Journal of Helminthology (2015) **89,** 415–427
doi:10.1017/S0022149X14000182

Steinernema innovationi n. sp. (Panagrolaimomorpha: Steinernematidae), a new entomopathogenic nematode species from South Africa

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(Received 10 October 2013; Accepted 20 February 2014; First Published Online 3 April 2014)

Abstract

Morphological and molecular sequence data were combined with cross-hybridization studies and used to identify a new *Steinernema* sp. from Free State, South Africa. Molecular and morphological data indicate that the new species belongs to the '*glaseri*-group' of *Steinernema* spp. Key morphological diagnostic characters for *S. innovationi* n. sp. include the morphometric features of the third-stage infective juveniles: total body length = 1054 (1000–1103) μm, tail length = 108 (97–117) μm, location of the excretory pore = 88 (82–91) μm, and D% = 58 (54–63), E% = 115 (104–137) and H% = 43 (37–46). Additionally, the morphology of the spicules and gubernaculum of the first-generation males are considered key diagnostic traits. *Steinernema innovationi* n. sp. was also characterized by analysis of both rDNA and mitochondrial gene sequence data, which further indicate the uniqueness of this *Steinernema* species.

Introduction

Several surveys have been conducted to document the diversity of entomopathogenic nematodes (EPN) (Steinernematidae and Heterorhabditidae) in South Africa (Malan *et al.*, 2006, 2011, 2012; Hatting *et al.*, 2009). The rationale for such work was driven by the need to find locally adapted species and/or isolates that could be considered for control of native insect pests. The first EPN species found in this country was *Heterorhabditis bacteriophora*, which was isolated from an infected maize beetle, *Heteronychus arator* (Spaull, 1988). Subsequently, focused surveys were conducted in the provinces of KwaZulu-Natal (Spaull, 1990, 1991) and Western Cape (Malan *et al.*, 2006, 2008; Nguyen *et al.*, 2006). As a result, several uncharacterized novel *Steinernema* and *Heterorhabditis* species were isolated, including *S. khoisanae* Nguyen, Malan and Goezel, 2006; *Heterorhabditis safricana* Malan, Nguyen de Waal and Tiedt, 2008; *Steinernema citrae* Stokwe, Malan, Nguyen, Knoetze and Tiedt, 2011; and *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt, 2013. Currently, many of these endemic species are being implemented in successful pest management programmes (Malan & Manrakhan, 2009; De Waal *et al.*, 2010; Van Niekerk & Malan, 2012).

The survey conducted by Hatting *et al.* (2009) was the first systematic sampling of indigenous EPN species in South Africa. This study encompassed all seven geographic regions of this country and also considered sampling of diverse habitats (natural and human-impacted) as well as different soil types. Five per cent of all samples taken in this survey yielded EPNs. Specifically, four *Steinernema* species, including *S. khoisanae* and three undescribed species, and one *Heterorhabditis* species, *H. bacteriophora*, were collected (Hatting *et al.*, 2009).

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Herein, we describe one of these new *Steinernema* isolates, originally labelled as 'species 1' (Hatting *et al.*, 2009). We utilized differential interference contrast optics (DIC) and scanning electron microscopy (SEM) for morphological observation and morphometric analysis, in addition to DNA sequence analysis and cross-hybridization methods, to fully describe and illustrate this novel *Steinernema* species.

Materials and methods

Nematode isolation and rearing

Steinernema isolate SGI-60 was recovered from a grain field at the Small Grain Institute in Bethlehem, Free State province (Hatting *et al.*, 2009). Nematodes were recovered directly from soil samples using the insect-baiting method of Bedding & Akhurst (1975). Cadavers with positive signs of nematode infection were placed in modified White traps (Kaya & Stock, 1997) to recover infective juvenile (IJ) progeny. A 30% bleach solution was used to surface sterilize the nematodes for 15 min. Surface sterilized nematodes were used to infect fifth-instar *Galleria mellonella* larvae (100 IJs per insect) to confirm Koch's postulates for pathogenicity (Kaya & Stock, 1997). Emerging IJ progeny were stored in 250-ml tissue-culture flasks for subsequent identification and establishment of cultures, following the procedures described by Stock & Goodrich-Blair (2012).

Morphological characterization

Morphometrics

Third-stage infective juveniles and adult stages (first and second generation) were randomly collected from White traps and infected cadavers, respectively. Twenty-five randomly selected specimens of each nematode stage were examined after heat killing and relaxation in M9 buffer in a water bath heated to 60°C. Heat-killed specimens were fixed in formaldehyde–acetic acid solution (FA 4:10) (Franklin & Goodey, 1949), slowly dehydrated and processed to anhydrous glycerin (Seinhorst, 1959). Specimens were mounted on glass slides using Pliobond® industrial contact cement to both seal and provide cover glass support (Lee *et al.*, 2009). An Olympus BX51 microscope equipped with differential interference contrast optics and Olympus Microsuite software (Soft Imaging System Corp., Lakewood, Colorado, USA) was used to obtain morphometric data of each nematode specimen. Morphological characters measured were based on the recommendations of Hominick *et al.* (1997). Line drawings were prepared from digitized camera lucida and/or from video images.

Scanning electron microscopy (SEM)

Male and IJ specimens were heat-killed in M9 buffer and then fixed in 8% glutaraldehyde buffered in cacodylate at pH 7.30 overnight. Fixed nematodes were rinsed in distilled water three times, post-fixed in osmium tetroxide for 1 h, rinsed again in distilled water before being subjected to a serial dehydration process in ethanol (McClure & Stowell, 1978). Specimens were critical point dried in liquid carbon dioxide, mounted on SEM stubs and coated twice with gold. Observations and image recordings were made at 5 kV accelerating voltage on a Hitachi S-4800 Type II series microscope equipped with a digital camera (Hitachi, Clarksburg, Maryland, USA).

Molecular characterization

Nematodes were molecularly characterized using two rDNA genes: the internal transcribed spacer region (ITS) and the 28S large subunit region, inclusive of the D2D3 domain. Total genomic DNA isolation, polymerase chain reaction (PCR) amplification (reaction, cycling conditions and primers) followed protocols described by Stock *et al.* (2001a) and Nguyen *et al.* (2001). Sequence data from rDNA and mitochondrial genes were compared to an existing library of more than 60 *Steinernema* spp. (P. Stock's laboratory, University of Arizona, USA) and available sequences found in GenBank.

