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Heterorhabditis amazonensis n. sp. (Rhabditida: Heterorhabditidae) from Amazonas, Brazil

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Summary – In a survey of entomopathogenic nematodes in Brazil, a nematode isolate of the genus *Heterorhabditis* was found. The nematode was collected from soil by the insect-baiting technique and maintained in the laboratory on last instar *Galleria mellonella* (L.) larvae. Morphological and molecular studies of the isolate showed that the nematode is a new species. Light and scanning electron microscopy, DNA characterisation and phylogeny were used for this description. *Heterorhabditis amazonensis* n. sp. is morphologically similar to *H. baujardi*, *H. floridensis*, *H. mexicana* and *H. indica*, and can be distinguished from these species mainly by male and female characters. Fifty percent of *Heterorhabditis amazonensis* n. sp. males have two pairs of bursal papillae in the terminal group; 25% with two papillae on one side and one papilla on the other side and 25% with one pair of papillae. Twenty percent of the population has a curved gubernaculum. The percentage of the gubernaculum to spicule length (GS%) is lower than that of *H. mexicana* (50 *vs* 56), and the length of the spicule relative to anal body diam. (SW%) is lower than that of *H. mexicana* (152 *vs* 167) and *H. baujardi* (152 *vs* 182). The female can be differentiated from related species by its unique vulva pattern. In a phylogenetic tree, the new species, *H. floridensis*, *H. baujardi* and *H. mexicana* form a monophyletic group, a sister group to *H. indica.* The new species has evolved three autapomorphic nucleotide character states, differing from its sister taxa *H. mexicana* at 22, and *H. baujardi* at 15, aligned positions.

Keywords – description, entomopathogenic nematodes, ITS rDNA, morphology, morphometrics, SEM, systematics, taxonomy.

Entomopathogenic nematodes (EPN) of the family Heterorhabditidae and Steinernematidae have been used successfully for biological control of insects (Klein, 1990; Shapiro-Ilan *et al.*, 2002). Currently, over 48 species of *Steinernema* and ten species of *Heterorhabditis* (Nguyen, 2006) have been validly described, and the number of nominal species is increasing rapidly. Hominick (2002) reported that the number of EPN species described up to 2002 was 34, of which 23 were described between 1989 and 2002. The nematodes seem to be present all over the world.

In Brazil, several species have been discovered and identified. The first record of EPN in Brazil was by Pizano *et al.* (1985). These authors reported that infective juveniles of *Neoaplectana glaseri* Steiner, 1929 (now called *Steinernema glaseri*), were found in an egg of *Migdolus fryanus* (Westwood) in Usina Amalia, São Paulo. In 1990, Poinar reported *Heterorhabditis bacteriophora* Poinar, 1975 in Pernambuco state. Hominick (2000) reported that *S. carpocapsae* was found in Brazil. Recently, Valle *et al.* (2005) collected an isolate of *H. baujardi* Phan, Subbotin, Nguyen & Moens, 2003 from Rondonia state, and Machado *et al.* (2005) isolated *S. glaseri* in Araras, São Paulo and *H. indica* Poinar, Karunakar & David, 1992 in Itapetininga, São Paulo.

In a survey of entomopathogenic nematodes in Benjamin Constant, Amazonas state, Brazil, a nematode isolate of the genus *Heterorhabditis* Poinar, 1975 was found. Morphological characters, morphometrics and molecular data show that this nematode is a new species. The new species is described herein as *Heterorhabditis amazonensis* n. sp.

Materials and methods

Nematodes were collected from soil by the insect-baiting technique (Bedding & Akhurst, 1975) using *Galleria mellonella* (L.) larvae and maintained in the laboratory on the last instar of this insect (Dutky *et al.*, 1964). For morphological studies, ten larval *G. mellonella* were

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exposed to *ca* 300 infective juveniles (IJ) in a Petri dish (100 × 15 mm) lined with two moistened filter papers. The first generation hermaphrodites, second generation females and males were obtained by dissecting infected insects at 3-4 days and 6-7 days, respectively, after the insect died. Third-stage infective juveniles were obtained during the first 2 days after emerging from insect cadavers (Nguyen & Smart, 1995a).

Light microscopy

For light microscope observations, 20 males, 20 females and 20 infective juveniles were examined alive. Additional specimens of different stages were killed and fixed in either triethanolamine formalin (TAF) as suggested by Courtney *et al*. (1955) or lactophenol (Franklin & Goodey, 1949). These nematodes were used when more observations were needed to confirm the morphology or variation of some structures. Nematodes fixed in TAF were processed to glycerin using the Seinhorst (1959) method. Type specimens were mounted in glycerin. Cover glass supports were used in all cases to avoid flattening of the specimens.

Morphology of bursa

The preparation of the bursa was done using the method reported by Nguyen *et al.* (2004). The nematodes were reared and collected as stated above. Males were transferred into a small drop of lactophenol (with 0.002% acid fuchsin) on a hot plate at 65-70°C. After 30 min, a male was transferred to a drop of lactophenol on a glass slide. The anterior three-fourths of the body was severed and removed. A cover glass was placed on top of the drop of lactophenol containing the posterior part of the nematode. The cover glass was moved slowly by a needle to rotate the posterior part of the nematode to a ventral view. Twenty four males were observed.

Scanning electron microscopy

Adults and IJ were fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.2 for 24 h at 8°C. They were post-fixed with 2% osmium tetroxide solution for 12 h at 25°C, dehydrated in a graded ethanol series, critical point dried with liquid CO_2, mounted on SEM stubs, and coated with gold. Spicules and gubernacula were prepared as suggested by Nguyen and Smart (1995b).

Molecular characterisation

The *Heterorhabditis* species used in this study were: *Heterorhabditis amazonensis* n. sp.; *H. bacteriophora* strain HP88; *H. baujardi* strain Vietnam; *H. downesi* Stock, Griffin & Burnell, 2002 strain K122; *H. floridensis* Nguyen, Gozel, Koppenhöfer & Adams, 2006; *H. indica* strain India; *H. marelatus* Liu & Berry, 1996 strain OH10; *H. megidis* Poinar, Jackson & Klein, 1987 strain AGC; *H. mexicana* Nguyen, Shapiro-Ilan, Stuart, McCoy, James & Adams, 2004 strain MX4; and *H. zealandica* Poinar, 1990 strain Florida. All of these sequences were from Nguyen *et al.* (2004, 2006). All accession numbers are cited on the phylogenetic tree.

DNA was extracted from a single hermaphroditic female as described previously (Nguyen *et al*., 2001, 2004, 2006) and the entire internally transcribed spacer regions (ITS) were PCR amplified using the primers 18S: 5′-TTGATTACGTCCCTGCCCTTT-3′ (forward) and 26S: 5′-TTTCACTCGCCGTTACTAAGG-3′ (reverse). All PCR reactions were conducted in a Gradient Thermocycler, PTC-200 (MJ Research, Inc., Watham, MA, USA) with the cycle profile: 1 cycle of 94°C for 7 min followed by 35 cycles at 94°C for 60 s; 45°C for 60 s; and 72°C for 60 s. The last step was 72°C for 10 min. PCR products were sequenced bi-directionally in their entirety. Internal primers used for sequencing were: 58P = 5′-ACGAATTGCAGACGCTTAG-3′ (forward) and H58R = 5′-GTGCGTTCAAAACTTCACC-3′ (reverse) (Nguyen *et al.*, 2004). Sequences of the complete ITS array were aligned to previously published sequences of the ITS region (Nguyen *et al.*, 2006) using the profile alignment option of Clustal X (Thompson *et al.*, 1997), then optimised manually in MacClade 4.05 (Maddison & Maddison, 2002).

