Phasmarhabditis akhaldaba sp. n. associated with a slug *Deroceras reticulatum* in Lesser Caucasus mountains in Republic of Georgia

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Summary. A new nematode species, *Phasmarhabditis akhaldaba* sp. n., was isolated in Borjomi municipality (Republic of Georgia). The species was characterised by the presence of two strains, the first isolated from the slug *Deroceras reticulatum* and another one from the cadaver of *Galleria mellonella* larva used as a bait for entomopathogenic nematodes in soil. Strains designated as 'quick' and 'slow' differed in the body lengths of dauer juveniles and the visually different speed of movement during migration. Morphologically, the adult stages are characterised by a head with six low, rounded lips bearing a ring of six labial papillae, four cephalic papillae and pore-like amphids situated on top of lateral lips, males with a moderately wide bursa and spicules 65-67 µm long lacking holes at the distal tips. Average length of an ensheathed dauer juvenile of *P. akhaldaba* sp. n. is 862 µm in the strain 'quick' and 702 µm in the strain 'slow'. The molecular analysis based on partial sequences of ITS rDNA regions of both strains has shown its identity. Both morphologically and genetically, the new species is close to another species from Caucasus, *P. circassica* Ivanova, Geraskina & Spiridonov, 2020.

Key words: description, ITS rDNA sequences, molecular, Mollusca, morphology, morphometrics, new species, *Pellioditis*, phylogeny, SSU, taxonomy.

Two isolates of rhabditid nematodes were recovered during a collection trip in Borjomi municipality in the central part of Republic of Georgia. The isolate recovered from a slug *Deroceras reticulatum* (O.F. Müller, 1774) was designated 'quick' due to the rapid movement of infective juveniles during migration into the water. The isolate 'slow' showed visibly lower speed of migration and was recovered from the surface of the cadaver of *Galleria melonella* (Linnaeus, 1758) larvae used as an insect trap for entomopathogenic nematodes in the soil sample. An integrative approach was used to identify both isolates. Both morphological and molecular analyses confirmed that the nematodes of both isolates belong to the genus *Phasmarhabditis* Andrássy, 1976. Morphological examination has shown the presence of certain differences in morphology between the isolates, while the ITS rDNA sequences obtained for both isolates were nearly identical. Currently, the genus *Phasmarhabditis* includes 14 species from all over the world with two species recently described from an area close to the present species location, *i.e.* Caucasus. All but one species of the genus was found in association with terrestrial gastropods. Using the name *Phasmarhabditis*, we follow the opinion expressed by Nermuť *et al.* (2016a), Ross *et al.* (2018) and Ivanova *et al.* (2020) on the current taxonomic status of the genus. Description of *Phasmarhabditis akhaldaba* sp. n. is presented below.

MATERIAL AND METHODS

Both isolates originated from a private hazelnut garden at the village of Akhaldaba (Borjomi municipality, Republic of Georgia), coordinates 41.908 N 43.520 E at 760 m a.s.l. 5 June 2018.

A specimen of *Deroceras reticulatum* collected in the garden was rinsed in water and then dissected in the laboratory and the nematode juveniles found were cultivated on the slug body parts to obtain all

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developmental stages (isolate 'quick'). A soil sample collected in the garden was inoculated by larvae of the greater wax moth *G. melonella* to attract entomopathogenic nematodes according to Hominick *et al*. (1997). After a week in soil, dead wax moth larvae were removed and examined under a stereoscopic microscope. One specimen of wax moth larvae was found covered in multiplying rhabditid nematodes. These nematodes were designated as 'slow' and were cultivated onwards on freeze-killed *Helix* sp. either on 2% agar with the addition of 5% of pig kidney. Some of the progeny of both isolates was collected and preserved in hot 4-5% formalin for morphological examination and several specimens were also frozen for DNA extraction. Live nematodes from the culture were photographed using a Leica microscope equipped with a digital camera. Nematodes preserved in formalin were processed to anhydrous glycerin for light microscopy as described by Seinhorst (1959). Light microscopic studies and drawings were done using Nikon Eclipse 200 microscope equipped with a drawing attachment. Scale bars are given in micrometres (μm). Abbreviations: V% – distance from anterior extremity to vulva to body length in %; a, b, c – de Manian indices. Illustrations were prepared using WACOM Intuos A4 USB drawing tablet and Adobe Illustrator CS5. For the SEM studies, formalin-preserved material was dehydrated, critical point dried and coated with gold. Images were taken on a Tescan CamScan MV 2300 and Mira 3Tescan.

Molecular characterisation and phylogenetic analysis. For DNA extraction, juveniles recovered from the snail and mature females and males from cultures were frozen individually in 0.7 ml Eppendorf tubes. DNA was extracted according to the method described by Holterman *et al.* (2006). The worm-lysis solution (950 μl of a mixture of 2 ml of 1 M NaCl, 2 ml of 1 M Tris-HCl, pH 8 plus 5.5 ml of deionised water plus 10 μl of mercaptoethanol and 40 μl of proteinase K, 20 mg ml^{-1}) was prepared directly before DNA extraction. Aliquots of 25 μl of sterile water and 25 μl of worm-lysis solution were added to each tube with a nematode and incubated at 65°C for 90 min. The tubes containing the homogenate were then incubated at 99°C for 5 min to deactivate proteinase K. About 1.0 μl of homogenate was used as PCR template.

PCR reactions were performed using Encyclo Plus PCR kit (Evrogen®, Moscow, Russia) according to the manufacturer's protocol. A pair of primers LSU391 (5'-AGC GGA GGA AAA GAA ACT AA-3') and LSU501 (5'-TCG GAA GGA ACC AGC TAC TA-3') was used to amplify a 1100 bp long sequence of D2-D3 expansion segment of 28S rDNA (Nadler *et al.*, 2000). PCR cycling parameters included denaturation at 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 54°C for 35 s, and 72°C for 70 s.

A pair of primers 18S (5'-TTG ATT ACG TCC CTG CCC TTT-3') and 26S (5'-TTT CAC TCG CCG TTA CTA AGG-3') was used to amplify approx. 1100 bp long sequence of ITS region of ribosomal DNA (Vrain *et al.*, 1992; Curran & Driver, 1994). PCR cycling parameters included primary denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 45 s, 54°C for 60 s and 72°C for 70 s.