Phylogenetic analysis

SeqEdit software (DNA Star Inc., Madison, Wisconsin, USA) was used to perform contig assembly and sequence ambiguity resolution. Sequences were aligned using ClustalW v2.1 (Larkin *et al.*, 2007) under default alignment parameters, and alignment inconsistencies were corrected by hand in Mesquite v2.75 (Maddison & Maddison, 2011). *Caenorhabditis elegans* was used as the outgroup taxon for all analyses, according to criteria described by Nadler *et al.* (2006). Ribosomal and mitochondrial DNA sequences for the new *Steinernema* species were deposited in GenBank (accession numbers KJ578793 and KJ578794 for ITS and 28S rDNA genes, respectively). ITS and 28S sequence data were analysed separately. Each dataset was analysed by unweighted maximum parsimony (MP). MP methods were performed in PAUP* v.4.0b10 (Swofford, 2002) following standards described by Stock *et al.* (2001a) and Nadler *et al.* (2006).

Cross-hybridization

Reproductive compatibility of the new species was tested using the modified hanging-blood assay described by Kaya & Stock (1997). Two morphologically similar and close relatives of *S. innovationi* n. sp., *S. khoisanae* Nguyen *et al.*, 2006 and *Steinernema glaseri* (Steiner, 1929), were crossed for assessing reproductive compatibility of this new species. Additionally, another South African isolate recovered from the survey conducted by Hatting *et al.* (2009), ROOI-352 (which represents another novel undescribed *Steinernema* sp.), was used for the cross-breeding experiments. Controls consisted of crosses with male and single female nematodes of the same species, as well as a single female only. There were ten replicates per cross and tests were repeated twice.

Results and discussion

Description of Steinernema innovationi *n. sp.*

First-generation male

Body slender, ventrally curved posteriorly, J-shaped when heat-killed (figs 1A, 2A). First-generation male larger (average 1896 μm) than second-generation male (average 1322 μm). Cuticle smooth under light microscopy. Lateral field and phasmids inconspicuous under light microscopy. Head truncate to slightly round, continuous with body (figs 1B, 2B). Six lips amalgamated but tips distinct, with one labial papilla each. Four conspicuous cephalic papillae (fig. 2B). Amphidial apertures small, located posterior to lateral labial papillae (fig. 2B). Stoma reduced (cheilo-, gymno- and stegostom vestigial), short and wide, with inconspicuous sclerotized walls. Pharynx muscular, with a cylindrical procorpus and a metacorpus slightly swollen and non-valvate. Isthmus indistinct followed by pyriform basal bulb with reduced valve. Nerve-ring usually located about mid-isthmus level or on the anterior part of the basal bulb (fig.1A). Excretory pore opening circular, located anterior to nerve ring at anterior third of metacorpus (fig. 2C). Testis monorchic, ventrally reflexed (figs 1A, 2A).

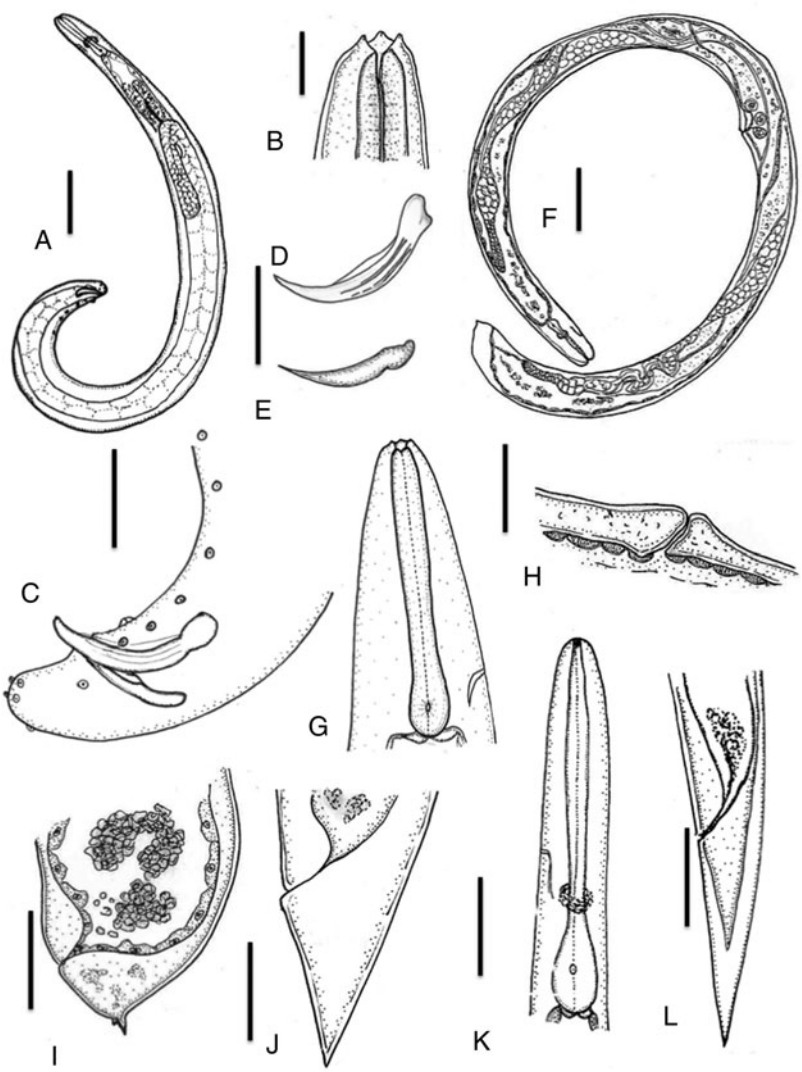

Fig. 1. *Steinernema innovationi* n. sp., line drawings. First-generation male (A–E), first-generation female (F–I), second-generation female (J) and third-stage infective juvenile (K, L). First-generation male: (A) full body, lateral view; (B) anterior end (lateral view), showing stoma region; (C) tail (lateral view), showing genital papillae, spicules and gubernaculums; (D) spicule, lateral view; (E) gubernaculum, lateral view. First-generation females: (F) full body, lateral view; (G) anterior end (lateral view), showing pharyngeal region and excretory canal; (H) vulva, lateral view; (I) tail, lateral view. Second-generation female: (J) tail, lateral view. Third-stage infective juvenile: (K) anterior end (lateral view), showing pharyngeal region, nerve ring and excretory canal; (L) tail, lateral view. Scale bars: A = 2 μm; B = 3 μm; C, G = 50 μm; D, E = 30 μm; F = 190 μm; H = 70 μm; I = 55 μm; J, K, L = 40 μm.