Phylogenetic analysis was performed using PAUP* (Swofford, 2002). For the parsimony analysis, the shortest trees were obtained using the branch and bound algorithm. Gaps in the matrix were treated as either missing data or as a fifth base. *Pellioditis typica* and *Caenorhabditis elegans* were used as outgroup taxa and to root the tree. For ML analysis, models of sequence evolution were evaluated using Modeltest 3.07 (Posada & Crandall, 1998). Modeltest utilises likelihood ratio tests which favoured the TVM + G model with a 0.8845 gamma distribution shape parameter. The maximum likelihood tree was inferred using a neighbour-joining starting tree, with heuristic searching of tree space by TBR branch swapping. Branch support was estimated by bootstrap analysis (1000 replicates for MP and 100 replicates for ML) us-

ing the same parameters as the original search. For further species delimitation, we traced the phylogenetic relationships among *Heterorhabditis* species and map of ITS character states that could be polarised unambiguously (MacClade 4.05). This process shows the numbers of transition/transversion sites and autapomorphies (unique, derived characters) that, when fixed within and among lineages, indicate lineage independence.

Heterorhabditis amazonensis* **n. sp.**
(Figs 1-8)

MEASUREMENTS

See Table 1.

DESCRIPTION

Male

Body curved ventrally when killed by gentle heat. Head truncate, sometimes slightly swollen. Face view with six labial papillae, cephalic papillae not observed. Amphidial apertures prominent. Stoma with sclerotised rod- or barrel-shaped cheilorhabdions; other rhabdions not distinguishable, forming a funnel-shaped structure posterior to cheilorhabdions. Pharynx with cylindrical corpus, metacorpus sometimes slightly enlarged. Nerve ring surrounding isthmus just anterior to basal bulb. Basal bulb with reduced valve. Cardia present, protruding into intestine. Excretory pore usually located posterior to basal bulb. Testis monorchic, reflexed; distance from end of basal bulb to testis flexure variable. *Vas deferens* well developed. Spicules paired, separate, rostrum absent, ventral side flattened. Gubernaculum well curved ventrally in 20% of the population, slightly curved ventrally in 20%. Bursa peloderan. Bursal papillae numbering 7-8 pairs. From anterior to posterior, pair 1 well anterior with papilla tips extending beyond bursal rim; pairs 2 and 3 in a group anterior to cloaca, also extending beyond bursal rim. Pairs 4, 5 and 6 forming a group, situated posterior to cloaca, pair 4 usually not curved outward (laterally). Number of genital papillae from one to six (from anterior end) typical for *Heterorhabditis* spp. (Nguyen *et al*., 2004). Pairs 7 and 8 forming a terminal group of papillae at end of bursa. Number and distribution of papillae in terminal group variable. Observation of terminal group papillae in 24 bursas gave following results: 50% with two pairs of papillae; 25% with two papillae on one side and one papilla on other side; and 25% with one pair of papillae. Tail conoid, slightly curved ventrally.

Female

Hermaphroditic female: C-shaped after killing with gentle heat, body robust, always with many eggs in young females and many eggs and juveniles in mature females. Cuticle smooth under light microscope but finely annulated with SEM. Lateral field and phasmid not observed. Head region tapering anteriorly. Labial region with six prominent lips, each lip with a terminal labial papilla. Cephalic papillae not observed with SEM. In face view, mouth hexagonal in shape. Amphidial aperture pore-like. Stoma with refractile cheilorhabdions. Posterior part of stoma funnel-shaped, enclosed by anterior part of pharynx. Pharynx with cylindrical corpus. Isthmus present. Nerve ring located at middle of isthmus. Basal bulb prominent with inconspicuous valve, but lumen of pharynx in basal bulb well sclerotised. Cardia present. Gonads didelphic, amphidelphic. Vulva in form of a transverse slit, located on a slightly protuberant area, anterior to mid-body. In ventral view, vulva top smooth, elliptical, surrounded by unique pattern. Pattern in form of a rough area radiating from vulva opening. Close to opening, many wart-like structures present. Vagina short. Tail longer than anal body diam., conoid with pointed terminus. Tail sometimes widening near end then narrowing to a pointed tip. Post-anal swelling present, sometimes (20%) with two papilla-like structures. Phasmid inconspicuous.

Amphimictic female: Similar to hermaphroditic female but smaller. Vulva rarely protuberant, usually (80%) covered with exudates or copulation plug. Post-anal swelling much smaller.

Infective juvenile

Body elongate. Sheath (second-stage cuticle) present immediately after harvesting, but many infective juveniles (IJ) losing sheath in storage. Ensheathed IJ with body length close to that of *H. bacteriophora* and *H. mexicana*. Labial region with seven annules, lacking longitudinal incisures. Anterior part of body (*ca* 3-5 body diam. from anterior end) with tessellate pattern. Posterior part with longitudinal ridges. Tail long, pointed. Exsheathed IJ body annulated, without longitudinal ridges. Labial region with dorsal tooth, labial and cephalic papillae absent. Amphidial aperture prominent. Excretory pore located posterior to nerve ring but just anterior to base

* The species epithet is derived from *Amazonas*, Brazil, the state from where the species was obtained.

