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Description of a new species of entomopathogenic nematode, *Steinernema ramanai* sp. n. from Kerala, India

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Abstract

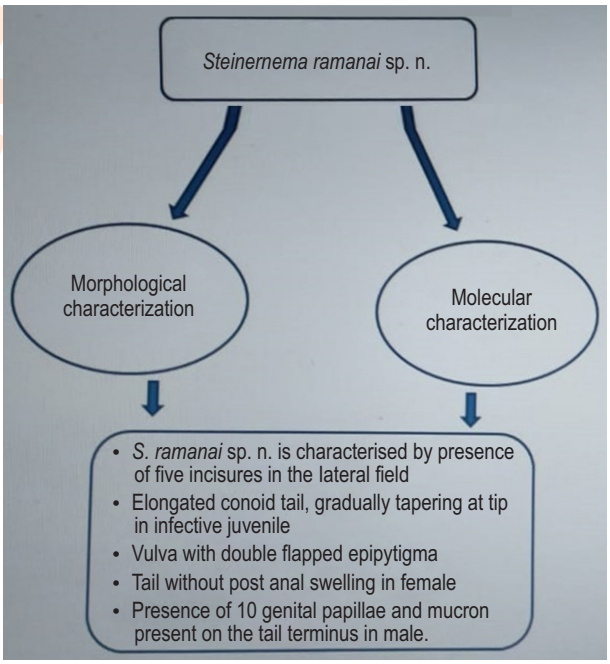
Aim: A novel species of entomopathogenic nematode (EPN) belonging to genus *Steinernema* was discovered in the rhizosphere of ginger grown at Kozhikode, Kerala, India.

Methodology: Morphological and molecular characterization of new species of *Steinernema* and its phylogenetic relationships with other *Steinernema* species were investigated using DNA extracted and amplified rDNA of the ITS region using primers 18S (FORWARD) 5' TTGATTACGTCCCTGCCCTTT 3' and 26S (REVERSE) 5' TTTCACTCGCCG TTACTAAGG 3'.

Results: New species described based on the length of infective juvenile, which was the smallest species among the described *Steinernema* species. *S. ramanai* sp. n. has five incisures in the lateral field, an elongate conoid tail that gradually tapered at the tip in infective juveniles; vulva with double flapped epipytigma and tail without post anal swelling in females; body 'J' shaped upon fixation, ten genital papillae, and a mucron on the tail terminus in males. This new species is distinguished genetically by its unique rDNA ITS region nucleotide sequence. The ITS sections of ribosomal DNA were sequenced to confirm this new species.

Interpretation: Native EPNs may yield species and/or strains that are better suited for inundative release against local pests. This logic has prompted coordination of several surveys in the search for novel species and strains, particularly in areas where EPNs had previously remained undetected.

Key words: Biocontrol, Morphology, Molecular characterization, *Steinernema*, Taxonomy



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Introduction

Entomopathogenic nematodes (EPNs) are effective biological control agents against a wide range of insect pests due to their wide host range, ease to handle, short life cycle and environmental safety (Ali *et al.,* 2005a; Pervez *et al.,* 2007; Pervez and Rao, 2021). These are symbiotically associated with bacteria *Xenorhabdus* and *Photorhabdus* (Akhurst, 1982; Boemare, 2002; Pervez *et al.,* 2020). Third stage infective juveniles penetrate into host's body through natural openings and release the symbiotic bacteria that cause septicaemia and death of the insect (Shapiro and Mc Coy, 2000). They are being employed as biopesticides to combat a number of major insect pests around the world. As a result, the efficient application of entomopathogenic nematodes as biopesticides is dependent on the isolation of native species, which are adapted to local environment and climatic conditions, as well as accurate identification of these species (Ali *et al.,* 2005b; Pervez *et al.,* 2013; Pervez and Rao, 2018).

