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MORPHOLOGICAL CHARACTERISTICS AND INSECT KILLING POTENTIAL OF A SOIL DWELLING NEMATODE, *ACROBELOIDES* CF. *LONGIUTERUS* FROM SRI LANKA

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ABSTRACT

A free-living nematode was isolated from soils of coastal areas of Northern Sri Lanka using an insect-baiting technique. The morphometrics and the potential of the nematode to control the insect pest, red flour beetle (*Tribolium castaneum*) and white grubs (*Phyllophaga ephilida*) were evaluated. The female nematode had a mean body length of 720 μm longer than male (607 μm) with an annulated cuticle and narrow stoma with rhabditoid esophagus. Female with monodelphic, dextral, reflexed with post uterine sac and protuberance vulva at the mid-body, showed sexual dimorphism with male which had a single and reflexed testis. The identification of the nematode was confirmed as *Acrobeloides* cf. *longiuterus* (Nematoda: Cephalobidae). The propensity of this nematode to kill insect pests was subsequently tested against *T. castaneum* and *P. ephilida* under laboratory conditions. In Petri dish trials, the nematode caused significant mortality of larvae, pupae and adults of *T. castaneum* within 48 hours. LC_{50} for larvae ranged from 3.8-5.9 infective juveniles (IJs)/larva 1.1 and 7.1 IJs/insect for pupae and adults respectively. In soil-based bioassays, the LC_{50} for *T. castaneum* larvae was 69.46 IJs/larva, LT_{50} (Larvae) under the exposure time assay was 4.43 hours, whereas in pot experiment mortality of *P. ephilida* was recorded 69%. The experimental results showed that *A. cf. longiuterus* is a potential biocontrol agent and further studies are suggested in this regard.

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INTRODUCTION

Nematodes belonging to families Steinernematidae and Heterorhabditidae are considered potential insect biocontrol agents. They enter into the insect hosts via natural openings, release their symbiotic bacteria which multiply on haemolymph and produce toxins, resulting in the death of the host insect (Askary, 2010). However, there are other free-living nematodes other than these

two entomopathogenic nematodes (EPN) families, which have shown capacity to kill insects, *Oscheius shamimi* (Khanum and Shahina, 2010) and *Caenorhabditis briggsae* (Dillman et al., 2012) as examples. This could, potentially, expand the taxonomic breadth of the EPN group and, increase the number of different nematode species available as inundative biocontrols. Interest in the insect-killing capacity of nematodes lies primarily in

their potential as environmentally benign pest control agents, in order to reduce the reliance on chemical insecticides; although the production of antimicrobial compounds by EPN, particularly from the bacterial symbionts that they associate with and aspects of coevolution and speciation have also received attention (Edgington et al., 2010). This interest has led to numerous surveys hoping to find native species, from the equator to sub-polar territories, complemented, systematically, with improved identification techniques, including molecular ones (Hunt, 2010).

The first record of an EPN from Sri Lanka was in 1993 (Amarasinghe and Hominick, 1993a) when both *Steinernema* and *Heterorhabditis* isolates were obtained. Since then, several more surveys have revealed further isolates of both genera including, at the species level, *Heterorhabditis indicus* (Amarasinghe, 2008; Amarasinghe and Hominick, 1993a, 1993b; Amarasinghe et al., 1994). Other countries in the Indian subcontinent including India (Bora et al., 2015; Lalramliana and Kumar, 2010; Razia and Sivaramakrishnan, 2014) and Nepal (Khatri-Chhetri et al., 2010) have revealed further EPN isolates from a range of habitats. Of note, however, none of the surveys have revealed nematodes outside the two 'true' EPN families. The present study reports on a survey for EPN from soils of Northern Sri Lanka, together with efficacy tests using a nematode obtained from the surveys, which was not a 'true' EPN.

MATERIALS AND METHODS

Isolation of nematodes

A survey was done in Northern Sri Lanka (09°12'N 80°25'E) from 2015 to 2017 across an area of around 8200 km². Random sampling method was used for the soil sampling, from agricultural and natural habitats (Stuart et al., 2006). Extraction of nematodes from the samples was done through an insect baiting technique described by Kaya and Stock (1997). The insect-bait (*T. castaneum* larvae) were observed for death after 3-4 days and kept over White traps to check for nematode emergence into the water bath. Extracted nematodes were stored, in water, in the fridge, as well as routinely culturing through *T. castaneum*.

