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Steinernema pui sp. n. (Rhabditida, Steinernematidae), a new entomopathogenic nematode from Yunnan, China

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Abstract

A new species of entomopathogenic nematode, herein described as *Steinernema pui* **sp. n.** was recovered from a soil sample collected from Xiao-jie town, Jing-hong city, Xi-shuang-ban-na district in Yunnan province, the People's Republic of China in December 2002. Both morphological and molecular evidence show congruently that *S. pui* **sp. n.** belongs to the *S. glaseri* group. It can be separated from all described *Steinernema* species by a combination of morphological and morphometrical characters of adult and juvenile stages, including spicule and gubernaculum shape of the first generation males (spicule bearing an aperture on the tip and an irregular-shaped concave on ventral side of the lamina close to the tip; gubernaculum with a short needle-shaped cuneus); the tail and vulva shape of the first generation females (tail conoid and pointed with a mucron; vulva with a short double flapped epiptygma) and the body and tail length, distance from anterior end to excretory pore and to the base of pharynx of infective juveniles. The new species can also be distinguished from other *Steinernema* species by DNA sequences of either a partial 28S rDNA or the internal transcribed spacer (ITS) regions of rDNA, and from the closely related species *S. longicaudum* and *S. guangdongense* by cross-breeding tests.

Key words: 28S rDNA sequence, entomopathogenic nematode, identification, rDNA ITS sequence, *Steinernema pui* **sp. n.**, taxonomy

Introduction

Entomopathogenic nematodes (EPN) of the families Steinernematidae Travassos, 1927 and Heterorhabditidae Poinar, 1976 have been used as biopesticides for controlling insect pests in niche markets (Bedding, 1998). The Steinernematidae currently comprise two genera, *Steinernema* Travassos, 1927, with more than 60 recognized species and *Neosteinernema* Nguyen & Smart, 1994 with only one species, *N. longicurvicauda* Nguyen & Smart, 1994 (Adams & Nguyen, 2002). Although diversity of EPN in China has not been done exhaustively, at least thirteen EPN species have been described originally from the nematodes collected from China until now (Shen & Wang, 1991; Xu *et al.*, 1991; Liu 1994; Jian *et al.*, 1997; Cutler & Stock, 2003; Qiu *et al.*, 2004, 2005a, b, c; Nguyen *et al.*, 2006; Chen *et al.*, 2006; Mráček *et al.*, 2006; Mráček *et al.*, 2009). In addition to this, several named species, such as *H. indica* Poinar, Karunakar & David, 1992, *H. bacteriophora* Poinar, 1976, *S. glaseri* (Steiner, 1929) Wouts, Mráček, Gerdin & Bedding, 1982 and *S. carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982 have been reported in China (Hominick, 2002), indicating that this country has rich EPN diversity. The State Key Lab of Biocontrol (SKLB) of Zhongshan University has carried out several systematic EPN surveys mainly in Guangdong and Yunnan provinces of China since 2001, which have recovered more than 400 isolates of insect parasitic nematodes. Preliminary examinations show that many of these nematodes are likely to be new EPN species. Herein, we describe an isolate collected from Yunnan province as a new species *Steinernema pui* **sp. n.** after detailed morphological, molecular and cross breeding studies.

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Material and methods

Origin of the nematodes used in this study: The *Steinernema pui* **sp. n.** (isolate, YNd393) was isolated from a soil sample collected from a rubber plantation in the suburb of Xiao-jie town, Jing-hong city, Xi-shuang-ban-na district, Yunnan province in December 2002 using Galleria larvae as bait (Mráček, 1980). The soil type was red sandy loam. *S. longicaudum* Shen & Wang, 1990 CWL05 strain was collected in Shangdong province by J. Liu in1986, who also described it as *S. serratum* in his PhD thesis (Liu, 1990). *S. serratum* was recently synonymised as *S. longicaudum* (Qiu *et al.*, 2004).

Nematode culture: All nematodes used in this study were produced in *Galleria mellonella* larvae. Fifteen *G. mellonella* larvae were exposed to about 2000 infective juveniles (IJ) in a Petri dish (60 x 15 mm) lined with two moistened filter papers kept at 23ºC. After they died, the insect cadavers were transferred to a White trap (White, 1927) and incubated at 23ºC until IJ emerged. The first and second generation adults were obtained by dissecting infected insects 3 to 4 days and 6 to 7 days, respectively, after the death of insects. The infective juveniles used for measurements were collected 3 days after the first emergence of the IJ.

Morphological characterization with light microscopy: All nematode samples, including IJs, the first and second generation males and females, were killed by gentle heat and then fixed in TAF (Courtney *et al.*, 1955) and processed to anhydrous glycerol using the method described by Seinhorst (1959). Permanent slides were made using glass slides and cover-glass supports were used in all cases to avoid flattening of specimens. Measurements were conducted using a Nikon inverted microscope equipped with 10x, 20x or 40x differential interference contrast objection. Some fine characters, such as stoma, cheilorhabdions and prorhabdions of first generation males or females, morphology of the anterior part and tail of IJs were observed under a 100x plan objective lens.

Morphological characterization with scanning electron microscopy: For scanning electron microscopic (SEM) examination, the first generation adults and IJs were fixed in 3% glutaraldehyde buffered with 0.1 M Phosphate buffer at pH 7.2 for at least 2 days at 4–8º C (Nguyen & Smart, 1995). They were post-fixed with 2% osmium tetroxide at 23º C overnight, dehydrated in a graded ethanol series, critical point dried with liquid CO_2, mounted on SEM stubs, and coated with gold and examined under a Hitachi S-520 scanning electron microscope. Spicules and gubernacula were prepared as suggested by Nguyen & Smart (1997).

