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Isolation and Identification of Native Isolates of Entomopathogenic Nematodes from Anand Gujarat, India

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ABSTRACT

A survey was conducted to collect soil samples to isolate the native entomopathogenic nematodes from different habitats, viz., fruit crops, field crops and fallow land during *kharif* and *rabi* 2020-21from the Anand Agricultural University campus, Anand (GS) India. Out of 103, three samples were found positive and were collected from three different locations i.e., Horticultural farm (Mango), the Veterinary College Garden and the International Agri-Business Management College. The frequency of occurrence of these nematodes was very low (2.9%). All three isolates belonged to the genus *Steinernema*. Among three, *Steinernema* sp. (AAU St-1) was successfully maintained under laboratory conditions. Based on morphometric characters, *Steinernema* sp. (AAU St-1) was identified and designated as *Steinernema ritteri.*

Keywords Entomopathogenic nematodes, Habitats, *Steinernema ritteri*.

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INTRODUCTION

Entomopathogenic nematodes (EPNs) are parasites of insects. This group of nematodes is characterized by carrying specific symbiotic bacteria of the genus *Xenorhabdus* or *Photorhabdus* in their intestine. Symbiotic bacteria play an essential role in the pathogenicity of the nematode-bacteria complex to insect hosts and the subsequent reproduction of the nematodes in the hosts. Entomopathogenic nematodes are currently used as biopesticides for managing several important insect pests worldwide (Shapiro-Ilan *et al.* 2002).

Nematodes associated with insects also called entomophilic, entomogenous or entomopathogenic, are known to parasitize, cause disease and kill the insects within 24 to 48 h due to septicaemia. Biological control of insects with EPNs progressed since 1932 with the discovery of *Steinernema glaseri* (Glaser 1932) infecting Japanese beetle (*Popillia japonica*, Newman). The primary objective of biological control is to reduce pest population below the economic threshold level by using natural enemies. They are remarkably more virulent against lepidopterous and coleopterous pests.

The EPNs of the families Steinernamatidae and Heterorhabditidae are potentially useful for biological control in agriculture systems. The infective juveniles of these families are free-living, non-feeding and can search out their hosts. They have the potential for

Table 1. Soil samples collected from AAU campus, Anand. Note : + = Positive, - = Negative.