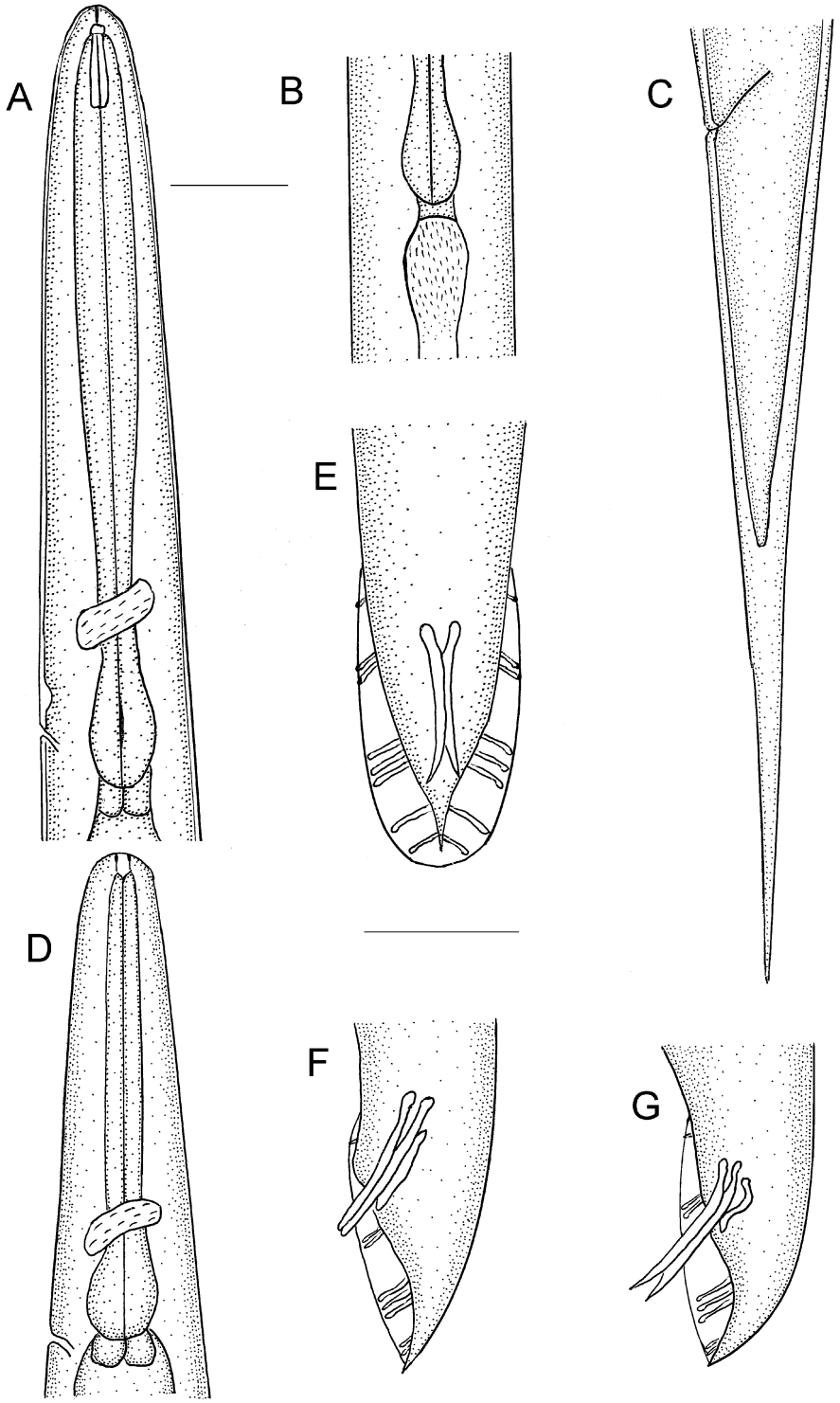

Fig. 1. Heterorhabditis amazonensis *n. sp. A-C: Infective juvenile. A: Anterior region; B: Basal bulb region; C: Posterior region; D-G: Male. D: Anterior region; E: Posterior region, ventral view; F, G: Variation of tail and gubernaculum. (Scale bars: A-C = 14 μm; D-G = 41 μm.)*

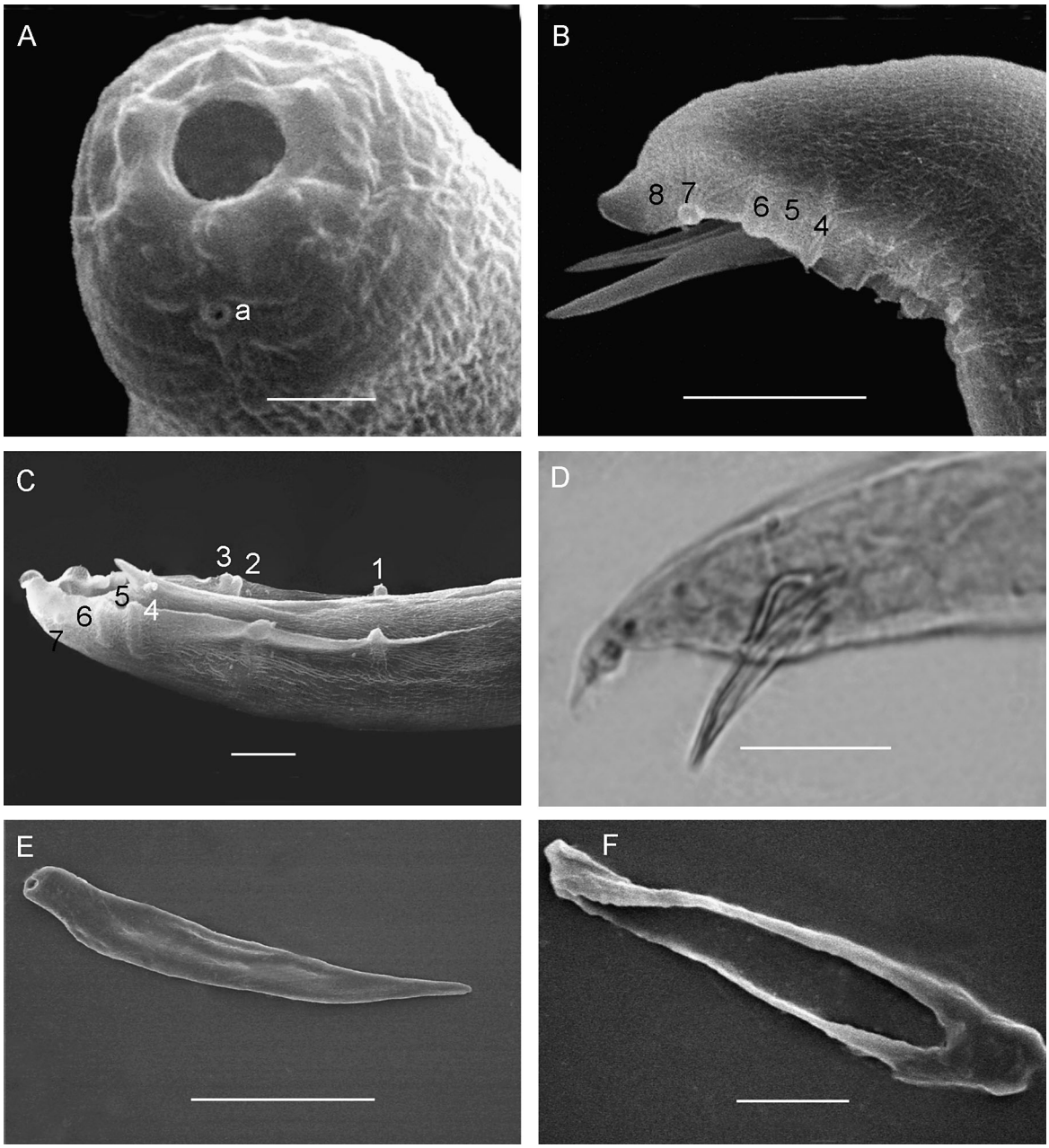

Fig. 2. Heterorhabditis amazonensis *n. sp. A-C, E, F: SEM photographs of male. A: Head region showing mouth opening, six labial papillae and an amphidial aperture (a); B: Posterior region showing protruding spicules and bursa with papillae 4-8; C: Posterior region showing bursa with papillae 1-7, note that papilla 4 does not curve laterally; E: Spicule; F: Gubernaculum; D: Light microscope photograph of posterior region showing a well-curved gubernaculum. (Scale bars: A = 2.4 μm; B = 16.8 μm; C = 10 μm; D = 25.8 μm; E = 16.8 μm; F = 4.3 μm.)*