As a result, collecting native entomopathogenic nematodes may yield species and/or strains that are better suited for inundative release against local pests. This logic has prompted coordination of several surveys in the search for novel species and strains, particularly in areas where entomopathogenic nematode has remained undetected. So far, 100 *Steinernema* and 21 *Heterorhabditis* species have been identified globally (Bhat *et al.,* 2020). Several surveys have revealed the natural occurrence of entomopathogenic nematodes associated with different ecosystems in India such as Meghalaya (Lalramlina and Yadav, 2010), Andaman and Nicobar islands (Prasad *et al.,* 2001), Gujarat (Vyas, 2003), Tamil Nadu (Josephrajkumar and Sivakumar, 1997), Uttar Pradesh (Pervez and Ali, 2007; Kaushal *et al.,* 2000; Pervez, 2022) and South Kerala (Banu *et al.,* 2004; 2005; Pervez *et al.,* 2014a). However, information on the entomopathogenic nematodes associated with Kozhikode districts of Kerala is meagre. In this backdrop, an extensive survey for entomopathogenic nematode was conducted in the northern regions of Kerala state, India.

Materials and Methods

Insect source: The greater wax moth, *Galleria mellonella* L. was grown on an artificial diet as per the procedure described by David and Kurup (1988). Yeast tablets were grounded into a fine powder and mixed with corn flour, wheat bran and milk powder (Part A). Glycerin and honey were mixed separately (Part B). Finally parts A and B were mixed together thoroughly and a homogenous mixture was prepared in a container. Eggs of *G. mellonella* were released in container and incubated at room temperature. The larvae were ready for use within 3 weeks. Some of the *G. mellonella* larvae were left to complete their life cycle and emerge as a moth. These moths were collected and placed in a separate jar in which several small pieces of honey combs were provided with hanging folded paper strips which served as dark areas for hiding and as substrate for egg laying. The neonate larvae in honey combs or eggs collected from paper strips were then put on artificial diet.

Origin of entomopathogenic nematode: At a depth of 10-20 cm, soil samples were obtained at random from the rhizosphere of ginger cultivated at the ICAR-Indian Institute of Spices Research Experimental farm, Peruvannamuzhi, Kozhikode, India. The atmospheric temperature of sample collection site at the time of sampling was 36°C, annual rainfall 681-6917 mm, laterite soil and pH 6.2. To prevent moisture loss, the samples were packed in polyethylene bags.

Isolation of entomopathogenic nematode: Insect-baiting (Bedding and Akhurst, 1975) and modified white trap (White, 1927) method were used to isolate entomopathogenic nematode (Kaya and Stock, 1997). Five live *G. mellonella* larvae were introduced into a plastic pot with the composite soil sample to bait out the entomopathogenic nematode. The presence of entomopathogenic nematodes in the soil sample was checked continuously for seven days. If any dead larvae were identified, they were placed on a modified white trap for two weeks at 28°C to check the emergence of any entomopathogenic nematodes.

Morphological characterization: Nematodes were killed in warm water at 60°C, fixed in triethnolamine formaldehyde (Courtney *et al.,* 1955), dehydrated using a slow evaporation process (Seinhorst, 1959), and permanently mounted in anhydrous glycerin. A Leica light microscope was used to take measurements and examine the morphology. A holotype male, 20 paratype infective juveniles, and 20 each first-and second-generation males and females were all measured using an ocular micrometre. The study employed morphometric characters, according to Hominick *et al.* (1997). The line diagrams of new species were drawn using a Leica microscope drawing tube.

Molecular characterization: DNA extracted and the isolated DNA was amplified of the ITS region of the rDNA using 18S (FORWARD) 5' TTGATTACGTCCCTGCCCTTT 3' and 26S (REVERSE) 5' TTTCACTCGCCG TTACTAAGG 3' primers and sequenced (Vrain *et al.,*1992; Nguyen *et al.,* 2001). The Neighbor-joining approach was used to compare evolutionary relationships with other *Steinernema* species (Saitou and Nei, 1987). The maximum composite likelihood approach was used to calculate the evolutionary distances (Tamura *et al.,* 2004). MEGA X was used to undertake evolutionary analysis (Kumar *et al.,* 2018).

Results and Discussion

Morphometric characterization: Measurements of the holotype male, 20 each paratype first and second generation of males and females, and 20 infective juveniles of new species of *Steinernema ramanai* are given in Table 1.