Identification

Specimens were heat-killed, fixed in Triethanolamine formalin (TAF) and then transferred to glycerin using the method described by Woodring and Kaya (1988). Images of each stage were captured using a microscope

mounted camera (Labomed and Olympus) and drawings were made with the help of a Camera Lucida. Morphometric measurements were taken for 15 specimens of each IJ, male and female nematodes using the criteria of Nguyen and Smart Jr (1996). Scanning electron microscopy observations were done as described by Nguyen and Smart Jr (1995). Images were captured through a ZEISS EVO LS 15 camera at the International Research Centre, University of Peradeniya, Sri Lanka.

Genus and species level identification was initially done morphometrically in-house (Figures 1-3 and Table 1) with keys to the family Cephalobidae and genus *Acrobeloides* (Abolafia and Peña-Santiago, 2003, 2017; Nguyen, 2009; Tarjan et al., 1977), with nematode samples, microscopic photographs and drawings. These details were sent subsequently to the University of Jaen (Spain) and the Swedish Museum of Natural History for confirmation.

Profiling of nematodes

One isolate of *Acrobeloides* cf. *longiuterus* (Rashid and Heyns, 1990) and (Siddiqi et al., 1992) obtained from the survey was assessed for efficacy against two insect species (*T. castaneum* and *P. ephilida*).

Tribolium castaneum in vitro bioassays

The trials used 5th, 6th and 7th instar *T. castaneum* larvae, pupae, and adults. For each life stage, 10 specimens were put into a Petri dish (9 cm diameter) containing moistened filter paper. Infective juveniles were pipetted directly onto the insects at concentrations of 50, 100 and 150 IJs/dish in 1 mL water. Control insects were treated as above but the 1 mL water contained no nematodes. There were four dishes per treatment, hence 40 insects in total per treatment. The dishes were maintained at 27±1°C in darkness. Mortality of insects was recorded 24, 48 and 72 hours after inoculation. Insects were deemed dead if they did not move following gentle prodding with a needle. The experiment for each insect stage was done twice and each experiment was arranged in a completely randomized design.

Tribolium castaneum exposure study

This study followed the method of Glazer (1992). Ten 7th instar larvae were put into a Petri dish containing moistened filter paper. Each dish was inoculated with IJ at a concentration of 0 and 200 IJs/dish in 1 mL water, applied topically to the insects. The dishes were kept at 27±1 °C in darkness. At 2, 5, 10, 17 and 24 hours following inoculation, the insects were removed, rinsed

with distilled water (to remove any IJs from the body surface) and placed in Petri dishes lined with moistened filter paper and kept at $29\pm 3^{\circ}\text{C}$. Insect mortality was checked every 24 hour to a maximum of 5 days. This study consisted of four dishes each containing 10 insects and was done on two occasions.

***Tribolium castaneum* soil based bioassay**

These tests were carried out following the method of Bedding (1981). Twenty grams of sterilized soil was placed in a plastic chamber (250 cm^3). Ten 7th instar larvae were put onto the soil surface in the test chamber. Infective juveniles were inoculated into the soil of each chamber at concentrations of 0, 150, 300, 450, 600, 1000, 2000 and 6000 IJs/chamber in 1 mL water. There were four chambers per treatment, hence 40 insects in total per treatment. The chambers were kept at $27\pm 1^{\circ}\text{C}$ in darkness. Insect mortality was recorded 96 h post inoculation, with gentle probing of the insect to confirm if dead or not. The experiment was arranged in a completely randomized design.

***Phyllophaga ephilida* soil based bioassay**

Five kilograms of sterilized soil was put into a plastic pot and 10 cm long piece of sweet potato yam was planted and allowed for the growth up to two weeks. Eight grubs of *P. ephilida* of similar age were released into the pot on the soil surface. Infective juveniles were inoculated onto the soil surface at concentrations of 0, 1000, 2500, 3500, 4500, 5500, and 7000 IJs/pot in 5 mL of water. The pots were kept in the field at $31.2\pm 3.5^{\circ}\text{C}$ and adequate watering was done thrice a week. There were four pots per treatment. Larvae were assessed 15 days following inoculation by tipping the soil out. All the larvae were transferred individually to a Petri dish lined with moistened filter paper and kept at $27\pm 1^{\circ}\text{C}$ for 3-4 days and insect mortality was tested with gentle probing of the insect to confirm if dead or not.

Data analysis

Data were analyzed using the SAS package and Duncan mean separation was done for the CRD experiments. Exposure assay and pot experiment's data were analyzed using one-way ANOVA and mean separation was done according to the Fisher LSD method at 95% confidence interval, using the software Minitab 17. LC_{50} and LC_{90} values were calculated according to the Probit analysis.