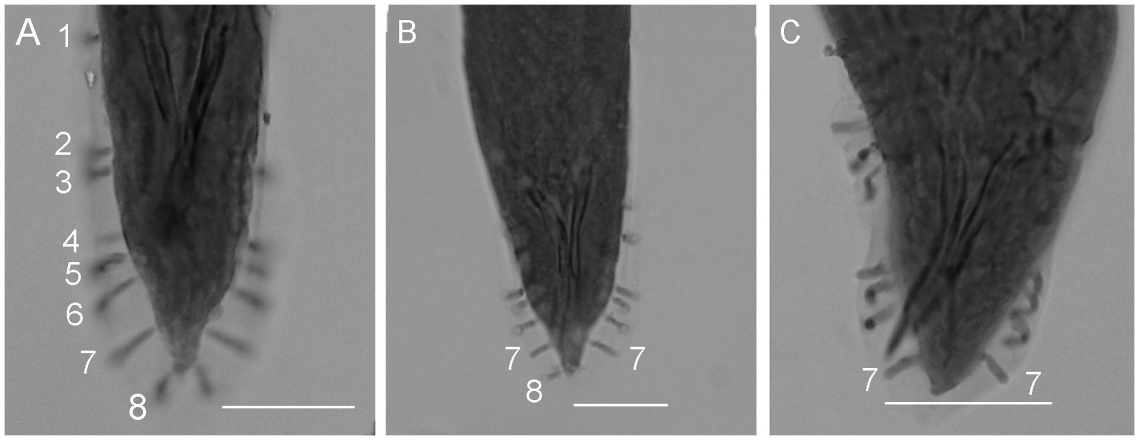

Fig. 3. Heterorhabditis amazonensis *n. sp. Male tails with bursal papillae. A: Bursa with two pairs of bursal papillae in terminal group; B: Bursa with two bursal papillae on one side and one papilla on other side in terminal group; C: Bursa with one pair of bursal papillae in terminal group. (Scale bars: A = 28.7 μm; B = 27 μm; C = 31.3 μm.)*

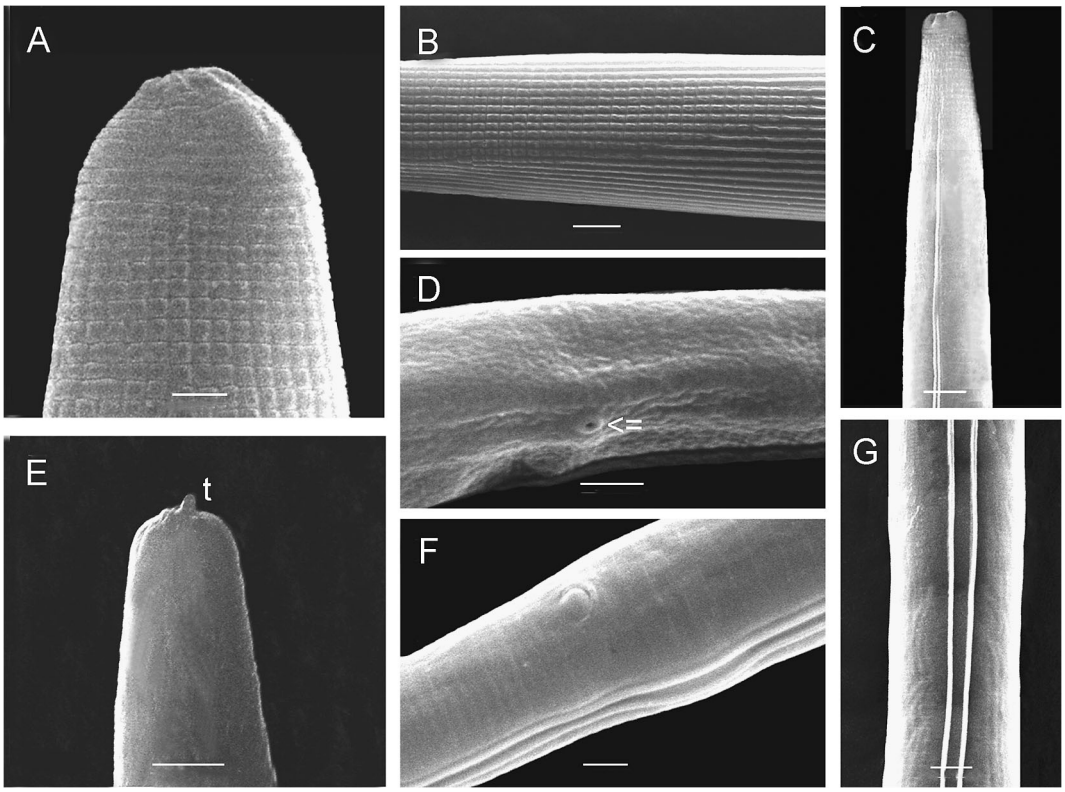

Fig. 4. Heterorhabditis amazonensis *n. sp. SEM photographs of infective juvenile. A: Anterior region of an ensheathed infective juvenile (IJ) showing head with seven annules and body with tessellate pattern; B: Portion of body showing tessellate pattern and longitudinal ridges; C: Anterior region of exsheathed IJ showing lateral field in anterior region with one ridge, then changing to two ridges; D: Phasmid near mid-tail of infective juvenile (arrow); E: Anterior region of exsheathed IJ showing dorsal tooth (t); F: Lateral field and anus in posterior region; G: Lateral field at mid-body with two ridges. (Scale bars: A, B, C, E, G = 4 μm; D, F = 2 μm.)*

