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Steinernema tbilisiensis sp. n. (Nematoda: Steinernematidae)—A New Species of Entomopathogenic Nematode from Georgia

Oleg Gorgadze[1], Manana Lortkhipanidze[1], Jean-Claude Ogier[2, 3], Patrick Tailliez[2, 3] and Medea Burjanadze[4]

1. Institute of Zoology of Ilia State University, Tbilisi 0162, Georgia

2. INRA, UMR 1333 Diversité, Génomes & Interactions Microorganismes-Insectes (DGIMI), Montpellier F-34000, France

3. Université Montpellier 2, UMR 1333 Diversité, Génomes & Interactions Microorganismes-Insectes (DGIMI), Montpellier F-34000, France

4. Agricultural University of Georgia, Tbilisi 0103, Georgia

Abstract: A new species of entomopathogenic nematode (EPN), *Steinernema tbilisiensis* sp. n. is described. The species was isolated from soil samples of the deciduous forest located in the Tbilisi area. Morphological and morphometric data as well as phylogenetic analysis show that *S. tbilisiensis* sp. n. belongs to the group *S. affine*/*intermedium*. *S. tbilisiensis* sp. n. has been attributed to the group *S. affine*/*intermedium* on the basis of spicule and gubernaculum structure. The new species differs from other species of *S. affine/intermedium* group in the following diagnostic characters: the spicule of *S. tbilisiensis* sp. n. is the smallest and the gubernaculum of *S. tbilisiensis* sp. n. is shorter than other species of the *S. affine/intermedium* group. Infective juveniles (IJs) of *S. tbilisiensis* sp. n. are distinguished by a relatively long body (L = 866 µm), the position of excretory pore (EP = 72 µm), the length of the esophagus (ES = 140 µm) and the length of the anal body width (ABW = 25 µm). IJs of *S. tbilisiensis* sp. n. have four lateral lines like *S. beddingi*, but the number of lines is six in *S. affine*, *S. sichuanense* and *S. intermedium*. Also the analysis of rDNA (28S and ITS) gene sequences depicts this *Steinernema* species as a distinct and unique entity. The symbiotic bacteria of *S. tbilisiensis* sp. n. was isolated and found to be *Xenorhabdus bovienii* using a multigene approach.

Key words: New species, *Steinernema*, morphology, phylogeny, EPN.

1. Introduction

The genus *Steinernema* nematodes Travassos [1] are obligate and lethal endoparasites that have a symbiotic relationship with gram-negative y-proteobacteria in the genus *Xenorhabdus* [2]. This nematode-bacteria complex represents a mutualistic association, where the nematodes (third stage infective juveniles (IJs)) vector the symbiotic bacteria between insects in a specialized intestinal receptacle [3]. Once the bacteria are released in the insect's hemocoel, the bacteria kill the insect host and create a favorable environment within the host cadaver for nematode growth and development. This pairing is pathogenic for a wide range of insects and has successfully been implemented in biological control and integrated pest management (IPM) programs worldwide [4-6].

At present, four local species of Steinernematidae are registered in Georgia: *Steinernema georgica* [7], *S. thesami* [8], *S. disparica* [9] and *S. gurgistana* [10]. Their bio-efficiency against pests of the agricultural and forest plants has been established [11-13].

The biological control as a pest control technology is becoming more desirable. This takes important place in bio-protection and food safety strategy. Biological formulation on basis of entomopathogenic nematodes (EPNs) is one of the effective means for protecting agricultural and forest plants from the

Corresponding author: Oleg Gorgadze, Ph.D., research field: nematology. E-mail: oleg.gorgadze@iliauni.edu.ge.

harmful insects and were successfully used in practices.

Nowadays, the use of EPNs as biological control agents is a key component in IPM.

Study of local EPNs species is very important from the viewpoint of biological diversity and practical application, because local nematodes are adapted to the local climatic conditions and more effective for biological control. Tbilisi and its environs are characterized by diverse biotopes, such as different forests, parks and also adjacent agrarian territories, where agricultural activities are accomplished.

Issuing from the above, the research aimed at the identification of EPNs on the mentioned territories, which may be applied for the biological control of the main pest insects of agricultural and forest plants, occurring on agrarian territories, adjacent to Tbilisi or in different regions of Georgia. With the aim of obtaining EPNs, soil samples from the deciduous forests of the Tbilisi area were examined. Morphological study, morphometric and phylogenetic analysis of the *Steinernema* nematode isolated from this territory showed that *S. tbilisiensis* sp. n. is a new species.

2. Materials and Methods

2.1 Isolation and Nematode Propagation

S. tbilisiensis sp. n. was isolated from soil samples of the deciduous forest situated in the grounds of Tbilisi ethnographic museum in June, 2011, using *Galleria mellonella* (L.) [14] as bait. Dead larvae were placed into White traps [15]. The third stage IJs were collected and used to infect live *G. mellonella* larvae [16]. Living populations of the nematode are deposited in the Institute of Zoology of the Ilia State University in Tbilisi.

2.2 Morphological Observations

Twenty specimens from each stage (adults and IJs) were randomly collected from 10 *G. mellonella* cadavers. Nematodes were examined live or heat-relaxed in Ranger's solution at 60 °C [17]. Nematodes were fixed in triethanolamine formalin (TAF) [18] and processed to anhydrous glycerol for mounting [19]. Specimens were mounted on glass slides, and the coverslip was supported by glass rods to avoid flattening. Selection of morpholometric characters was done according to Hominick et al. [20].

2.3 Light Microscopy

Measurements were conducted using a light biological research microscope (Motic-DMB1) equipped with 10×, 20×, 40× or 100× objectives. Some fine characters of the nematodes, such as the oocyte of the first generation (G1) adults and the morphology of the dissected spicule and gubernaculums, were also observed using a 100× plan objective lens. Drawings and pictures of nematodes fixed on glass slides were performed using a digital video camera Genius (G-Shot) DV 1110.