Morphological characterization: Diagnostic characters of male, female and infective juveniles are enumerated below:

Male: Cuticle with tiny transverse striae, body ventrally curved, J shaped after fixation. With separate labial papillae, the lip region was continuous. Behind the lateral papillae, there was an amphid

Table 1: Measurements (µm) of the holotype male, infective juveniles and paratype of first and second generation male and female of *Steinernema ramanai* sp. n.

Characters	Holotype	Infective juvenile	Paratype			
			First generation		Second generation	
			Male	Female	Male	Female
N	Male	20	20	20	20	20
Total length	646.99	377.0±42.08 (334.65-420.01)	672.53±22.12 (646.99-685.79)	822.23±44.13 (777.14–866.21)	687.40±40.83 (659.6– 734.21)	757.89±81.4 (672.2–834.2)
Greatest width	38.81	21.34±1.18 (20.37-23.28)	43.65±4.85 (38.8 – 48.5)	63.37±7.84 (56.26 – 71.78)	48.18±1.46 (46.56 – 49.4)	58.52±3.40 (55.29 – 62.08)
Stoma length	12.23	5.49±0.56 (4.85-5.82)	13.09±0.68 (12.61 – 13.58)	14.04±0.48 (13.58 – 14.55)	12.93±2.24 (11.63 – 15.52)	15.19±1.12 (14.55 – 16.49)
Stoma width	10.67	-	11.31±0.56 (10.67 – 11.64)	12.28±2.24 (9.70 – 13.58)	12.28±2.01 (10.67 – 14.55)	14.87±1.48 (13.58 – 16.49)
EP	49.47	34.59±2.24 (32.01 – 35.89)	50.76±1.48 (49.47 – 52.38)	34.59±6.45 (29.1 – 41.71)	53.34±2.91 (50.44 – 56.26)	49.47±2.91 (46.56 – 52.38)
EPW	23.28	14.06±0.68 (13.58-14.55)	24.25±0.97 (23.28 – 25.22)	19.4	24.25	28.45±1.12 (27.16 – 29.1)
ES	126.10	77.49±10.33 (69.82 – 89.24)	133.53±6.60 (126.1–138.71)	147.1±5.60 (140.65– 150.35)	130.3±8.47 (123.1–139.68)	142.59±7.93 (135.8 – 151.32)
Testis reflection	19.21	-	19.2	-	14.5	-
ABD	24.25	12.61±1.68 (10.67 – 13.58)	23.60±4.88 (18.43 – 28.13)	24.57±0.56 (24.25 – 25.22)	27.16±1.68 (25.22 – 28.13)	25.22±2.56 (23.28 – 28.13)
Tail	19.93	42.03±3.67 (37.83 – 44.62)	18.92±2.11 (16.49 – 20.37)	37.83±1.68 (36.86 – 39.77)	19.4±3.36 (17.46 – 23.28)	35.56±2.80 (33.95 – 38.8)
Spicule length (SL)	53.35	-	53.99±3.92 (50.44 – 58.2)	-	51.70±2.00 (49.47 – 53.39)	-
Gubernaculam length	29.41	-	28.87±0.66 (28.13 – 29.4)	-	28.61±0.68 (28.13– 29.1)	-
Anterior to vulva	-	-	-	483.7±33.8 (447.7 – 514.1)	-	441.02±85.32 (347.26– 514.1)
Vulva to anus distance	-	-	-	300.7±12.83 (291.0 – 315.25)	-	281.3±9.7 (271.6 – 291.0)
A	16.67	17.81±3.16 (14.37 - 20.16)	15.49±1.28 (14.12 – 16.67)	13.07±1.40 (11.45 – 13.95)	14.26±0.64 (13.57 – 14.86)	12.92±1.20 (11.55 – 13.80)
B	5.13	4.87±0.22 (4.70 – 5.12)	5.03±0.10 (4.93 – 5.13)	5.57±0.50 (5.40 – 6.15)	5.29±0.60 (4.78 – 5.96)	5.31±0.47 (4.77 – 5.65)
V(%)	-	-	-	58.77±0.71 (57.98 – 59.35)	-	57.94±5.47 (51.65 – 61.62)
C	33.35	9.0±1.19 (7.66 – 9.94)	36.17±4.68 (33.35 – 41.58)	21.78±2.01 (19.56 – 23.5)	35.86±3.74 (31.54 – 38.27)	21.3±1.41 (19.8 – 22.6)
c’	0.82	3.34±0.17 (3.21 – 3.54)	0.83±0.26 (0.58 – 1.10)	1.54±0.09 (1.46 – 1.64)	0.71±0.10 (0.62 – 0.82)	1.40±0.04 (1.37 – 1.45)
D (EP/ES)%	39.2	45.06±5.74 (40.2 – 51.4)	37.8±1.45 (36.3 – 39.2)	23.4±3.78 (20.6 – 27.7)	40.96±2.90 (38.1 – 43.9)	34.7±1.65 (33.1 – 36.4)
E (EP/Tail)%	248.51	82.4±2.10 (80.4 – 84.6)	271.25±40.17 (247.6 – 317.64)	91.0±13.02 (78.9 – 104.8)	281.4±56.74 (216.6 – 322.2)	139.46±5.45 (135.0 – 145.7)
SW (SL/ABD)	2.21	-	1.90±0.05 (1.85 – 1.96)	-	2.33±0.35 (2.06 – 2.73)	-