Results

Isolation of nematodes

Nematodes were obtained from 3 out of 423 samples

surveyed in Northern Sri Lanka. Nematode positive samples were in Jaffna (Kamparmalai) and Mullaithivu (Alampil and Semmalai) districts. Nematodes isolated from the Jaffna soil sample were able to infect and multiply on target insect pests and were used in further studies; while the other two isolates failed to multiply in insects.

Identification

Visual assessment

The nematode was identified as *A. cf. longiuterus* (in-house and later confirmed by external sources).

Descriptions

Acrobeloides cf. longiuterus (Figures 1-3 and Table 1)

Female (Figures 1a-m, Figures 2b, c)

Cuticle annulated (Figures 1c, k). Excretory pore observed. Head truncate, three lips partially fused and forming a triangular-shaped mouth (Figure 1k). Lateral field observed (Figure 1k). Stoma narrow (Figures 1b, c), lateral view of anterior part indicated two large sclerotized dots (Figures 1b, c). Esophagus rhabditoid in form with slightly swollen metacarpus, narrow isthmus and nerve ring located around anterior part of isthmus (Figures 1a-d). Well swollen basal bulb with distinct valve plate. Esophagus intestinal valve present, conoid or rounded tail tip without prominent phasmids (Figures 1e-g).

A ventral post anal swelling observed (Figure 1g). Reproductive system, monodelphic, dextral, reflexed, often containing single or many eggs (Figures 1j, i). Post uterine sac present (Figure 2c). Vulva with a transverse slit at the mid region of the body and protuberance, vulva covered with mucus like substance (Figure 1h). Females observed were oviparous and sometimes ovoviviparous (Figure 1m) (juveniles sometimes observed within the female body). The adult female larger than other stages and size varying significantly compared to male (Table 1), (Figures 2a, b).

Male

Body slender (Figure 2a, d, h), smaller than female (Figure 2a, b), cuticle annulated (Figure 2e), ventrally curved, J-shaped upon fixation (Figure 2d, h). Lateral field starts in anterior part of body with three incisures, with five incisures at mid-body (Figure 2j). Esophagus similar to that of female. Testis observed as single and reflexed (Figure 2a, d). Spicules paired; gubernaculum long (Figure 2d, f, g, i). Tail tip rounded, digitate and not murconate (Figure 2d, f, g, i).