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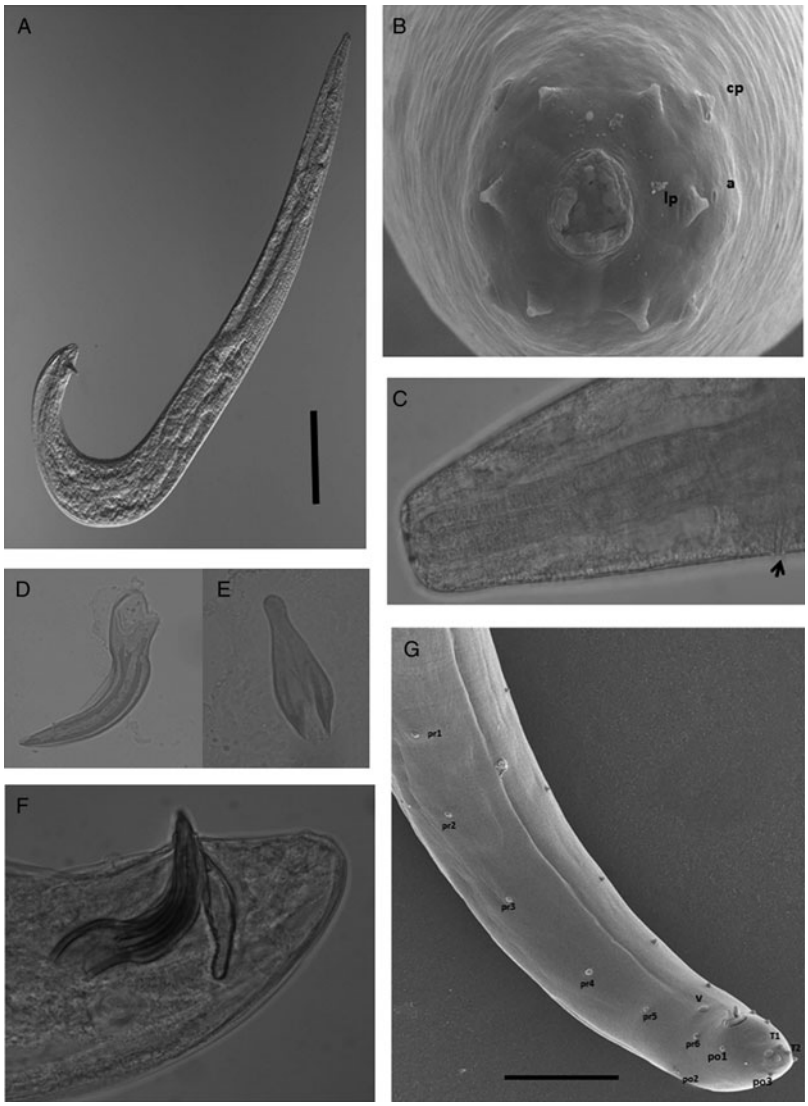

Fig. 2. *Steinernema innovationi* n. sp., light and scanning electron microscope (SEM) photographs. First-generation male: (A) full body, lateral view; (B) SEM of anterior end, face view (lp, labial papillae; cp, cephalic papillae; a, amphid); (C) anterior end, lateral view showing location of excretory canal (arrow); (D) spicule, lateral view; (E) gubernaculum, lateral view; (F) tail, lateral view; (G) tail, SEM, ventrolateral view, showing arrangement of genital papillae (pr, precloacal; po, postcloacal; v, ventral; t, terminal). Scale bars: for light microscopy, the scale bar is given in (A), and for SEM images, in (G). A = 150 μm; B = 5 μm; C = 35 μm; D, E, F = 40 μm; G = 100 μm.

Spicules paired, symmetrical, curved, with ochre–brown colouration (figs 1D, 2D). Manubrium rhomboidal (fig. 1D). Shaft distinct. Blade with rostrum or retinaculum and two internal ribs. Velum present, narrow (fig. 1D). Blade terminus blunt (figs 1D, 2D). Gubernaculum boat-shaped or arcuate, *c.* 3/4 length of spicules. Manubrium of gubernaculum curved ventrally (figs 1E, 2E). Tail conoid and non-mucronate, with no bursa (figs 1C, 2F). There are 23 genital papillae (11 pairs and one single) arranged as follows: six precloacal pairs, of which five pairs are subventral and one pair is lateral, and one single ventral papilla, and five pairs of postcloacal, of which one pair is subventral, two pairs are subdorsal and two pairs are terminal (figs 1C, 2G).

Second-generation male

General morphology similar to that of first-generation males, but smaller in size. Tail with or without mucron. Spicules with manubrium morphology similar to those of first-generation male. Gubernaculum more slender and longer than that of first-generation male.

First-generation female

Lip region, stoma and pharyngeal region as in male (figs 1F, 3A). Body C-shaped when heat-relaxed. Cuticle smooth under light microscope, with slight annulations under SEM. First-generation females larger (average 4070 μm) than second-generation females (average 2063 μm). Excretory pore located about mid-procorpus

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level or surrounding isthmus (fig. 1G). Genital system didelphic, amphidelphic. Ovaries opposed, reflexed in dorsal position; oviduct well developed; glandular spermatheca and uterus in ventral position. Vagina short, with muscular walls. Vulva located near middle of body with slightly protruding lips and mostly symmetrical (figs 1H, 3B). First-generation female tail blunt, conoid and with a digitated tip (figs 1I, 3C). Postanal lips non-protruding or slightly protruding (figs 1H, 3C).

Second-generation female

Body open C-shaped when heat-killed. Similar to first-generation female but smaller. Vulva shape and lips similar to first-generation female. Tail conoid, without postanal swelling (fig. 1J).