Molecular characterization. The sequences of the following two fragments of nucleic DNA were used as molecular markers to differentiate *S. pui* **sp. n.** from other described *Steinernema* species: a fragment of 28S rDNA (D2D3 region), and the internal transcribed spacer (ITS) regions of rDNA, including complete ITS1, ITS2 and 5.8S rDNA subunit and partial 18S and 28S rDNA subunit. They were PCR amplified and sequenced using protocols described previously (Nguyen *et al.*, 2001; Stock *et al.*, 2001). The sequences obtained were deposited in GenBank under accession numbers GU395636 and GU395618. Previously published 28S rDNA sequences and ITS region sequences of the described *Steinernema* species were used as reference sequences for comparison. The sequences were aligned using the default parameters (gap opening penalty 10 and gap extension penalty 5) of Clustal X (Thompson *et al.*, 1997) and then optimized manually. Based on the aligned sequence data set of either the partial 28S rDNA or rDNA ITS regions, a phylogenic tree was constructed by neighbor joining method (Saitou & Nei, 1987) using Software MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007). *Caenorhabditis elegans* and *Panagrellus redivivus* were used as out group taxon to resolve relationships among the nematode species examined. The pairwise distances between closely related species in both data sets were also calculated (Wilbur & Lipman, 1983) using Software MagAlign.

Cross-breeding. Cross-breeding test was performed between *S. pui* **sp. n.** YNd393, *S. guangdongense* GDc339 and *S. longicaudum* CWL05 using the haemolymph hanging drop technique (Poinar, 1967). For the test, one IJ from each of two nematode populations to be tested was transferred into a drop of haemolymph of *G. mellonella* larvae on a glass slide using a hair probe under a dissecting microscope. Self-cross controls were also conducted in the same way except that the two IJ were from the same nematode population. Fifteen replicates were made for each treatment. Slides were then incubated in Petri dishes lined with moist filter paper at 23ºC for 2 weeks. The development of the inoculated IJs into adults and the reproduction of the nematodes was observed and recorded during the experimental period. The experiment was repeated once.

Results

Steinernema pui sp. n. *description*

Measurements. Morphometrics of the holotype (the first generation male), allotype (the first generation female) and paratypes of IJs and the first- and second- generation males and females are listed in Table 1.

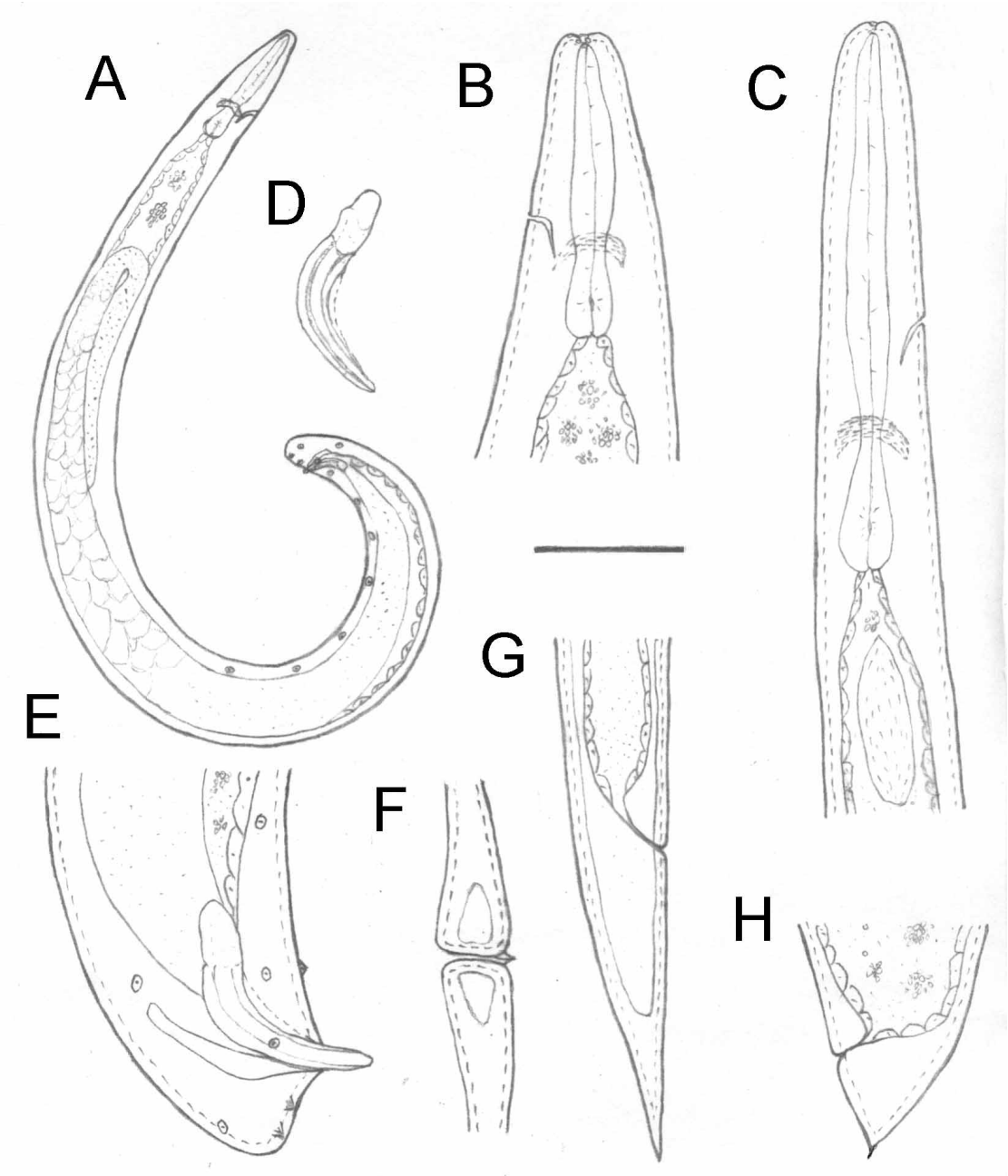

FIGURE 1. *Steinernema pui* **sp. n.** in lateral view. A, D and E: the whole body (A), Spicule (D) and tail (E) of first generation male; B, F and G: the anterior region (B), epiptygma (F) and tail (G) of the first generation female; C and F: the anterior region (C) and tail (F) of the infective juvenile. Scale bar (in μm): A = 320; B = 100; C = 32; D = 32; E = 65; F = 78; G = 28 and H = 90.