PCR products were visualised in agarose gel and bands were excised for DNA extraction with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). Samples were directly sequenced using the same primers as used for primary PCR. The sequences were combined and aligned using the Clustal_X program after the addition of sequences from the GenBank (Thompson *et al.*, 1997). Subsequently, the sequences were edited using the Genedoc 2.7 program (Nicholas *et al.*, 1997), to prepare a file for the analysis in MEGA5 (Tamura *et al.*, 2011). Phylogenetic trees were obtained with different methods (MP – maximum parsimony, NJ – neighbour joining and ML – maximum likelihood) and pairwise nucleotide differences were calculated. Obtained sequences were deposited in GenBank (MZ265149 for the ITS rDNA sequence and MZ265148 for the 28S rDNA sequence.

Etymology. The specific epithet reflects the location of the new nematode finding.

DESCRIPTION

Phasmarhabditis akhaldaba sp. n.
Figs 1-6

Measurements. See Table 1 & 2.

Adults. Body length from 1 mm to 3 mm. Body straight when relaxed, slightly tapering to anterior end. Cuticle *ca* 1 μm thick, finely annulated and longitudinally striated. Lateral field 3-5 μm wide comprising central elevated double ridge flanked by marginal single ridge at each side. Along with marginal ridges, coarse longitudinal striae forming four fine bands at each side. Head truncate, mouth opening triangular. Lip region relatively wide, comprising six low lips. Lips continuous with body contour ('slow') or slightly offset ('quick') bearing two circles of papillae. Six lips grouped in pairs, each

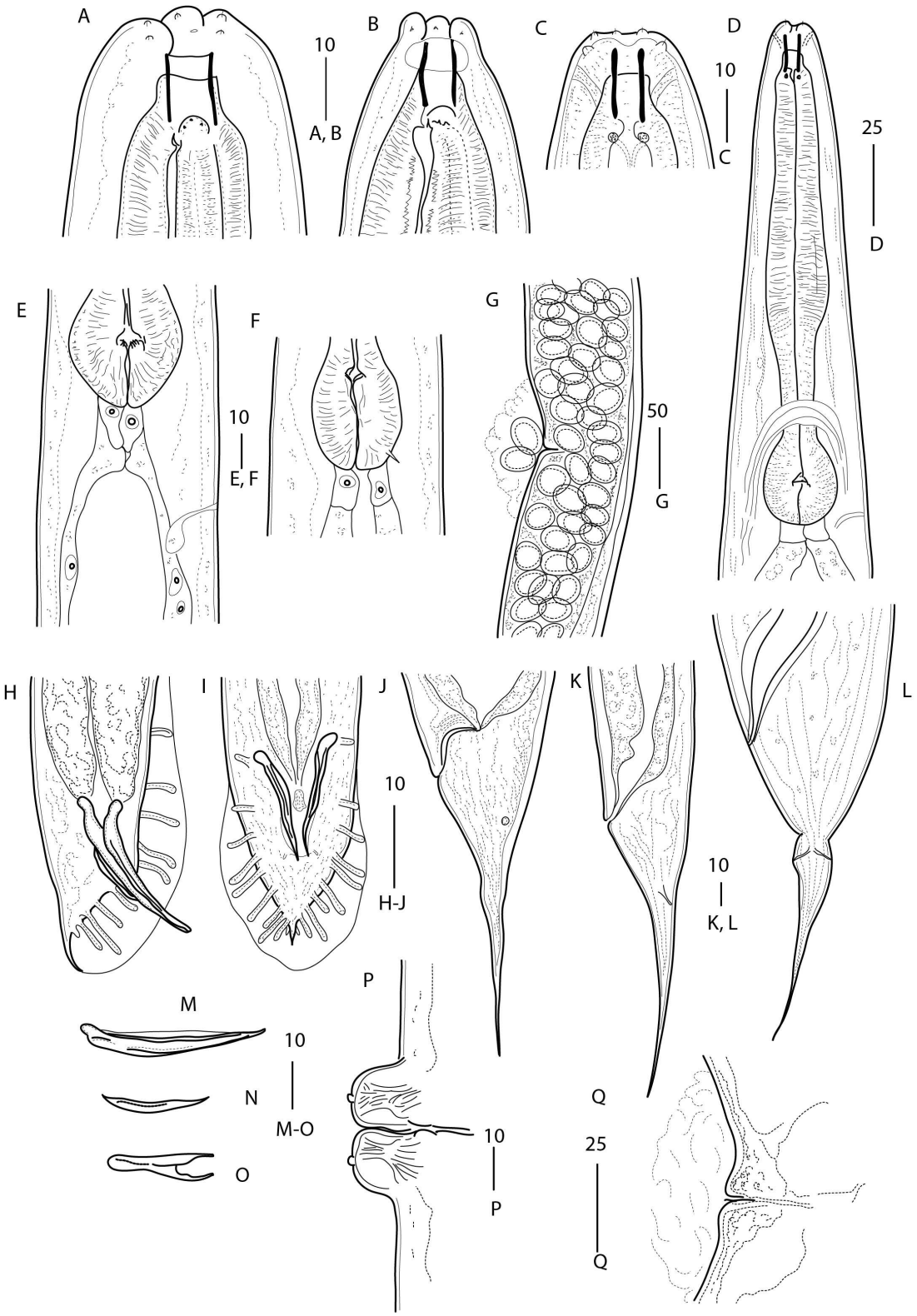

Fig. 1. *Phasmarhabditis akhaldaba* sp. n. Adults. A-C, cephalic end. A, female 'slow' dorsal; B, male 'quick' subdorsal; C, female, lateral; D, anterior end, female, lateral; E & F region of basal bulb; E, female, showing excretory pore; F, female, showing deirid; G, female, midbody region; H-I, male tale, H lateral, I ventral; J-L, female tail, J and K slimmer ('quick' strain) (lateral) and L 'slow' sublateral; M spicule lateral; N gubernaculum lateral, O gubernaculum ventral; P-Q – vulva region. Scale bars in µm.