Third-stage infective juvenile

Body of heat-relaxed specimens almost straight, slender, gradually tapering posteriorly. Cuticle with fine transverse striae. Head region continuous with body, slightly truncate (figs 1K, 3D). Six lips each bearing one small labial papilla. Four conspicuous cephalic papillae. Amphidial apertures pore-like (fig. 3G). Lip region smooth, continuous; stoma closed (fig. 3G). Lateral field begins anteriorly with one line at fourth or fifth annule and splits posteriorly into two additional lines forming

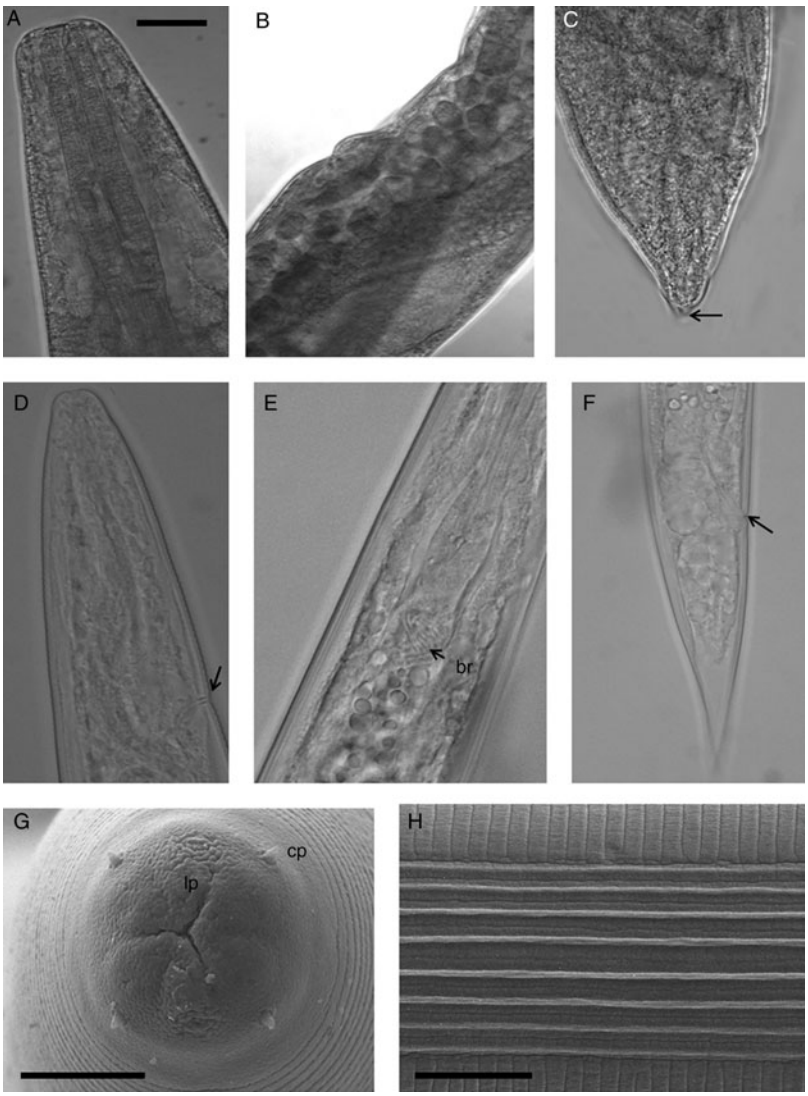

Fig. 3. *Steinernema innovationi* n. sp., light and scanning electron microscope (SEM) photographs. First-generation female (A–C) and third-stage infective juvenile (D–H). First-generation female: (A) anterior end (lateral view), showing procorpus region of pharynx; (B) vulva, lateral view; (C) tail (lateral view), showing digitate mucron (arrow). Third-stage infective juvenile: (D) anterior end (lateral view), showing pharynx and excretory canal (arrow); (E) close-up of basal bulb and bacterial receptacle (br); (F) tail (lateral view), showing rectum (arrow) and hyaline portion; (G) anterior end (face view), showing position of labial (lp) and cephalic papillae (cp); (H) lateral field pattern in mid-body region. Scale bars (based on the scale bar in (A), except for SEM images): A = 40 μm; B = 54 μm; C, E = 20 μm; D = 25 μm; G = 5 μm; F, H = 10 μm.

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Table 1. Morphometric characters of male, female and third-stage infective juvenile of *Steinernema innovationi* n. sp.; n = number of examined specimens; measurements in μm include mean values ± standard deviation and ranges.

Type material	Holotype	Paratypes				
Stage	Male	Males		Females		Infective juveniles
Generations	1st, $n = 1$	1st, $n = 24$	2nd, $n = 25$	1st, $n = 25$	2nd, $n = 25$	$n = 25$
TBL	1844.8	1896 ± 159 1664–2273	1322 ± 54 1220–1435	4070 ± 474 3398–5423	2063 ± 152 1837–2281	1054 ± 27 1000–1103
MBW	125.0	139 ± 21 100–171	80 ± 7 70–94	173 ± 15 144–202	149 ± 14 123–175	37 ± 5 31–50
PH	165.5	170 ± 6 162–190	148 ± 3 142–154	192 ± 6 179–202	174 ± 2 170–179	150 ± 5 142–160
EP	137.6	140 ± 12 115–164	116 ± 6 105–125	147 ± 10 125–168	127 ± 5 116–136	88 ± 3 82–91
NR	126.6	127 ± 10 112–158	103 ± 6 93–114	135 ± 8 126–154	122 ± 5 113–129	108 ± 4 97–117
TL	49.7	51 ± 8 42–76	39 ± 2 34–41	58 ± 3 52–66	65 ± 5 57–74	76 ± 3 66–80
ABD	53.7	58 ± 7 45–73	45 ± 2 42–51	65 ± 6 49–83	50 ± 4 41–57	23 ± 3 19–30
SpL	79.6	81 ± 5 74–91	59 ± 0.8 58–61	–	–	–
GuL	59.0	58 ± 3 54–63	41 ± 1 39–43	–	–	–
SW%	1.5	140 ± 20 110–190	130 ± 10 120–140	–	–	–
GS%	0.7	70 ± 10 60–80	70 ± 0.0 70	–	–	–
V	–	–	–	2167 ± 228 1729–2761	1124 ± 84 981–1243	–
a	–	–	–	–	–	29 ± 3 21–34
b	–	–	–	–	–	7 ± 0.3 6.5–7.6
D%	83.1	82 ± 7 67–95	79 ± 4 71–88	–	–	58 ± 2 54–63
E%	277	279 ± 40 196–325	301 ± 15 277–344	–	–	115 ± 7 104–137
H	–	–	–	–	–	32 ± 2 29–36
H%	–	–	–	–	–	43 ± 3 37–46