First-generation males: Body of heat-relaxed specimens C-shaped and curved posteriorly. Head truncated and slightly swollen anteriorly. Six lips fused at base, each with a papilla. Four cephalic papillae present. Two amphidial apertures distinct, located behind lateral labial papillae (Fig. 2A). Stoma shallow, circular anteriorly and triradiate internally. Pharynx extends nearly to mouth opening. Both cheilorhabdions and prorhabdions small but distinct.

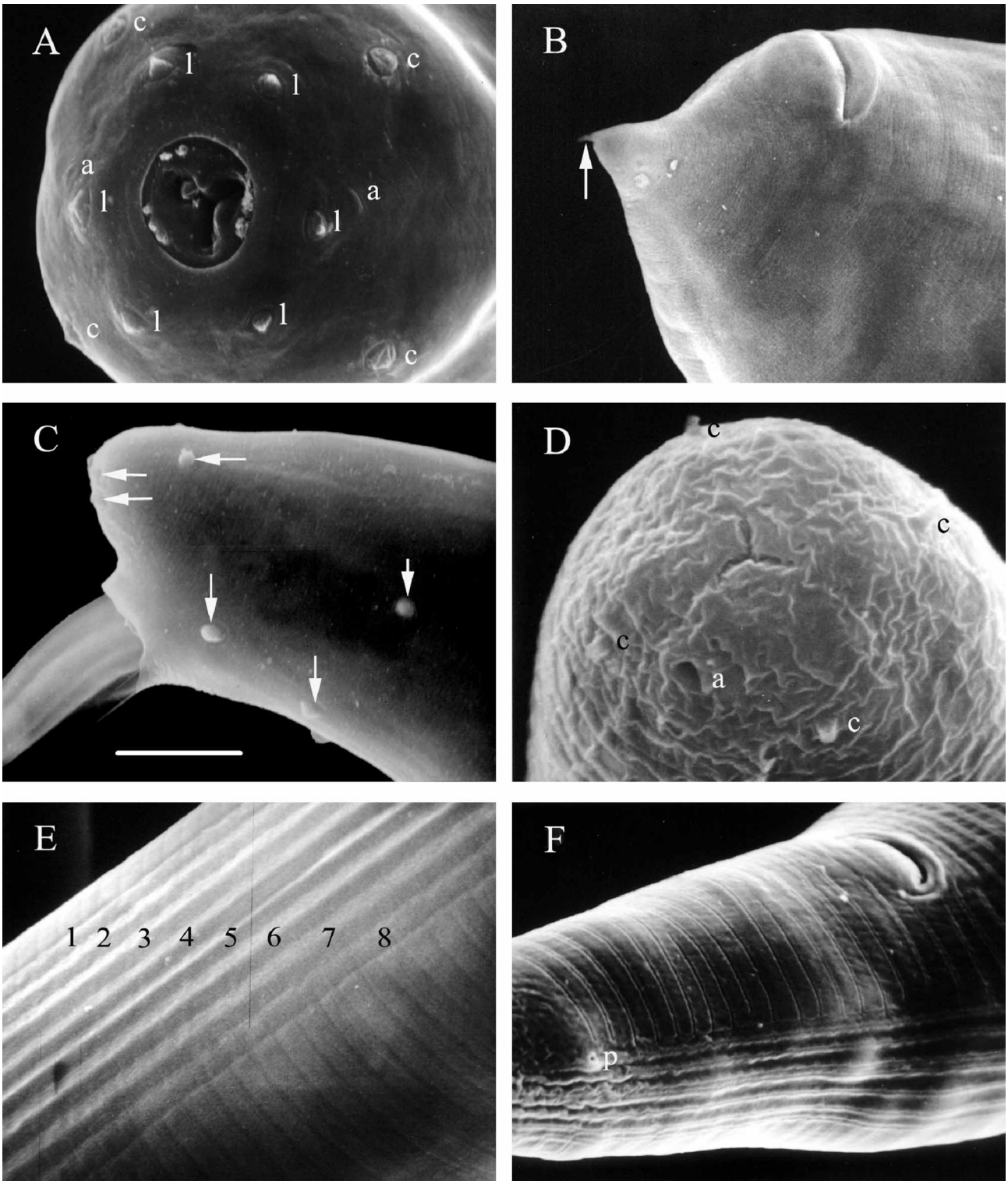

FIGURE 2. SEM micrographs of *Steinernema pui* **sp. n.** A: face view of a first generation male showing 6 labial papillae (l), 4 cephalic papillae (c) and 2 amphids (a); B: the tail of a first generation female showing a mucron (arrow) and anus; C: the tail of a first generation male showing the shape (conoid, short without mucron) and the distribution of genital papillae (arrows); D: the head of an ensheathed infective juvenile (IJ) showing 4 cephalic papillae (c) and an amphid (a); E: the middle portion of an IJ showing lateral field pattern; F: the tail of an IJ showing lateral field pattern and phasmids (p). Scale bar (in μm): A = 11; B = 30; C = 25; D = 7; E and F = 8.

Pharynx muscular, with a cylindrical procorpus, metacorpus somewhat swollen, isthmus well defined, basal bulb slightly enlarged. The maximum diameter of metacorpus is equal to that of basal bulb. Nerve ring surrounds anterior portion of basal bulb. The excretory pore opening located slightly anterior to nerve ring. Lateral fields and

phasmids not observed. Gonad single, reflexed. Spicules paired with a light brown color, moderately curved. The spicule length/width of the mature male is about 5.2 (Fig. 3A and B). Spicule head longer than wide with length/width ratio of about 1.7 (1.5–2.0). Lateral and dorsal limb distinct, starting from head and extending to the spicule tip. Ventral limb starts from head and end well before the spicule tip. The tip of spicule blunt, bearing an aperture located on the tip with a circular shape and a slit-like concave located on the ventral side of the blade closed to the tip (Fig. 3D). This character was observed clearly in all of 4 specimen in posterior and ventral view (the only position that enables this character to be observed) among about 20 dissected spicules examined under SEM. Velum moderately developed, starting from anterior portion of ventral limb and ending at the tip of the ventral limb. Gubernaculum is boat-shaped in lateral view. In ventral view, it tapers anteriorly and ends at a slightly enlarged, ventrally bent head (Fig. 3E and F). Cuneus is needle-shaped and short. Spicules of the young males (Fig. 3C) are slightly different from those of the mature ones (Fig. 3A and B). Twelve pairs and a single precloacal genital papillae distributed in a pattern showed in Fig. 1A and E and Fig. 2C, including seven pairs preanal papillae, one pair adanal, one pair dorsolateral and three pair postanal (two being subterminal and one subdorsal). Tail conoid and short, without mucron.