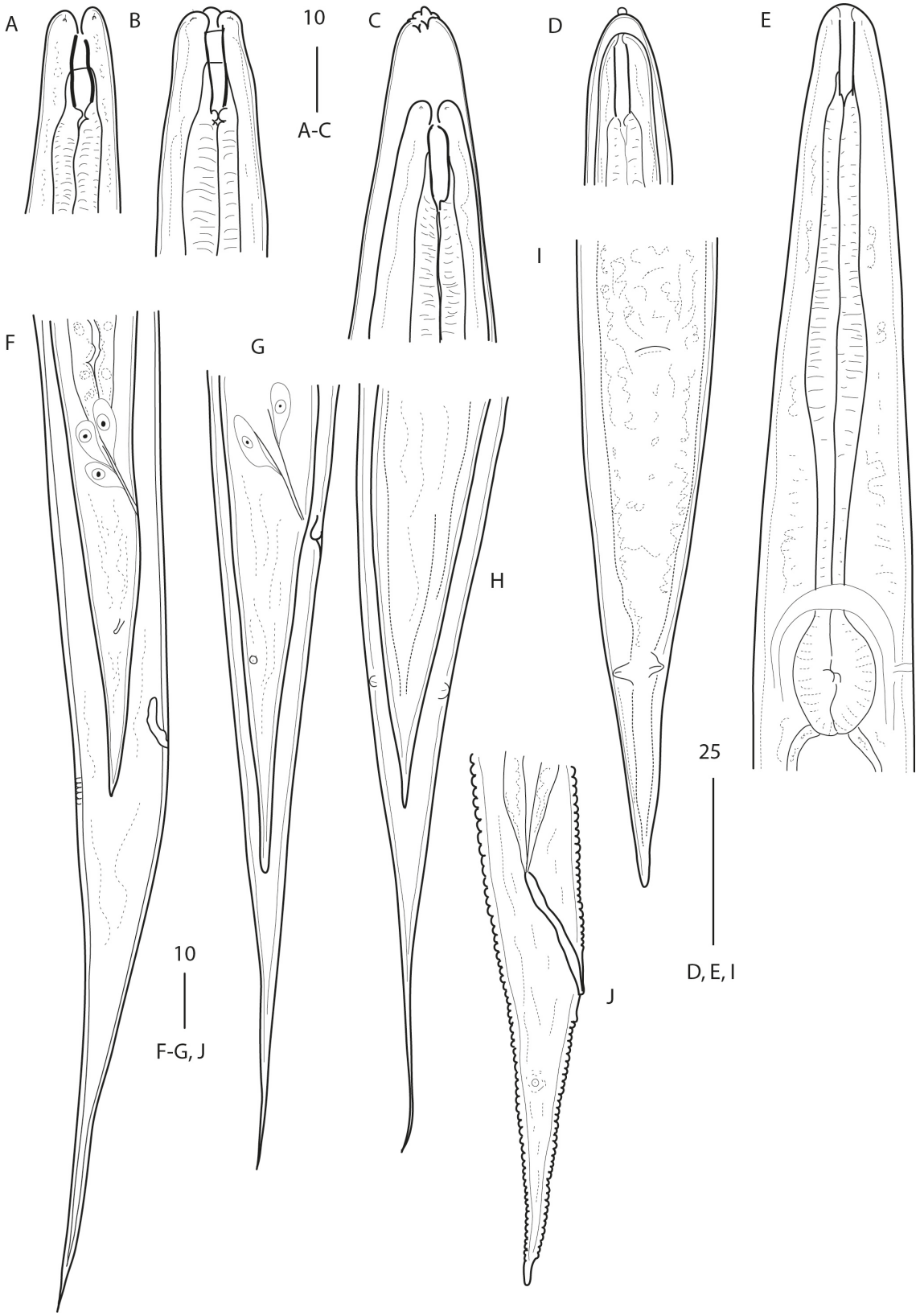

Fig. 2. *Phasmarhabditis akhaldaba* sp. n. Infective juveniles (IJ). A-D, cephalic end: A & B, exsheathed IJ, lateral; C & D, ensheathed IJ, lateral; E, anterior end, exsheathed IJ, lateral; F-J, tail: F-H, ensheathed IJ, F & G lateral, H, ventral; I-J, exsheathed IJ: I, ventral, J, lateral. Scale bars in µm.

Table 1. Morphometric data of two strains of *Phasmarhabditis akhaldaba* sp. n.
All measurements are in µm and in the form: mean ± s.d. (range).

