, mounted on SEM stubs, and coated with gold and examined under the JEOL JSM-5410 scanning electron microscope (SEM). Infective juveniles (IJs) were fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.2 for 24 h at 8 °C (in the refrigerator) (Nguyen and Smart 1990). All nematode stages were post-fixed with 2% osmium tetroxide solution for 12 h at 25 °C and dehydrated in a graded ethanol series, critical point dried with liquid CO_2, mounted on SEM stubs, and gold coated. All specimens were examined with SEM operating at 5 kV.

Laboratory testing of isolated EPNs for virulence against the fall armyworm (FAW)

Experimental design

The efficacy of the new EPN isolated species of *H. alii* n. sp. in addition to two other isolates of EPN, one foreign isolate of *Heterorhabditis bacteriophora* (HP88), and one local, *Heterorhabditis indica* (Mango 2) against FAW larvae was determined. Nematodes infective juveniles were tested against 3rd, 4th, 5th, and 6th larval instars of the FAW. A completely randomized design (CRD) was used for each isolate. All experiments were replicated three times. Treatments consisted of three different concentrations of each EPN per ml. of sterile distilled water 100, 250, and 500 IJs per ml. and the same volume of sterile distilled water without EPN was used as controls.

Treatment of the new EPN species against the fall armyworm larvae

Fall armyworm (FAW) larvae were in vivo cultured to the 3rd, 4th, 5th, and 6th larval instars and three larvae were placed into a Petri dish (5.5 cm in diameter) and fed on castor leaves. Using a micropipette, 1 ml. of the infective nematode juvenile suspension was mixed with 1 ml. of sterile distilled water and the mixture was added to cover the leaf and the larvae. As a control, 2 ml of sterile distilled water without nematode IJs was applied. Treated larvae then were incubated at 25 ± 2 °C under 60 ± 5% RH at 14:10 (light: dark) photoperiod. Food was changed every 24 h, each treatment was repeated three times.

Assessment of mortality and data collection

Mortality was assessed 24, 48, and 72 h post-treatment. Insect larvae were detected and counted as dead when they did not respond to the touch of the forceps. For the white trapping technique (White 1927), within 7–10 days, insect cadavers were observed for the emergence of infective nematode juveniles. Only larvae with emerging infective nematode juveniles were recorded as nematode-killed larvae.

Statistical analysis

Analysis of variance (ANOVA) was applied on the collected data. ANOVA was performed on the number of dead insect larvae with different nematode isolates and different IJs concentrations. Means were compared using the least significant differences (LSD) test to detect

differences between treatments at ($p \leq 0.05$) using the SPSS software.

Results

Due to the limited numbers of heterorhabditid species in comparison with other EPNs belong to the genus *Steinernema* and relatively low variations of morphological differences among the heterorhabditids, molecular approach was adopted first in this publication to identify the heterorhabditid nematode isolates found by the authors. The present molecular work confirmed that the strain SBIH2 is a new heterorhabditid species. Furthermore, the nematode examination with both the light and scanning electron microscopes supports the new identity of this novel species.

Identification of the isolated entomopathogenic nematodes based on molecular analysis

Five EPNs isolates were found in the soil samples under grape trees et al.—Khatatba Center, Menoufia Governorate, Egypt.

Numerous species of *Heterorhabditis* EPNs were globally identified based on morphometrical and molecular data. Morphology was not the only decisive criterion to surely identify a certain nematode species and that is why it was depended first on molecular analysis to classify the collected nematode isolates.

The results of applying our molecular techniques indicate that four out of the five sequenced isolates were identified as *H. indica* due to the high identity and coverage score of their 18S rRNA with those of *H. indica* species. Interestingly, a single isolate was found to be relatively different from other sequenced isolates. Since the sequence could place this isolate under a different *Heterorhabditis* species, it could not exclusively classify as *H. indica* based on nucleotide similarity of its 18S rRNA (Fig. 1). It was found that the sequenced forward strand of this isolate had a similarity score to multiple *Heterorhabditis* species, such as *H. indica, H. hawaiiensis, H. brevicaudis* and *H. sp.* MM7 at equal E-score of 0.0, equal coverage of 62%, and percentage identity more than 97.70%. The closest NCBI-deposited strain was *Heterorhabditis* sp. isolate SBIH2 (Sankaranarayanan et al. 2017) with an E-score of 0.0, coverage of 100%, and percentage identity of 86.86% to the sequenced forward strand of our isolate. Therefore, this isolate was identified as a new *Heterorhabditis* species and its sequence was deposited at the National Center for Biotechnology Information (NCBI) and registered under accession no. (OP555450) with the name of *Heterorhabditis alii* (isolate SHM). New nematode isolate requires further morphometrical and Scanning Electron Microscopy investigations as well as detailed behavioral studies as a novel heterorhabditid nematode species.

Morphometrical and morphological identification of *Heterorhabditis alii* n. sp. measurements

No males were found when the cadavers of the infected last instar larvae of *G. mellonella* were dissected starting from the 7th up to the 11th days post-infection.

Infective 3rd stage Juvenile (Fig. 2) ($n=20$): $L=497.64$ (474.26–526.45) greatest diameter = 19.51 (17.80–22.33) pharynx length = 110.37 (100.95–118.00) Tail length = 95.96 (88.50–102.10) (Table 1).

Hermaphroditic female (Fig. 3) (n = 20): L = 3532.67 (2646.28–4760.54) Distance from head to vulva opening = 1565.44 (1264.81–2054.81) Great diameter = 185.47 (157.59–207.84) Esophagus length = 171.06 (165.00–204.43) Tail length = 116.35 (98.30–137.52) Body diameter at anus = 48.02 (40.67–56.34) (Table 2).