Sl. No.	Habitat	Location	Latitude - longitude	Result
		Fruit crops		
1	Mango	In front of MVRS	22°31′37′′N 72°57′56′′E	-
2	Mango	The near Mango orchard – RRS	22°32′16′′N 72°58′45′′E	-
3	Mango	Livestock Research Station	22°32′16′′N 72°58′4′′E	-
4	Jamun	Backside of AMUL parlour	22°32′07′′N 72°58′3′′E	-
5	Lemon	Horticulture farm – backside of the aeroponic unit	22°31′5′′N 72°58′1′′E	-
6	Sapota	The near Horticulture college	22°31′5′′N 72°58′0′′E	-
7	Guava	Horticulture farm	22°31′5′′N 72°58′1′′E	-
8	Mango	Right side of Apiculture	22°32′2′′N 72°59′0′′E	-
9	Sapota	Sapota farm	22°31′56.2′′N 72°58′09′′E	-
10	Guava	Guava farm	22°31′58′′N 72°58′14.4′′E	-
11	Jamun	Jamun farm	22°31′54.9′′N 72°58′11′′E	-
12	Banana	In front of Vidhya dairy	22°32′06′′N 72°57′48′′E	-
13	Mango	In front of the guest house	22°32′1′′N 72°58′4′′E	-
14	Mango	Horticulture farm	22°31′47.′′N 72°58′02′′E	+
15	Mango	AAU main gate	22°31′02′′N 72°58′19.9′′E	-
16	Mango	AAU guest house road	22°32′02.1′′N 72°58′21.0′′E	-
17	Mango	AAU guest house road	22°32′00′′N 72°58′22.6′′E	-
18	Mango	AAU guest house road	22°31′58′′N 72°58′23.3′′E	-
19	Mango	Staff quarter New B 22-23-24	22°53′38.1′′N 72°97′25.4′′E	-
20	Mango	Staff quarter New B 19	22°53′38.1′′N 72°97′25.4′′E	-
21	Mango	Staff quarter New B 24	22°53′36.1′′N 72°97′39.26′′E	-
22	Mango	Staff quarter New B 26	22°53′36.1′′N 72°97′39.2′′E	-
23	Mango	Staff quarter Old B 22- 25	22°53′50.0′′N 72°97′54.76′′E	-
24	Mango	Staff quarter D	22°53′43.7′′N 72°97′56.4′′E	-
25	Custard apple	Horticulture farm	22°41′4′′N 72°58′11′′E	-
		Vegetable crops		
1	Cucumber	Nematology polyhouse	22°31′48′′N 72°58′1′′E	-
2	Brinjal	Biotechnology farm	22°32′1′′N 72°58′4′′E	-
3	Okra	MVRS (C-10) plot	22°32′0′′N 72°58′00′′E	-
4	Tomato	In front of Vidhya dairy	22°32′0′′N 72°57′4′′E	-
		Field crops		
1	Sorghum	Near Library	22°32′1′′N 72°58′4′′E	
2	Castor	Near Railway track	22°32′23′′N 72°58′5′′E	-
3	Cotton	The Near Mango orchard – RRS	22°32′1′′N 72°58′4′′E	-
4	Castor	Entomology farm	22°32′16′′N 72°58′45′′E	-
5	Maize	Veterinary field	22°32′1′′N 72°57′4′′E	-
		Horticultural crops		
1	Jasmine	Jasmine farm	22°31′52.3′′N 72°58′10′′E	-
2	Sandal	Sandal farm	22°31′51.9′′N 72°58′10.9′′E	-
3	Rose	In front of MVRS	22°32′0′′N 72°57′51′′E	-
		Trees		
1	Neem	MVRS parking	22°32′1′′N 72°57′56′′E	-
2	Neem	Staff quarter old C 1-8	22°53′42.21′′N 72°97′55.7′′E	-
3	Neem	In front of Hanuman Temple	22°32′0′′N 72°57′50′′E	-
4	Neem	Nematology farm	22°32′0′′N 72°57′56′′E	-
		Fallow land		
1	Fallow field	International Agri-Business Management college building	22°32′06.9′′N 72°58′07.2′′E	-

Table 1. Continued.