Species	*P. akhaldaba* sp. n. isolate 'quick'			*P. akhaldaba* sp. n. isolate 'slow'	
Character	Female		Male	Female	Male
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes
n	–	11	13	10	15
L	2240	1983 ± 406 (1320-2940)	1271 ± 198 (980-1930)	2712 ± 376 (1780-3080)	2163 ± 237 (1480-2410)
a	23.1	22.2 ± 3.2 (19.3-28.7)	24 ± 2.4 (21.3-29.2)	20.4 ± 2.8 (17-26.1)	25.0 ± 3.3 (19.8-30.6)
b	7.6	7.5 ± 1.2 (6.1-9.1)	6.4 ± 0.7 (5.4-7.9)	9.9 ± 1 (7.5-10.8)	8.9 ± 0.7 (7.5-9.9)
c	17.9	17.0 ± 4.2 (9.1-24.5)	38.9 ± 9.2 (31.6-66.5)	20.6 ± 4 (12.7-28)	57.1 ± 7 (47.9-74.7)
c'	2.7	3.4 ± 0.9 (2.2-5.2)	1.1 ± 0.2 (0.8-1.4)	2.6 ± 0.7 (2-4.4)	1.1 ± 0.1 (0.9-1.2)
V	50.0	52.3 ± 0 (49.2-57.2)	–	51.1 ± 0 (49.3-52.9)	–
Max. body diam	97	91 ± 22 (60-140)	53 ± 8 (42-78)	135 ± 25 (74-155)	87 ± 12 (58-102)
Anal body diam.	47	37 ± 8 (28-54)	30 ± 4 (22-35)	53 ± 9 (32-63)	36 ± 4 (30-40)
Lip region diam.	17	16 ± 2 (13-20)	13 ± 2 (10-16)	21 ± 2 (18-25)	18 ± 2 (15-20)
Stoma total length	24	21 ± 2 (17-24)	17 ± 1 (14-19)	23 ± 1 (20-25)	21 ± 2 (18-24)
Cheilostom length	5	4 ± 1 (3-6)	4 ± 1 (2-6)	6 ± 1 (5-7)	4 ± 1 (3-5)
Gymnostom length	4	5 ± 1 (4-7)	5 ± 1 (3-7)	5 ± 1 (3-6)	5 ± 1 (4-6)
Stegostom length	15	12 ± 2 (8-15)	9 ± 2 (6-12)	12 ± 1 (10-14)	12 ± 1 (10-14)
Stoma max. diam.	7	7 ± 1 (5-8)	7 ± 1 (5-8)	8 ± 1 (7-9)	6 ± 1 (5-8)
Corpus length	172	157 ± 15 (125-172)	117 ± 9 (104-140)	163 ± 11 (140-172)	147 ± 9 (123-158)
Mid-corpus diam.	25	20 ± 4 (13-25)	13 ± 2 (10-15)	22 ± 3 (18-26)	18 ± 1 (16-20)
Metacorpal expansion	35	33 ± 4 (24-38)	22 ± 1 (20-25)	38 ± 5 (26-42)	29 ± 2 (26-32)
Isthmus length	82	65 ± 11 (45-82)	46 ± 4 (40-55)	58 ± 5 (50-69)	51 ± 5 (40-61)
Isthmus diam.	17	16 ± 4 (10-25)	11 ± 2 (9-14)	17 ± 1 (14-19)	14 ± 1 (10-15)
Bulb length	56	45 ± 10 (28-59)	33 ± 4 (26-40)	50 ± 4 (43-55)	45 ± 5 (35-56)
Bulb diam.	35	34 ± 5 (26-44)	24 ± 2 (22-28)	38 ± 3 (30-42)	34 ± 3 (27-38)
Pharynx total length	296	263 ± 24 (215-296)	197 ± 11 (180-220)	273 ± 17 (238-287)	242 ± 14 (198-260)
Corpus to pharynx (%)	58.1	59.6 ± 0 (55.2-63.0)	59.2 ± 0 (54.2-63.6)	59.7 ± 0 (56.4-62.6)	60.9 ± 0 (58.7-64.2)
Apex to excretory pore	300	260 ± 33 (195-300)	183 ± 29 (129-243)	283 ± 22 (245-317)	232 ± 29 (167-285)
Apex to nerve ring	208	190 ± 13 (165-210)	149 ± 16 (125-175)	207 ± 15 (179-230)	189 ± 12 (152-205)
Apex to anterior ovary reflexion	700	604 ± 172 (320-720)	–	625 ± 199 (420-1150)	–
Tail extremity to posterior ovary reflexion	550	555 ± 34 (310-750)	–	637 ± 93 (513-800)	–
Vagina length	67	67 ± 3 (65-70)	–	71 ± 16 (48-90)	–
Cardia length	8	10 ± 4 (6-18)	9 ± 1 (7-11)	11 ± 3 (7-15)	11 ± 2 (7-15)
Egg length	–	53 ± 4 (50-57)	–	53 ± 6 (45-62)	–
Egg diam.	–	29 ± 2 (27-30)	–	34 ± 4 (28-38)	–

Table 1 (continued). Morphometric data of two strains of *Phasmarhabditis akhaldaba* sp. n. All measurements are in µm and in the form: mean ± s.d. (range).

Rectum length	55	48 ± 7 (38-55)	–	49 ± 16 (30-70)	–
Tail length	125	120 ± 19 (92-147)	33 ± 4 (26-38)	134 ± 14 (110-162)	38 ± 4 (30-43)
Anus to phasmid	55	44 ± 9 (27-55)	–	69 ± 10 (55-80)	–
Apex to testis reflexion	–	–	308 ± 46 (222-390)	–	398 ± 46 (350-500)
Testis reflexion length	–	–	197 ± 51 (118-290)	–	373 ± 82 (220-500)
Spicule length (chord)	–	–	65 ± 6 (54-77)	–	67 ± 4 (61-75)
Gubernaculum length	–	–	38 ± 4 (30-44)	–	37 ± 3 (32-42)

lip bearing a small, short labial papilla in apical position. Each sublateral lip bearing small cephalic papilla located slightly posterior to labial one. Slit-like amphids situated close to labial papillae on top of lateral lips. Mouth aperture triangular. Stoma typical for the genus, tubular, wide and short (about as long as lip region diam.). Cheilostom: gymnostom: stegostom ratio 1:2:3. Cheilostom not cuticularised. Gymnostom walls parallel, thickened. Stegostom with glottoid apparatus, isomorphic, isotropic, metarhabdions thickened, warts prominent. Pharyngeal collar narrow, covering two thirds of stoma length. Pharynx wide, muscular, procorpus with pronounced metacorpal expansion, short isthmus and rounded terminal bulb as wide as metacorpal expansion. Terminal bulb with valve and haustrulum. Corpus corresponding to 60 (54-64)% of pharynx length. Nerve ring surrounding posterior of isthmus. Excretory pore distinct, at level of bulb base or slightly posterior to it, excretory duct short, weak. Cardia well-developed, projecting into intestine. Intestine expanded posterior to pharynx base. Deirids at level of pharynx base. Rectal glands present.

Male. Body shorter and slimmer than that of female. Cheilostom, gymnostom and stegostom mean lengths 4 µm, 5 µm and 9 µm, respectively, in strain 'quick' and 4 µm, 5 µm and 12 µm in strain 'slow'. Spermatocytes rounded, arranged in two rows. *Vas deferens* wide, filled with large immature sperm cells *ca* 5-6 µm in diam., ejaculatory duct separating from latter by constriction. Testis flexure length variable, from mean 197 µm in strain 'quick' to 373 µm ('slow'). Tail short, tail tip reaching edge of bursa. Bursa peloderan, anteriorly open, wide, genital papillae (GP) formula 5/4 or 1+2+2/1+3 + ph. GP5 and 9 opening on dorsal side of bursa. Phasmids located posterior to GP9. Spicules usually non-cephalate, length variable, gradually tapering to pointed distal tips. Three pericloacal papillae: prominent precloacal papilla on anterior cloacal lip in median position and two process-like papillae at lateral margins of cloacal opening. Spicules nearly straight, not distinctly cephalate, uniformly thick for most of spicule length and tapering to pointed distal tips. Velum indiscernible. Gubernaculum boat-shaped, about half as long than spicules; dorsal and ventral processes not observed.