ABD, anal or cloacal body diameter; D% = (EP/PH) × 100; E% = (EP/TL) × 100; EP = distance from anterior end to excretory pore; GS% = (GuL/SpL) × 100; GuL = gubernaculum length; H = length of hyaline portion of tail; H% = H as % of TL; MBW = maximum body width; NR = distance from anterior end to nerve ring; PH = distance from anterior end to base of pharynx; SpL = spicule length (measured *in situ*, along the curvature in a line along the centre of the spicule); StL = stoma length; StD = stoma diameter; SW% = (SpL/ABD) × 100; TBL = total body length; TL = tail length; V = distance from anterior end to vulva; a = TBL/MBW; b = TBL/PH.

Table 2. Comparative morphometric traits of third-stage infective juveniles and males of *Steinernema innovationi* n. sp. with other members of the *S. glaseri*-group (clade V); measurements in μm include mean values with ranges; see table 1 for abbreviations.

	Third–stage infective juveniles					Males				
	TBL	EP	TL	D%	E%	SpL	GuL	D%	SW%	GS%
S. aciari [1]	1113	95	78	65	123	86	64	76	204	65
	975–1250	87–100	68–88	60–70	113–134	80–94	47–73	69–88	180–240	57–77
S. arenarium [2]	1134	83	75	55	119	76	53	60	NA	70
	724–1408	76–86	64–84	52–59	106–130	63–93	45–63	38–126		NA
S. australe [3]	1316	110	103	65	107	72	45	71	172	62
	1162–1484	95–125	92–114	57–78	94–122	55–78	36–51	59–87	118–196	46–71
S. boemarei [4]	1101	91	86	63	106	79	52	85	170	70
	1005–1323	82–111	77–109	56–68	94–122	64–96	43–65	68–99	120–240	50–90
S. braziliense [5]	1157	94	88	63	106	83	47	65	192	57
	1023–1284	87–102	80–104	58–70	95–118	75–89	41–56	57–80	158–208	48–65
S. costaricense [6]	1696	77	63.5	53	85	92	46	56	160	49
	1600–1773	75–82	54.5–68.5	45–60	82–91.5	81–101	40.5–50.5	50.5–66	150–170	45–55
S. cubanum [7]	1283	106	67	70	160	58	39	70	141	70
	1149–1508	101–114	61–77	NA	NA	50–67	37–42	NA	NA	NA
S. diaprepesi [8]	1022	74	83	84	90	79	54	80	180	69
	880–1133	66–83	65–91	30–70	78–114	71–90	45–61	68–86	150–200	59–79
S. ethiopiense [9]	898	78	73	56	107	73	49	57	164	67
	768–1010	65–84	64–80	51–58	91–116	69–77	46–57	54–61	154–175	63–70
S. glaseri [10]	1130	102	78	65	131	77	55	70	210	70
	864–1448	87–110	62–87	58–71	122–138	64–90	44–59	60–78	164–243	60–85
S. guangdongense [11*]	**1055**	**80**	**91**	**59**	**88**	**86**	**64**	**70**	**175**	**75**
	987–1145	**71–85**	**82–103**	**54–65**	**74–100**	**80–94**	**47–73**	**67–78**	**152–216**	**59–82**
S. hermaphroditum [12]	*928.5*	*65*	*77*	*50*	*85*	68	48	48	150	71
	700–950	*62.5–82.5*	*65–82.5*	*47–55*	*76–100*	*65–70*	*47–50*	44–50	140–160	70–75
S. khoisanae [13*]	**1075**	**93**	**85**	**138**	**111**	**85**	**56**	**85**	**203**	**70**
	904–1214	**84–100**	**69–98**	**115–147**	**95–123**	**70–88**	**50–63**	**71–99**	**167–227**	**60–80**
S. lamjungense [14*]	**832**	**68**	**88**	**54**	**79**	**87**	**57**	**61**	**173**	**65**
	690–950	**60–73**	**67–103**	**48–63**	**69–97**	**81–94**	**50–66**	**44–91**	**151–193**	**56–78**
S. longicaudum [15*]	**1043**	**82**	**94**	**57**	**87**	**91**	**60**	**75**	**161**	**66**
	929–1170	**74–92**	**79–105**	**52–63**	**76–104**	**72–108**	**54–65**	**56–92**	**116–225**	**56–88**
S. phyllophagae [16]	1289	99	89	70	110	72	51	75	187	71
	1133–1395	84–120	77–109	58–100	70–139	65–77	46–56	68–80	136–223	61–77
S. puertoricense [17]	1171	95	94	66	101	78	40	77	152	51
	1057–1238	90–102	88–107	62–74	88–108	71–88	36–45	NA	NA	NA
S. scarabaei [18]	918	77	76	60	100	75	44	66	170	60
	890–959	72–81.5	71–80	50–75	90–110	67–83	36–50	53–77	150–200	50–65
S. vulcanicum [19]	1204	93	86	59.6	109	71	50	69.7	140	70
	1081–1323	77–107	74–100	47–68	83–135	46–88	32–65	42–90	90–210	60–90
***S. innovationi* n. sp.**	**1054**	**88**	**76**	**58**	**115**	**81**	**58**	**58**	**140**	**70**
	1000–1103	**82–91**	**66–80**	**54–63**	**104–137**	**74–91**	**54–63**	**54–63**	**110–190**	**60–80**

Entries in bold and with * are the most closely related species to *S. innovation* n. sp.