2.4 Phylogenetic Position of Nematodes and Their Symbiotic Bacteria

Nematode genomic DNA was extracted from IJs. IJs were placed in Eppendorf tubes at -80 °C for 30 min and then incubated at 65 °C for 15 min. The pellets of IJs were then collected after centrifugation (13,000 ×g for 10 min), resuspended in 180 µL of lysis buffer T1 included in the NucleoSpin®Tissue kit (Macherey-Nagel, Germany), supplemented with 25 µL of proteinase K and incubated at 56 °C for at least 3 h to achieve cell lyses. DNA purification was then performed in accordance to the manufacturer's recommendations. A 850 bps fragment corresponding to the internal transcribed spacer (ITS) region of the ribosomal DNA was amplified in a 50 µL reaction solution containing the Taq polymerase according to the manufacturer's protocol (Invitrogen, France). The amplification primers were ITS (forward) 5'-GGACTGAGCTGTTTCGAGA-3' targeted the 3'-terminus of the small subunit (SSU) rDNA; ITS

(reverse) 5'-TACTGATATGCTTAAGTTCAGCG-3' targeted the 5'-terminus of the large subunit (LSU) rDNA. Polymerase chain reaction (PCR) was carried out in a Bio-Rad thermocycler (Bio-Rad, France) programmed for 30 cycles of amplification: after an initial 4 min denaturation step at 94 °C, each cycle consisted of 30 s at 94 °C, 30 s at 52 °C and 90 s at 72 °C, followed by a last step at 72 °C for 5 min. Amplification of LSU rDNA that included the D2 and D3 domains was performed as described previously. To confirm size and yield, PCR fragments were run by electrophoresis in agarose gel (1% agarose in 1× TAE buffer). DNA fragments were then purified using a high purity purification kit (Roche Diagnostic, France) and sequenced by the sequencing service of MWG (Eurofins MWG Operon, Germany), using the primers listed above and those described by Stock et al. [21] as sequencing primers.

Xenorhabdus cells were obtained from the infective stages of the nematode by the hanging-drop technique [22] and isolated by plating on nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride (TTC) and 0.0025% (w/v) bromothymol blue (NBTA medium) at 28 °C [23] for 48 h. Bacterial DNA extraction, gene fragment amplification (16S rDNA, *recA*, *gyrB*, *dnaN*, *gltX* and *infB*) and sequencing were as previously described [24].

Phylogenetic trees were produced as previously described [25], using individual gene sequences and on concatenated sequences (LSU fragment + ITS for nematodes), using the "concatenate" function, which was included in the multiplatform graphical user interface SeaView [26].

2.5 Cross-Hybridization

Cross-breeding of *S. tbilisiensis* sp. n. with *S. affine* and *S. intermedium*, the only closely related species available in the collection, were performed using modified hanging-blood assays [16]. *S. affine* [27] and *S. intermedium* [28], which two are morphologically similar and phylogenetically close relatives of *S. tbilisiensis* sp. n., were used to assess reproductive compatibility of this new species.

3. Results and Discussion

3.1 Description of Steinernema tbilisiensis sp. n.

Light microscope (LM) photographs of *Steinernema tbilisiensis* sp. n. are presented in Figs. 1-3. Morphometrics of the holotype male (G1), paratype male (G1, G2), paratype females (G1, G2) and infective juveniles of the new nematode are presented in Tables 1 and 2.

3.1.1 First Generation (G1) Male

Slim body is narrower in the anterior part of the head (Figs. 1a, 2a and 2e), ventrally curved and J- or C-shaped. Cuticle smooth is 3-4 μm wide. Head is rounded anteriorly with clearly protruded labial papillae (Fig. 1e). Six labial papillae surround the stoma and four cephalic papillae occur at the level of the bottom of the stoma. Shape of the stoma ranges from square to funnel-shaped. Length and width of stoma is almost equal to 6.8 μm × 6.7 μm in the fixed state. The posterior—concave part of the stoma reaches the triple ribbed lumen of the esophagus. Corpus of esophagus is muscular, cylindrical (Figs. 1b, 2a and 2e) and significantly widened posteriorly. Anterior part of procorpus includes proximal part of stoma. At this level, diameter of procorpus is 21.5 μm, elsewhere it is 15.5-19.2 μm in diameter. Metacorpus is wider than procorpus and maximum diameter is 19-29 μm. Basal bulb is almost circular (length 41 μm, width 38 μm), with valve that can be seen in living individuals. Isthmus is narrowed, surrounded by nerve-ring located between metacorpus and basal bulb. Excretory pore opens behind the metacorpus. Excretory duct is less sclerotized than excretory pore. Distance between the basal bulb and gonad is 478.5 μm. Gonad is mainly located on a dorsal side of nematode corpus. Germinative zone, located in the distal part of a gonad, is always bent at a back side. Gonad consists of three zones: spermatogonium, zone of spermatocyte growth and zone of sperm maturation.

Distal part of a gonad is filled with small and big size (15-26 μm in diameter) mature spherical spermatocytes (Fig. 2c). Sperm cytoplasm contains elongated granules. Spicules are paired, symmetrical without a certain color (Figs. 1c, 1d, 1f, 2b and 2f) and strongly ventrally curved. Apex is rounded (13.5 μm × 12 um) and distal end is also rounded. Membrane on spicule is wide (Figs. 3a and 3b) in the central part, 26-36 μm long and does not reach distal end. Gubernaculum is of medium size; distal end is ventrally curved and forked (Figs. 1g, 1h, 3c and 3d). Proximal part of gubernaculum is distinctly wider than distal. There are 11 pairs of genital papillae and one big single papilla (Figs. 1c and 1d), arranged as one pre-anal papilla located medially; five pre-anal pairs located ventro-laterally; one adanal pair located ventro-laterally; two post-anal pairs located ventro-laterally; one post-anal pair located ventrally and one pair laterally. The terminus of tail is without mucron.