Female. Body obese. Cheilostom, gymnostom and stegostom mean lengths 4 µm, 5 µm and 12 µm, respectively, in strains 'quick' and 6 µm, 5 µm and 12 µm, 'slow'. Ovoviviparous. Gonads amphidelphic, ovaries reflexed on dorsal side. Anterior and posterior ovary branches equally long. Oocytes rounded, large, arranged in two or three rows. Oviduct short. Spermathecae filled with sperm cells. Uterus spacious, containing 20-30 eggs with thin, smooth shells. Vagina straight, muscular, less than half corresponding body diam. long. Vulva median, a wide transverse slit. Epyptigma present. Vulval lips flat in mature specimens and inflated in younger ones. Massive copulatory plug over vulva present in fertilised specimens. Rectum inflated, about corresponding body diam. long. Anus an arcuate slit. Tail shape variable depending on body size: from conoid in slimmer specimens to cupola-shaped with a spine. Spine (terminal part of tail) half-tail length long. Thereafter, nearly 100% of females from the strain 'quick' possess a conical tail, while about 80% of 'slow' females are characterised by a cupola-shaped tail with a spine. Phasmids thin, long, slightly projecting, located at one-third tail length from anus.

Dauer juvenile (ensheathed). Body straight when heat-killed, slender, tapering towards head end. Cuticle loose, transversally and longitudinally striated; longitudinal striations appearing posterior to 20-22nd annulus. Lateral field 7-8 µm wide at mid-body, with central band *ca* 2 µm wide flanked with three slightly elevated, even ridges at each side. Head rounded, distinct apical cuticular cap present, lip region low, not offset from body contour, not divided

into lips; six cephalic papillae, amphidial apertures prominent, situated posterior to circle of cephalic papillae. Mouth aperture enclosed. Stoma long, narrow; promesorhabdions slightly curved inwards at both ends. Pharynx comprising straight, narrow corpus with slightest metacorpal expansion, isthmus narrow, half as long as corpus and pear-shaped, valvate basal bulb only slightly wider than metacorpal expansion. Nerve ring surrounding isthmus. Cardia prominent, elongated, protruding into intestine. Excretory pore poorly discernible. Deirids inconspicuous. Intestine walls filled with fat globules. Genital primordium short, located at mid-body. Tail elongate conoid with filamentous terminus. Phasmids pore-like, situated anterior to mid-tail.

Dauer juvenile (exsheathed). Similar to ensheathed dauer juvenile in internal morphology. Cuticle without longitudinal striations. Lateral fields

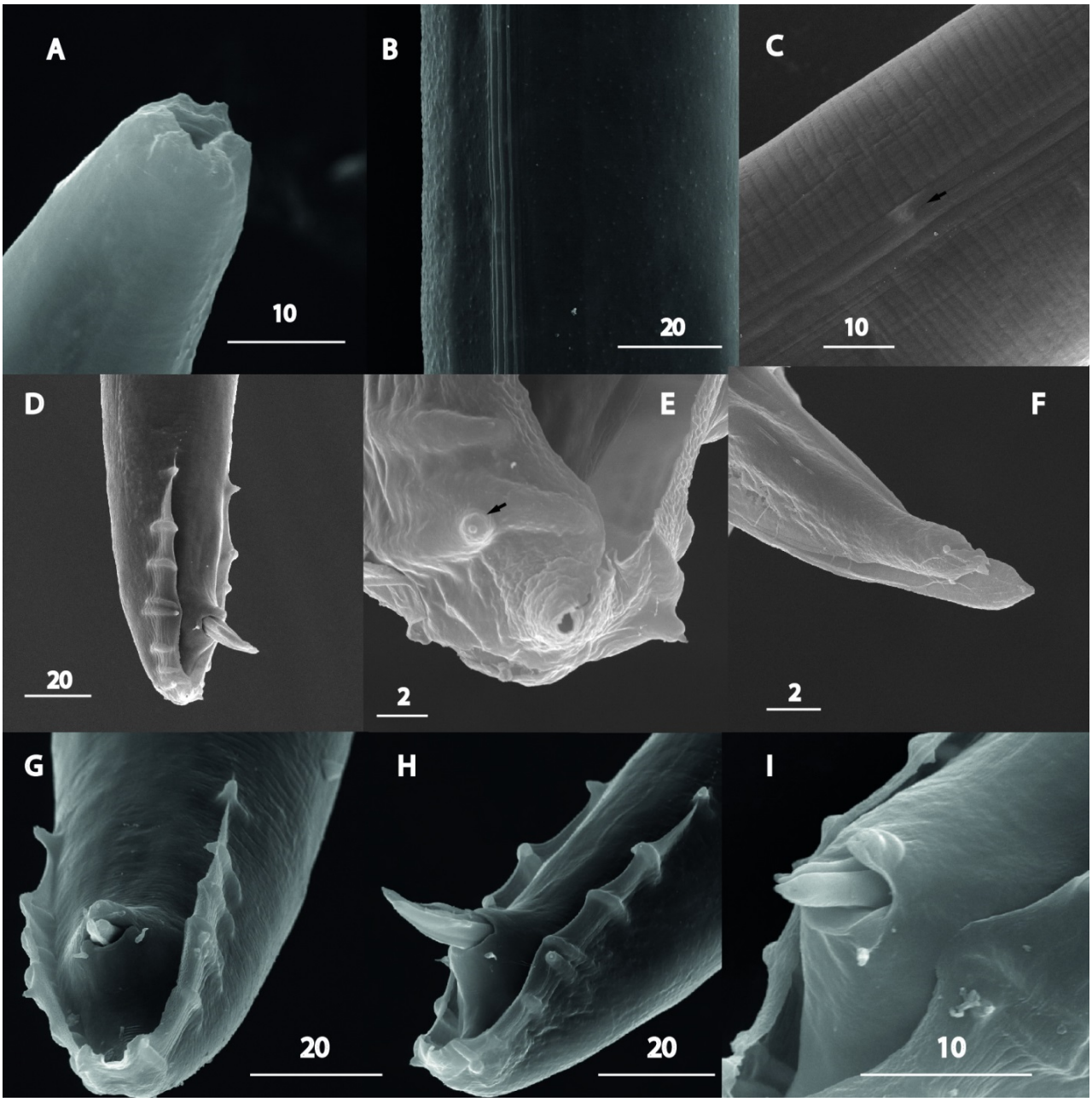

Fig. 3. *Phasmarhabditis akhaldaba* sp. n. SEM images, male. A, cephalic end; B-C, lateral field (arrow indicating deirid); D-I, tail region (arrow indicating GP9 opening dorsally); F, spicule tips. Scale bars in µm.