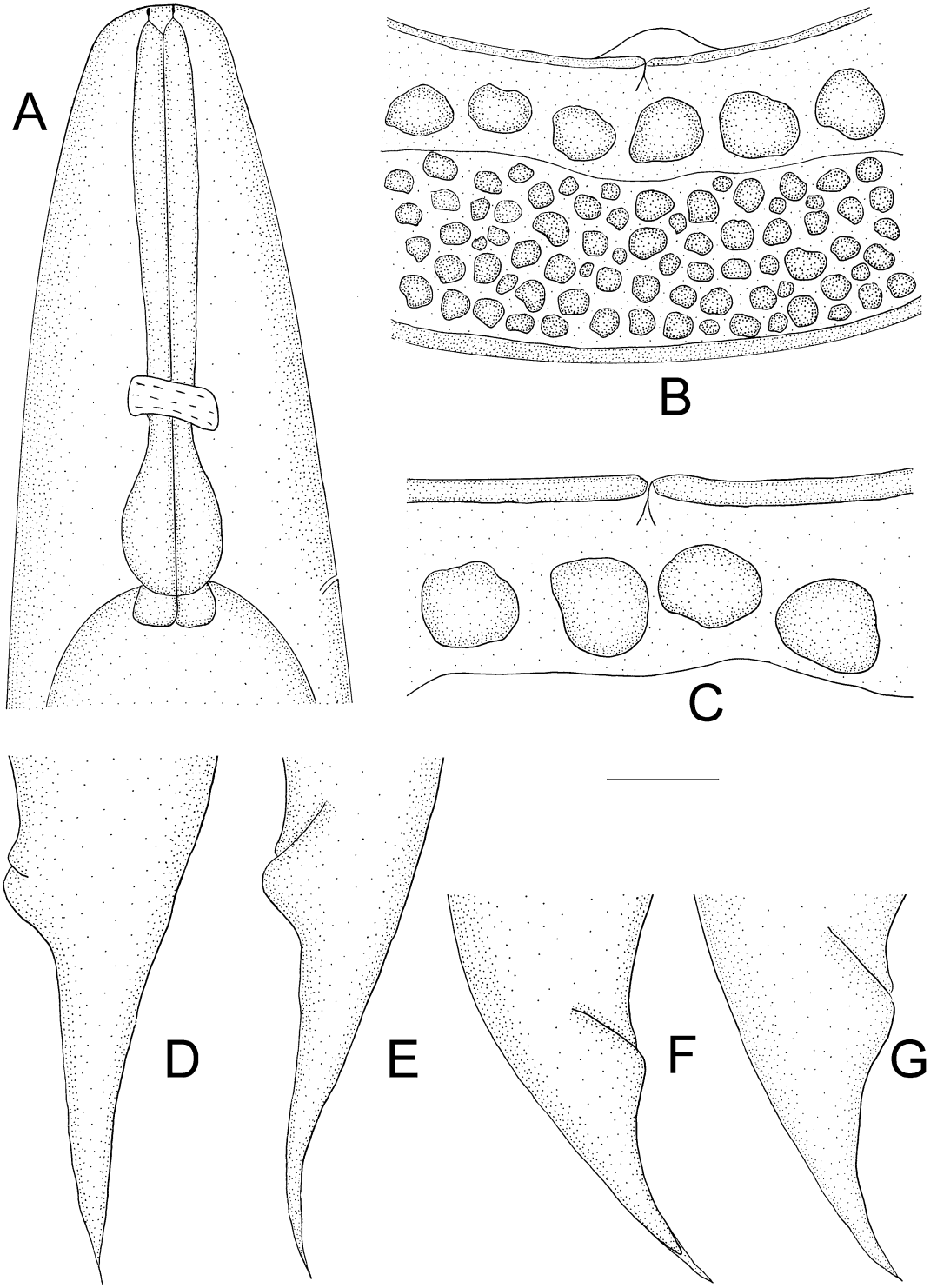

Fig. 5. Heterorhabditis amazonensis *n. sp. female. A: Anterior region; B: Vulval region of amphimictic female showing exudates covering vulva; C: Vulval region of hermaphroditic female; D, E: Variation of tail of hermaphroditic females; F, G: Variation of tail of amphimictic females. (Scale bar: A-G = 25 μm.)*

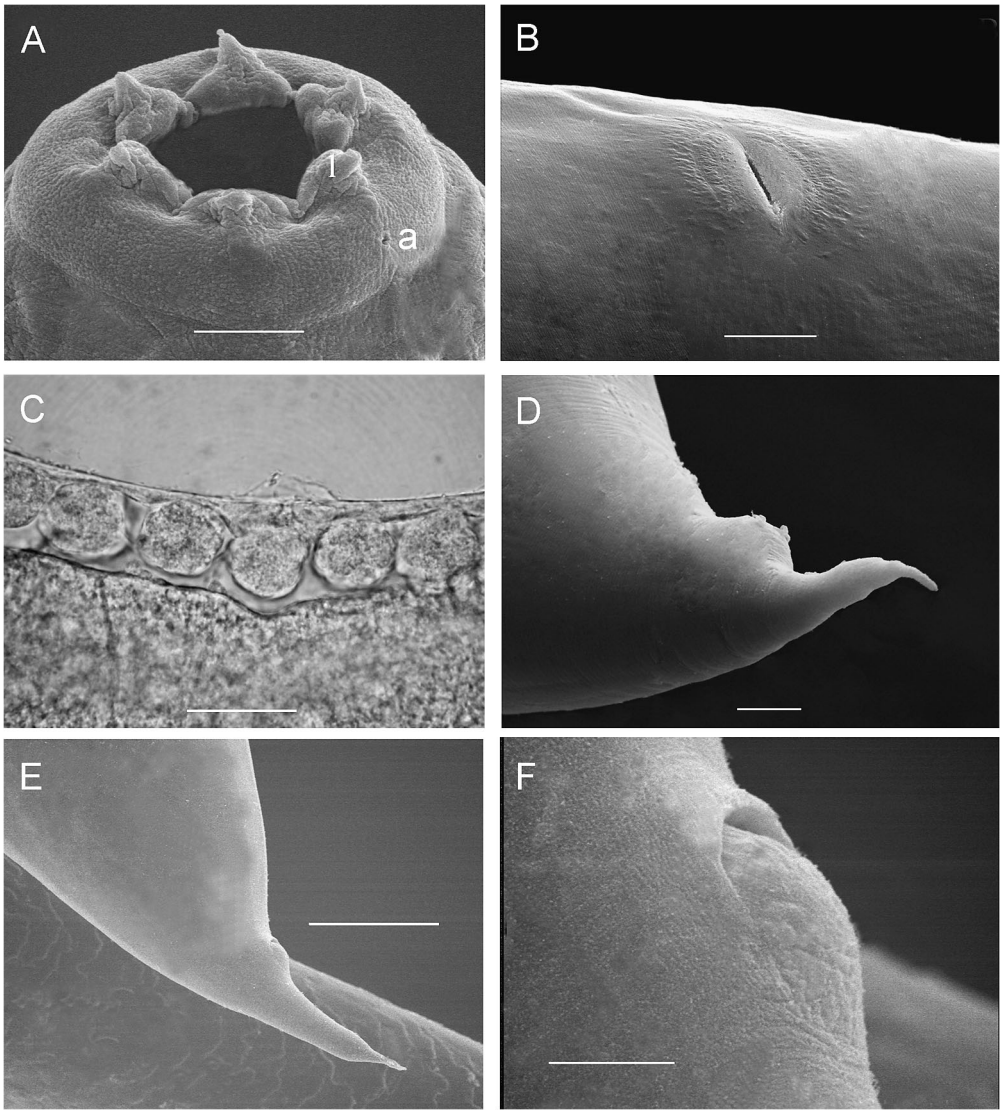

Fig. 6. Heterorhabditis amazonensis *n. sp. A, B, D: SEM photographs of hermaphroditic female. A: Anterior region showing six prominent lips (l), six labial papillae and an amphidial aperture (a); B: Vulva showing elliptical pattern; D: Tail showing postanal swelling with two papilla-like structures; C: Light microscope photograph of vulva of amphimictic female covered with exudates or copulation plug; E: Variant tail shape of hermaphroditic female; F: Anus of hermaphroditic female. (Scale bars: A = 8.6 μm; B = 20 μm; C = 23 μm; D = 10 μm; E = 93 μm; F = 4.9 μm.)*

of pharynx. Hemizonid, when observed, located 2-3 annules anterior to excretory pore. Pharynx typical for *Heterorhabditis*. Lateral field beginning anteriorly with one ridge, but a short distance posteriorly (2-3 body diam), a second ridge occurs, thus forming two ridges. Two-ridge lateral field pattern unchanged for remainder of body. Phasmid in lateral field, pore-like, prominent, located near mid-tail. Tail elongate conoid with pointed terminus. Tail length without sheath *ca* 65% of tail length with sheath.

Type host and locality

Natural host unknown. Nematode collected by baiting from a forest in the northern part of the state of Amazonas, near the city of Benjamin Constant, Brazil.