3.1.2 Second Generation (G2) Male

Male of G2 is smaller in size than G1 (Table 1), but

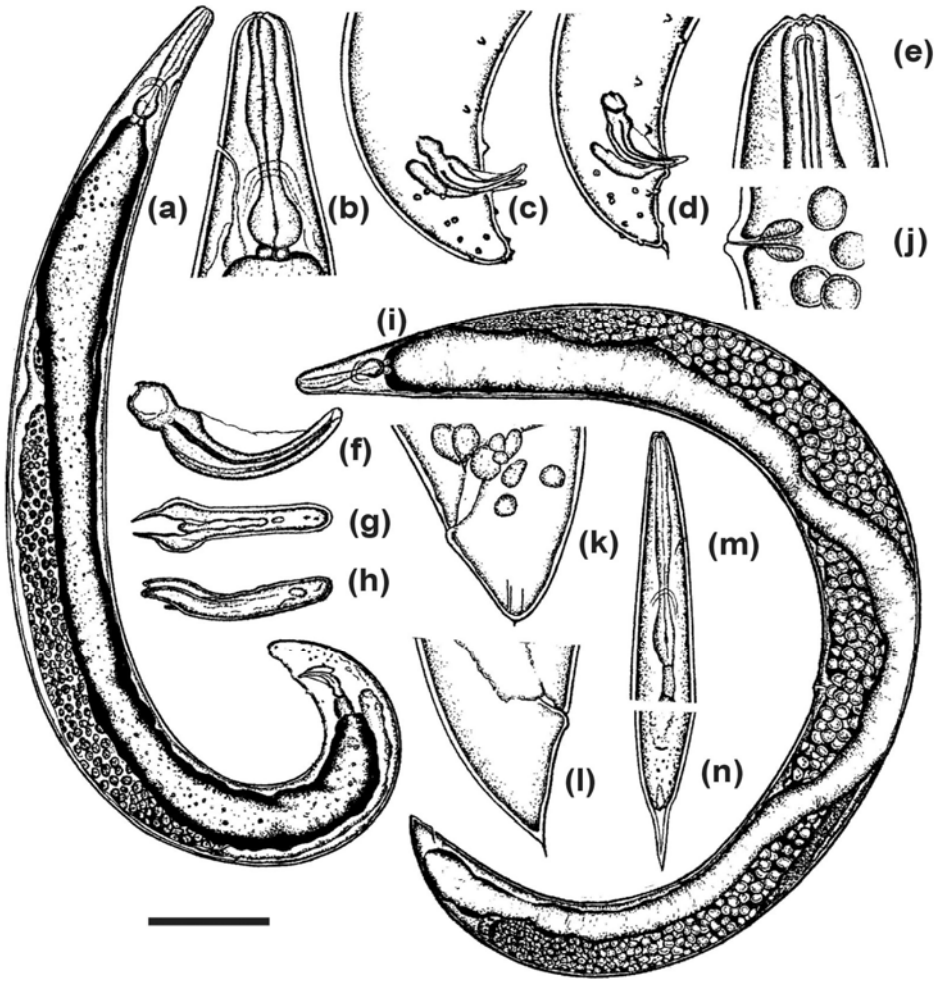

Fig. 1 Light microscope (LM) photographs of *Steinernema tbilisiensis* sp. n..

First generation male (a, b, c, e, f, h) in lateral view and (g) in ventral view: (a) entire body; (b) anterior portion with pharynx and excretory pore; (c) tail with spicule, gubernaculum and of genital papillae; (e) cephalic end; (f) spicule, (g) and (h) gubernaculums.

Second generation male (d) in lateral view: (d) tail with mucron.

First generation female (i, j, k) in lateral view: (i) entire body; (j) vulval region; (k) tail with mucron.

Second generation female (l) in lateral view: (l) tail with mucron.

Infective juvenile (m) in lateral view and (n) in ventral view: (m) anterior region; (n) tail and hyaline portion.

Scales bars are based on scale-bar in a: a = 200 μm; b, k = 100 μm; f, g, h = 25 μm; c, d, e, l, m, n = 50 μm; i = 400 μm; j = 21 μm.