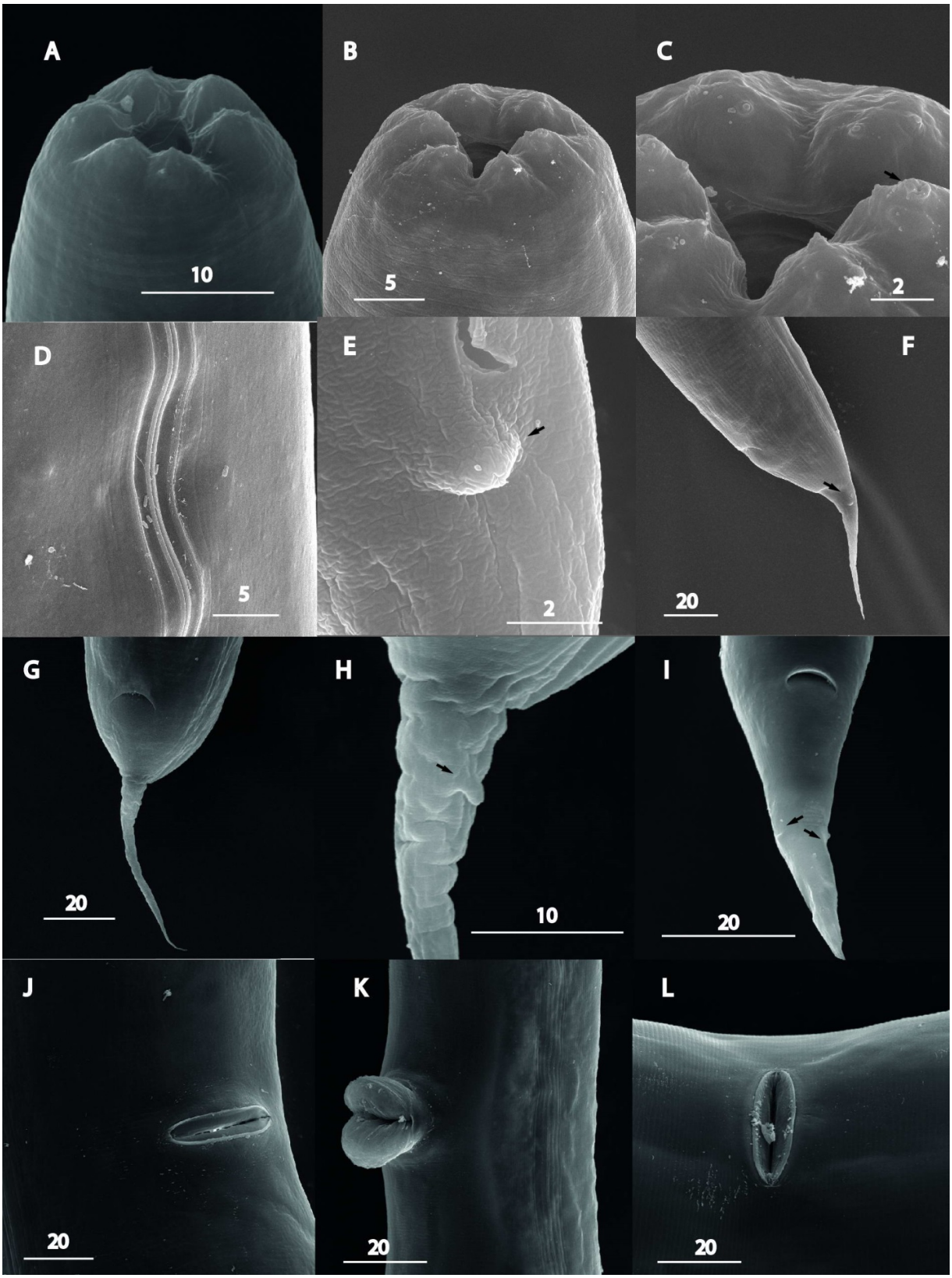

Fig. 4. *Phasmarhabditis akhaldaba* sp. n. SEM images, female. Adults. A-C, cephalic end. A, 'quick', B-C, 'slow'; D, lateral field; E, phasmid; F-I, tail region; J-L, vulva region. Arrows indicating amphid (C) and phasmid (E, F, H, I). Scale bars in μm.

Table 2. Morphometric data of dauer juveniles (DJ) of *Phasmarhabditis akhaldaba* sp. n. 'quick' and 'slow' strains. All measurements are in µm and in the form: mean ± s.d. (range).