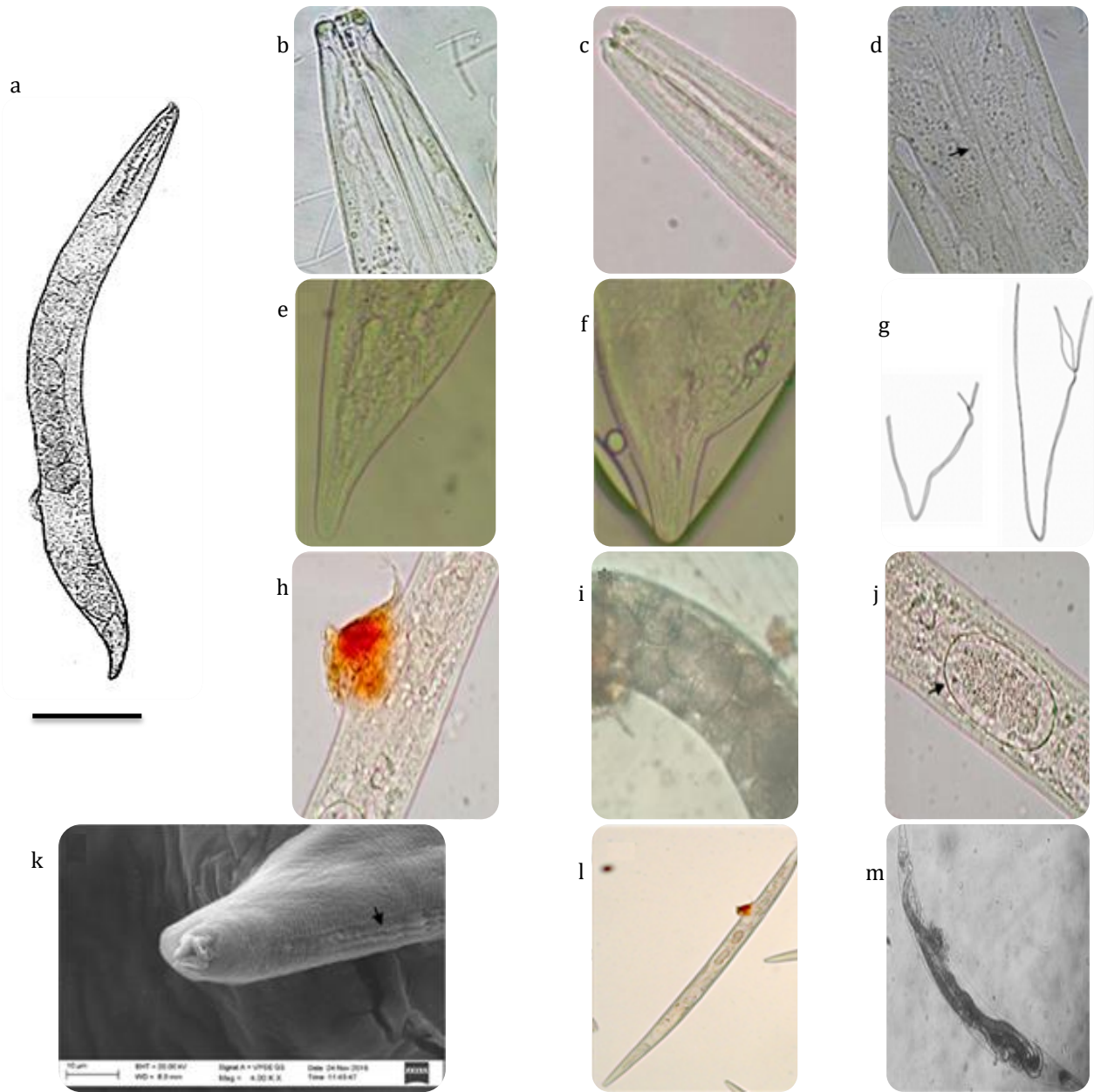


Figure 1. Morphology of female *Acrobeloides cf. longiuterus*. a, l. entire female. b, c, k. Anterior region (arrow mark indicating lateral fields). d. isthmus with nerve ring. e to g. Tail shapes. h. Valval region. i. Many eggs. j. Single egg. m. Juveniles inside the female body. (Scale bar. a =50 μ m. b to j = 12 μ m. l, m = 100 μ m).

Infective juvenile

Body slender, smaller than adult male and female (Table 1), cuticle annulated (Figure 3b). Esophagus similar in form to female and male; intestine not clear (Figure 3a). Excretory pore not distinct. Tail conoid in shape (Figure 3c).

Bioassay

Mortality of all stages of *T. castaneum* following exposure to *A. cf. longiuterus* is shown in Table 2. All

nematode treatments were significantly different from the control. Complete larval mortality was recorded at 150 IJs/dish two days' post inoculation. Pupal and adult mortality was recorded as 90%, 1 and 3 DAI, respectively at the 150 IJs/dish concentration. LC₅₀, determined at 2 DAI for the 5th, 6th and 7th instar larvae was 5.9, 4.0 and 3.8 IJs/larva, respectively. LC₅₀ of pupae and adult was determined as 1.1 IJs/pupa 1 DAI and 7.1 IJs/adult 3 DAI respectively.

Table 1: Comparisons of morphometric measurements of adults of both sexes and juvenile of the nematode.

Measurement (μ)	Female (n=15)	Male (n=15)	Juvenile (n=15)
Body length (L)	720 (672-765)	607 (540-700)	556 (470-640)
Greatest diameter (W)	45 (30-75)	42 (30-50)	34 (20-70)
Tail length (T)	62 (55-70)	51 (40-62.5)	48 (30-72.5)
Distance from anterior end to the Excretory pore (EP)	144 (135-172)	132.5 (115-165)	113 (87.5-167.5)
Pharynx length (ES)	174 (160-192)	154 (130-190)	138 (107.5-195)
Distance from the anterior end to the Nerve ring (NR)	147 (136-175)	-	-
Anal body diameter (ABD)	28 (25-30)	28 (22.5-35)	-
Vulva Distance from anterior end (VL)	540 (520-550)	-	-
Spicule length (SL)	-	41.4 (35-50)	-
Spicule width (Sw)	-	7.5 (5-12.5)	-
Gubernaculam length (GL)	-	33.3 (25-45)	-
Murcon	-	Absent	-
a (L/W)	16 (10.2-23.3)	14.7 (12.4-18.3)	17.5 (8.7-25)
b (L/ES)	5 (4.9-5.3)	4 (3.6-4.7)	4.1 (3.1-4.7)
c (L/T)	11.6 (10.6-12.7)	12.1 (9.2-17.5)	12 (8.4-18.6)
c' (T/ABD)	2.2 (1.8-2.3)	1.82 (1.6-1.95)	-
D % (EP/ES) x 100	82.9 (79-87.6)	86 (81.7-92.3)	82.2 (71.4-90)
E % (EP/T) x 100	232 (217-245)	264.1 (200-412.5)	241.8 (182-400)
SW (SL/ABD) x100	-	150.4 (121-189)	-
GS (GL/SL) x100	-	80 (69-94.4)	-