*Mean ± standard deviation (range)

pore-like structure. At the base, the stoma was shallow, somewhat collapsed, and triangular. Cheilorhabdions were separated by a thick sclerotized ring. Muscular pharynx with a cylindrical procorpus that spaned the entire width of the oral opening. The non-valvate metacorpus was nearly indistinguishable, with a thin isthmus and a circular basal bulb with valve plates. Just above the basal bulb, a nerve ring surrounded the isthmus. The conoid of the pharyngeal-intestinal valve was symmetrical on both sides. Excretory pore was located near the base of the metacorpus, above the nerve ring. Testis had with a single reflex. The spicule was golden dark

Table 2: Comparison of the morphometrics of *S. ramanai* sp. n. and other closely related Steinernema species.

Character	*S. simkayai*	*S. tami*	*S. seemae*	*S. ramanai* sp. n.
Infective Juvenile				
L	446 (398 – 495)	530 (400-600)	380 (400 – 428)	377 (334 – 420)
EP	35 (29 - 38)	36 (34-41)	35 (24 – 31)	34 (32 – 36)
ES	94 (80 – 107)	117 (110-123)	76 (81 – 84)	77 (69.8 – 89.2)
Tail	35.5 (31 - 41)	50 (42-57)	37 (33 – 38)	42 (38 – 45)
D (%)	37 (31 - 43)	31 (28-34)	46 (40 – 54)	44 (40 – 51)
Male				
L	1135 (1035-1278)	1286 (1194-1308)	915 (718-1528)	672 (646-685)
SL	77.5 (75 - 80)	77 (71-84)	41 (45 – 46)	54 (50 – 58)
SW	1.7 (1.4 - 2.2)	2.0 (1.4-3.0)	1.5 (1.4 – 1.6)	2.4 (1.8 – 3.1)
Female				
L	1836 (1410-2560)	1904 (1523-2674)	1405 (1241-1538)	822 (777-867)
V (%)	56 (53-56)	52 (48-54)	56 (53-59)	59 (58 - 60)

*Mean ± standard deviation (range)