References: [1]after Qui *et al.*, 2005; [2]Artyukhovsky *et al.*, 1997; [3]after Edington *et al.*, 2009; [4]after Lee *et al.*, 2009; [5]after Nguyen *et al.*, 2010; [6]after Uribe–Lorío *et al.*, 2007; [7]after Mráček *et al.*, 1994; [8]after Nguyen & Duncan, 2002; [9]after Tamiru *et al.*, 2012; [10]after Poinar, 1990; [11]after Qui *et al.*, 2004; [12]after Stock *et al.*, 2004; [13]after Nguyen *et al.*, 2006;[14] after Khatri–Chhetri *et al.*, 2011; [15]after Stock *et al.*, 2001b; [16]Nguyen & Buss, 2011; [17]after Román & Figueroa, 1994; [18]Stock & Koppenhöfer, 2003; [19]after Clausi *et al.*, 2011.

two ridges. More posteriorly, the number of ridges increases to six. At the mid-body region there are nine distinct lines (= eight ridges) evenly spaced and developed (fig. 3H). Ridges reduced to six near the anus and to two near the phasmid region. Lateral line formula is 2, 6, 8, 6, 2. Pharynx long, narrow, with slightly expanded procorpus, narrower isthmus and pyriform basal bulb with valve (figs 1K, 3D). Nerve-ring located at isthmus level (figs 1K, 3D). Excretory pore located about mid-corpus (figs 1K, 3D). Anterior portion of intestine with small bacterial receptacle (fig. 3E). Intestine filled with numerous fat globules, lumen of intestine narrow. Rectum long, straight; anus distinct (figs 1L, 3F). Genital primordium evident. Tail conoid with pointed terminus. Hyaline portion occupying *c.* 32% of tail length (figs 1L, 3F).

Type material

Holotype male, first generation; five paratype males, first generation; five paratype females, first generation; five paratype third-stage infective juveniles deposited in the USDA Nematode Collection, Maryland.

Five paratype males, first generation; five paratype females, first generation; five paratype third-stage infective juveniles deposited at the University of California Davis Nematode Collection, Davis, California, USA.

Dimensions of holotype and paratype specimens are provided in table 1.

Type host. The natural host of this novel species is unknown.

Type locality. Isolate SGI-60 was collected from a grain field at the Agricultural Research Center, Small Grain Institute in Bethlehem, South Africa.

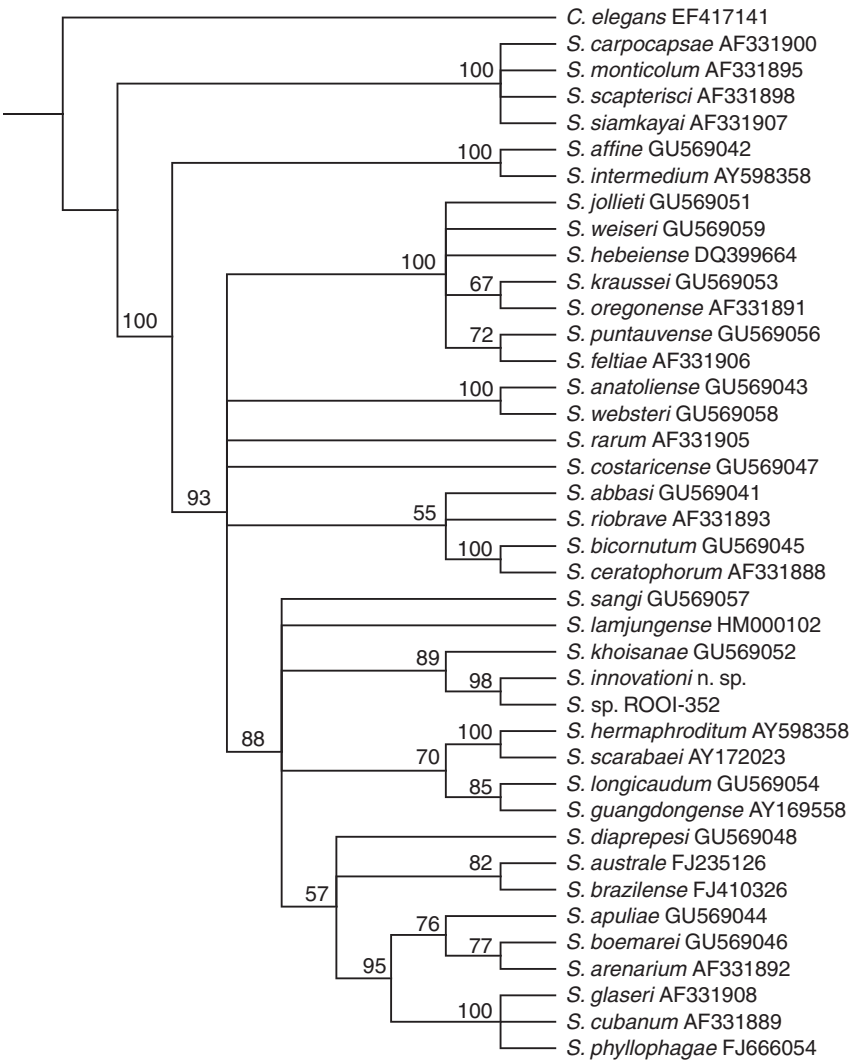

Fig. 4. Evidence of 28S rDNA (inclusive of D2D3 domain) lineage independence for *S. innovationi* n. sp. based on maximum parsimony, with bootstrap values above 50% shown.

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Etymology. This species is dedicated to the 'Innovation Foundation' that provided financial support for the survey conducted in 2009 to isolate entomopathogenic nematode species in South Africa.

Cross-hybridization results

Cross-hybridization assays between males and females of *S. innovationi* n. sp. with *S. khoisane, S. glaseri* and another South African *Steinernema* sp. isolate ROOI-352 yielded no progeny. No progeny were observed in the single female control plates.

Diagnosis and relationships

Based on morphological and morphometric traits, the new species is considered a member of the '*glaseri*-group'. Nematodes in this group are characterized by having the largest third-stage infective juveniles (average total body length (TBL) $\geq 1000\,\mu m$) and by the lateral field in infective juveniles, which possess eight ridges in the mid-body region.

Phylogenetic analyses of the ITS and 28S genes dataset also confirmed affiliation of *S. innovationi* n. sp. in clade V (as depicted by Spiridinov *et al.*, 2004) which encompasses taxa of the '*glaseri*-group'. Within this clade, the new species is closely related to the four described *Steinernema* species: *S. khoisanae* Nguyen *et al.*, 2006, from South Africa and three Asian species: *S. longicaudum* Shen & Wang, 1992, *S. lamjungense* Khatri-Chhetri *et al.*, 2011 and *S. guangdongense* Qiu *et al.*, 2004. However, the novel species can also be distinguished from these taxa by several morphological and morphometric characters, which are listed below.