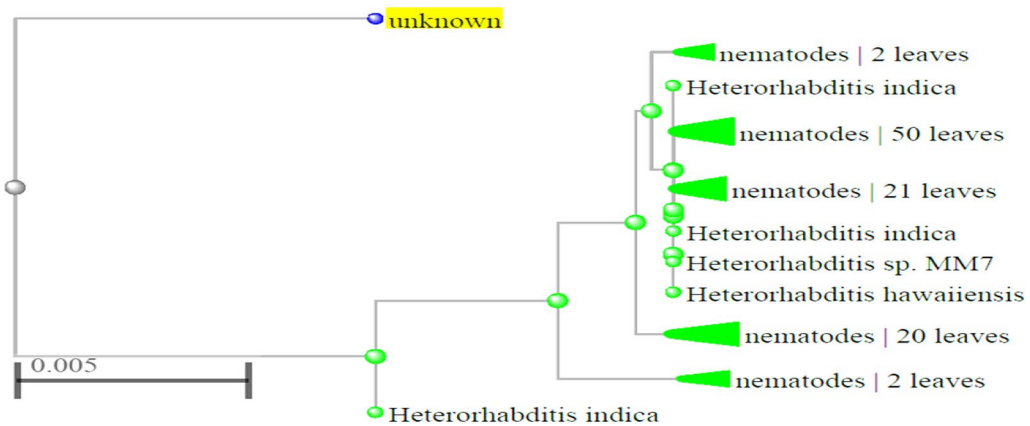

Fig. 1 Drawn cladogram using the Fast Minimum Evolution method with 0.75 maximum sequence differences of our novel isolate # 2 and other similar species retrieved from NCBI BLAST

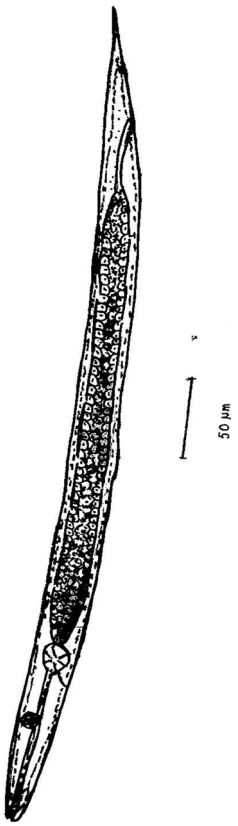

Fig. 2 The third infective juvenile stage of *Heterorahbditis alii*, **a** the whole body; **b** Anterior end; **c** Posterior end

Table 1 Morphometrics in micrometers of infective nematode juveniles of the novel species, *Heterorhabditis alii*

Measured individuals	Characters			
	L	W	PHL	TL
1	485.10	18.50	107.00	92.43
2	510.60	21.90	110.90	102.10
3	512.60	22.27	113.45	101.10
4	480.50	17.50	110.40	90.90
5	501.30	18.60	112.00	99.15
6	526.45	22.33	100.95	100.87
7	487.20	19.00	107.50	93.00
8	494.60	19.40	109.20	96.00
9	476.42	18.24	105.30	90.00
10	480.04	17.99	114.20	91.70
11	517.50	20.30	117.30	99.00
12	490.54	19.35	116.10	95.10
13	505.25	19.20	112.80	100.10
14	474.29	19.11	103.00	88.50
15	481.80	17.80	105.10	91.00
16	495.41	19.59	109.90	96.15
17	499.50	18.20	111.70	97.90
18	518.30	21.89	118.00	99.80
19	500.44	18.11	111.80	98.56
20	515.10	21.00	110.90	95.90
Average	497.64	19.51	110.37	95.96

L = length; W = greatest body width.; PHL = pharynx length; TL = tail length

Description

Cadavers of the nematode infected greater wax moth were dissected and examined for five generations, with no males or amphimictic females were found in this new species. The only available stages found out in the life cycle of *H. alii* were the hermaphroditic females and the third juvenile stages. This phenomenon is a unique character in *H. alii* as a new EPN species. Neither the males nor amphimictic females found in this novel species, cross hybridization between this species nor other closely related nematode species could not be performed.

Hermaphroditic females: with paired, amphidelphic reflexed ovaries with sperm occurring in the proximal portion of the ovotestis; anal region conspicuously swollen. The anus occurred approximately at the anterior third of the anal swelling. The rectum is heavily cuticularized and a conspicuous valve separates it from the intestine. In comparison with other species that belong to the genus *Heterorhabditis*, the novel species *H. alii* is a medium size species in most measurements.

Third-stage infective juveniles: The infective juveniles (third-stage juveniles inside second stage cuticles). This species is one of the shortest among the previously described species of the genus *Heterorhabditis* The lips of the third-stage juvenile contain a large dorsal tooth and two smaller but distinct subventral teeth. The stoma walls are opened and not collapsed at the base. The outer second stage cuticle is strongly ribbed longitudinally and is closely oppressed to the third-stage cuticle. The hemizonid is quite distinct and located just anterior to the excretory pore. The smaller less distinct hemizonion is located in the anterior intestinal region. The ventricular portion of the intestine was devoid of intestinal cells and forms an intestinal pouch which was filled with the symbiotic bacteria. These bacteria also occurred in the lumen of the intestine, mainly in the anterior portion.

The described new species was preliminary registered in the ZooBank under the registration link of: LSID urn: lsid: zoobank.org: act: 306F9D57-CC30-4B8E-8B19-4F0E42B08F34.

The present research article was also preliminary registered in the ZooBank under the following link: LSID urn: lsid: zoobank.org: pub:541C0F91-8EA9-4660-A0B0-CA00FA7621D3.

This novel species will have an official status on the website of the ZooBank after the publication of the present manuscript.