Range of measurement is given within the parenthesis

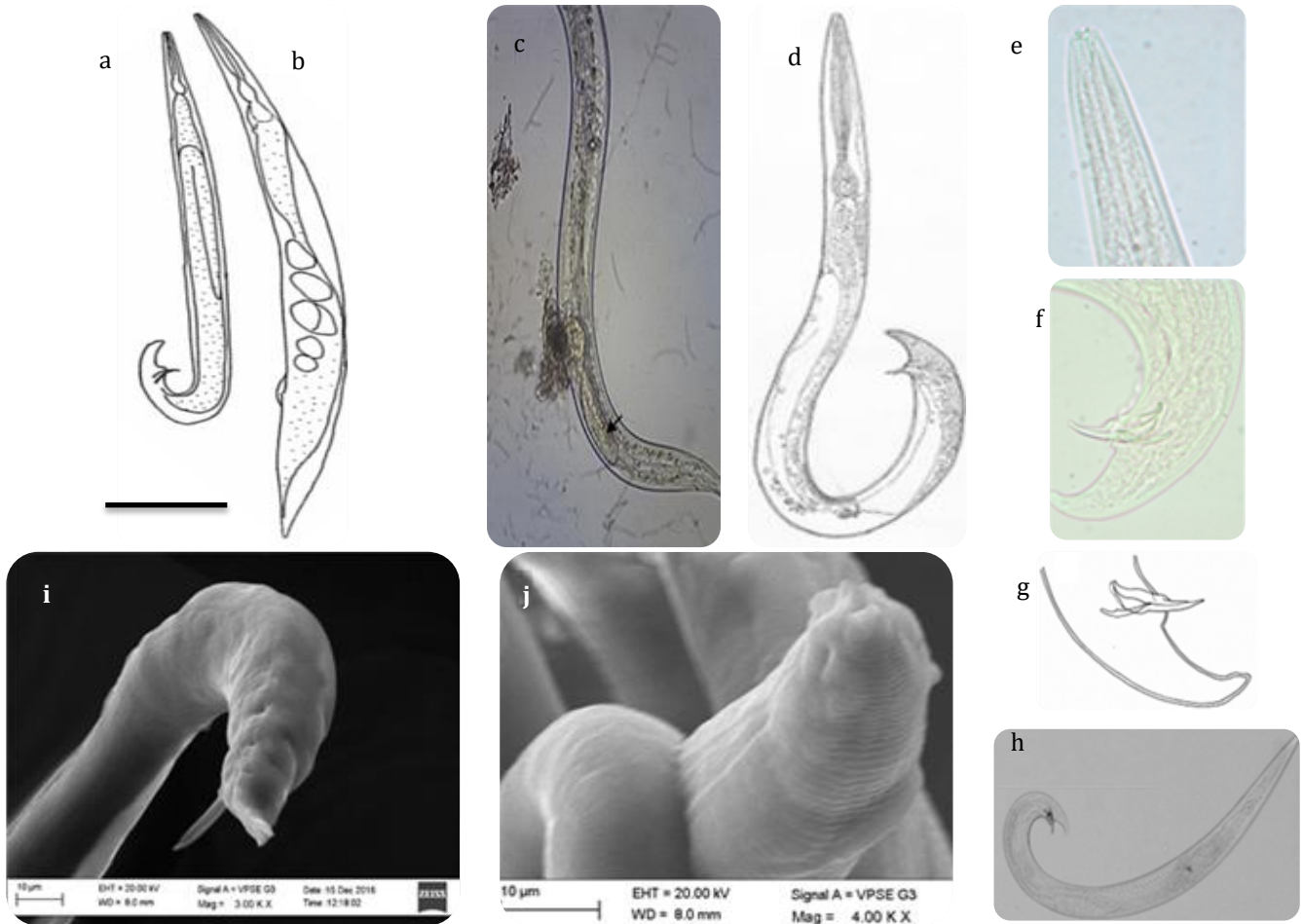


Figure 2. Female and male morphology of *Acrobelloides cf. longiuterus*. a. Male reproductive system. b, c. Female reproductive system (dextral) (arrow mark indicating Post valval sac). d, h. Male. e, j Anterior region of male. f, g, i. Posterior region of male. (Scale bar a to d, h = 100 µm, e to g = 20 µm).