Sl. No.	Habitat	Location	Latitude - longitude	Result
2	Fallow field	Behind Canteen	22°32′09.2′′N 72°58′33.6′′E	-
3	Fallow field	Behind medicinal nursery	22°32′12.4′′N 72°58′10.9′′E	-
4	Fallow field	Entrance of medicinal nursery	22°32′11.2′′N 72°58′56.′′E	-
5	Fallow field	The near the agronomy farm	22°32′26′′N 72°58′46′′E	-
6	Fallow field	BACA garden	22°32′09′′N 72°58′55.4′′E	-
7	Fallow field	Veterinary college gate	22°32′27.′′N 72°57′37.7′′E	-
8	Fallow field	Veterinary college road	22°32′13.′′N 72°58′03.0′′E	-
9	Fallow field	In front of FPT college	22°32′08.′′N 72°58′06.′′E	-
10	Fallow field	AMUL parlour, near university bhavan	22°32′06.0′′N 72°58′32.5′′E	-
11	Fallow field	AMUL parlour, near university bhavan	22°32′07′′N 72°58′33′′E	-
12	Fallow field	AMUL parlour, near university bhavan	22°32′04.9′′N 72°58′34.5′′E	-
13	Fallow field	AMUL parlour, near university bhavan	22°32′05′′N 72°58′36′′E	-
14	Fallow field	In front of the girl's hostel	22°31′59.2′′N 72°58′26.3′′E	-
15	Fallow field	Polytechnic college gate	22°32′01.0′′N 72°58′25.3′′E	-
16	Fallow field	Near Electric pole (FPT)	22°32′15.0′′N 72°58′02′′E	-
17	Fallow field	Near Electric pole (MVRS)	22°32'11.7"N 72°58'06.4"E	-
18	Fallow field	Near Electric pole (IABMI)	22°32′08.4′′N 72°58′08′′E	-
19	Fallow field	Near Electric pole (AMUL)	22°32′07.3′′N 72°58′31.4′′E	-
20	Fallow field	Animal husbandry road	22°31′43.1′′N 72°58′12.3′′E	-
21	Fallow field	Animal husbandry road	22°31′45.1′′N 72°58′18.3′′E	-
22	Fallow field	Horticulture farm office	22°31′50.3′′N 72°58′24.7′′E	-
23	Fallow field	Horticulture farm office	22°31′50.0′′N 72°58′26′′E	-
24	Fallow field	The near horticulture farm office	22°31′52.6′′N 72°58′25.1′′E	-
25	Fallow field	RRS farm	22°31′50.6′′N 72°57′53.8′′E	-
26	Fallow field	In front of animal husbandry food store	22°31′39′′N 72°57′56′′E	-
27	Fallow field	RRS farm	22°31′33′′N 72°57'57.0′′E	-
28	Fallow field	RRS farm	22°31′28.4′′N 72°58′00.5′′E	-
29	Fallow field	RRS farm	22°31′41.5′′N 72°58′06.4′′E	-
30	Fallow field	RRS farm	22°31′36.2′′N 72°58′12.9′′E	-
31	Fallow field	Near Hanuman temple	22°31′58.0′′N 72°58′49′′E	-
	Fallow field	AMUL parlour, near university bhavan	22°32′05.4′′N 72°58′36.8′′E	-
32	Fallow field	Veterinary college	22°32′22.2′′N 72°57′42.3′′E	-
33	Fallow field	Veterinary college	22°32′22.6′′N 72°57′34.2′′E	-
34	Fallow field	Veterinary college	22°32′26.4′′N 72°57′38.7′′E	-
35	Fallow field	International Agri-Business Management college building	22°32′07.6′′N 72°58′08.4′′E	+
36	Fallow field	In front of Vidhya dairy–near shed	22°32′04′′N 72°57′50′′E	-
37	Fallow field	In front of Vidhya dairy	22°32′05′′N 72°57′50′′E	-
38	Fallow field	Near girl's hostel	22°32′16′′N 72°58′45′′E	-
39	Fallow field	BTRS	22°32′17′′N 72°58′06′′E	-
40	Fallow field	Near library entomology farm	22°32′12′′N 72°58′41′′E	-
41	Fallow field	The backside of Medicinal and Aromatic	22°32′11′′N 72°58′59′′E	-
42	Fallow field	Horticulture nursery	22°32′07′′N 72°58′49′′E	-
43	Fallow field	Agronomy farm (PCP plots)	22°32′27′′N 72°58′45′′E	-
44	Fallow field	The backside of Nematology polyhouse	22°31′47′′N 72°58′16′′E	-
45	Fallow field	In front of the Infrastructural Livestock Research Station	22°32′17′′N 72°57′45′′E	-
46	Fallow field	In front of the boy's hostel	22°32′04.0′′N 72°58′45.8′′E	-
47	Fallow field	Railway track	22°32′30.1′′N 72°58′57.7′′E	-
48	Fallow field	Observatory	22°32′05.1′′N 72°58′27.3′′E	-
49	Fallow field	Agronomy farm	22°32′24.0′′N 72°58′43.4′′E	-
50	Fallow field	Agronomy farm	22°32′25.5′′N 72°58′47.7′′E	-
51	Fallow field	Near college Canteen	22°32′08.3′′N 72°58′56.1′′E	-
52	Fallow field	Veterinary college	22°32′24.′′N 72°57′39.′′E	+

Table 2. Samples possessing entomopathogenic nematodes. Note: + = Positive, - = Negative.

Sl. No.	Habitat	Location	Latitude-longitude	Genera identified
1	Mango	Horticulture farm	22°31′47.9′′N 72°58′02.9′′E	*Steinernema* sp. (AAU St-1)
2	Fallow field	International agri-business management college building	22°32′07.6′′N 72°58′08.4′′E	*Steinernema* sp. (AAU St-2)
3	Fallow field	Veterinary college (Near garden)	22°32′24.1′′N 72°57′39.2′′E	*Steinernema* sp. (AAU St-3)

long-term establishment in the soil through recycling infected insect larvae.

Many surveys have revealed the natural occurrence of EPNs associated with different ecosystems and agroclimatic regions (Ganguly and Rathour 2004, Ganguly 2006, Maru *et al.* 2007). The objective of this study was to survey the presence of EPN in natural habitats and identify the recovered based on morphometric of the infective stage juveniles (IJs) and other stages in their life-cycle.