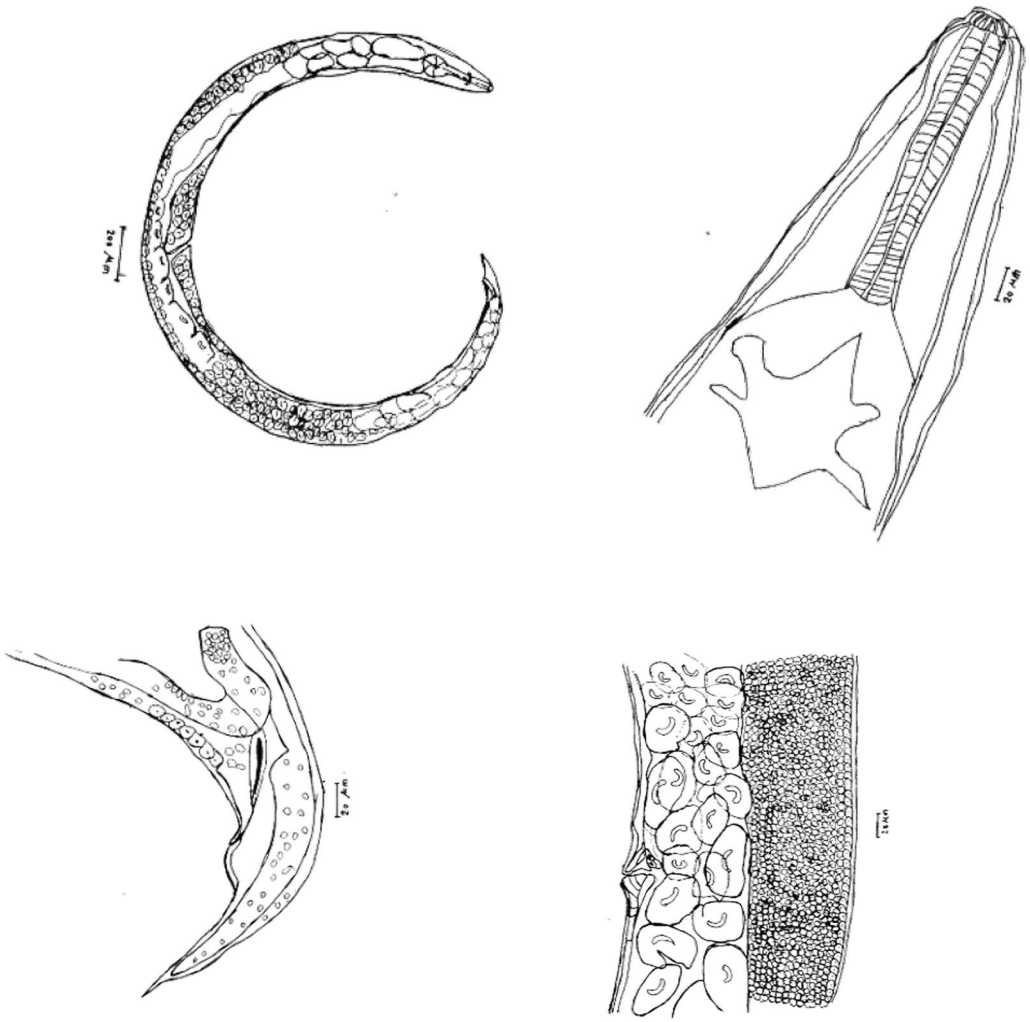

Fig. 3 Hermaphroditic female of the novel species *Heterorhabditis alii*, **a** The whole female body; **b** Anterior end; **c** Region of the vulva; d) posterior end

Scanning electron microscope examination

The nematode infective juveniles were prepared for scanning electron microscope to demonstrate the morphological differences between *H. indica* and the novel species of *H. alii*. Ridges exists on the ventral side of the infective juveniles of *H. alii* were obvious while all IJs of *H. indica* had no ridges on their ventral side (Figs. 4, 5, and 6).

Etymology

The new species of *H. alii* described here is named after the late Prof. Mohamed A. M. Ali of the Department of Plant Protection, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt, in recognition of his endeavors to start the field of Entomonematology as early as the year 1968 in Egypt.

Laboratory bioassay of the new species against the FAW larvae

Virulence of the novel *H. alii* species against the FAW larvae was tested in comparison with one local EPN species, *H. indica* (Mango 2) and another foreign species of *H. bacteriophora* (HP88) using three different concentrations of 100, 250, and 500 IJs of the nematode infective juveniles (IJs) per 1 ml. of nematode suspension. The concentration screening assay showed that the new species, *H. alii*, with all tested instar larvae caused the highest mortality rate followed by *H. bacteriophora* (HP88), while *H. indica* (mango 2) caused the least mortality among the three tested nematodes (Table 3 and Figs. 7, 8, 9, and 10).

After 24 h post-infection and when the novel species of *H. alii* was used at a concentration of 500 IJs/ml., 100% mortality rate was recorded for the 3rd, 4th, and 5th larval instars while the same concentration caused 78% with

Table 2 Morphometrics in micrometers of the hermaphrodite female of the novel species, *Heterorhabditis alii*