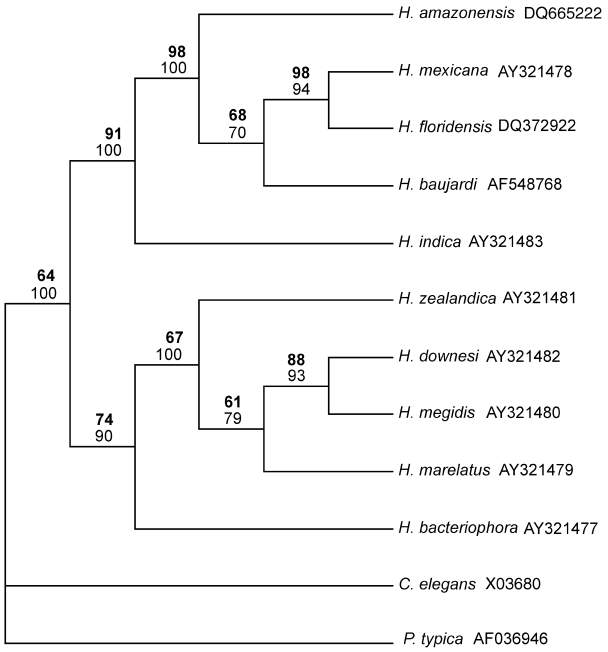

Fig. 7. *Phylogenetic relationships of ten species of* Heterorhabditis *based on analyses of ITS rDNA regions. Maximum parsimony and maximum likelihood analyses produced identical trees. Bootstrap support indices for maximum parsimony (1000 replicates) are in regular font and maximum likelihood (100 replicates) indices are in bold.*

TYPE MATERIAL

Holotype (male), allotype (female) and paratypes (hermaphrodites, females, male and infective juveniles in 3% formalin) isolated from haemocoel of *G. mellonella* deposited in the United States Department of Agriculture Nematode Collection (USDANC), Beltsville, MD, USA. Slide of one male and one female paratype of the second generation, and several infective juveniles deposited in the California Collection of Nematodes, University of California-Davis Nematode Collection, Davis, CA, USA. Several paratype hermaphrodites, females, male, and all stages in 3% formalin deposited in the Entomology and Nematology Department, University of Florida, Gainesville, FL, USA.

DIAGNOSIS AND RELATIONSHIPS

Heterorhabditis amazonensis n. sp. is characterised by a combination of morphological and morphometric characters of the male, female and infective juvenile. For males, the number of papillae in the terminal group of bursa is variable either with two pairs of papillae (50%; Fig. 4A), with two papillae on one side and one papilla on the other side, (25%; Fig. 4B), or with one pair of papillae (25%; Fig. 4C). Twenty percent of the population has a well-curved gubernaculum. The percentage of the gubernaculum to spicule length (GS%) is 51, similar to *H. baujardi* but lower than *H. mexicana* (50 *vs* 56), and the length of the spicule relative to anal body diam. (SW%) is 152, lower than that of *H. mexicana* (167) and *H. baujardi* (182). The D% of the three nematodes is also different. Females possess a typical vulva pattern (Fig. 6B). This pattern is different from other closely related species as in Figure 5 of Nguyen *et al.* (2006). The infective juvenile has a body length of 589 (567-612) μm, EP = 107 (89-115) μm, ES = 121 (107-132) μm, tail length = 107 (98-115) μm, and a = 26 (24-29).

Heterorhabditis amazonensis n. sp. is morphologically similar to *H. floridensis*, *H. baujardi*, *H. mexicana* and *H. indica*, and can be distinguished from these species mainly by male and infective juvenile characters (Tables 2, 3). The new species can be distinguished from *H. bacteriophora*, *H. indica*, *H. megidis*, *H. zealandica* and *H. marelatus* by the body length, pharynx length of the IJ, D% (distance from anterior end/pharynx length × 100), GS% and SW% of males. This new species can be further characterised by molecular characteristics of ITS regions of ribosomal DNA.

MOLECULAR ANALYSIS

DNA characterisation

The ITS rDNA regions, flanked by primers 18S and 26S, of *Heterorhabditis amazonensis* n. sp. are characterised by the sequence lengths (1010 base pairs (bp), ITS1 = 395 bp, ITS2 = 211 bp), and nucleotide usage composition (Table 4). Compared to sequences of nine other species in the genus *Heterorhabditis*, the sequence length of the new species is longer than that of six other species, the same length as that of *H. mexicana* and shorter than that of two species, *H. bacteriophora* (1021 bp) and *H. floridensis* (1012 bp). The ITS1 sequence length of the new species (195 bp) is longer than that of all other species (1-25 bp longer); it is only one bp longer than that of *H. mexicana* (ITS1 = 394 bp). The sequence length of the ITS2 region of the new species, 211 bp, is the same as that of *H. baujardi* and *H. marelatus* but shorter than that of all other species (Table 4). The nucleotide composition of all ten species of *Heterorhabditis* is presented in Table 4. The reconstructed nucleotide character transformations (Fig. 8) show that the new species differs from its closest taxon *H. baujardi* at 15 aligned positions, three