MATERIALS AND METHODS

The studies were carried out in the Department of Nematology, BA College of Agriculture, Anand Agricultural University, Anand (Gujarat) during *kharif* and *rabi* 2020-21. The details of materials used and methods employed during experimentation are described here under different headings.

Isolation of entomopathogenic nematodes

Rearing of bait insect, rice moth, corcyra cephalonica

In the present study, *C. cephalonica* was used for the mass culturing of isolates obtained from the AAU campus, Anand. The eggs of *C. cephalonica* were collected from AICRP on Biological Control of Crops Pests, ICAR unit-9, AAU, Anand. The larvae were reared on an artificial diet prepared from the following ingredients.

(i) Sorghum whole grains : 2.5 kg

(ii) Broken sorghum grains : 0.5 kg

(iii) Streptomycin : 0.5 g

(iv) Yeast extract powder : 1 g

The healthy sorghum whole grains, broken sorghum grains, streptomycin and yeast extract powder were mixed and kept in round aluminum containers (33×12 cm). Then, 0.5cc of *C. cephalonica* eggs (1cc = 16,000 eggs) were sprinkled and maintained for the hatch out. After that, the containers were covered with a black cloth and tied with thread. The eggs hatched in four days and the larvae preferred broken grains for feeding. The larvae were generally creamy white. After twenty days, they fed on whole grain and made silken cocoons among infested grains.

The pupal stage was about ten days. Moths started emerging out during the peak period of thirty-five days. The eggs were collected, cleaned and used for re- inoculation.

Collection of soil samples

Soil samples were collected from different habitats, viz., fruit crops, field crops and fallow land of the AAU campus, Anand, during *kharif* and *rabi* 2020-21 as per the methodology described by Kaya and Stock (1997). Since EPNs are confined to top layers, samples were drawn from 10–15 cm depth.

Sub-samples of 500 g soil were collected and mixed thoroughly to form a composite sample from each sampling site. Samples were packed in plastic bags and kept away from sunlight. Each sample was labeled with a tag containing information such as place of sampling, GPS coordinates, date and cultivated crop were recorded. The samples were packed

Table 3. Morphometrics of the isolate *Steinernema* sp. (AAU St-1). Note: Measurements are in µm and data are expressed in the form of mean ± SD (range).

Characters	Juveniles	Male	Female
L	581.61 ± 56.57 (479.75- 666.31)	872.79 ± 64.67 (727.68 - 974.12)	848.25 ± 68.66 (763.13 - 995.38)
W	31.25 ± 4.16 (21.64 - 38.62)	54.90 ± 7.91 (33.36 - 65.50)	52.14 ± 6.83 (37.48 - 67.41)
EP	113.66 ± 11.33 (94.30 - 130.40)	121.66 ± 9.60 (105.85 - 138.02)	122.09 ± 14.63 (103.55 - 149.11)
NR	125.98 ± 10.46 (104.52 - 140.11)	132.67 ± 7.69 (119.23 - 142.80)	133.90 ± 16.47 (108.87 - 159.66)
ES	153.42 ± 9.68 (127.61 - 168.00)	173.51 ± 8.03 (156.02 - 184.48)	166.92 ± 20.70 (140.89 - 205.51)
T	37.14 ± 2.86 (32.12 - 41.09)	42.54 ± 2.83 (36.86 - 47.88)	42.58 ± 4.54 (31.09 - 50.79)
AV	–	–	593.76 ± 60.38 (446.79 - 693.52)
V	–	–	70.00 ± 87.93 (56.06 - 76.06)
SL	–	58.05 ± 4.85 (48.05 - 65.13)	–
ABW	–	29.35 ± 3.07 (23.33 - 34.86)	–
SW	–	1.99 ± 0.21 (1.66 - 2.66)	–
GS	–	0.57 ± 0.06 (0.47 - 0.67)	–
a	18.82 ± 2.17 (14.53 - 24.18)	16.16 ± 2.08 (14.08 - 23.45)	16.44 ± 1.65 (14.10 - 21.08)
b	3.80 ± 0.33 (3.09-4.39)	5.04 ± 0.37 (4.45 - 5.66)	5.12 ± 0.40 (4.49 - 6.21)
c	15.70 ± 1.51 (12.29 - 18.38)	20.56 ± 1.49 (17.87 - 22.88)	20.05 ± 1.83 (17.50 - 25.55)
D%	74.08 ± 5.59 (63.29 - 82.62)	70.17 ± 5.21 (62.45 - 80.90)	73.34 ± 4.75 (65.86 - 86.45)
E%	307.22 ± 33.00 (253.25 - 369.93)	287.22 ± 29.97 (243.42 - 358.20)	288.01 ± 31.23 (245.84 - 391.72)
L = Total body length		T = Tail length	D% = EP/ESx100
W = Greatest body width		ABW = Anal body width	E% = EP/Tx100
EP = Distance from the anterior end to the excretory pore		SL = Spicule length (male only)	SW = SL/ABW (male only)
NR = Distance from the anterior end to the nerve ring		GL = Gubernaculum length (male only)	GS = GL/SL (male only)
ES = Esophagus length		AV = Distance from the anterior end to the vulva (female only)	V = AV/Lx100 (female only)
a = L/W		b = L/ES	c = L/T