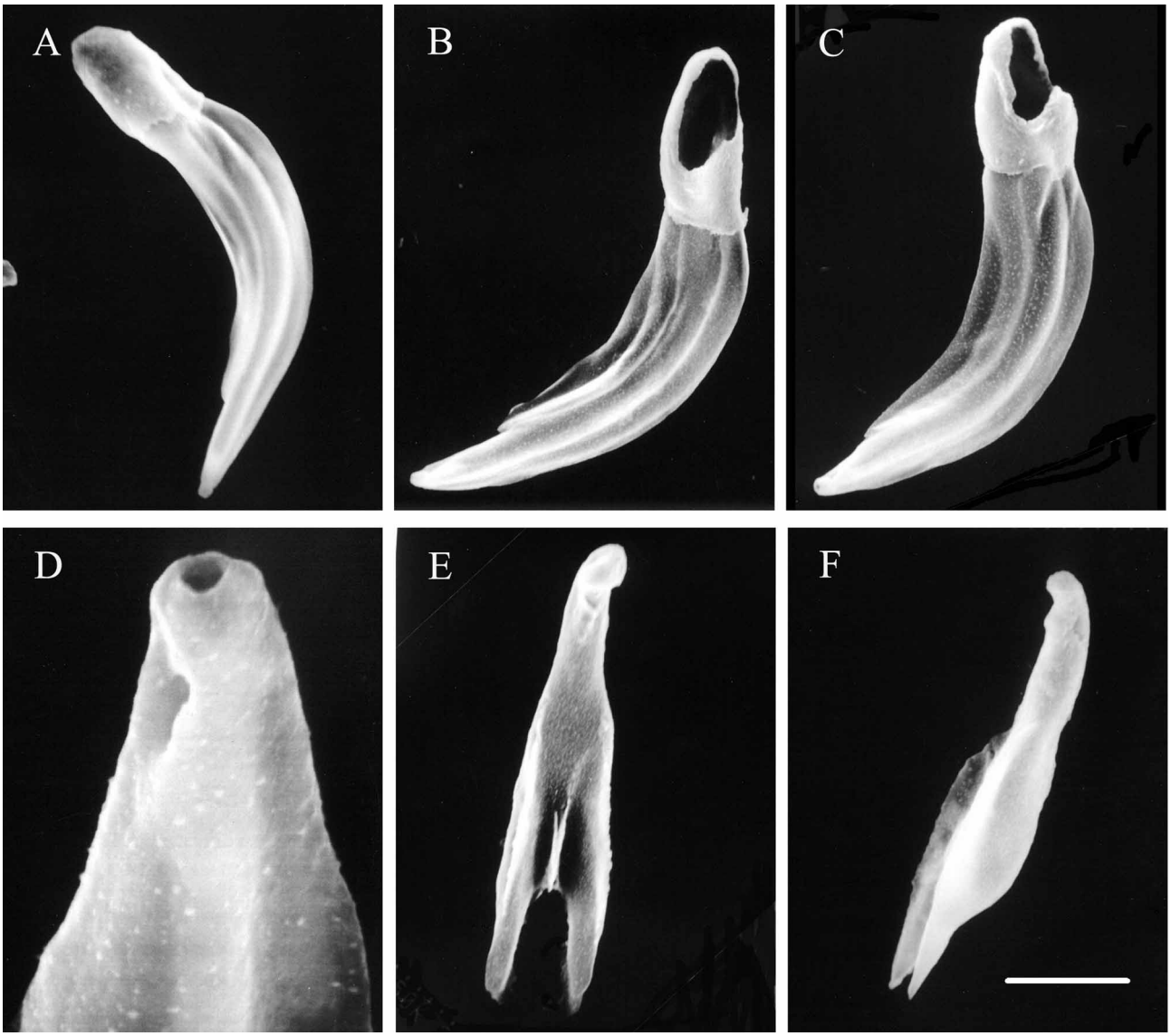

FIGURE 3. SEM micrographs of spicules and gubernacula of *Steinernema pui* **sp. n.** A and B: spicules of mature males; C: spicule of a young male; D: spicule tip showing an aperture and an irregular shaped concave; E and F: gubernacula in ventral (E) and lateral view (F). Scale bar (in μm): A = 15; B = 13, C = 12; D = 2.6; E and F = 12.