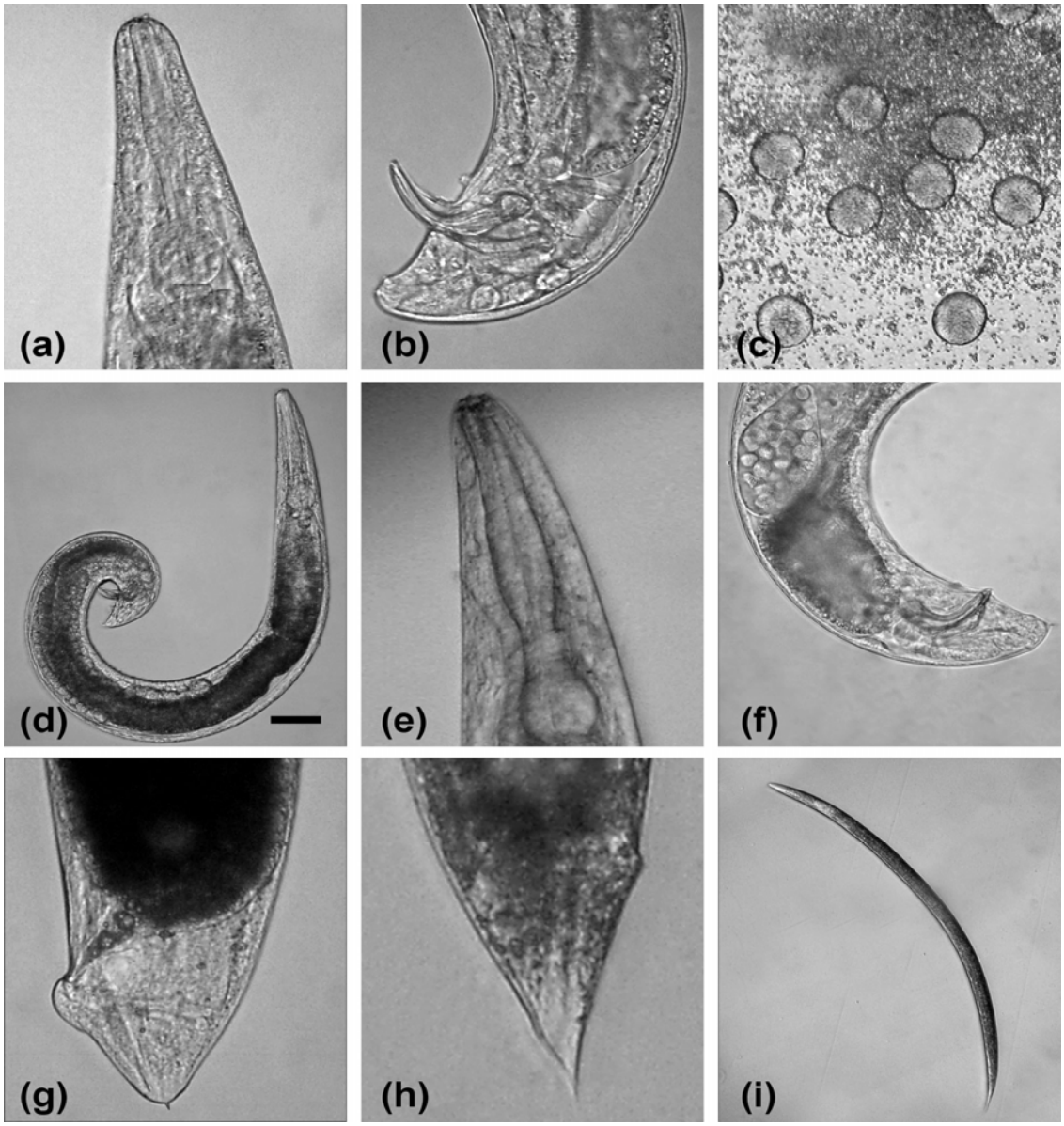

Fig. 2 Light microscope (LM) photographs of *Steinernema tbilisiensis* sp. n..

First generation male (a-c) in lateral view: (a) esophageal region; (b) posterior view with spicule; (c) oocyte.
Second generation male (d-f) in lateral view: (d) entire body; (e) esophageal region; (f) posterior end.
First generation female (g) in lateral view: (g) posterior end, tail terminus with mucron.
Second generation female (h) in lateral view: (h) posterior end tail.
Infective juvenile (i) in lateral view: (i) entire body.
Scales-bars are based on scale-bar in d: a, b, e, f = 26 μm; c = 10 μm; d = 66 μm; g, h = 22 μm; i = 100 μm.

the shape of body is similar. Lips are more prominent than in G1 male. Excretory duct is sclerotized, and can be seen 3-4 μm from the excretory pore. Gonad is located in the dorsally part of the body. Mature spermatocytes are similar to those of G1 male, but a little smaller (17-24 μm in diameter). Spicule and gubernaculum are of the same color and shape as in G1 male. Subventral terminal papillae at the terminal part of the tail are fused with mucro 7-14 μm long (Figs. 1d and 2f), which emerges between the last pair of papillae. Number and location of all other papillae is not the same as in the male of G1 generation.