Infective juveniles of *S. innovationi* n. sp. are wider (average 37 μm versus 33 μm) and slightly shorter than those of *S. khoisanae* (average 1054 μm versus 1075 μm). The excretory pore in the new species is more anteriorly

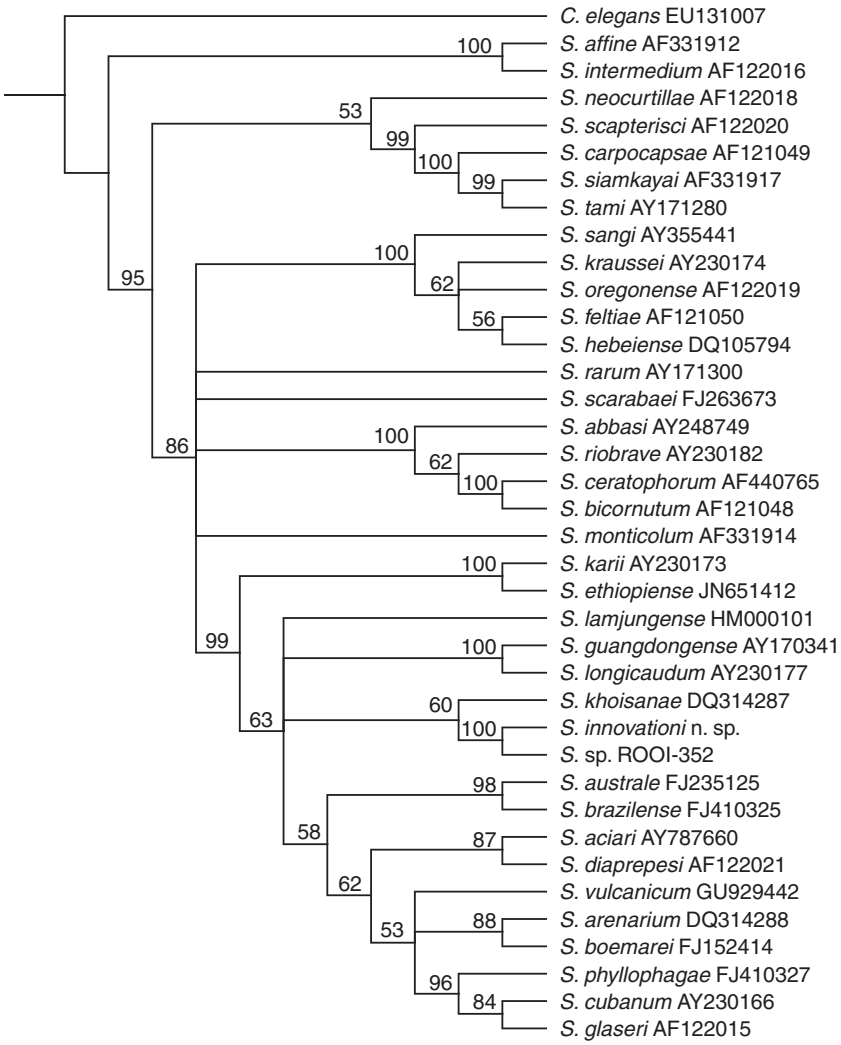

Fig. 5. Evidence of internal transcribed spacer region (ITS) rDNA lineage independence for *S. innovationi* n. sp. based on maximum parsimony analysis, with bootstrap values above 50% shown.

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located (average 88 μm versus 93 μm), and the tail is usually shorter than that of *S. khoisanae* (average 76 μm versus 85 μm). Additionally, differences exist in the values of D% and E% (table 2). Males of *S. innovationi* n. sp. can be distinguished from *S. khoisanae* by the D% (average: 83 versus 88) and SW ratio (average: 1.4 versus 1.9). In *S. khoisanae* the number of pairs of genital papillae may differ between 11–12 pairs plus one single papilla; however, in the new species a consistent number of 11 pairs plus one single papilla was observed in all specimens examined. Moreover, the value of SW% between these two species is quite different (table 2). Females of *S. innovationi* n. sp. have a digitated tail, similar to those of S. *khoisanae.* However, females of the new species lacked the postanal swelling or, if present, this was slightly protruding when compared to *S. khoisanae*.

Third-stage infective juveniles of *S. innovationi* n. sp. differ from those of *S. longicaudum* by the location of the excretory pore (average 88 μm versus 82 μm), and by having a shorter tail (average 76 μm versus 94 μm). Males of *S. innovationi* n. sp. can be separated from those of *S. longicaudum* by the morphology of the spicules which are more robust and smaller than those of *S. longicaudum* (see table 2). Moreover, the lamina of the spicules in *S. longicaudum* is more slender than that of the new species. The gubernaculum in *S. innovationi* n. sp. is shorter than that of *S. longicaudum.* Differences also exit in the values of SW% and GS% (see table 2). Additionally, first-generation males of the new species have a longer tail than those of *S. longicaudum* (average 51 μm versus 30 μm).

Steinernema innovationi n. sp. can be distinguished from *S. lamjungense* by the size of the IJs, which are larger in the new species (table 2). Moreover, the excretory pore in *S. lamjungense* is more anteriorly located than that of *S. innovationi* n. sp. The tail of the IJs in the new species is slightly shorter than that of *S. lamjungense.* Additionally, differences exist in the values of D% and E% (table 2). Males of the novel species differ from *S. lamjungense* in the values of D% and SW%. Furthermore, the morphology of the spicules in *S. innovationi* n. sp. is different than that of *S. lamjungense.* In particular, the shape of the manubrium in *S. lamjungense* is usually round compared to that of *S. innovationi* n. sp. which is rhomboidal. The gubernaculum in the new species is boat-shaped to arcuate, whereas in *S. lamjungense* it varies from boat-shaped to almost straight, and with the anterior part slightly recurved. Differences also exit in the values of D%, SW% and GS% (table 2).