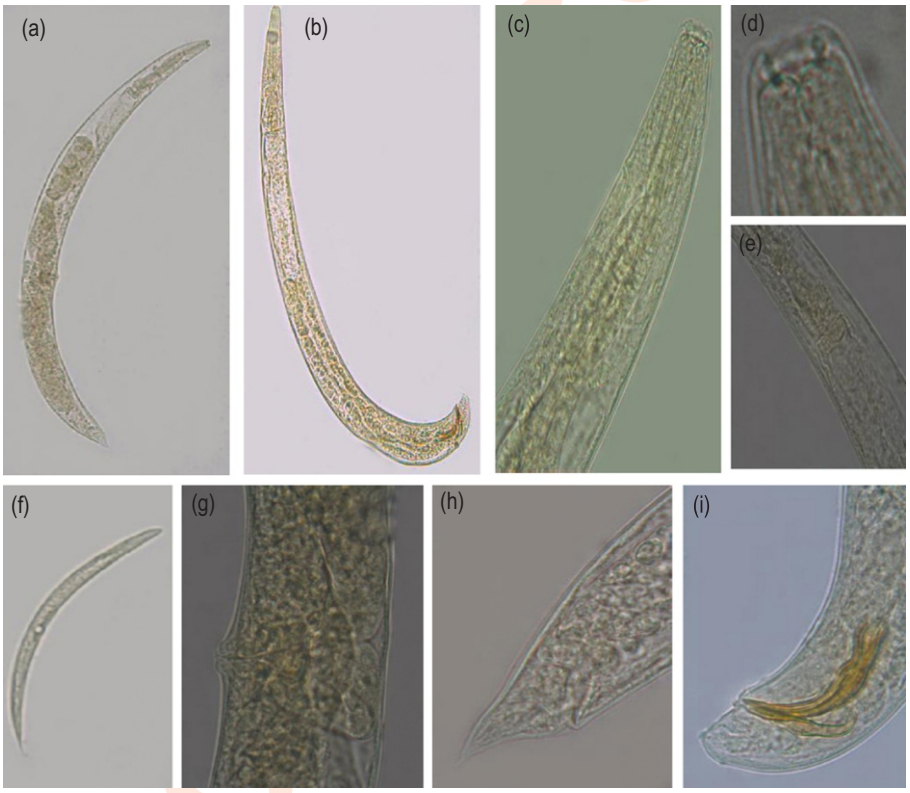

Fig. 1: *Steinernema ramanai* sp. n.: A- entire female; B- entire male; C- anterior region; D- cephalic region; E- excretory pore; F- infective juvenile; G- vulval region; H- female tail; I- male tail. Photographs A, B, F at 10x and C, D, E, G, H, I at 100x.

yellow coloured and paired. The spicule head (manubrium) accounted roughly 25-27 % of the spicule's length. The shaft (calomus) was nearly non-existent, and the blade (lamina) was thick and tapered about three times the length of the head. Each spicule had two internal ribs and a velum. Gubernaculum was present 50-70 % of the way down the spicule. Bursa was absent. Ten genital papillae were present. The tail was short, rounded conoid that measured 72-89% of the anal body width and had a mucron on the terminus (Fig. 1B, C, D and I; Fig. 2B,C,F and H).

Female: The female body was robust with an open 'C' shape after fixation. Cuticle had fine transverse striae. With six separate labial