TABLE 1. Morphometrics of *Steinernema pui* **sp. n.**

Character[1]	First generation				Second generation		Infective
	Holotype	Male	Allotype	Female	Male	Female	Juvenile
n		20		20	10	10	20
L	2025	2059±124[2]	5200	6081±1151	1425±67	2495±278	1004±75
		(1800–2350)		(4850–8875)	(1300–1500)	(2070–2950)	(900–1120)
Greatest	130	137±15	238	228±32	84±7	138±10	36±2
body diam		(118–180)		(155–275)	(70–90)	(125–150)	(33–40)
EP	140	152±13	133	147±17	138±10	126±6.1	85±4
		(130–180)		(125–188)	(120–150)	(120–137)	80–95
NR	150	157±10	150	166±17	110±12	137±9.3	117±7
		(143–188)		(150–218)	(93–125)	(125–152)	(100–125)
ES	193	196±12	225	236±21	141±13	183±16	144±8
		(175–228)		(213–288)	(120–162)	(162–207)	(130–150)
Tail length (T)	34	32 ± 2	55	57±10	27±3.7	60±3.7	69±5
		(29–38)		(43–75)	(21–30)	(53–63)	(60–80)
Anal body	55	55±3	90	98±13	38±4	56±5	22±1
diam. (ABD)		(48–63)		(85–125)	(35–48)	(50–65)	(20–25)
Spicule	80	84±4			65±4.3		
length (SP)		(78–88)			(58–70)		
Gubernaculum	65	62±2			44±4.4		
length (GU)		(58–65)			(35–50)		
a	15	15±1.3	22	27±5	17±3	18± 4	28±1.7
		(14 –16)		(20–35)	(14–20)	(14–22)	(26–31)
b	10	11± 1.4	23	26±3	10.1±0.2	14±3	7.03±0.74
		(9.5–12)		(22–31)	(9.6–11)	(11–18)	(6.00–8.15)
c	60	64±6	94	107± 14	53±4	42±3	15±0.8
		(58–70)		(96–118)	(48–66)	(37–46)	(13–16)
c’	0.62	0.58±0.06	0.61	0.58±0.07	0.71±0.06	1.07±0.1	3.1±0.2
		(0.53–0.61)		(0.53–0.61)	(0.53–0.61)	(0.53–0.61)	(2.9–3.3)
H%							45±3.7
							(40–50)
D% =EP/ES x 100	73	77±5.8	59	62.3±4.5	98±12	68.9±6.7	59.5±3.04
		(70–91)		(54–71)	(77–114)	(57–81)	(55.00–66.04)
E% = EP/T x 100	412	465±48	241	258±35	511±48	210±45	125±8.0
		(403–512)		(201–312)	(389–621)	(139–272)	(109–142)
SW=SP/ABD	1.45	1.52±0.10			1.71±0.20		
		(1.40–1.84)			(1.32–1.93)		
GS=GU/SP	0.81	0.74±0.04			0.68±0.08		
		(0.69–0.81)			(0.52–0.78)		
V			51	50±5		52±2.3	
				(37–55)		(50–57)	

[1]EP, NR and ES: distance from anterior end to excretory pore, nerve ring and the base of pharynx, respectively; H%: hyaline tail length in % of total tail length. [2]Mean ± SD and range in µm.

Second-generation males: Similar to that of the first generation male except that the excretory pore is located much more posterior and most morphometrics, such as spicule and gubernaculum length, are smaller. Tail conoid without mucron.

First generation female: Body C-shaped when killed by gentle heat. Cuticle smooth or with faint annules. Head rounded and slightly truncated anteriorly (Fig. 1B). The morphology of the anterior part and pharynx is similar to those of males. The excretory pore opening is anterior to nerve ring. Lateral fields and phasmids not observed. Gonads didelphic, reflexed. Vagina muscular and short. Vulva a transverse slit, slightly protruding from body surface and with a short double flapped epiptygma. Tail conoid, pointed, with a short projection (Fig. 1G; Fig. 2B).

Second-generation female: Similar to the first generation female but smaller. Vulva asymmetric, protruding from body surface. Short double flapped epiptygma present in the first generation female indistinct or absent in the second generation females. Tail longer than anal body width, tapering to a pointed end.

Infective juveniles: Body slender. Mouth and anus closed. Anterior end rounded, slightly truncated and continuous with body contour (Fig. 1C, Fig. 2D). Labial region smooth, papillae not seen. Amphidial apertures distinct, slit –shaped. Four cephalic papillae prominent. Pharynx long and narrow, isthmus distinct and surrounded by nerve ring, basal bulb slightly elongated, cardia indistinct (Fig. 1C). Excretory pore located anterior to nerve ring, in slightly posterior position of pharynx. Bacterial pouch located in the anterior portion of the intestine. Cuticle with prominent transverse striations. Lateral field begins anteriorly with one line and increases to 9 posterior to the base of pharynx, making a total of 8 ridges (Fig. 2E). The nine-line pattern extends posteriorly to a position closed to anus where the number of ridges reduced to five followed by two and then disappeared (Fig. 2F). Phasmid distinct, located on the posterior end of the first ridge from ventral side. Tail short, attenuate and tapering evenly (Fig. 1F). Hyaline portion occupying 46% of the tail length.

Type locality and host. The soil sample was collected from a rubber plantation at Xiao-jie town, Jing-hong city, Xi-shuang-ban-na district (22.01° N, 100.47° E), Yunnan province, People's Republic of China. The type host of this nematode in nature is unknown as it was recovered from soil using *Galleria* larvae as bait.

Type specimens and etymology. The slides of holotype (first generation male), allotype (first generation female) and paratypes of about 60 infective juveniles on three slides, 10 each of the first and second generation males and females on 20 slides (one male and one female from the same generation on each slide) of *S. pui* **sp. n.** were deposited in the State Key Lab for Biocontrol (SKLB), School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China. Additional paratypes (one each of the first and second generation male and female and about 20 IJs on three slides) will be deposited in the Nematode Collection of the Department of Agriculture, USA. Living infective juveniles are also preserved in liquid nitrogen in the nematode collection of SKLB, Zhongshan University.

This species is named after the late Professor Zhelong Pu, a distinguished scientist in the area of biological control and the founder and first director of SKLB, Sun Yat-sen University.

Cross-breeding. Normal offspring were observed on the majority of slides on which two inoculated IJs developed into opposite sex adults in the self-cross controls of CWL05, GDc339 and YNd339. However, no offspring were observed from any slide in the cross-breeding treatments of YNd339 x CWL05 and YNd339 x GDc339.

Molecular characterization. The lengths of ITS and partial 28S rDNA of *S. pui* **sp. n.** amplified using the above mentioned primers were 977bp (235A, 255G, 291T and 195C) and 800bp (193A, 247G, 196T and 164C), respectively. The phylogenetic tree of the partial 28S rDNA (Fig. 4) shows that *S. pui* **sp. n.** forms a monophyletic group with *S. khoisanae*, *S. longicaudum*, *S. guangdongense*, *S. scarabaei* and *S. hermaphroditum*. This is a subgroup of the *S. glaseri* group that comprises EPNs with long IJ body and adopted a cruiser host finding strategy (Campbell *et al.*, 2003). The sequence alignment of the *S. pui* **sp. n.** group (Fig. 5) shows that it has 22 diagnostic characters (= number of base pairs, in the same column of the alignment, present in one sequence but not in others) in the group and differs from its closest nematode, *S. khoisanae* by 33 characters while there are only 11 different characters between *S. guangdongense* and *S. longicaudum*. Pairwise distances (Table 2) clearly differentiate it from all other nematodes in the group. The phylogenetic tree of the rDNA ITS regions (Fig. 6) support the relationship of *S. pui* **sp. n.** with other *Steinernema* species revealed by the partial 28S rDNA sequences. The pairwise distances of the ITS sequences (Table 3) can also be used to differentiate the new species from other nematodes in the group.