Number of individuals	Characters						
	TBL	HV	ES	W	VW	ABW	TL
1	3743.55	1624.90	172.75	178.71	183.88	52.19	117.42
2	3650.20	1480.60	166.80	150.40	164.30	41.80	135.90
3	3200.50	1460.85	171.95	190.60	195.00	45.00	111.10
4	3368.16	1504.11	166.63	201.96	207.84	51.74	112.57
5	4267.20	1806.55	169.33	181.03	189.54	49.55	116.88
6	4050.90	1750.90	168.00	179.00	188.90	48.00	114.90
7	2646.28	1264.81	171.01	193.88	197.37	55.47	119.18
8	4100.40	1760.20	170.30	183.50	190.00	56.30	118.90
9	4760.54	2054.81	204.43	190.69	203.37	56.34	122.35
10	2800.80	1300.00	173.00	195.70	199.00	54.00	120.80
11	3020.00	1410.70	165.00	185.30	190.70	42.80	109.90
12	3596.74	1630.27	173.87	170.90	179.30	45.20	117.33
13	3142.94	1455.34	170.32	187.37	193.79	44.56	110.38
14	3700.70	1590.00	170.90	178.00	184.30	50.00	116.40
15	3702.13	1570.00	167.23	153.00	165.40	40.67	137.52
16	3400.60	1610.30	171.50	168.60	178.80	44.95	116.90
17	4270.10	1815.40	170.10	184.70	190.00	50.80	118.90
18	3122.53	1378.07	165.58	155.25	161.62	45.04	102.33
19	2990.90	1380.70	163.70	183.35	188.85	42.00	109.15
20	3118.28	1460.43	168.92	150.94	157.59	44.08	98.30
Average	3532.67	1565.44	171.06	178.14	185.47	48.02	116.35

TBL = Total body length (from tip of head to end of tail); HV = Distance from head to vulva opening; ES = Esophagus length (distance from head to base of esophagus (Basal bulb); W = Body width; VW = Vulva body width; ABW = Anal body width; TL = Tail length (from anus/cloaca opening to end of tail)

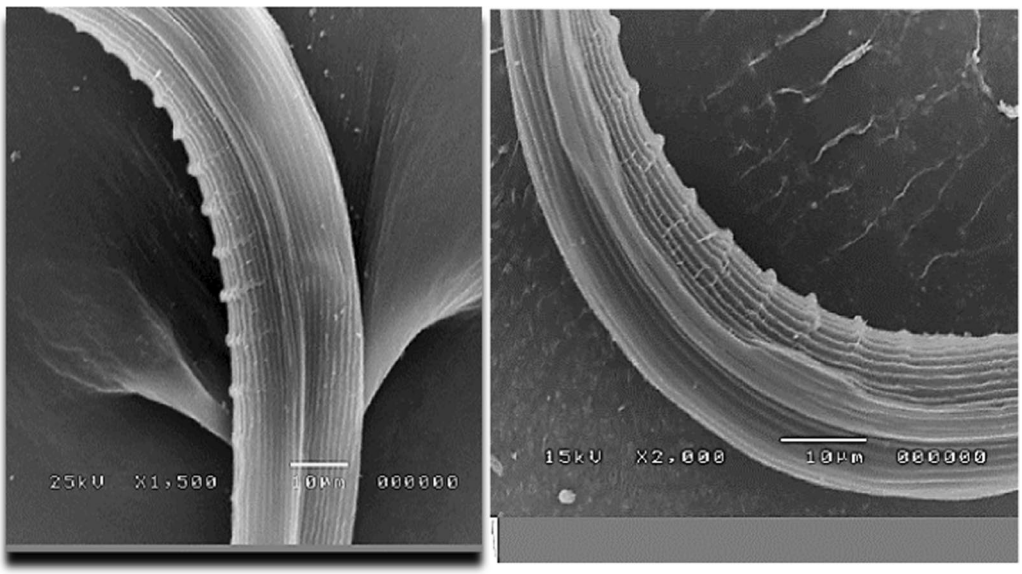

Fig. 4 Scanning electron photomicrographs of *Heterorhabditis alii* infective juveniles show the transverse ridges exist on the nematode ventral side

the 6th instar larvae (Table 3). In comparison, *H. bacteriophora* (HP88) caused less mortality rates of 44, 22, 22, and 11% on the 3rd, 4th, 5th, and 6th larval instars, respectively. As *H. indica* (Mango 2) when used at a concentration of 500 IJs/ml against the 3rd instar larvae. Mortality rates was the least and almost stable at 22, 11,

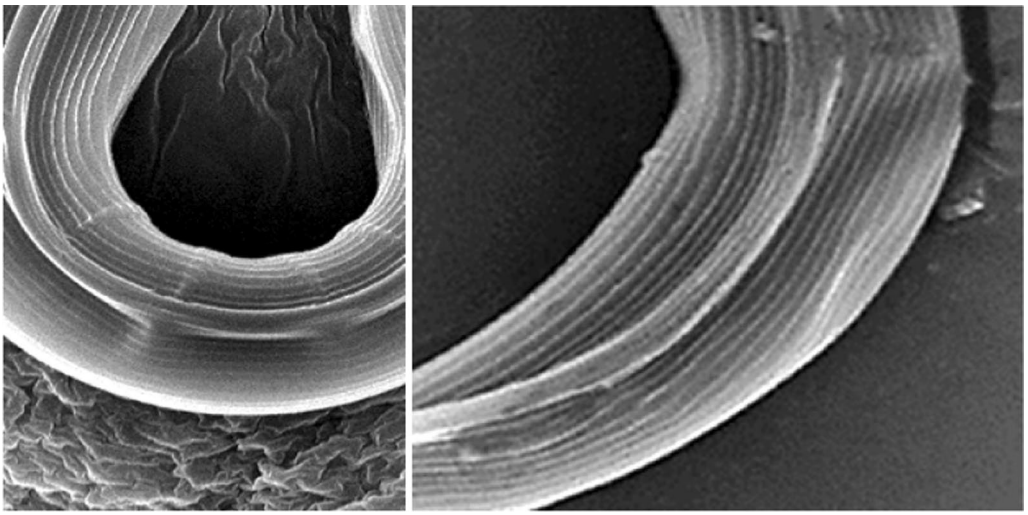

Fig. 5 Scanning electron photomicrographs of the infective juvenile ventral side of *Heterorhabditis indica* (right side of the graph) without transverse ridges and *Heterorhabditis alii* (left side of the graph) with transverse ridges