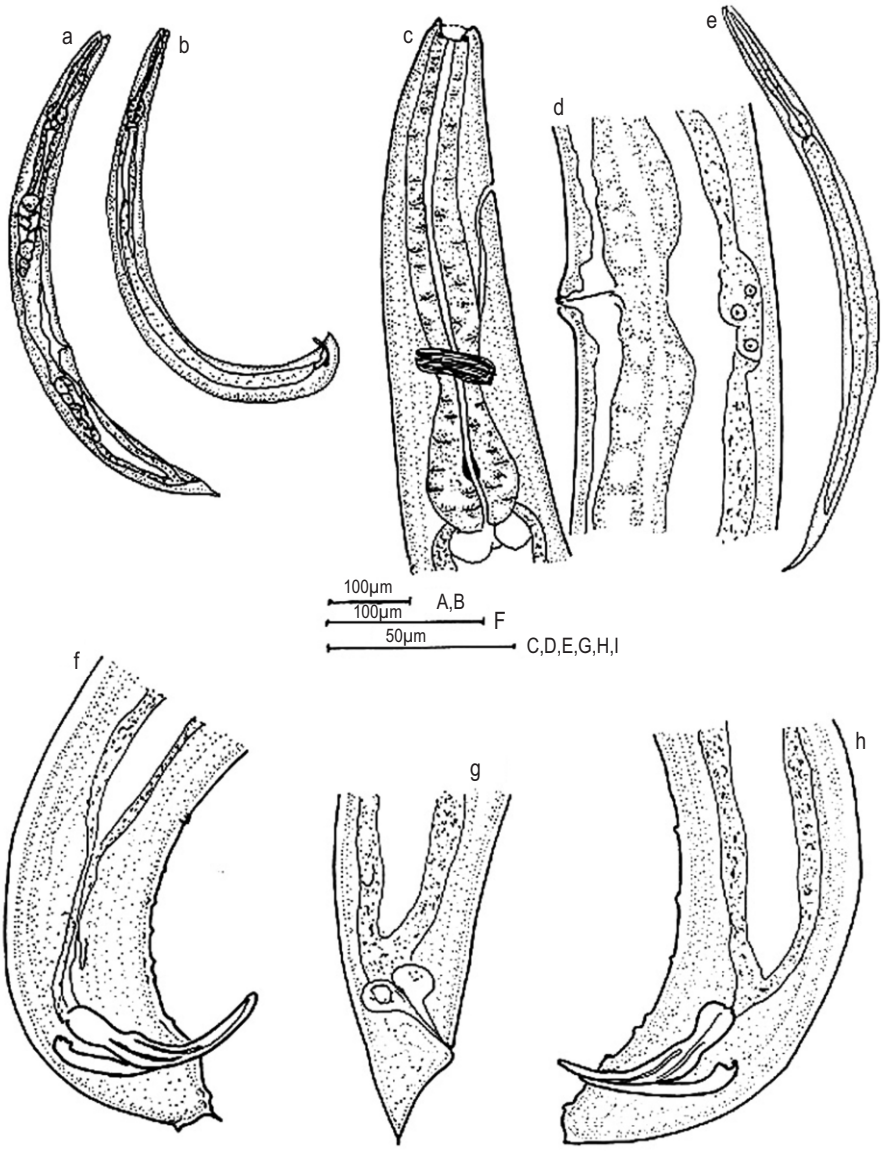

Fig. 2: Line diagram of the *Steinernema ramanai* sp. n.: A- entire female; B- entire male; C- oesophageal region; D- vulval opening; E- infective juvenile; G- female tail; F, H- male tail.

papillae, the lip region was round and continuous with the body. At the base, the stoma was shallow and triangular. Cheilorhabdians that had partially collapsed were well sclerotized. Muscular pharynx with cylindrical procorpus at the anterior end covered the entire width of the body, there were non-valvate metacorpus, comparatively thin isthmus, and rounded basal bulb with prominent valve plates. Cardia was conical and short. Nerve ring was present just above the basal bulb. Excretory pore was present at the metacorpus level. Gonads were amphidelphic, reflexed, and often held a large number of eggs. The vulva had a transverse slit and a double flapping epiptygma. Vaginal sclerotization was 19.4-21.34 µm deep and 35 % of the equivalent body width. Tail was short conoid with a pointed tip (Fig. 1A, E, G and H; Fig. 2A, D and G).

Infective juvenile: The infective juvenile body was slender, elongated, straight, with a continuous lip area and a lateral field with five incisures. Near the base of the metacorpus, there was an excretory pore. At the same level, the distance between the anterior end and the excretory pore was more than the body width. Pharynx had cylindrical procorpus. Just above the basal bulb, there was a nerve ring. The tail was elongated and attenuated, with a spine-like protrusion near the tip (Fig. 1F; Fig. 2E).