FIGURE 4. Phylogenetic relationship of *Steinernema pui* **sp. n.** with other *Steinernema* species based on the D2D3 domain of 28S rDNA sequence.

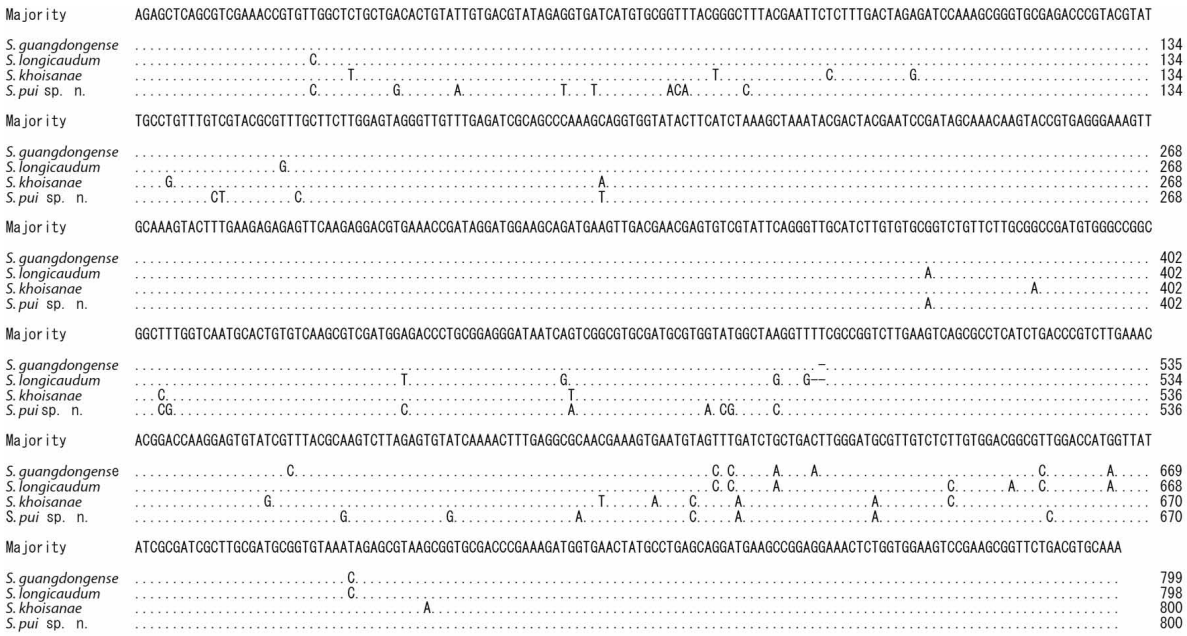

FIGURE 5. Alignment of the D2D3 domain of 28S rDNA sequence of *Steinernema pui* **sp. n.** with those of closely related *Steinernema* species.

TABLE 2. Percentage of similarity (upper triangle) and genetic distance (lower triangle) on the sequences of D2D3 domain of 28S rDNA of *Steinernema pui* **sp. n.** with other closely related *Steinernema* species**.**

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *S. boemarei*	***	95.0	92.1	89.2	92.4	93.1	92.4	94.0	92.7	90.2	90.7	90.8	89.4
2. *S. arenarium*	2.7	***	91.7	89.4	93.0	92.2	92.6	91.9	91.4	90.1	90.0	90.2	89.9
3. *S. glaseri*	6.0	5.2	***	97.1	92.1	92.0	92.0	90.5	90.9	88.2	88.9	89.9	88.0
4. *S. cubanum*	6.6	5.7	0.9	***	91.7	91.0	91.1	89.1	89.7	87.5	88.3	89.0	87.6
5. *S. puertoricense*	5.1	4.0	6.3	6.4	***	97.6	95.9	94.2	95.7	91.5	93.6	94.7	91.9
6. *S. diaprepesi*	5.0	4.7	6.4	6.9	2.2	***	96.2	94.7	96.2	92.9	93.6	94.6	91.0
7. *S. khoisanae*	4.8	4.8	6.3	6.7	3.2	2.8	***	95.5	96.1	95.4	94.4	95.4	93.5
8. *S. longicaudum*	4.9	5.4	7.8	8.3	5.1	4.7	3.9	***	98.0	94.2	94.0	95.2	92.2
9. *S. guangdongense*	5.5	5.8	7.6	8.0	4.1	3.7	3.2	1.4	***	94.5	94.5	95.7	93.0
10. S. pui **sp. n.**	6.9	7.4	9.7	10.1	7.0	6.2	4.5	4.7	4.7	***	92.8	93.6	92.1
11. *S. hermaphroditum*	5.9	5.9	8.0	8.4	4.7	4.4	3.4	3.5	3.9	4.9	***	99.2	91.6
12. *S. scarabaei*	6.1	5.9	7.9	8.3	4.6	4.4	3.3	3.4	3.6	4.8	0.0	***	91.8
13. *S. karii*	7.6	7.8	9.4	9.5	6.7	7.2	5.8	6.8	6.2	7.2	5.7	6.5	***

Diagnosis. *S. pui* **sp. n.** is characterized by its unique nucleotide sequences of either the partial 28S rDNA or the rDNA ITS regions. Morphologically, it is characterized by the combination of the features of various developmental stages of the nematode. For IJs, the combination of the following characters: body length (1004 ± 75 μm); distance from anterior end to excretory pore (85 ± 4 μm), to the base of pharynx (114 ± 8 μm); tail length (69 ± 5 μm); E% (77 ± 4.5); lateral field with eight evenly distributed and identical ridges at the middle body portion; and tail short and attenuate with a hyaline portion occupying 40–50% of the tail length can be used to differentiate the new species from other *Steinernema* species. For the first generation males, the new species can be recognized by spicule length (84 ± 4 μm, measured along the arch); GS (0.74 ± 0.04); SW (1.52 ± 0.10); D (77 ± 5.8); spicule possesses an aperture on the tip and a pit on the ventral side of the lamina close to the tip; gubernaculum with a short needle-shaped cuneus; tail conoid and short without mucron. For female, *S. pui* **sp. n.** is recognized by a conoid and pointed tail with a short mucron on the tip and a slightly protruding and symmetrical vulva with a short double flapped epiptygma.