Fig. 6 Scanning electron micrographs of the infective juveniles to compare between *Heterorhabditis indica* (upper two pictures) and *Heterorhabditis alii* (bottom two pictures). Notice the transverse ridges in the novel species of *H. alii* (bottom pictures)

Table 3 Mortality rate of *Spodoptera frugiperda* for four larval instars induced by three concentrations of infective juveniles (IJs) for two native and one foreign heterorhabditid species in three time intervals

Nematode species	Time in hours								
	24			48			72		
	IJs/ml./3 larvae*			IJs/ml./3 larvae*			IJs/ml./3 larvae*		
	100	250	500	100	250	500	100	250	500
	***S. frugiperda* 3rd instar larvae**								
Heterorhabditis. alii	67^{bcd}	78^{bcd}	100^{a}	89^{ab}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Heterorhabditis. bacteriophora (HP88)	22^{fgh}	33^{efg}	44^{def}	67^{bcd}	67^{bcd}	89^{ab}	100^{a}	100^{a}	100^{a}
H. indica (Mango 2)	11^{gh}	22^{fgh}	22^{fgh}	56^{cde}	56^{cde}	67^{bcd}	100^{a}	100^{a}	100^{a}
LSD value at 0.05	22.909								
	4th instar larvae								
H. alii	56^{cde}	78^{abc}	100^{a}	89^{ab}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
H. bacteriophora (HP88)	22^{fgh}	33^{efg}	22^{fgh}	67^{bcd}	56^{cde}	67^{bcd}	89^{ab}	100^{a}	100^{a}
H. indica (Mango 2)	11^{gh}	22^{fgh}	11^{gh}	56^{cde}	44^{def}	78^{abc}	89^{ab}	100^{a}	100^{a}
LSD value at 0.05	22.874								
5th instar larvae									
H. alii	44^{def}	67^{bcd}	100^{a}	89^{ab}	100^{a}	100^{a}	89^{ab}	100^{a}	100^{a}
H. bacteriophora (HP88)	11^{gh}	11^{gh}	22^{fgh}	44^{def}	44^{def}	56^{cde}	78^{abc}	100^{a}	100^{a}
H. indica (Mango 2)	0^{h}	11^{gh}	11^{gh}	33^{efg}	44^{def}	44^{def}	89^{ab}	89^{ab}	100^{a}
LSD value at 0.05	22.807								
6th instar larvae									
H. alii	33^{f}	44^{ef}	78^{bc}	78^{bc}	100^{a}	100^{a}	89^{ab}	100^{a}	100^{a}
H. bacteriophora (HP88)	0^{g}	11^{g}	11^{g}	44^{ef}	44^{ef}	56^{de}	78^{bc}	100^{a}	100^{a}
H. indica (Mango 2)	0^{g}	0^{g}	11^{g}	33^{f}	44^{ef}	44^{ef}	67^{cd}	89^{ab}	100^{a}
LSD value at 0.05	21.333								

* Numbers followed by different letters indicate significant differences

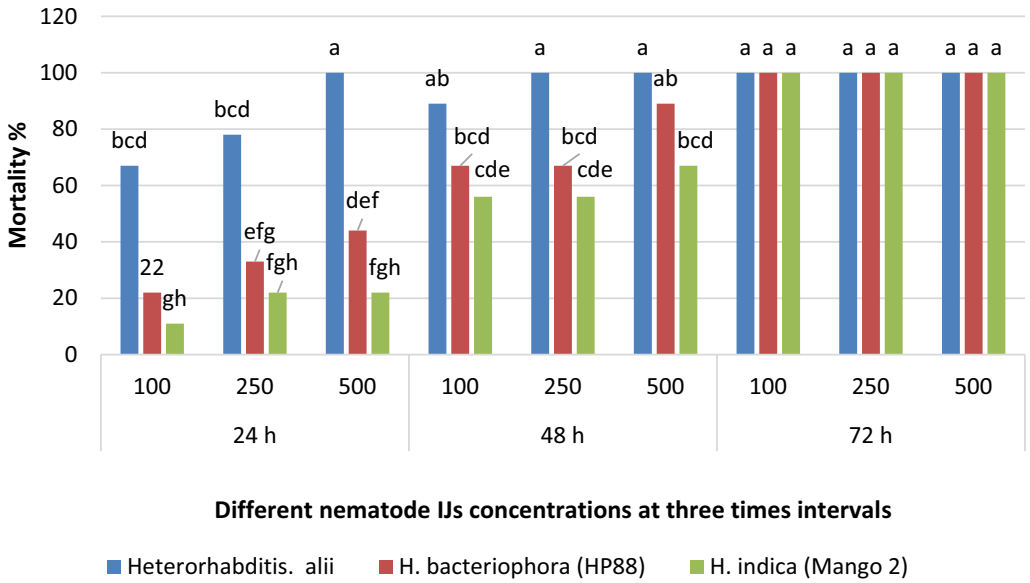

Fig. 7 Effect of *Heterorhabditis alii*, *H. bacteriophora* (HP88), and *H. indica* on the 3rd instar larvae of *Spodoptera frugiperda*. Different letters on the bars indicate significant statistical differences