3.1.3 First Generation (G1) Female

Female is 2.5 times longer than male. The body

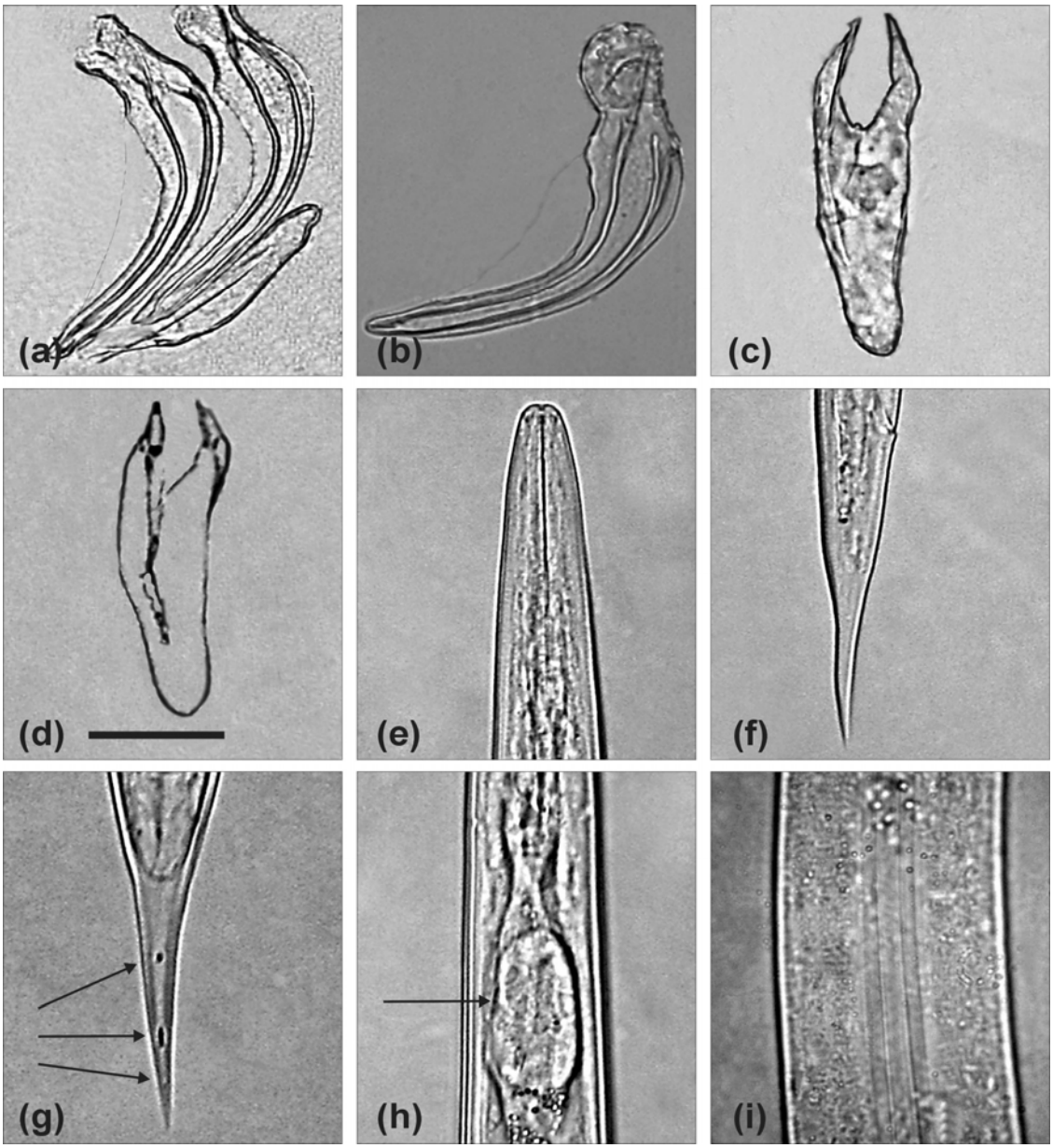

Fig. 3 Light microscope (LM) of *Steinernema tbilisiensis* sp. n..

First generation male (a-d): (a) spicules and gubernaculums, in lateral view; (b) spicule, in lateral view; (c) gubernaculums, in ventral view; (d) gubernaculums, in dorsal view.

Infective juvenile (e-i): (e) anterior portion; (f) tail with anus and hyaline in lateral view; (g) ventral view of tail with hyaline part and sometimes droplet formations (arrows point), (h) bacterial vesicle is located in the anterior region near basal bulb (arrow point), (i) lateral field in mid-body.

Scales-bars are based on scale-bar in d: a = 20 μm; b, c, d, e, f, h = 17 μm; g = 14.3 μm; i = 10 μm.

slightly spiral or C-shaped when heat-relaxed (Fig. 1i). Head is roundish. Six labial and four cephalic papillae are located on the anterior end. Shape of the stoma ranges from square to funnel-shaped, like male stoma. Cuticle is smooth, 7 μm thick. Lateral field is not seen. Excretory pore is located anterior to nerve-ring. Morphologically, stoma and esophagusas are as in the male. Stoma is 9-11 μm long and 9.3 (9-12) μm wide. Nerve-ring surrounds isthmus, or is located above basal bulb. Basal bulb often is submerged into anterior

part of the intestine and fixed in this position. Basal bulb is spheroid, 56.5 μm in diameter. Vulva of transverse section shape is located posterior to mid-body length. Vulval lips may be asymmetrical and slightly protruded (Fig. 1j). Vagina is long with straight, muscular walls. Reproductive tract of a female is didelphic, mainly has two ducts—right and left. Anterior reproductive tract is in area of basal bulb; posterior tract is in the region of rectum. Eggs are roundish-oval in shape, 45-56 μm in length. Tail length is less than body width at anus; tail tip is blunt-conoid (Figs. 1k and 2g). Mucron of 2-3 μm in length always presents on apical part of tail.

3.1.4 Second Generation (G2) Female

The morphology of G2 female is similar to that of G1 female, but 2.4 times smaller than G1 (Table 1). Excretory duct is sclerotized and can be seen up to 5-8 μm from the excretory pore. Basal bulb is more obvious than in G1 females, especially when ovaries are not well developed. Tail is slightly longer than in G1 females. Terminal part of the tail, 2 μm long, resembles a mucron. Insignificant post-anal swelling is present.

3.1.5 Third-Stage IJs

Body is ventrally curved both in relaxed and fixed states (Fig. 2i). Cuticle with thin envelope is 2 μm wide. Apical end of the head is usually rounded (Fig. 3m), rarely blunt. Semi-concave stoma is 2 μm long. Anterior concave part of esophagus is funnel-shaped, short, 3 μm in length. Posterior part of esophagus is cylindrical, concave, narrower with thinner walls, 4-5 μm long. At the terminal part of the head, the transparent membranous shed cuticle of the second stage larva is well seen. On the membrane, six pairs of papillae can be seen, radially arranged around stoma at 3 μm distance. IJs have six obvious labial tubercles. Stoma and anus are closed. Esophagus comprises the narrow corpus, with isthmus as its narrowest section, surrounded by the nerve-ring. Basal bulb is pyriform, 22-24 μm long and 12-13 μm in diameter; valve is not seen. Excretory pore is located near end of isthmus. Intestine is filled with granules along its length. Rectum is thin, 9 μm long. Tail is short, pointed, slightly curved on dorsal side (Fig. 3f). Hyaline part of tail clear comprises 45% of total tail length. Phasmid is distinguishable in anterior part of tail. Droplet formations (Fig. 3g) may be present in hyaline part of tail.

3.2 Type Location

The new nematode species was discovered in Georgia, Tbilisi city, in the soil of a deciduous forest adjacent to the Kus Tba (Turtle Lake). Altitude is 550 m above sea level, and coordinates are N 41°42.645′, E 044°45.708′. The host insect was not identified. The symbiotic bacteria of the species is *Xenorhabdus bovienii*. During the process of development on the nutrient medium the nematode emits a characteristic scent.

3.3 Molecular Analysis and Phylogenetic Position of S. tbilisiensis sp. n.

The phylogenetic analysis of the 28S rDNA (Fig. 4a) and ITS (Fig. 4b) sequences showed that *S. tbilisiensis* belongs to the *S. affine/S. intermedium* group [29, 30]. Within this group, *S. intermedium* and *S. tbilisiensis* have the highest similar sequences (28S: 98.2% on 433 nucleotides, ITS: 92.6% on 773 nucleotids, length which corresponds to the shortest sequence—*S. beddingi*, used to calculate the tree). The analysis based on the concatenated sequences (28S + ITS; Fig. 3c) suggested that *S. intermedium* and *S. tbilisiensis* probably have a direct common ancestor (bootstrap value at the node = 86%).