in polythene bags and transported to the laboratory. Soil samples were maintained at low temperature in the refrigerator till further processing.

Baiting of soil samples

The EPNs were extracted from soil samples using the insect baiting method described by Bedding and Akhurst (1975) using the last instar *C. cephalonica* larvae as bait organism. Soil samples were taken out from polythene bags in a plastic tray and remove debris like rocks/stones, pieces of wood or bark leaves from the soil samples. Soil samples were placed in a plastic container (15×6 cm) and baited five last instar *C. cephalonica* larvae at the bottom. The containers were covered tightly with lids and the top was arable.

Samples were kept at room temperature (25-30ºC). The larval mortality was recorded daily for up to ten days to monitor infected and dead larvae. Cadavers with a brown to black were usually parasitized by steinernematids, whereas heterorhabditids parasitize brick red cadavers. The signs of the dead larvae were recorded and removed from the containers for further use.

Isolation and extraction of EPNs

Dead larvae from each sample were rinsed once with 0.1% formalin, thrice with sterile distilled water for surface disinfection and placed on a modified White trap (White 1927). White traps were made by placing a single layer of Whatman's No. 1 filter paper on the concave side of the watch glass in a glass Petri plate (90×10 mm). The filter paper was moistened with sterile distilled water. The dead larvae were placed on the filter paper over the edge of the watch glass with a small quantity of sterile distilled water and covered with a lid. The EPNs emerged out from the cadaver were harvested in a beaker using sterile distilled water.

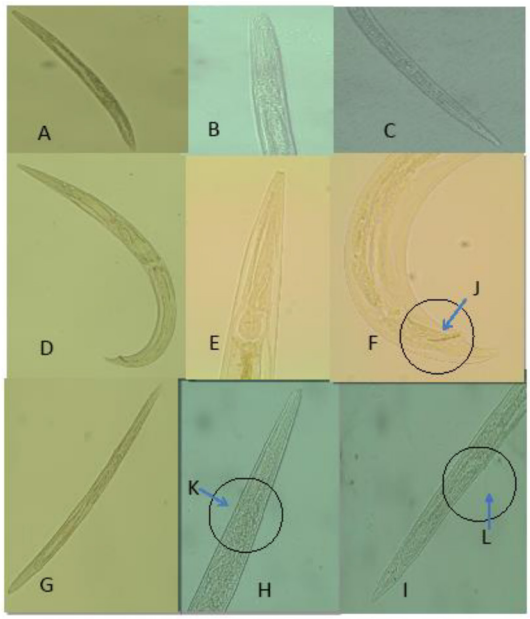

Plate 1. Photomicrograph of Steinernema sp. (AAU St-1). A= Infective juvenile, B= Head region of IJs, C= Tail region of IJs, D= Male, E= Head region of male, F= Tail region of male, G= Female, H= Head region of female, I= Tail region of female, J= Spicule of male, K = Excretory pore, L= Vulval opening.

The collected EPNs were rinsed thrice with sterile distilled water. Then allowed to settle at the bottom of the beaker and the supernatant suspension was separated. This process was repeated four times to obtain apparent nematode suspension. The nematode suspension was stored in a beaker at room temperature and checked periodically as the shelf life of EPNs is variable.