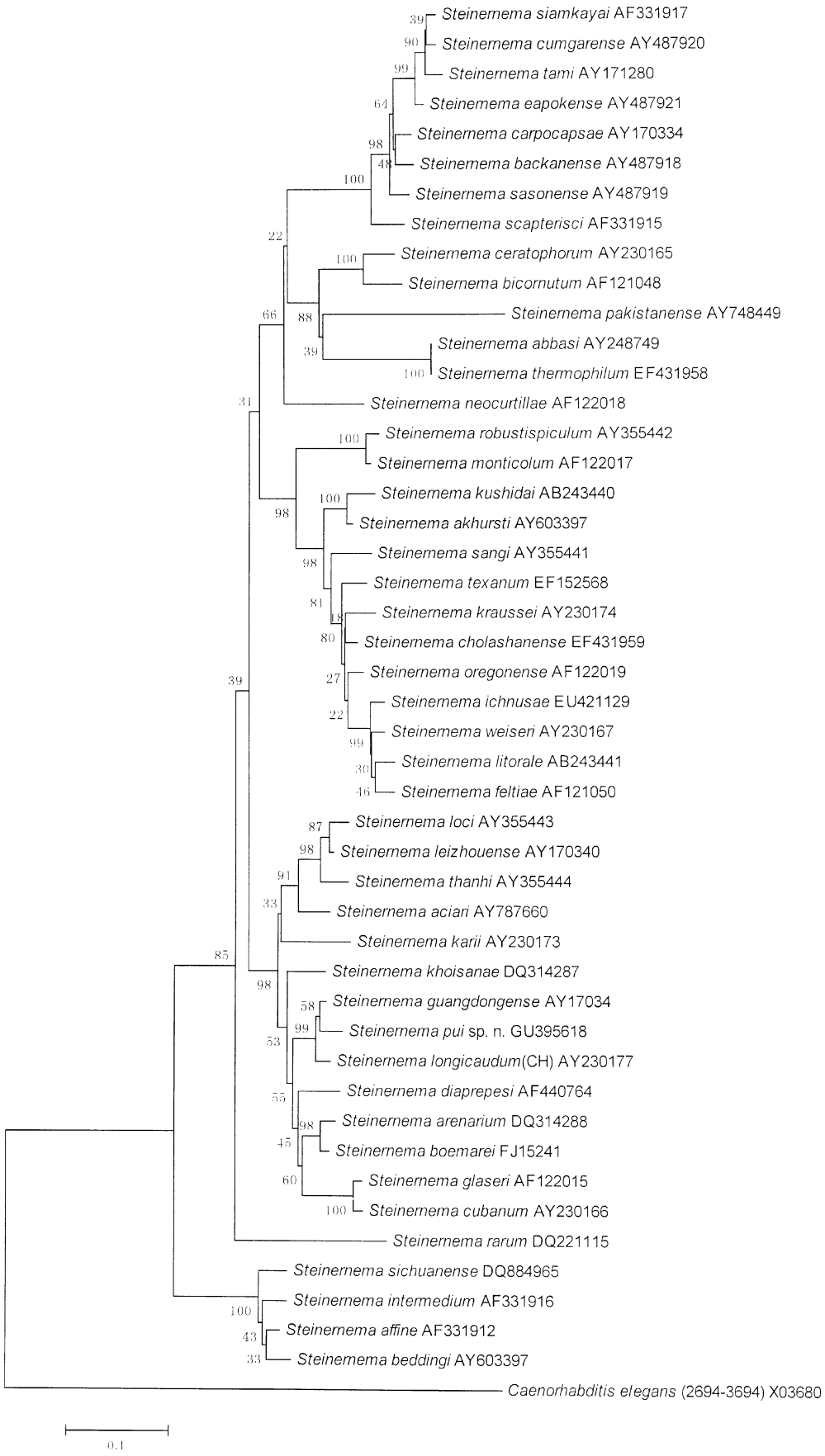

FIGURE 6. Phylogenetic relationship of *Steinernema pui* **sp. n.** with other *Steinernema* species based on rDNA sequence of the internal transcribed spacer regions.

TABLE 3. Percentage of similarity (upper triangle) and genetic distance (lower triangle) on the rDNA internal transcribed spacer region sequences of *Steinernema pui* **sp. n.** with other closely related *Steinernema* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *S. loci*	***	82.7	58.8	62.5	61.3	47.9	50.2	52.1	56.9	58.5	54.1	55.0	58.7	55.0
2. *S. leizhouense*	4.3	***	63.8	63.8	54.3	52.3	59.3	55.0	53.9	58.2	56.3	59.8	64.6	54.2
3. *S. thanhi*	8.3	6.6	***	52.5	52.8	45.8	44.0	49.3	52.5	47.6	50.1	50.4	51.5	47.8
4. *S. aciari*	19.9	18.7	18.7	***	63.8	62.3	61.0	60.3	62.7	65.3	64.0	61.0	62.7	58.0
5. *S. diaprepesi*	20.3	19.1	21.1	18.3	***	57.0	63.1	62.0	58.1	59.2	68.9	55.4	58.9	61.6
6. *S. glaseri*	30.0	28.3	26.9	25.0	25.2	***	89.3	59.1	65.5	56.2	60.4	63.4	61.5	59.3
7. *S. cubanum*	29.5	28.4	27.5	27.4	27.5	4.4	***	60.1	63.3	61.8	61.0	62.2	61.8	59.0
8. *S. boemarei*	31.1	29.0	28.0	28.9	29.7	18.2	19.5	***	68.1	52.3	56.4	53.9	60.5	54.4
9. *S. arenarium*	28.1	26.0	24.2	27.3	25.8	16.6	17.3	12.6	***	59.2	60.2	58.2	63.7	58.3
10. *S. longicaudum*	24.5	23.0	21.6	21.1	17.5	22.6	24.1	25.6	22.0	***	84.0	77.2	67.1	65.2
11. *S. guangdongense*	23.7	22.9	21.5	21.5	15.7	22.3	24.0	23.9	21.6	4.3	***	80.0	65.3	63.5
12. S. pui **sp. n.**	23.5	22.3	21.9	23.3	18.0	22.1	24.5	23.9	21.8	8.9	7.2	***	63.2	62.9
13. *S. khoisanae*	27.5	27.1	27.6	26.0	22.3	29.5	31.1	27.5	26.4	17.5	15.4	17.3	***	60.4
14. *S. karii*	26.9	27.5	25.1	22.9	20.4	28.6	29.1	27.1	25.6	19.8	17.8	19.0	23.0	***