3.4 Biological Observation

The terms of activity and development of *S. tbilisiensis* sp. n. have been studied at optimum, minimum and maximum temperatures, respectively. Each larva of *G. melonella* was infested with 20 nematodes. In experiments, 22-23 °C turned out to be optimum temperature for the development of nematodes.

Table 1 Morphometric of *S. tbilisiensis* sp. n..

Character	Males			Females		Infective juveniles
	First generation		Second generation	First generation	Second generation	
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes	Paratypes
n	1	20	20	20	20	31
L	2,001	1,652 ± 254 (1,911-2,140)	1,222 ± 153 (974-1,452)	4,241 ± 480 (3,297-5,176)	1,785 ± 293 (1,479-2,557)	866 ± 53.1 (713-948)
W	156	127 ± 18 (98-156)	92 ± 8 (78-107)	238 ± 22 (200-274)	146 ± 20 (117-191)	36 ± 2.3 (34-41)
EP	137	118 ± 11 (10-142)	100 ± 9 (88-117)	150 ± 14 (121-176)	146 ± 20 (117-191)	72 ± 3.4 (68-79)
NR	147	134 ± 12 (117-156)	127 ± 12 (93-142)	174 ± 10 (161-196)	151 ± 15.8 (132-176)	107 ± 3.7 (98-112)
ES	196	186 ± 10 (166-200)	185 ± 8 (171-200)	241 ± 12 (221-269)	216 ± 10.9 (196-234)	140 ± 5.6 (132-151)
ABW	73	56 ± 8 (44-73)	48 ± 5 (44-58)	91 ± 13 (69-112)	59 ± 6.8 (50-73)	25 ± 5.9 (17-36)
TL	53	48 ± 6 (39-58)	49 ± 5 (39-53)	80 ± 10 (63-98)	82 ± 6.0 (73-98)	82 ± 4.4 (70-93)
SL	73	67 ± 3 (58-73)	52 ± 3 (49-58)			
SW	19	19 ± 0.3 (18-19)	17 ± 0.3 (16-18)			
GL	53	44 ± 4 (34-53)	37 ± 2 (34-49)			
a	12	13.0 ± 1.7 (10.1-17.3)	13.3 ± 1.1 (11.7-15.5)	17.7 ± 1.1 (15.3-19.2)	55.4 ± 1.8 (52.1-58.7)	23 ± 1.9 (19.9-27.8)
b	9.3	9.0 ± 1.0 (7.5-11.0)	6.5 ± 1.0 (5.0-7.4)	17.3 ± 1.6 (14.2-20.1)	8.1 ± 1.0 (7.2-10.6)	6.1 ± 0.4 (5.2-6.7)
c	36	33.0 ± 6.0 (25.5-43.0)	25.0 ± 1.9 (22.0-28.0)	52.8 ± 5.4 (46.4-57.7)	21.5 ± 2.4 (17.9-26.3)	10.8 ± 0.9 (9.6-12.9)
c^1	0.7	0.7 ± 0.03 (0.7-0.8)	0.7 ± 0.05 (06-0.8)	0.5 ± 0.02 (0.4-0.6)	0.8 ± 0.01 (0.7-0.8)	4.1 ± 0.6 (3.5-4.8)
HT						37 ± 3.2 (31-48)
H%						45 ± 0.2 (43-57)
V%				54.3 ± 2.1 (50.6-58.0)	55.4 ± 1.8 (52.1-58.7)	
D%	62	62 ± 0.3 (54-72)	54 ± 0.4 (48-62)	62 ± 0.4 (50-72)	52 ± 0.4 (45-62)	51 ± 0.2 (47-55)
E%	249	235 ± 22 (194-310)	206 ± 17 (165-252)	182 ± 21 (150-221)	135 ± 16 (112-175)	90 ± 5.0 (81-100)
SW%	100	115 ± 3.5 (93-149)	107 ± 1.6 (97-135)			
GS%	58	66 ± 4.5 (57-75)	67 ± 5.0 (58-73)			

All measurements in μm and in the form: mean ± SD (range), but body length of males and females are in mm.

L = length; W = greatest width; V = vulva; EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = oesophagus length; ABW = anal body width; TL = tail length; SL = spicula length; SW = spicula width; GL = gubernaculum length; a = L/W; b = L/ES; c = L/TL; c^1 = body length ÷ body width at the anus; HT = hyaline tail length; H% = HT/TL × 100; V%= V/L × 100; D% = EP/ES; E% = EP/TL × 100; SW% = SL/ABW × 100; GS% = GL/SL × 100.