Identification of isolated EPNs

Identification based on the morphometric was made according to Kaya and Stock (1997), Shahina *et al.* (2001), Uribe-Lorio *et al.* (2005).

The measurements of IJs, males and females are necessary to identify the nematodes. The key characters of twenty specimens were measured using a trinocular upright microscope (ZEISS AX 10). DeMan's (De Man 1880) formula was used for each generation with the help of ZEN Axio Cam ICc5 software.

Identification parameters were described as follows

Infective juvenile

Total body length, greatest body width, distance from the anterior end to excretory pore, distance from anterior end to nerve ring, distance from anterior end to esophagus and tail length.

Male

Total body length, greatest body width, distance from anterior end to excretory pore, distance from anterior end to nerve ring, distance from anterior end to esophagus, tail length, spiculae length, gubernaculum length.

Female

Total body length, greatest body width, distance from anterior end to excretory pore, distance from anterior end to nerve ring, distance from anterior end to esophagus, tail length, distance from head end to vulva. Finally different ratios were calculated.

RESULTS AND DISCUSSION

Isolation of entomopathogenic nematodes

There were 103 soil samples collected from the various locations of the AAU campus, Anand in which, 26 fruit crops, four vegetable crops, five field crops, three horticultural crops, four trees and 52 from fallow lands (Table 1). Out of these, three samples were found positive from EPNs. The frequency of occurrence of these nematodes was 2.9%. These native EPNs were isolated from the Horticultural farm (Mango), the Veterinary College Garden and the International Agri-Business Management college building (Table 2).

Similar findings were recorded by Dannyelle *et al.* (2020). They have taken in a total of 200 soil samples. Out of them, they found two samples positive of EPNs. Results of the present experiment confirm the finding of (Devi *et al.* 2016), who reported that out of 140 soil samples, they found eight

samples positive. In which 2.1 and 3.5% *Heterorhabditis* and *Steirnernema* were isolated, respectively. The findings made similar studies in this regard of Maru *et al*. (2007) collected 97 soil samples from the rhizosphere of various crops grown in various agro-climatic zones of Rajasthan. Only six samples were collected from wheat rose, guava and forest trees yielded entomopathogenic nematodes belonging to genera *Steinernema* and *Heterorhabditis*. Similarly, Chand *et al.* (2016) also collected 105 soil samples, and out of them, three samples were found positive.

Identification of the entomopathogenic nematodes

Brown color was observed in *C. cephalonica* cadavers infected with all three isolates. That suggested the isolated EPNs belong to the genus *Steinernema*. Identification of the isolates up to species was done at the Department of Nematology, BACA, AAU, Anand. The populations recovered were critically examined based on morphological characters and body dimensions for the identification up to species level by comparing them with the original descriptions of the different known species. Nematode cultures from different locations were considered isolates and designated *Steinernema* sp. (AAU St-1), *Steinernema* sp. (AAU St-2) and *Steinernema* sp. (AAU St-3). Among three, *Steinernema* sp. (AAU St-1) was successfully maintained under laboratory conditions.

Based on morphometric characters, these were identified and designated as *Steinernema ritteri* (Doucet and Doucet 1990). The average total body length of infective juvenile 581.61 ± 56.57 (479.75–666.31) µm whereas a, b and c ratio was 18.82 ± 2.17(14.53-24.18), 3.80 ± 0.33 (3.09–4.39) and 15.70 ± 1.51 (12.29-18.38) µm, respectively, similar to *S. ritteri*. The male spicule length 58.05 ± 4.85 (48.05–65.13) and gubernaculum length 32.63 ± 2.57 (27.24–37.99) µm were found similar to *Steinernema ritteri.* Mucron was absent in the male. The female of *Steinernema* sp. (AAU St-1) distance of excretory pore from the anterior end and distance of nerve ring position from the anterior end were 122.09 ± 14.63 (103.55–149.11) and 133.90 ± 16.47 (108.87–159.66) µm, respectively were also found similar to *S. ritteri* (Table 3) (Plate 1).

The results obtained from morphological characteristics were similar to (Doucet and Doucet 1990). They found similar characteristics of length a, b, c ratio, spicule length and gubernaculum length, similar to present findings.

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