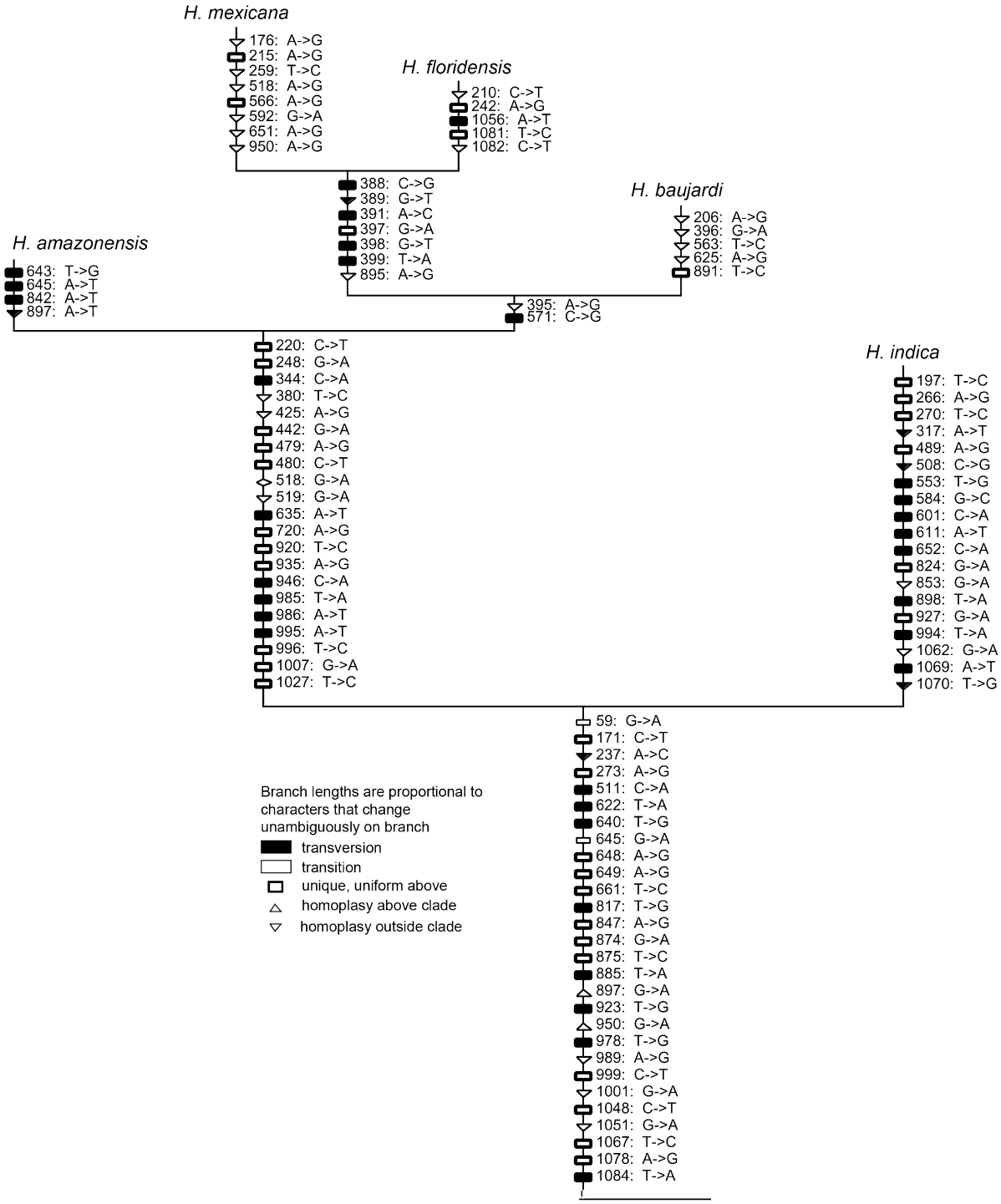

Fig. 8. *Clade containing* H. amazonensis *n. sp. and nearest neighbours in the* indica*-group (*H. amazonensis *n. sp.,* H. baujardi, H. floridensis, H. indica, H. mexicana*). Phylogeny reconstructed from ITS rDNA sequences (maximum parsimony and maximum likelihood solutions were congruent). Mapped character states (unambiguous changes only; MacClade 4.05) identify character support and autapomorphies (= unique, derived characters; rectangular boxes at the positions 643, 645 and 842 of the transformation series), all of them are transversions.*

Table 1. *Morphometrics of* Heterorhabditis amazonensis *n. sp. Measurements are in μm and in the form: mean ± sd (range).*

Character	Male		Hermaphrodite	Female	Infective juvenile
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes
n		20	20	20	20
L	843	752 ± 43 (692-826)	4734 ± 664 (3517-5587)	1580 ± 180 (1279-2070)	589 ± 12 (567-612)
a					26 ± 1.3 (24-29)
b					4.9 ± 0.3 (4.4-5.5)
c					5.5 ± 0.2 (5.1-6.1)
V			44 ± 1.5 (42-47)	48 ± 1 (46-50)	
Max. body diam.	45	41 ± 2.3 (36-43)	282 ± 25 (220-316)	100 ± 13 (70-122)	23 ± 1.2 (20-24)
Stoma length			13.8 ± 2.7 (10.1-17.4)	8.8 ± 1 (7.2-10.1)	
Stoma diam.			12.7 ± 1.6 (11.6-16)	8.1 ± 1 (7.2-8.7)	
EP	109	109 ± 6 (96-116)	211 ± 14 (184-238)	114 ± 7.8 (103-126)	107 ± 6.1 (89-115)
NR		79 ± 5 (71-88)	149 ± 12 (128-171)	85 ± 9 (68-100)	85 ± 4.9 (76-93)
ES	109	105 ± 5 (97-114)	206 ± 15 (180-225)	130 ± 7.1 (119-142)	121 ± 6.6 (107-132)
Testis reflexion	75	59 ± 10 (42-85)			
Tail length with sheath (T)					107 ± 4.7 (98-115)
Tail length without sheath	30.6	33 ± 2.7 (29-41)	128 ± 14 (104-154)	74 ± 12 (58-91)	69 ± 4.4 (59-74)
Anal body diam. (ABD)	23	27 ± 2.6 (23-33)	70 ± 6.5 (59-83)	30 ± 3.7 (25-38)	14 ± 1.4 (13-17)
Spicule length (SP)	40.6	41 ± 2.9 (35-45)			
Gubernaculum length (GU)	20	21 ± 1.5 (19-23)			
D% = EP/ES × 100	104	103 ± 3.7 (95-109)			88 ± 2.7 (83-92)
E% = EP/T × 100					100 ± 6.0 (89-109)
SW% = SP/ABD × 100	177	152 ± 20 (120-187)			
GS% = GU/SP × 100	49	51 ± 3.2 (44-56)			
Hyaline/tail × 100					64.7 ± 5.3 (52-74)

EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = distance from anterior end to end of pharynx.

Table 2. *Comparative morphometrics (μm) of male of* Heterorhabditis amazonensis *n. sp. and other related species.*

Character	FLO	MXA	BAU	IND	BAC	AMA
n	20	20	14	12	15	20
L	862 ± 44	686 ± 38	889 ± 45	721 ± 64	820	752 ± 43
	(785-924)	(614-801)	(818-970)	(573-788)	(780-960)	(692-826)
Max. body diam.	47.6 ± 2.2	42 ± 3	49 ± 2	42 ± 7	43	41 ± 2.3
	(43-50)	(38-47)	(43-53)	(35-46)	(38-46)	(36-43)
EP	117 ± 6	124 ± 10	81 ± 7	123 ± 7	121	109 ± 6
	(104-128)	(108-145)	(71-93)	(109-138)	(114-130)	(96-116)
NR	80 ± 5	71 ± 6	65 ± 7	75 ± 4	72	79 ± 5
	(73-90)	(61-83)	(54-77)	(72-85)	(65-81)	(71-88)
ES	105 ± 4	96 ± 5	116 ± 10	101 ± 4	103	105 ± 5
	(97-111)	(89-108)	(105-132)	(93-109)	(99-105)	(97-114)
Tail length	34 ± 5.8	27 ± 4	33 ± 3	28 ± 2	28	33 ± 2.7
	(29-40)	(21-36)	(28-38)	(24-32)	(22-36)	(29-41)
Anal body diam (ABD)	26.3 ± 3	24 ± 1.3	22 ± 1	23 ± 8	23	27 ± 2.6
	(20-31)	(23-27)	(20-24)	(19-24)	(22-25)	(23-33)
Spicule length (SP)	42 ± 3.5	41 ± 3.8	40 ± 3	43 ± 3	40	41 ± 2.9
	(36-46)	(30-47)	(33-45)	(35-48)	(36-44)	(35-45)
Gubernaculum length (GU)	23 ± 3.7	23 ± 3	20 ± 1.5	21 ± 3	20	21 ± 1.5
	(17-30)	(18-32)	(18-22)	(18-23)	(18-25)	(19-23)
D% = EP/ES × 100	112 ± 4	129 ± 9	70	122	117	103 ± 3.7
	(105-119)	(114-149)	–	–	–	(95-109)
SW% = SP/ABD × 100	157 ± 25	167 ± 2	182 ± 18	187	174	152 ± 20
	(133-209)	(130-196)	(138-208)	–	–	(120-187)
GS% = GU/SP × 100	53.8	56 ± 7	50 ± 5	50 ± 10	50	51 ± 3.2
	(47-65)	(43-70)	(44-61)	(40-60)	–	(44-56)

EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = distance from anterior end to end of pharynx; FLO = *H. floridensis*; MXA = *H. mexicana*; BAU = *H. baujardi*; IND = *H. indica*; BAC = *H. bacteriophora*; AMA = *H. amazonensis* n. sp.

of which are unambiguous, polarised autapomorphies. It is most divergent from *Heterorhabditis zealandica* (184 aligned positions; Table 5). Genetic distances between the new species, which are presented in Table 5, also can be used to differentiate species in the genus *Heterorhabditis*.