Molecular characterization: The novel species was

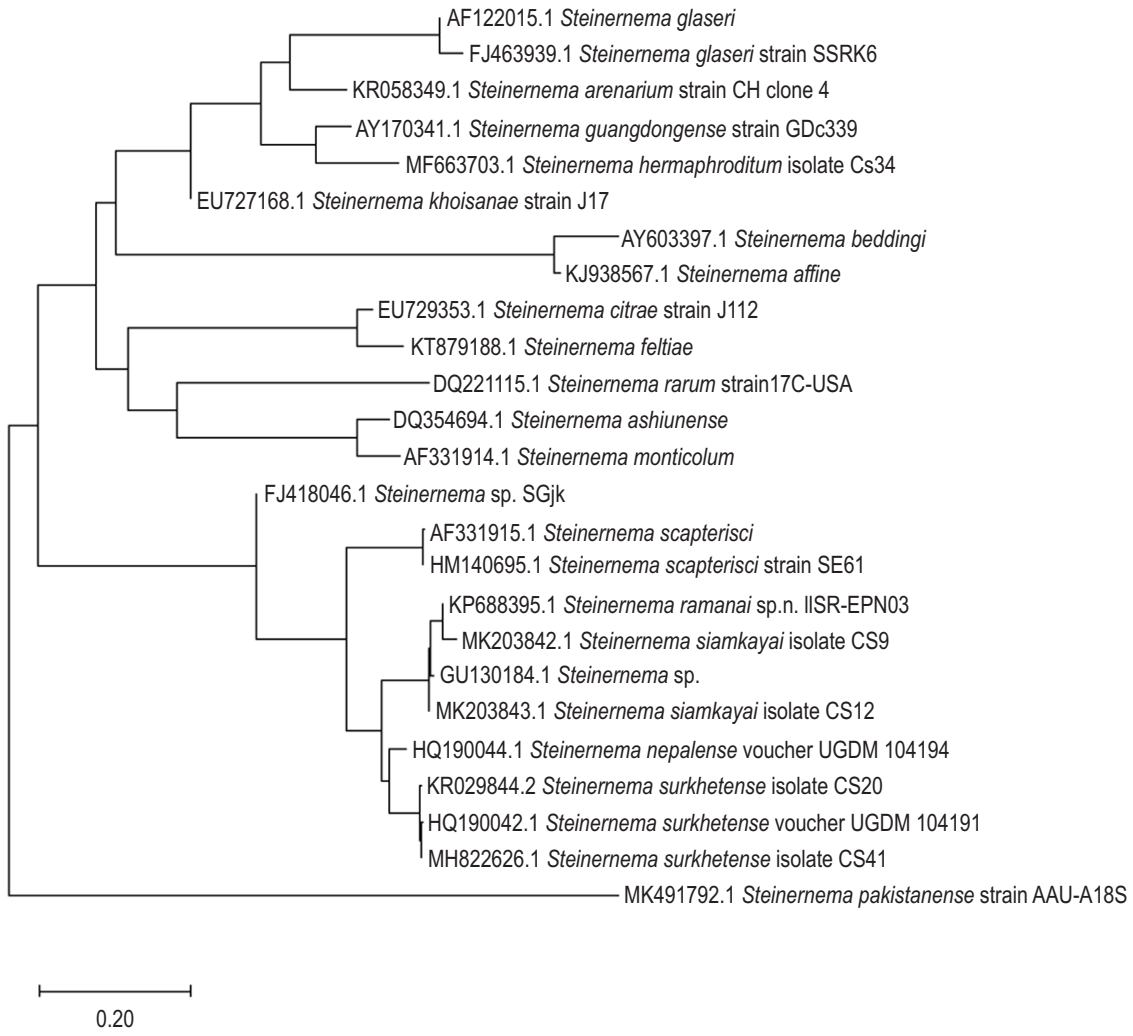

Fig. 3: Phylogenetic relationships of *S. ramanai* sp. n. with the other *Steinernema* species based on rDNA sequence of the ITS regions.

distinguished genetically by its unique rDNA ITS region nucleotide sequence. The acquired rDNA sequence (659 bp) was deposited in the NCBI GenBank database under the accession number KP688395. The evolutionary connections based on ITS rDNA polymorphism between *S. ramanai* sp. n. and other closely related *Steinernema* species are shown in Fig. 3.