Species	***Phasmarhabditis akhaldaba* sp. n. 'quick'**		***Phasmarhabditis akhaldaba* sp. n. 'slow'**	
Character	**DJ ensheathed**	**DJ exsheathed**	**DJ ensheathed**	**DJ exsheathed**
n (paratypes)	18	11	16	8
L	862 ± 47 (760-940)	667 ± 119 (520-895)	702 ± 67 (520-830)	669 ± 31 (620-710)
a	25.6 ± 2.6 (22.6-34.5)	27.7 ± 4.1 (23.1-35.8)	26.2 ± 2.1 (23.1-30.4)	26.6 ± 5.0 (20.9-35.6)
b	5.9 ± 0.2 (5.5-6.2)	4.7 ± 0.4 (3.9-5.4)	5.2 ± 0.5 (4.3-6.2)	4.9 ± 0.2 (4.6-5.1)
c	7 ± 0.5 (5.8-7.7)	11.6 ± 2 (7.8-13.9)	6.2 ± 0.7 (4.1-7.3)	10.6 ± 1.4 (8.3-12.5)
c'	6.3 ± 1.2 (5.3-10.8)	4.9 ± 0.9 (3.5-6)	6.9 ± 1.4 (5.3-11.7)	3.7 ± 0.7 (2.6-4.8)
Max. body diam.	34 ± 4 (22-38)	24 ± 4 (18-32)	27 ± 3 (22-32)	26 ± 6 (18-34)
Anal body diam.	20 ± 2 (12-23)	13 ± 2 (8-16)	17 ± 2 (13-20)	18 ± 4 (14-22)
Lip region diam.	8 ± 1 (6-10)	8 ± 1 (6-10)	8 ± 1 (6-10)	7 ± 0 (7-8)
Stoma total length	19 ± 1 (16-22)	18 ± 2 (17-22)	20 ± 1 (18-21)	19 ± 1 (18-20)
Cheilostom length	4 ± 1 (3-5)	3 ± 1 (2-4)	4 ± 0 (3-5)	4 ± 0 (4)
Gymnostom length	4 ± 1 (3-5)	3 ± 1 (2-5)	4 ± 1 (3-6)	4 ± 0 (4)
Stegostom length	11 ± 1 (10-13)	12 ± 2 (10-15)	11 ± 1 (10-12)	11 ± 0 (11)
Stoma max. diam.	2 ± 0 (2-2)	2 ± 0 (2-3)	2 ± 0 (2)	2 ± 0 (2)
Corpus length	88 ± 6 (73-95)	85 ± 13 (73-115)	84 ± 6 (75-98)	87 ± 3 (80-90)
Mid-corpus diam.	7 ± 1 (6-8)	8 ± 1 (7-10)	6 ± 1 (5-7)	8 ± 2 (6-10)
Metacorpal expansion	10 ± 1 (8-12)	14 ± 3 (11-19)	8 ± 1 (7-9)	9 ± 1 (8-11)
Isthmus length	37 ± 4 (30-45)	34 ± 7 (26-48)	33 ± 4 (25-40)	38 ± 4 (28-42)
Isthmus width	5 ± 1 (3-6)	5 ± 1 (4-7)	5 ± 1 (4-5)	6 ± 1 (4-7)
Bulb length	21 ± 2 (18-26)	20 ± 3 (15-24)	18 ± 3 (13-24)	17 ± 2 (14-20)
Bulb width	13 ± 1 (11-15)	16 ± 5 (12-27)	10 ± 1 (8-12)	12 ± 3 (8-15)
Pharynx total length	146 ± 8 (123-157)	141 ± 24 (116-186)	134 ± 9 (120-152)	137 ± 5 (129-145)
Apex to excretory pore	130 ± 8 (120-145)	132 ± 2 (130-133)	115 ± 14 (99-140)	123 ± 5 (116-130)
Apex to nerve ring	108 ± 9 (84-125)	105 ± 24 (85-155)	96 ± 11 (80-122)	87 ± 7 (79-100)
Cardia length	8 ± 1 (6-9)	9 ± 1 (7-12)	7 ± 2 (5-10)	7 ± 1 (5-9)
Tail length	124 ± 8 (110-140)	58 ± 10 (42-72)	114 ± 18 (85-164)	64 ± 8 (54-75)
Anus to phasmid	34 ± 6 (20-46)	23 ± 6 (17-32)	27 ± 5 (20-33)	22 ± 6 (17-30)
Genital primordium length	73 ± 17 (50-110)	164 ± 43 (115-240)	85 ± 9 (68-107)	91 ± 8 (85-110)
Apex to hemizonid		144 ± 36 (100-192)		117 ± 5 (110-125)

with slightly sunken central band *ca* 3 µm wide flanked by protruding ridges. Lip region continuous with body contour, divided into six lip sectors, prominent amphid openings at base of lateral lip sectors, six papillae on top of lips. Excretory pore anterior to basal bulb. Tail conical, half as long as in ensheathed juvenile, tip rounded. Genital primordium well developed, longer than in ensheathed juvenile. Phasmids button-like, prominent, situated at mid-tail.

Type material. Holotype female accession no. 1328 and paratype female and male, accession nos 1329 and 1330 respectively are deposited in the Museum of the Helminthological Collections of the

Centre of Parasitology at the A.N. Severtsov Institute of Ecology and Evolution, RAS, Moscow.

Type host. *Deroceras reticulatum* (O.F. Müller, 1774) (Pulmonata, Agriolimacidae).

Type locality. Akhaldaba (Borjomi municipality, Republic of Georgia), coordinates 41.908 N 43.520 E at 760 m a.s.l., 5 June 2018.

Diagnosis and relationships. Comparison of the metric characteristics of *Phasmarhabditis akhaldaba* sp. n. showed that peculiarities of the strains include generally smaller body lengths of adult stages of the strain 'quick' *vs* strain 'slow' (see Table 1) but longer dauer juveniles (see Table 2). The rest of metric and morphological characters were similar in both strains. Adult stages of *P. akhaldaba* sp. n. are characterised by a head with six low, rounded lips bearing a ring of six labial papillae, four cephalic papillae and pore-like amphids situated on top of lateral lips. Males feature a moderately wide bursa and spicules 65-67 µm long lacking holes at the distal tips. The present species is also characterised by a lateral field in adults comprising central elevated double ridge flanked by marginal single ridge at each side and that of an ensheathed dauer juvenile, with central band flanked with three slightly elevated, even ridges at each side. Dauer juveniles (ensheated) are recognised by the presence of a small cephalic cap, average length 862 µm and 702 µm in 'quick' and 'slow' strains and the characteristic pattern of a lateral field, while exsheathed ones are recognised by a conical tail with a rounded distal tip and prominent phasmids.

The morphology of the present species is very much in accord with the morphology of other species of the genus *Phasmarhabditis* sharing similarity in the structure of cuticle, stoma, pharynx and reproductive system, general body proportions and association with terrestrial gastropods. However, the molecular analysis supported its position on the phylogenetic tree as a new species of the genus and the combination of morphological and metric characters allows its differentiation from the rest of the genus. As a gonochoristic species, *P. akhaldaba* sp. n. differs from hermaphroditic *P. hermaphrodita* (Schneider, 1859) Andrássy, 1983 and *P. californica* Tandingan De Ley, Holovachov, McDonnell, Bert, Paine & De Ley, 2016 in the presence of males in the population. Though the shape of a female tail of the present species is variable and in most obese specimens approaching the shape described as a cupola-shaped with a spine (Sudhaus, 2014), it can be distinguished from *P. bonaquense* Nermut', Půža, Mekete & Mráček, 2016b, *P. papillosa* (Schneider, 1866) Andrássy, 1976, *P. huizhouensis* Huang, Ye, Ren & Zhao, 2015, *P. safricana* Ross, Pieterse, Malan & Ivanova, 2018, *P. meridionalis* Ivanova & Spiridonov, 2017 and *P. zhejiangensis* Zhang & Liu, 2020 in the wider spine base, *i.e.* in having a conical *vs* filamentous terminal part of tail.