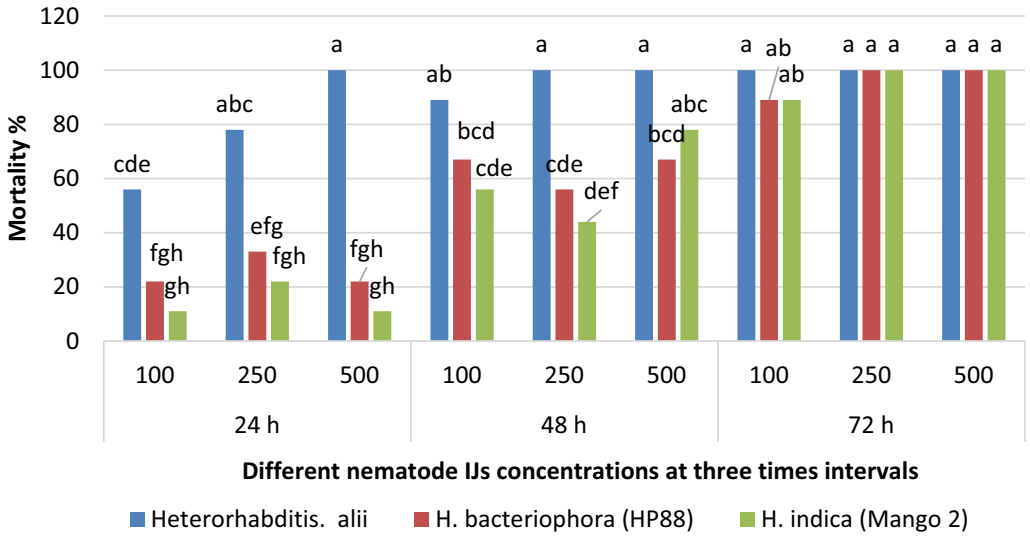

Fig. 8 Effect of *Heterorhabditis alii*, *H. bacteriophora* (HP88), and *H. indica* on the 4th instar larvae of *Spodoptera frugiperda*. Different letters on the bars indicate significant statistical differences

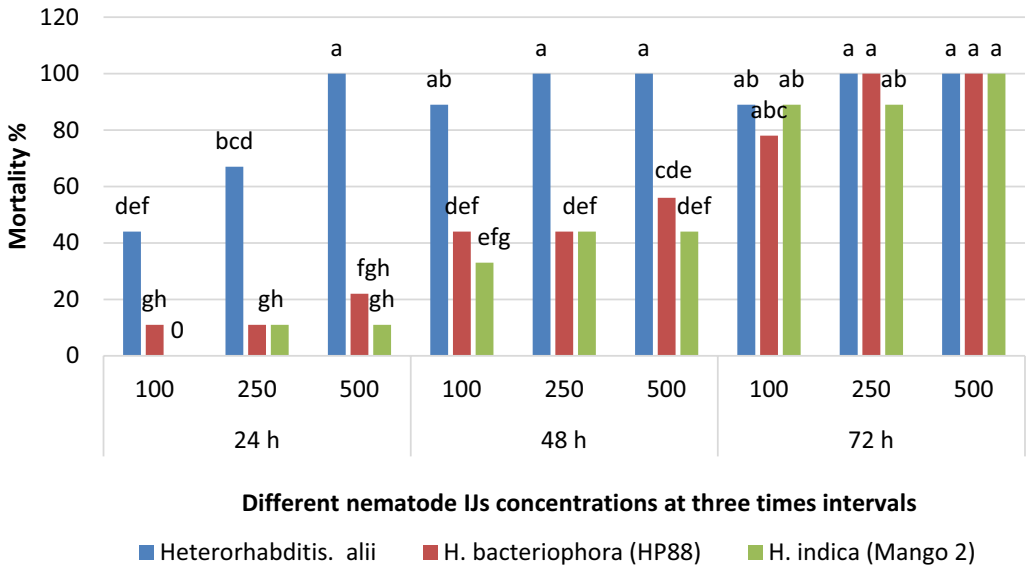

Fig. 9 Effect of *Heterorhabditis alii*, *H. bacteriophora* (HP88), and *H. indica* on the 5th instar larvae of *Spodoptera frugiperda*. Different letters on the bars indicate significant statistical differences

11, and 11% in the 3rd, 4th, 5th, and 6th larval instars, respectively (Table 3). The same trend was noticed with all tested nematode IJs concentrations.

The novel species of *H. alii* caused 100% mortality with the use of 500, 250, and 100 IJs/ml each treated after 24, 48, and 72 h post-infection, respectively, for all the tested larval instars. In comparison, *H. bacteriophora* (HP88) caused 100% mortality only in the 3rd instar larvae 72 h post-infection with all used nematode concentrations, while the two concentrations of 250 and 500 IJs/ml caused 100% mortality for the tested 4th, 5th, and 6th larval instars 72 h post-infection. When *H. indica* (Mango 2) was applied against the 3rd instar larvae of *S. frugiperda*, 100% mortality was occurred 72 h post-infection with all used nematode concentrations. When the same species was tested using 250 and 500 IJs/ml against the 4th instar larvae, 100% mortality occurred after 72 h post-infection, while the 5th and 6th larval instars were 100% killed after 72 h post-infection only when 500 IJs/ml were applied.