Diagnosis and relationship: *S. ramanai* sp. n. was distinguished by the length of the infective juvenile (377 µm), presence of five incisures in the lateral field, elongated conoid tail, gradually tapering at tip; female was 822 µm long, vulva with double flapped epipytigma and tail without post anal swelling; male was 672 µm long, body 'J' shaped upon fixation, presence of 10 genital papillae, mucron present on the tail terminus. *S. ramanai* sp. n. morphologically resembled *S. tami* (Luc *et al.,* 2000), *S. simkayai* (Stock *et al.,* 1998), and *S. seemae* (Ali *et al.,* 2005b) (Table 2). It was closely related to *S. tami*, but differed in the length of infective juvenile (vs L = 400–600 µm), the smaller 'a' value (vs a = 23.0), the larger 'b' value (vs b = 5.0), the smaller 'c' value (vs c = 10.0), the smaller spicule length (vs SL = 77), the larger 'SW' value (vs SW = 2.0) and presence of lesser number of lateral lines (vs LL = 6). The new species was similar to *S. simkayai* but differed in the size of infective juveniles (vs L = 398–495 µm), body posture (vs 'C' shaped), larger 'b' value (vs b = 4.7), smaller 'c' value (vs c = 11.3), tail shape (vs spicate tail), presence of less lateral lines (vs LL = 6-8), number of genital papillae (vs genital papillae = 23) and tail without post anal swelling (vs with post anal swelling). It differed from the most closely related species *S. seemae* by the infective juvenile's shorter average body length (vs L = 400–428 µm), larger tail size (vs 37 µm), larger 'a' value (vs a = 17.45), smaller 'b' value (vs b = 4.94), smaller 'c' value (vs c = 9.8), spicule shape (vs abruptly curved spicule), larger spicule length (vs SL = 41.11), larger 'SW' value (vs SW = 1.65), presence of lesser number of lateral lines (vs LL = 8), number of genital papillae (vs

genital papillae = 18), tail without post anal swelling (vs with post anal swelling) and presence of mucron on the tail (vs absent).

Type host and locality: *S. ramanai* sp. n. lackes a natural host. The nematode was caught using *G. mellonella* from the soil as bait. Soil samples were collected from the ginger rhizosphere at the ICAR-Indian Institute of Spices Research Experimental Farm at Peruvannamuzhi, Kozhikode, India.

Type material: The holotype along with 10 paratypes each of females, males, and infective juveniles (permanent mount) was deposited in the Nematodes Collection Centre, ICAR-Indian Institute of Spices Research, Ministry of Agriculture and Farmer's Welfare, Government of India, Kozhikode, India.

Etymology: This new species was named after Dr. K.V. Ramana, Principal Scientist and former Assistant Director General (Plantation Crops), Indian Council of Agricultural Research, New Delhi, Government of India in the recognition of his contributions to the advancement of nematology in India.

Exotic species of EPNs reported inconsistent results due to their poor adaptability to the local agro-climatic conditions. Hence, search for indigenous EPNs resulted three species of Heterorhabditis and 19 Steinernema species have been isolated and reported from India (Bhat *et al.,* 2021; Soni *et al.,* 2023; Patil *et al.,* 2023).

Our study revealed that, this newly encountered species as a new species based on the morphological and molecular characterization. This is the first report of *Steinernema* species from ginger rhizosphere. This new species is capable of killing hairy caterpillar (*Euproctis* sp.), shoot borer (*Conogethes punctiferalis*), root grub (*Holotrichia longipennis*) infesting ginger (Pervez *et al.,* 2012; Pervez and Rajkumar, 2018); leaf feeder (*Lema* sp.) infesting turmeric (Pervez *et al.,* 2014b); root grub (*Basilepta fulvicorne*) infesting cardamom (Pervez *et al.,* 2016); semi-looper (*Synegia* sp.) infesting black pepper (Pervez, 2018) within 24-72 h. This opens up the possibility of using *S. ramani* sp. n. as a biocontrol agent to control the insect pests of ginger, especially in the regions of Kerala because of their geographical adaption.

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Authors' contribution: R. Pervez: Research concept and design, collection and/or assembly of data, data analysis and interpretation, writing the article, critical revision of the article; **S.J. Eapen:** Research concept and critical revision of the article; **S. Devasahayam:** Research concept and critical revision of the article. The authors read and approved the final manuscript.

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