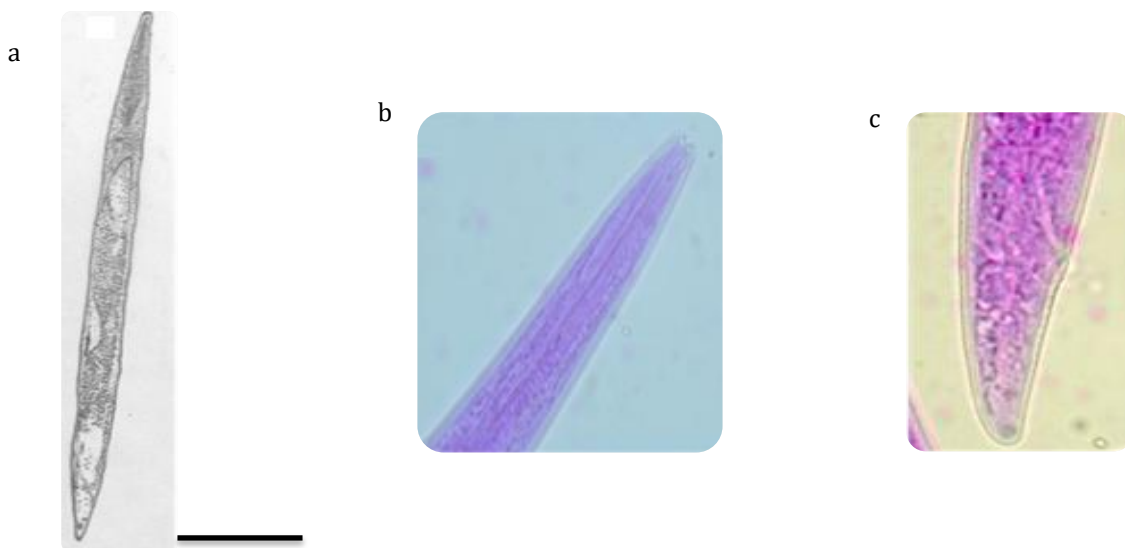


Figure 3. Infective juvenile morphology of *Acrobelloides cf. longiuterus*. a. Entire juvenile. b anterior region. c. Tail. (Scale bar a=100 µm, b, c = 20 µm).

Table 2: *In vitro* mortality of *Tribolium castaneum* against *Acrobelloides cf. longiuterus*.

Concentration (IJs/mL)	Mean Mortality				
	Larvae			Pupae	Adults
	5 th instar	6 th instar	7 th instar		
0	^d 0.75±0.5	^c 0.5±0.5	^c 0.75±0.5	^c 0.0±0.0	^c 0.5±0.5
50	^c 4±1.4	^b 6.5±1.0	^b 6.75±1.3	^b 7.75±0.5	^b 4.25±1.5
100	^b 8±0.8	^b 6.75±1.9	^a 8.5±1.7	^a 8.75±0.5	^b 4.5±1.0
150	^a 10±0.0	^a 10±0.0	^a 10±0.0	^a 9.0±0.8	^a 9.0±1.4

Mean mortality was recorded at larvae 2 DAI, pupae 1 DAI and adult 3 DAI. All values are means of four replicates (10 insects/replicate), figures having same letter in a column indicate the values are not significantly different according to Duncan's Multiple Range Test at 95% confidence interval.

Pathogenicity of *Acrobelloides cf. longiuterus* in soil based assay

Figure 4 shows the mortality of *T. castaneum* larvae when exposed to *A. cf. longiuterus* in the soil based assay. The 4 DAI larval mortality recorded a peak of 72.5% at 6000 IJs/dish. LC₅₀ of larva was 69.5 IJs/larva.

Exposure time assay

The mortality of *T. castaneum* with exposure time duration is shown in Figure 5. Larval mortality increased with longer exposure time. Highest larval mortality (90%) was achieved at 24 hours of exposure time followed by 17 hours which gave 80% larval mortality. LT₅₀ (Larvae) was recorded as 4.4 hours.

Efficacy of *Acrobelloides cf. longiuterus* on white grub; *Phyllophaga ephilida* (soil application)

Bioefficacy of the test nematode against the white grub (*P. ephilida*) is given in Table 3. The higher mortality of grubs was recorded as 68.8%, at a concentration of 7000

IJs/pot; a concentration of 5500 IJs/pot yielded grub mortality of 56.3%.