Phylogenetic relationships

Maximum parsimony (MP) analysis of ITS regions yielded a single most parsimonious tree (tree length = 847, CI = 0.8595, RI = 0.7959; Fig. 7). The parsimonious tree includes two monophyletic groups, which are well supported (⩾90%, regular numbers) by bootstrap analysis. The new species together with *H. baujardi*, *H. floridensis*, *H. indica* and *H. mexicana* comprise a monophyletic group (100% support) that we call the '*indica*-group' in which *H. indica* is a sister taxon to the group formed by the other four species, and *H. amazonensis* n. sp. is a sister taxon to the group formed by *H. floridensis*, *H. baujardi* and *H. mexicana*. All other species (*H. bacteriophora*, *H. downesi*, *H. marelatus*, *H. megidis* and *H. zealandica*) comprise another monophyletic group that we call the '*megidis*-group'. Maximum likelihood analysis of ITS regions generated a tree (Figs 7, 8) with a topology identical to that of the MP tree, but branch support (bold numbers) are weak for some nodes, especially in the *megidis*-group.

Morphological and molecular characteristics that we uncovered are sufficient to conclude that *H. amazonensis* is a new species.

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Table 3. *Comparative morphometrics (μm) of infective juveniles of* Heterorhabditis amazonensis *n. sp. and other related species.*

Character	FLO Nguyen *et al*., 2006	MXA Nguyen *et al*., 2004	BAU Phan *et al*., 2003	IND Poinar *et al.*, 1992	BAC Poinar, 1990	AMA Present study
n	25	25	25	25	25	20
L	562 ± 24 (554-609)	578 ± 23 (530-620)	551 ± 27 (497-595)	528 ± 26 (479-573)	558 (512-671)	589 ± 12 (567-612)
a	27.6 ± 5.2 (25-32)	25.8 (23.6-28.4)	28 ± 1 (26-30)	26 ± 4 (25-27)	25 (17-30)	26 ± 1.3 (24-29)
b	4.3 ± 2.1 (3.9-4.9)	4.6 (4.2-5.1)	4.8 ± 0.2 (4.5-5.1)	4.5 ± 0.34 (4.3-4.8)	4.5 (4-5.1)	4.9 ± 0.3 (4.4-5.5)
c	5.6 ± 2.4 (5.3-6.6)	5.9 (5.5-6.3)	6 ± 0.3 (6-6.7)	5.3 ± 0.5 (4.5-5.6)	6.2 (5.7-7)	5.5 ± 0.2 (5.1-6.1)
Max. body diam.	21.2 ± 4.6 (19-23)	23 ± 0.8 (20-24)	20 ± 2 (18-22)	20 ± 6 (19-22)	23 (18-31)	23 ± 1.2 (20-24)
EP	109 ± 10.4 (101-122)	102 ± 5.2 (83-109)	97 ± 3 (91-103)	98 ± 7 (88-107)	103 (87-110)	107 ± 6.1 (89-115)
NR	86 ± 9.2 (68-107)	81 ± 4.2 (74-88)	81 ± 3 (75-86)	82 ± 4 (72-85)	85 (72-93)	85 ± 4.9 (76-93)
ES	135 ± 11.6 (123-142)	122 ± 27 (104-142)	115 ± 3 (107-120)	117 ± 3 (109-123)	125 (10-139)	121 ± 6.6 (107-132)
Tail length with sheath (T)	103 ± 10.1 (91-113)	99 ± 4.2 (91-106)	90 ± 4 (83-97)	101 ± 6 (93-109)	98 (83-112)	107 ± 4.7 (98-115)
Tail length without sheath	63 ± 7.9 (48-68)	66 ± 3 (59-73)	– –	– –	– –	69 ± 4.4 (59-74)
Anal body diam.	14 ± 3.7 (12-16)	15 ± 1.2 (12-17)	13 ± 0.7 (11-14)	– –	– –	14 ± 1.4 (13-17)
D% = EP / ES × 100	81 ± 8.9 (71-90)	81 ± 3 (72-86)	84 ± 3 (78-88)	84 ± 5 (79-90)	84 (76-92)	88 ± 2.7 (83-92)
E% = EP / T × 100	105 ± 10.2 (95-134)	104 ± 5.2 (87-111)	108 ± 4 (98-114)	94 ± 7 (83-103)	112 (103-130)	100 ± 6.0 (89-109)

EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = distance from anterior end to end of pharynx; FLO = *H. floridensis*; MXA = *Heterorhabditis mexicana*; BAU = *H. baujardi*; IND = *H. indica*; BAC = *H. bacteriophora*; AMA = *H. amazonensis* n. sp.

Table 4. *Sequence length (base pairs = bp) and composition of ITS regions of ten species of* Heterorhabditis.

Species (sequence length)	ITS1 (bp)	5.8S (bp)	ITS2 (bp)	A	C	G	T
H. amazonensis n. sp. (1010 bp)	395	154	211	0.25941	0.20198	0.25545	0.28317
H. bacteriophora (1021 bp)	389	154	228	0.26347	0.1998	0.25661	0.28012
H. baujardi (795 bp)*	?	154	211	0.25732	0.19677	0.25089	0.29502
H. downesi (990 bp)	374	154	212	0.23838	0.21717	0.26162	0.28283
H. floridensis (1012 bp)	393	154	214	0.25988	0.20059	0.25494	0.28458
H. indica (988 bp)	370	154	215	0.26316	0.20547	0.25506	0.27632
H. marelatus (995 bp)	379	154	211	0.23417	0.21608	0.26432	0.28543
H. megidis (1005 bp)	384	154	220	0.23284	0.2199	0.27164	0.27562
H. mexicana (1010 bp)	394	154	213	0.25545	0.20198	0.2604	0.28218
H. zealandica (1003 bp)	387	154	212	0.22233	0.21834	0.27318	0.28614

* This sequence is not as complete as in other species.
? Not available.

Table 5. *Pairwise distances between taxa. Below diagonal: Total character differences; above diagonal: Mean character differences (adjusted for missing data).*

		1	2	3	4	5	6	7	8	9	10	11	12
1	*H. amazonensis* n. sp.	–	0.0685	0.0218	0.0189	0.1885	0.1596	0.1737	0.1607	0.1692	0.0198	0.44040	0.3102
2	*H. indica*	67	–	0.0786	0.09461	0.18352	0.15497	0.17140	0.15641	0.1533	0.07959	0.4289	0.3137
3	*H. mexicana*	22	77	–	0.0303	0.1965	0.16770	0.17872	0.16872	0.1773	0.0158	0.4379	0.3185
4	*H. baujardi*	15	72	24	–	0.2358	0.2059	0.2225	0.2056	0.21504	0.024	0.4897	0.40000
5	*H. zealandica*	184	176	192	179	–	0.0761	0.0996	0.086	0.1592	0.1973	0.4226	0.3566
6	*H. downesi*	154	148	162	154	75	–	0.0396	0.0374	0.1259	0.16960	0.41340	0.323
7	*H. megidis*	168	163	173	168	98	39	–	0.0589	0.1405	0.18060	0.4244	0.3314
8	*H. marelatus*	156	150	164	155	85	37	58	–	0.1251	0.1706	0.416	0.3243
9	*H. bacteriophora*	165	147	173	163	157	123	137	123	–	0.1791	0.4322	0.3178
10	*H. floridensis*	20	78	16	19	193	164	175	166	175	–	0.4415	0.3147
11	*C. elegans*	436	416	434	380	415	401	418	406	430	438	–	0.4344
12	*P. typica*	161	160	165	144	184	167	172	168	164	163	235	–

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