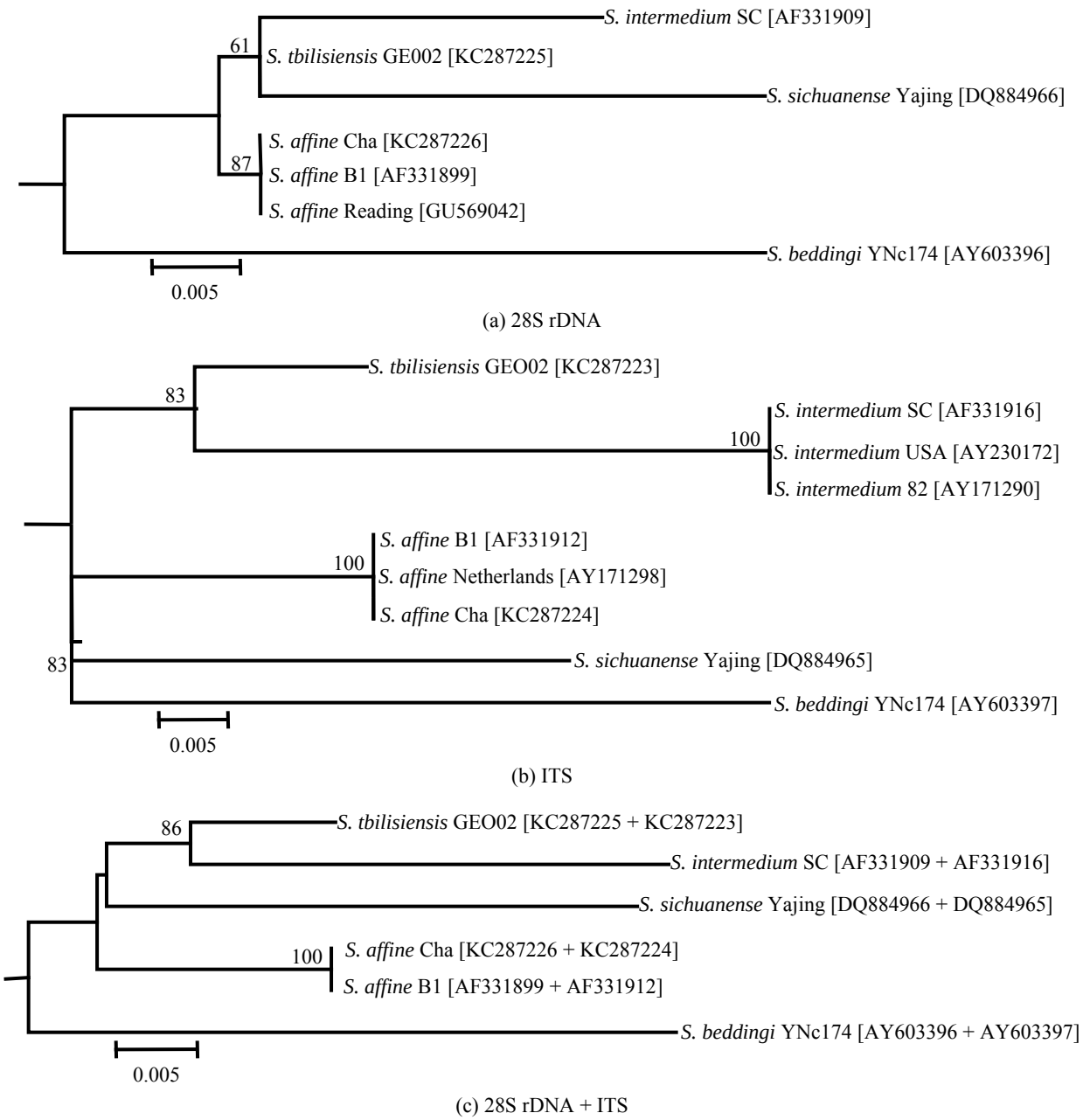

Fig. 4 Unrooted maximum likelihood trees showing the phylogenetic position of *S. tbilisiensis* sp. n. within the *S. affine*/*S. intermedium* group.

Bootstrap values > 50% are indicated at the nodes. Bars represent 0.5% divergence.

In Fig. 4, genbank accession numbers are indicated in brackets: (a) phylogenetic analysis based on 28S rDNA sequences (D3 domain); (b) phylogenetic analysis based on ITS sequences; (c) phylogenetic analysis based on the concatenation of 28S rDNA and ITS sequences.

Adult female and male nematodes were fixed on the day of insect's death. The 3rd instar larvae of G1 emerged after 24-36 days and on the 3rd day they started migration from the dead insects. The 1st and 2nd instar larvae of G2 on the 4th and 5th day developed in insect's body into invasive larvae; on the 8th day these larvae have left the insect's organism and on the 9th day their active migration has started.

Minimum temperature limit of the development of invasive larvae in insect's body was found to be 13 °C. At this temperature, the first invasive larvae started to emerge on the 16th day from insects' infection. Thus, invasive larvae have developed and emerged at 5-6 days later than at optimum temperature. As concern about maximum temperature, the development of nematodes and emergence of invasive larvae started at 1-2 days earlier as compared with optimum temperature.

3.5 Cross-Hybridization Tests

A major support for separation of species is the cross breeding with related species, in this case with *S. affine* and *S. intermedium*. There was no offspring between *S. tbilisiensis* sp. n. and these species. In the control, when all species were self crossed, males and females produced offspring normally. No progeny was observed in the single female control plates.

3.6 Type Specimens

Holotype male (G1), paratype male (G1, G2), paratype females (G1, G2) and infective third-stage juveniles are deposited in Tbilisi, in the collection stock of the museum of the Institute of Zoology of Ilia State University.

3.7 Classification of the Symbiotic Bacteria Isolated from S. tbilisiensis sp. n.

The five protein coding sequences tested (*recA*, KF946000; *gyrB*, KF946004; *dnaN*, KF946009; *gltX*, KF946014 and *infB*, KF946023) and 16S rDNA sequence (KF945996) of the symbiotic bacteria isolated from *S. tbilisiensis* sp. n. were highly similar to those of *X. bovienii* type strain T228T (98.6% for the five concatenated protein coding sequences on 4,363 nucleotide and 99.7% for the 16S rDNA sequence on 1,319 nucleotide). This result is consistent with the classification of *S. tbilisiensis* sp. n. in the *S. affine*/*S. intermedium* group. All *Steinernema* species of this group harbor the symbiotic bacteria of *X. bovienii* species [31-33].