DISCUSSION

The present study showed that a free-living nematode identified from Sri Lankan soil samples as *A. cf. longiuterus*, hence not from the 'classic' EPN genera (*Steinernema* and *Heterorhabditis*), could be extracted using a live insect bait and would also kill insects when exposed to them both in the soil and Petri dish environments. There are previous studies, which reported on *Acrobelloides* spp. associated with insects (Azizoglu et al., 2016). In addition, recent studies have shown that the capacity of nematodes to kill insects is not exclusive to *Steinernema* and *Heterorhabditis*, particularly the nematode genera *Acrobelloides* (K29), against *Zeuzera pyrina* in Iran (Elham et al., 2021), *Oscheius* (Torrini et al., 2015; Zhou et al., 2017) and *Caenorhabditis* (Dillman et al., 2012).

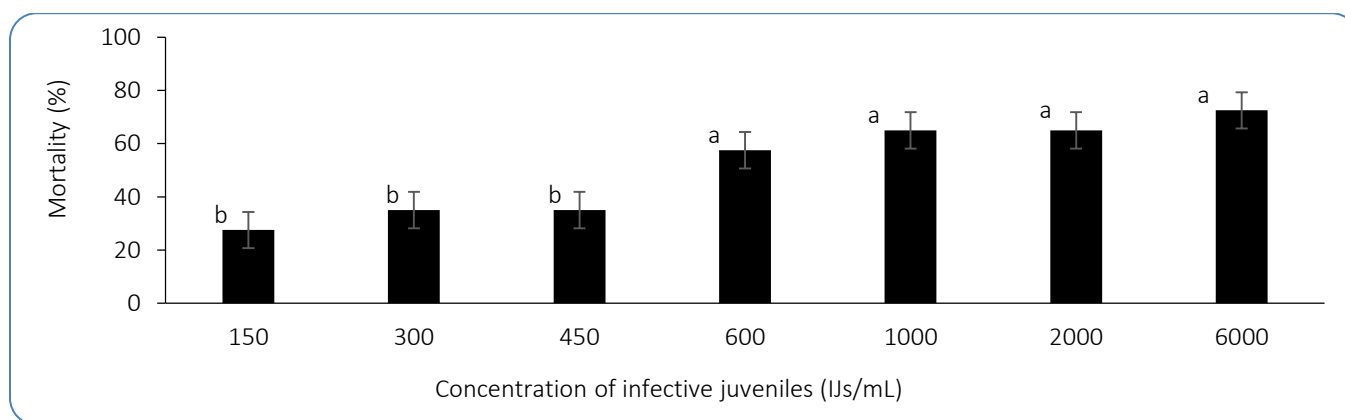


Figure 4. Entomopathogenicity of *Acrobelloides cf. longiuterus* on *Tribolium castaneum* larva in a soil-based assay. Mortalities with the same letters are not significantly different, according to the Duncan's Multiple Range Test at 95 % confidence interval.

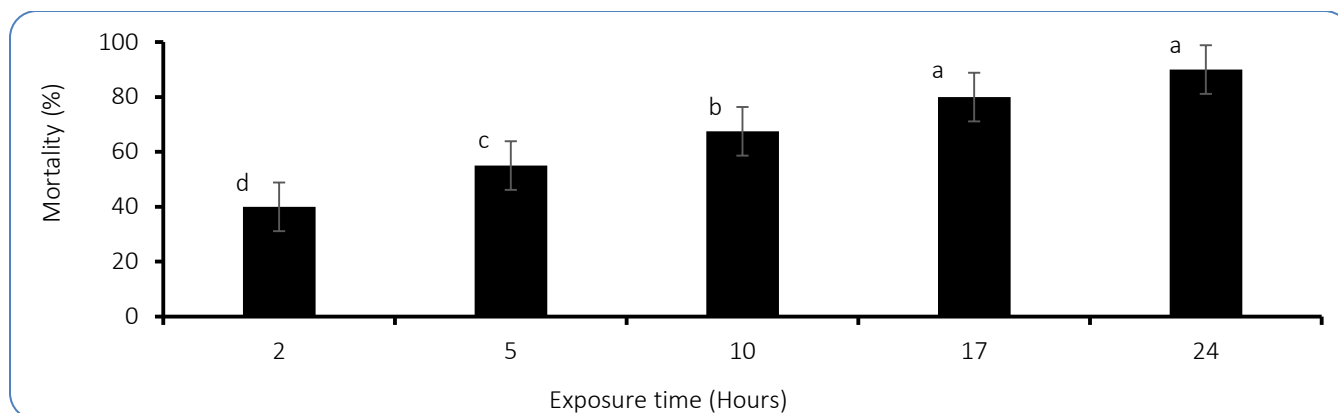


Figure 5. Mortality of *Tribolium castaneum* larvae with different exposure times of *Acroboloides cf. longiuterus*. The same letters in the figure are not significantly different according to the Fisher LSD mean separation method at 95% confidence interval (mortality 3 DAI).