Infective juveniles of the new species can be distinguished from *S. guangdongense* by the location of the excretory pore which is more posteriorly located in the new species (average 80 versus 88 μm). Additionally, the tail of the IJs in the new species is shorter than that of *S. guangdongense* (average: 76 versus 91 μm). Males of *S. innovationi* n. sp. can be discriminated from those of *S. guangdongense* by the values of D%, SW% and GS% (table 2). Moreover, the gubernaculum in the new species is shorter than that of *S. guangdongense.* Also, first-generation males have a mucronated tail, whereas no mucron is present in *S. innovationi* n. sp.

Molecular characterization and phylogenetic analysis

Maximum parsimony (MP) analysis of the 28S dataset yielded 401 parsimony informative characters out of 935 characters. A heuristic search of 5000 random addition replicates produced 126 equally parsimonious trees with a tree length of 1215 steps. Bootstrap MP analysis of this dataset placed *S. innovationi* n. sp. as a member of clade V, and strongly supported (98%) its placement as sister to the undescribed *Steinernema* isolate ROOI-352 (fig. 4). The two South African isolates are closely related to *S. khoisanae*, with 89% branch support. This clade comprises *Steinernema* spp. known to have infective juveniles with exceptionally large body size (average ≥ 1000 μm). Clading of '*glaseri*-group' individuals, including *S. innovationi* n. sp., was supported by

Table 3. Pairwise distance matrix of 28S rDNA (inclusive of D2D3 domain) for representative nematodes in the *S. glaseri*-group (clade V).

	1	2	3	4	5	6*	7	8	9	10	11	12	13	14	15	16	17	18
1. *S. boemarei*	–																	
2. *S. diaprepesi*	40	–																
3. *S. glaseri*	46	49	–															
4. *S. hermaphroditum*	49	34	60	–														
5. *S. khoisanae*	38	22	49	25	–													
6. *S innovationi* n. sp.*	38	28	54	33	10	–												
7. *S.* sp. ROOI-352	38	28	56	33	10	4	–											
8. *S. australe*	38	22	46	39	26	34	34	–										
9. *S. braziliense*	36	23	49	37	30	37	37	13	–									
10. *S. lamjungense*	25	20	30	19	12	12	13	24	23	–								
11. *S. guangdongense*	29	21	31	23	19	19	20	26	26	16	–							
12. *S. hebeiense*	52	45	52	43	42	40	43	47	46	43	46	–						
13. *S. cubanum*	52	52	9	62	51	55	57	48	50	29	31	51	–					
14. *S. arenarium*	17	39	42	48	39	44	44	34	36	25	28	49	48	–				
15. *S. phyllophagae*	54	52	18	66	55	60	60	53	55	31	33	56	20	52	–			
16. *S. pui*	54	48	75	38	34	38	38	55	54	18	24	53	76	59	76	–		
17. *S leizhouense*	46	36	64	40	35	38	38	40	37	22	29	46	68	45	68	58	–	
18. *S. longicaudum*	38	36	58	27	29	36	36	41	42	19	9	51	61	43	65	38	43	–

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Table 4. Pairwise distance matrix of ITS region for representative nematodes in the *S. glaseri*-group (clade V).

	1	2	3	4	5	6	7	8*	9	10	11	12	13	14	15	16	17	18	19	20
1. *S. cubanum*	–																			
2. *S. aciari*	170	–																		
3. *S. diaprepesi*	177	142	–																	
4. *S. arenarium*	150	157	150	–																
5. *S. boemarei*	168	164	165	113	–															
6. *S. longicaudum*	140	128	119	136	139	–														
7. *S.* sp. ROOI-352	176	153	152	141	165	91	–													
8. *S. innovationi* n. sp.*	201	186	181	165	196	114	58	–												
9. *S. phyllophagae*	143	151	165	148	158	133	162	180	–											
10. *S. glaseri*	94	182	176	148	149	129	167	182	121	–										
11. *S. australe*	161	159	147	153	160	111	138	164	147	143	–									
12. *S. braziliense*	171	163	158	156	165	116	150	184	177	170	116	–								
13. *S. karii*	175	142	151	161	159	112	135	159	159	168	134	124	–							
14. *S. ethiopiense*	164	127	139	139	154	107	132	165	153	159	116	133	38	–						
15. *S. lamjungense*	178	159	154	159	167	91	124	162	142	153	136	139	125	118	–					
16. *S. letzhouense*	172	150	160	153	151	112	147	175	172	170	161	152	130	116	130	–				
17. *S. guangdongense*	151	152	138	138	160	39	110	148	140	137	125	130	108	117	88	127	–			
18. *S. pui*	156	148	141	139	141	86	113	139	125	148	134	131	109	104	94	143	91	–		
19. *S. loci*	183	151	163	160	174	113	168	201	165	177	174	154	136	131	149	61	131	141	–	
20. *S. khoisanae*	198	175	174	158	170	100	116	147	147	160	141	142	137	123	132	149	110	135	159	–

bootstrap resampling (88%), for which a 50% majority rule consensus tree is given (fig. 4).

A heuristic search of the ITS rDNA sequences yielded two most parsimonious trees with tree length of 4356 steps. Of 1244 characters, 683 were parsimony informative. The MP bootstrap analysis of ITS data also placed *S. innovationi* n. sp. as a member of clade V, and (strongly, 100%) sister to the undescribed *Steinernema* isolate ROOI–352. The two South African isolates were also found to be closely related to *S. khoisanae* in this dataset; however, support was lower (60%). Bootstrap resampling provided strong (99%) support for the clade V *glaseri*-group assemblage, as seen in the 50% majority rule consensus tree (fig. 5).

The 28S rDNA pairwise distances between *S. innovationi* n. sp and *Steinernema* isolate ROOI-352 and *S. khoisanae*, close sister taxa, are 4 and 10, respectively. The considerably more variable ITS region yielded pairwise distances between *S. innovationi* n. sp. and the same two species of 58 and 147, respectively. Results of these analyses provide further evidence for the distinctiveness of this species (tables 3 and 4).

Financial support

This research was partially funded by the South African Agricultural Research Council (ARC) under Project GK05/14 to J.H. Training of H.Ç. in P. Stock's Laboratory was supported in part by NemaSym Research Coordination Network grant (PI P. Stock, NSF–IOS 0840932).

Conflict of interest

None.

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