Relationships. Both molecular (Figs. 4–6) and morphological characters (Table 1 and 4) showed congruently that *S. pui* **sp. n.** belongs to the *S. glaseri* group. 28S rDNA sequence shows that the new species is most closely related to *S. khoisanae*. However, the genetic distance between them is 3.2 (Table 2), greater than that of many closely related described species, such as *S. cubanum* and *S. glaseri* (0.9), *S. longicaudum* and *S. guangdongense* (1.4) and *S. boemarei* and *S. arenarium* (2.7). Several characters of various developmental stages can also separate the new species from *S. khoisanae*: IJ tail length of *S. pui* **sp. n.** is shorter (69 ± 5) vs (85 ± 5), the distance from anterior end to excretory pore is smaller (85 ± 4) vs (95 ± 5) and diameter greater (36 ± 2) vs (31 ± 2); vulva of the first generation females of *S. pui* **sp. n.** has a short double flapped epiptygma vs absence in *S. khoisanae*; SW (the ratio of spicule length to tail width) of the first generation male of the new species is 1.52 ± 0.1, lower than that of *S. khoisanae* (2.03 ± 0.5); an aperture were seen on the spicules tip of the first generation *S. pui* **sp. n.** but this character is not reported in *S. khoisanae*. *S. pui* **sp. n.** can be distinguished from the rest of *Steinernema* species of the *S. glaseri* group, including *S. khoisanae*, *S. guangdongense*, *S. longicaudum*, *S. scarabaei* and *S. karii* by morphological characteristics of the IJs and the first generation males as listed in Table 4.

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TABLE 4. Key morphometrics of infective juveniles of *Steinernema pui* **sp. n.** and other related *Steinernema* species.

Character [1]	*S. pui* **sp. n.** [2]	*S. khoisanae* [3]	*S. guangdongense*	*S. longicaudum*	*S. scarabaei* [3]
Infective Juveniles					
L	1004±75 (900–1120)	1062±63 (904–1159)	1055 (987–1145)	1043 (929–1170)	918 (890–959)
Diam.	36±2 (33–40)	31±2 [4] (27–34)	42 (30–48)	37 (34–40)	31 (25–34)
EP	85±4 (80–95)	95±5 (87–101)	80 (71–85)	82 (74–92)	77 (72–81)
NR	117±7 (100–125)	109±5 (101–118)	102 (88–111)	111 (98–129)	120 (116–127)
ES	144±8 (130–150)	140±7 (130–155)	134 (123–144)	142 (134–150)	156 (145–171)
Tail length	69±5 (60–80)	85±5 (76–97)	91 (82–103)	94 (79–105)	76 (71–80)
D%	60±3 (55–66)	68±4 (60–73)	59 (54–65)	57 (52–63)	60 (50–75)
E%	125±8 (109–142)	112±7 (95–128)	88 (74–100)	87 (76–104)	100 (90–110)
First generation males					
Spicule length	84±4 (78–88)	85±5 (70–88)	86 (80–94)	91 (72–108)	75 (67–83)
Gubernacu lum length	62±2 (58–65)	56±5 (50–63)	64.6 (47–73)	60 (54–65)	44 (36–50)
D	77±6 (70–91)	85±7 (71–99)	70 (67–78)	75 (56–92)	66 (53–77)
SW	1.52±0.10 (1.4–1.8)	2.03±0.15 (1.7–2.8)	1.75 (1.52–2.16)	1.61 (1.16–2.25)	1.7 (1.5–2.0)
GS	0.74±0.04 (0.69–0.81)	0.70±0.04 (0.60–0.80)	0.75 (0.59–0.82)	0.66 (0.56–0.88)	0.60 (0.50–0.65)
Infective Juveniles					
L	932 (876–982)	1034 (724–1408)	1106 (933–1269)	1283 (1149–1508)	1002 (880–1133)
Diam.	33 (31–35)	46 (28–77)	36 (34–41)	37 (33–46)	34 (30–42)
EP	74 (68–80)	83 (76–86)	82 (76–89)	106 (101–114)	74 (66–83)
NR	105 (97–112)	109 (100–120)	109 (104–126)	116 (106–130)	102 (74–109)
ES	134 (122–147)	138 (123–160)	156 (149–175)	148 (135–159)	138 (111–152)
Tail length	74 (64–80)	75 (64–84)	88 (80–100)	67 (61–77)	83 (65–91)
D%	57	55 (52–59)	52	70	54 (30–70)
E%	96	119 (106–130)	94	160	90 (78–114)
First generation males					
Spicule length	83 (73–91)	84 (81–91)	75	58 (50–67)	79 (71–90)
Gubernaculum length	57 (42–64)	55 (49–60)	52	39 (37–42)	54 (45–51)
D	66 (57–78)	93 (88–102)	71	70	80 (68–86)
SW		2.1	2.22	1.41	1.8 (1.5–2.0)
GS		0.65 (0.60–0.66)	0.70	0.70	0.69 (0.59–0.79)

[1] EP. distance from anterior end to excretory pore; NR. distance from anterior end to nerve ring; ES. distance from anterior end to the base of pharynx; D%. % of EP to ES; E%. % of EP to tail length. [2] mean ± SD and range in μm, n = 20; [3] mean ± SD and range in μm, all data come from Adam & Nguyen, 2002 and original authors. [4] underlined character means that it has significant difference ($p<0.5$) with that of *S. pui* **sp. n.**.