Table 3: Efficacy of *Acroboloides cf. longiuterus* on white grub in a pot experiment.

Treatment (IJs/pot)	*Mean Mortality	Mortality %
0	^d 00.00±000	00.00
1000	^{dc} 1.00±0.816	12.50
2500	^c 1.25±0.500	15.63
3500	^{bc} 2.00±0.816	25.00
4500	^b 2.75±0.957	34.38
5500	^a 4.50±1.290	56.25
7000	^a 5.50±0.577	68.75

*Values are mean of the four replicates (a replicate 8 grubs/pot). Figures in a column having same alphabet were not significantly different according to the fisher LSD at 95 % confidence interval.

Infestation has also been recorded in other hosts of arthropods, mollusks, and annelids (Grewal et al., 2003). Testing of *in vitro* pathogenicity of nematodes on insect pests is an important step before either initiating green/glass house or field level evaluation and, any mass production work for commercialization (Shapiro-Ilan and Gaugler, 2002; Shapiro-Ilan et al., 2002; Shapiro and McCoy, 2000). Bedding (1990) making it clear that *in vitro* experiments are primes and essential for success of field experiments. Therefore, laboratory bioassays used to examine nematode activity in the infection process are an essential early step in any product development. This study showed that mortality of *T. castaneum* larvae, pupae and adults was significantly different from the non-treated controls. Nematode infestation on insect stages was confirmed via dissecting the insect cadavers under microscopic examination. The exposure time assay indicated indirectly how quickly insects were infected by the nematodes. Mortality of the insect larvae increased with prolonged exposure times keeping with

the results of Selvan et al. (1993). These findings suggest that longer exposure times make it possible for more nematodes to penetrate their insect hosts.

Differences in mortality were apparent 24 and 48 hours after IJs had been applied; the differences were mostly between the very low and high IJs doses. These findings suggest that mortality increases with the number of IJs that penetrate an insect host; more IJs possibly/probably penetrated the hosts in cases where higher IJs doses were applied per insect, thus the higher mortality trend observed as early as 24 hours. The findings of the current study are in agreement with those of Anbesse et al. (2008) who reported that mortality increased with increased IJ concentrations. *Acroboloides cf. longiuterus* was exposed to different doses of IJs in the assays under *in vitro* conditions, it seems that the number of nematodes that invade a host is proportional to the exposure concentration, in keeping with studies of Selvan et al. (1993) and Shapiro-Ilan and Lewis (1999).

Pot experiments showed that 69% grub mortality whilst the control treatment yielded 0%, with nematode infection as the cause of grub mortality in the treatments confirmed through microscopic examination; demonstrating the capacity of *A. cf. longiuterus* to kill insects. Elham et al. (2021) reported similar capacity in Iran, with *Acrobeloides* sp. effective against *Z. pyrina* in laboratory and *in situ* conditions. However, many further tests and confirmation will need to be done before these nematodes can be declared as an entomopathogenic nematode with commercialization value. According to Dillman et al. (2012) entomopathogenic nematodes are defined by the association of symbiotic bacteria and its uniqueness, therefore, it is essential to identify the bacterial symbiosis and its characterization for the clear understanding of the phenomenon of the insect killing nature.

In future, it is essential to have a detailed study on the complete biology of the candidate nematode, its bacterial symbiosis and its effectiveness against pests, in different environmental conditions, such as green house or glass house or field level evaluation at least for two different seasons. If once these are successful, then there is the need to evaluate formulations and storage parameters/requirements. The present set of studies is early stage investigations into the potential to use a genus of nematode outside the standard entomopathogens as a biological control agent, either from an inundative or conservational perspective. Results from the trials would suggest that further studies are merited.

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AUTHORS' CONTRIBUTION

NT and GM conceived and designed the study; NT conducted the research work and wrote the initial draft; GM supervised and GTK co-supervised the work, GM, GTK, SE and AB reviewed and edited the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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