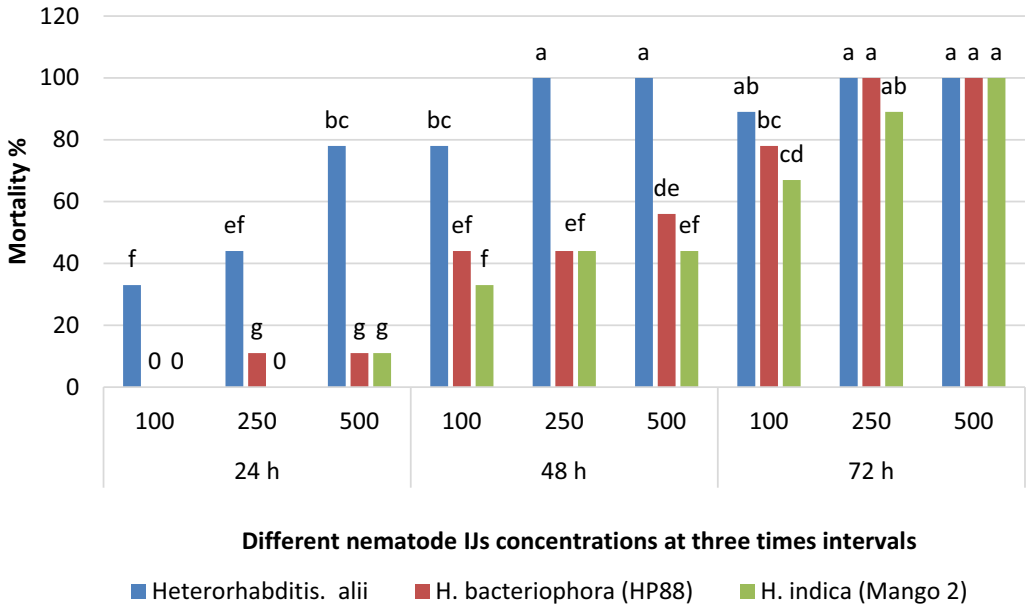

Fig. 10 Effect of *Heterorhabditis alii*, *H. bacteriophora* (HP88), and *H. indica* on the 6th instar larvae of *Spodoptera frugiperda*. Different letters on the bars indicate significant statistical differences

All used nematode IJs concentrations of *H. alii* caused 100% mortality rate after 48 and 72 h post-infection. The same results were obtained with the 3rd instar larvae of *S. frugiperda* after 72 h post-infection when *H. bacteriophora* (HP88) was used (Table 3 and Figs. 7, 8, 9, and 10). In contrast, *H. indica* (Mango 2) caused the least mortality rate with all tested instar larvae at all nematode IJs concentrations (Table 3 and Figs. 7, 8, 9, and 10). No mortality (0%) was observed in the non-treated control replicates.

Results of the conducted experimental work showed that our novel nematode species is promising for controlling the FAW invasive insect pest species and recently considered as one of the most destructive insect pests infesting corn in Egypt. All of the evaluated concentrations of this novel nematode species were effective and resulted in significant high mortality rates in all instar larvae of the FAW larvae at various time intervals comparing to the tested other two nematode species.

Discussion

The main objective of the present research efforts was to isolate and identify native species or isolates of EPN that can be used for the development of an efficient and sustainable biocontrol of the FAW, *Spodoptera frugiperda,* a devastative pest of corn in Egypt and other numerous global countries. Usually indigenous EPN species and/or isolates are more adapted to the local conditions compared to exotic ones, this will facilitate using them for direct commercial exploitation or as a source of genetic diversity in breeding programs aiming for production of improved beneficial nematodes (Choo et al. 1995).

The absence of males in the novel species of *H. alii* may attribute to a phenomenon called autotokous (producing progeny without males). Georgis and Gaugler (1991) stated that, however, heterorhabditid nematodes often perform better against certain insects than steinernematids but stable conditions for the production of *Heterorhabditis* spp. have not yet been achieved. In addition (Ehlers 1996), observed problems arise with the sensitivity of the nematodes to environmental extremes, e.g., high temperature, solar radiation, and desiccation, thus preventing exploitation of their maximal potential as control agents under field conditions. Strauch et al. (1994) assumed that in the genus *Heterorhabditis.*, the offspring of the first generation hermaphrodite can either develop into amphimictic adults or automictic hermaphrodites. The pathway leading to amphimictic adults is induced by favorable nutritional conditions, whereas the development into automictic hermaphrodites is induced by low concentrations of nutrients or the absence of symbiotic bacteria. It is possible to have continuous lines of heterorhabditid hermaphrodites. The effect of infection level on reproduction potential has also been reported by Abd El-rahman (2006) who dealt with different species of heterorhabditid nematodes.

The present study explains the isolation and identification of five EPN isolates of *Heterorhabditis* spp. in Egypt. Based on molecular characterization using sequence homology data of the rRNA including the ITS-1 region,

one isolate was identified as a new *Heterorhabditis* species. Our data are in agreement with Kajol et al. (2020) who reported that molecular characterization was essential for the validity of the taxonomic position as well as the phylogenetic relationships and authenticity among the species of a particular nematode genus. Moreover, the ITS-1 region is reportedly sufficient at assessing evolutionary relationships between species and differentiating them, particularly among *Heterorhabditis* spp. (Peat et al. 2009). Accurate identification of EPN has significant importance for the selection of species to be used in current and future biological control programs of insect pests. In addition, molecular identification of the present new species was supported by some differences in morphometrical and morphological observations when compared to *Heterorhabditis indica.*

EPNs occur naturally in the soil and show considerable promise as a biological control agent of insects (Bhairavi et al. 2021). The use of EPNs in insect pest management has been successfully demonstrated across the globe (Kumar et al. 2022). In a series of laboratory bioassays, when different species of EPNs that belong to the genera *Steinernema* and *Heterorhabditis* were used against different species of insect pests, mortality rate was also different throughout the years (Abbas, 2021).

The present study has evaluated the efficacy of two indigenous EPN species, *Heterorhabditis alii* and *H. indica* (mango 2) and one foreign EPN species, *H. bacteriophora* (HP88) against the FAW. A susceptibility test was performed by using different concentrations of each EPN species against this devastative pest. The result demonstrated that EPNs are capable of infecting and killing different instars of FAW larvae at different IJs concentrations in the laboratory.

The EPN virulence depends on their inherent characteristics and the type of their hosts (Acharya et al. 2020). Environment factors, stages of the host, the capability to find the host and the presence of the host lethal symbiotic bacteria, host penetration all influence the potentials of EPN were observed (Batalla-Carrera et al. 2010). Previous studies have also reported a positive relationship between IJs concentrations and mortality of treated insect larvae. There was a positive proportional increase in the mortality of the FAW larvae with an increase in density of infective juveniles per milliliter of sterilized distilled water.