3.8 Diagnosis and Relationships

Morphological and morphometric data as well as the phylogenetic analysis based on 28S rDNA and ITS sequences show that the new species *S. tbilisiensis* sp. n. belongs to other *S. affine*/*S. intermedium* group of EPNs, including *S. affine* [27], *S. intermedium* [28], *S. bedding* [33] and *S. sichuanense* [34]. The new species has been attributed to the group *S. affine/intermedium* on the basis of structures of spicule and gubernaculum: the presence of wide long membranes on their spicules, heavily curved shape of spicules, and resemblance of gubernaculum of *S. tbilisiensis* sp. n. to that of *S. affine* have been assumed as the main characters of the likeness of a new species with *S. affine*, *S. intermedium* and *S. sichuanense*. The above listed characters allow to distinguish the new species from other groups of *Steinernema*. Despite the resemblance of male genital organs of the new species with those of *S. affine*, *S. intermedium* and *S. sichuanense*, *S. tbilisiensis* sp. n. differs from them by a series of characters: the spicule of *S. tbilisiensis* (67 μm long) is the smallest among the species of the *S. affine*/*intermedium* group (Table 2) (*S. affine* = 70 μm, *S. beddingi* = 71 μm, *S. sichuanense* = 68 μm and *S. intermedium* = 93 μm); the gubernaculum of *S. tbilisiensis* sp. n. (44 μm) is shorter than that of *S. affine* (46 μm), *S. sichuanense* (47 μm), *S. intermedium* (62 μm), but longer than that of *S. beddingi* (43 μm). Males of *S. affine*, *S. intermedium* and *S. beddingi* (both generations) do not have a mucro at the tail terminus [34]. Mucron is marked in *S. sichuanense* and *S. tbilisiensis* sp. n. only in G2. Small size (2 μm) mucron of *S. sichuanense* is of papillated type, whereas that of *S. tbilisiensis* sp. n. is big in size 10 μm (7-14 μm). Adult females of G1 of the new species have a small size (2-3 μm long) mucron on the tail terminus, which is not found in female individuals of the *S. affine*/*intermedium* group. The new species differs from other species of the *S.*

Table 2 Comparative morphometric of infective juvenile and first generation male of *S. tbilisiensis* sp. n. and other closely related *Steinernema* species—*S. affine/intermedium* group.

Character	*S. tbilisiensis* sp. n.	*S. affine*	*S. beddingi*	*S. intermedium*	*S. sichuanense*
Infective juvenile					
L	866 (713-948)	712 (626-788)	743 (700-790)	680 (608-800)	710 (606-787)
W	36 (34-41)	30 (28-34)		28 (25- 35)	
EP	72 (68-79)	62 (51-69)	70 (64-75)	65 (61-69)	64 (57-68)
ES	140 (132-151)	126 (115-134)	125 (113-130)	121 (110-131)	131 (121-142)
TL	82 (79-93)	64 (52-68)	77 (72-83)	64 (53-72)	72 (64-76)
ABW	25 (17-36)	18 (16-19)		16 (13-18)	17 (14-19)
D%	51 (17-55)	49 (43-53)	57 (52-64)	51 (48-58)	49 (46-53)
E%	90 (81-100)	94 (74-108)	92 (84-103)	96 (89-108)	89 (79-99)
First generation male					
SL	67 (58-73)	70 (67-86)	71 (63-78)	93 (80-106)	68 (65-72)
GL	44 (34-53)	46 (37-56)	43 (38-48)	62 (48-96)	47 (40-51)
EP	118 (102-142)	94 (82-114)	114 (95-138)	137 (114-155)	95 (88-105)
ES	186 (116-200)	153 (136-174)	195 (175-218)	190 (155-209)	188 (172-199)
D%	62 (54-74)	61 (60-66)	58 (54-64)	67 (58-76)	51 (45-56)
E%	230 (190-310)	180 (145-230)	219 (179-266)	253 (197-319)	207 (168-251)
GS%	66 (57-75)	66 (60-75)	61 (55-66)	69 (63-77)	80 (60-85)
SW%	115 (93-149)	117 (96-145)	108 (88-132)	124 (103-139)	130 (120-140)

All measurements in μm and in the form: mean (range).

L = length; W = greatest width; EP = distance from anterior end to excretory pore; ES = oesophagus length; TL = tail length; ABW = anal body width; D% = EP/ES × 100; E% = EP/TL × 100; SL = spicula length; GL = gubernaculum length; GS% = GL/SL × 100; SW% = SL/ABW × 100.

affine/intermedium group by the measurements of IJs (Table 2): *S. tbilisiensis* sp. n. is distinguished by the big size of body L = 866 μm (*S. beddingi* L = 743 μm, *S. sichuanense* L = 710 μm, *S. affin*e L = 712 μm, *S.* intermedium L = 680 μm); by the position of excretory pore EP = 72 μm (*S. beddingi* EP = 70 μm, *S. intermedium* EP = 65 μm, *S. sichuanense* EP = 64 μm, *S. affine* EP = 62 μm); by the length of the oesophagus ES = 140 μm (*S. sichuanense* ES= 131 μm, *S. affine* ES = 126 μm, *S. beddingi* ES = 125 μm, *S. intermedium* ES = 121 μm); by the length of the tail TL = 82 μm (*S. beddingi* TL = 77 μm, *S. sichuanense* TL = 72 μm, *S. affine* TL = 64 μm, *S. intermedium* TL = 64 μm) and by the value of E% = 90 (*S. beddingi* E% = 92, *S. affine*

E% = 94, *S. intermedium* E% = 96).

The above listed morphological and morphometric diagnostic characters allow to single out *S. tbilisiensis* sp. n. as a new species.

4. Conclusions

As a result of the study, it was determined that a new *S. tbilisiensis* sp. n. species is EPN, belonging to *S. afine/intermedium* group. Male, female and IJs of the nematode were identified by modern morphological, morphometric and molecular methods: molecular analysis and phylogenetic position of *S. tbilisiensis* sp. n., phylogenetic position of nematodes and their symbiotic bacteria. It was specified that the nematode contains symbiotic bacteria of *Xenorhabdus* genus—*X. bovienii*. The pathogenicity and effectiveness of IJs of the nematode against *G. mellonella* and *Tenebrio molitor* test insects is proven. The results obtained prove that the identified species can be used for the biological control against harmful insects of agricultural and forest plants.

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