In agreement with the present results, Viteri et al. (2018) found that when *Heterorhabditis* sp. and *Steinernema arenarium* were used in a concentration of 200 IJs/5th instar larvae of *S. frugiperda*, the mortality rates were 96.07 and 100.00%, respectively. In addition, Garcia et al. (2008) reported that *Steinernema* sp. caused 100% mortality when 280 IJs were used against the 3rd instar larvae of *S. frugiperda.* In contrast, *H. indica* at a concentration of 400 IJs/ml against the 3rd instar *S. frugiperda* larvae caused 75.00% mortality at 48 h post-incubation.

In another publication, the concentrations of 50 and 100 IJs/ml of the nematode *Steinernema diaprepesi* were tested against last instar larvae of *S. frugiperda* achieved mortality rates of 93.00 and 100.00% at 144 h post-incubation (Caccia et al. 2014).

Several studies are in agreement with obtained results, they have recorded decreases in susceptibility to EPNs when insect age increases (Fallet et al. 2022). In support to our findings, Acharya et al. (2020) reported that younger larvae of the first three instar larvae of the FAW were more susceptible to the nematodes *H. indica* and *S. carpocapsae*, while the last three older instars were susceptible to *S. arenarium* and S. *longicaudum*.

Findings about high virulence of EPN against 3rd instar larvae of *S. frugiperda* are in agreement with our results and the findings of other researchers (Patil et al. 2022). The current and previous results also agree with the findings of (Wattanachaiyingcharoen et al. 2021) who discovered that 2nd instar larvae of the FAW was more susceptible to *H. indica* (AUT 13.2) and *S. siamkayai* (APL 12.3) than the 5th instar larvae.

Variations observed in mortality of the FAW larvae by the nematode isolates can be attributed to differences in the virulence of the tested nematode isolates which is determined by the presence of symbiotic bacteria and the ability of the nematode to find and flourish inside the host (Shapiro-Ilan et al. 2006).

Further studies have revealed that virulence of EPNs on the FAW larvae varies not only among nematode species but among isolates of the same species (Acharya et al. 2020). Meanwhile in laboratory studies where most environmental conditions were uniform, differences in mortality rates were attributed to the impact of different symbiotic bacteria in the nematode isolates which are responsible for killing the insect host. The genus of the symbiotic bacteria, *Photorhabdus*, is associated with all nematode species belonging to Heterorhabditidae family (Boemare et al. 1993). These species of bacteria are reported to release numerous toxins (Hinchliffe et al. 2010) that restrain the immune response of the target insect host or disrupt the normal immune development by disturbing host tissues (Dowling and Waterfield 2007).

The majority of insect pests at early developmental stages are susceptible to the infection by EPN and thus they are the best candidates to unravel pest problems (Dillon et al. 2006). Actually native isolated of EPN is more adapted to local environmental conditions (Sun et al. 2021). Consequently, and in support of the findings, the use of native nematode isolates may reduce the potential risk of applying genetically diverse alien isolates

and/or species as biological control agents in a certain region, which greatly reduces the damage to the native environment (Fallet et al. 2022).

Conclusions

The present study elucidates the discovery of a new species of the EPNs, *Heterorhabditis spp.* in Egypt. This new species named *Heterorhabditis alii* after the late Professor Mohamed Ali of Al-Azhar University. The results were confirmed by morphological using light and scanning electron microscopes (SEM) as well as molecular analysis of the DNA sequencing. The discovered new species lack the presence of males and amphimictic females which is a unique phenomenon. Three heterorhabditid species, including the newly isolated one, a foreign *Heterorhabditis bacteriophora* (HP88), and a local *H. indica* (Mango 2), were tested against four larval instars of the FAW, *Spodoptera frugiperda*. *H. alii* showed the highest mortality level in shorter times than *H. bacteriophora* (HP88) and *H. indica* (mango 2) against the FAW. Yet, more field studies are required to explore the usage of the new species as in the biological control programs against this devastative pest of corn in Egypt and other world countries.

Abbreviations

EPNs	Entomopathogenic nematodes
FAW	Fall armyworm
IJs	Infective juveniles
ITS	Internal transcribed spacer
NCBI	National Center for Biotechnology Information
LM	Light microscope
SEM	Scanning electron microscope

Acknowledgements

The authors are grateful for Randa Mohammed Abd El-Rahman, a Research Professor at the Pest Physiology Department, Plant Protection Institute, Agricultural Research Center (ARC), Egypt, for her efforts to assist in the identification of the novel Entomopathogenic nematode (EPN) species described in this publication and for Nehal Ahmed A. Othman, a Staff Research Assistant (SRA) at the Applied Center for Entomonematodes (ACE), Faculty of Agriculture, Cairo University, Egypt, for her relentless efforts to collect soil samples and isolate entomopathogenic nematodes from the collected samples.

Author contributions

The authors contributed equally in all parts of this study. M. N. Heikal did the field survey, collection, and morphological identification of the isolated nematodes. A. M. El-Awady did the interpretation of the collected molecular data to identify the novel nematode species as well as writing the molecular part of this manuscript. N. M. Abo-Shady performed data curation, supervised parts of the experimental work. M. S. M. Shamseldean conceptualized the idea behind this research work, designed the research experiments, and revised and edited the final version of this work. All authors have read and approved to submit the final version of this manuscript.

Funding

This study was funded by the Applied Center for Entomonematodes (ACE) at Faculty of Agriculture, Cairo University, Egypt.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors declared that there are no issues related to the journal policies.

Competing interests

All authors declare no conflict of interest and no competing interests exist regarding the publication of this manuscript.

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Received: 20 December 2023 Accepted: 10 February 2024
Published online: 14 February 2024

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