Also, whether female phasmids in the above-mentioned species are situated directly at the base of a terminus on the rounded part of tail, the phasmids of the present species are located more posteriorly, *i.e.* on the anterior part of a spine. The tail shape of slimmer females of the present species resembles that of *P. bohemica* Nermut', Půža, Mekete & Mráček, 2017; *P. hermaphrodita*, *P. neopapillosa* (Mengert, 1953) Andrássy, 1983, *P. tawfiki* Azzam, 2003, *P. apuliae* Nermut', Půža & Mráček, 2016a, *P. circassica* Ivanova, Geraskina & Spiridonov, 2020, *P. clausiliiae* Ivanova, Geraskina & Spiridonov, 2020 and *P. kenyaensis* Pieterse, Rowson, Tiedt, Malan, Haukeland & Ross, 2020. From *P. bohemica* the present species differs in having shorter spicules (65 µm and 67 µm, 'quick' and 'slow' *vs* 74 µm and 75 µm, CH1 and 185 strains) and longer dauer juveniles (862 µm and 702 µm, 'quick' and 'slow', ensheathed, *vs* 553 µm and 691 µm, CH1 and 185 strains). From *P. neopapillosa* it can be distinguished by much shorter dauer juveniles (*vs* 1010 µm) (Hooper *et al.*, 1999). From *P. tawfiki* it also differs in having the shorter dauer juveniles (*vs* 965 µm) and a shorter male tail (33 µm and 38 µm *vs* 49 µm). From *P. apuliae P. akhaldaba* sp. n. differs in having less separated and more flattened lips lacking papilla-like protrusions and shorter dauer juveniles (*vs* 812 µm and 986 µm, BAR and GRA strains). Males of *P. akhaldaba* sp. n. differ from *P. apuliae* by remarkably shorter spicules (*vs* 81 µm and 73 µm in BAR and GRA strains) and a narrower bursa. By the length of dauer juveniles *P. akhaldaba* sp. n. falls in the group with medium-sized (700-900 µm long) juveniles with *P. papillosa*, *P. circassica*, *P. clausiliiae*, *P. apuliae* and *P. meridionalis* but can be differentiated by the combination of characters.

From *P. kenyaensis P. akhaldaba* sp. n. is distinguished by much shorter dauer juveniles (*vs* 1232 µm).

It is differentiated from them by the larger body size, the more posterior position of female phasmids situated on the spine *vs* at its base, small sized *vs* prominent male phasmids, lack of the holes at distal spicule tips and slightly longer spicules (*vs* 58 µm and 54 µm). Morphologically and molecularly *P. akhaldaba* sp. n. is closest to *P. circassica* and, in less prominent way, to *P. clausiliiae*, both from Caucasus.

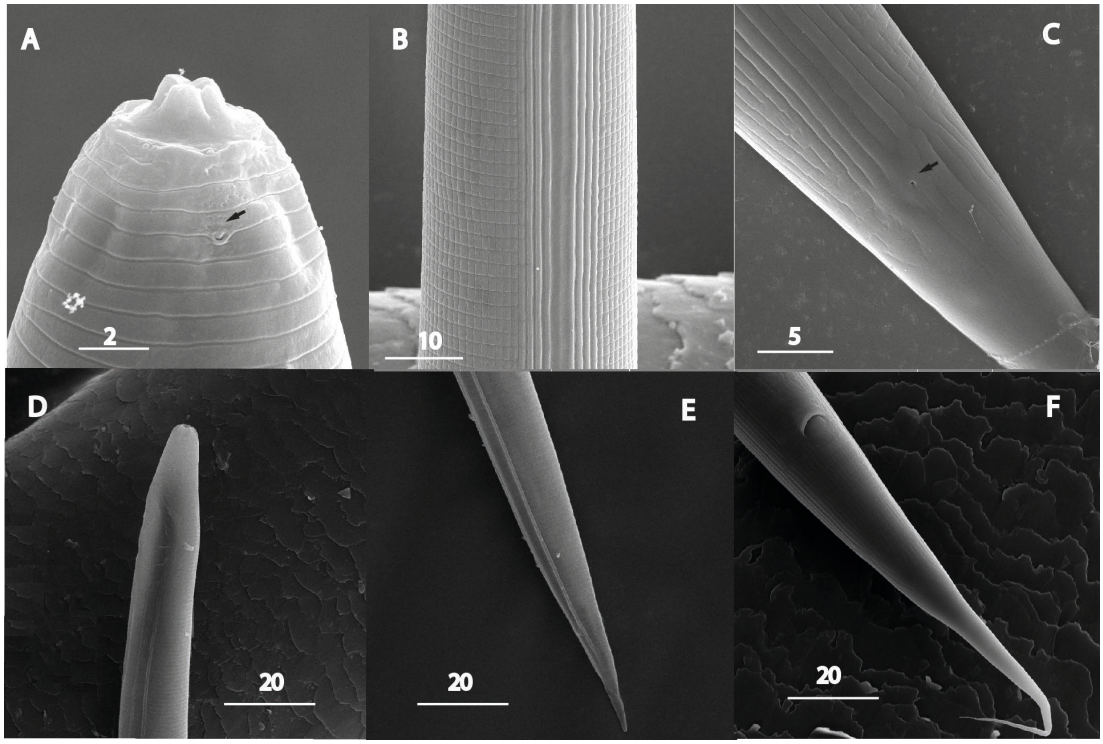

Fig. 5. *Phasmarhabditis akhaldaba* sp. n. SEM images, infective juvenules (IJ). A-C & F, ensheated IJ; D & E exsheathed IJ. A, head; B, lateral field; C, tail region; D, anterior end; E & F, posterior region. Arrows indicating amphid (A) and phasmid (C). Scale bars in µm.

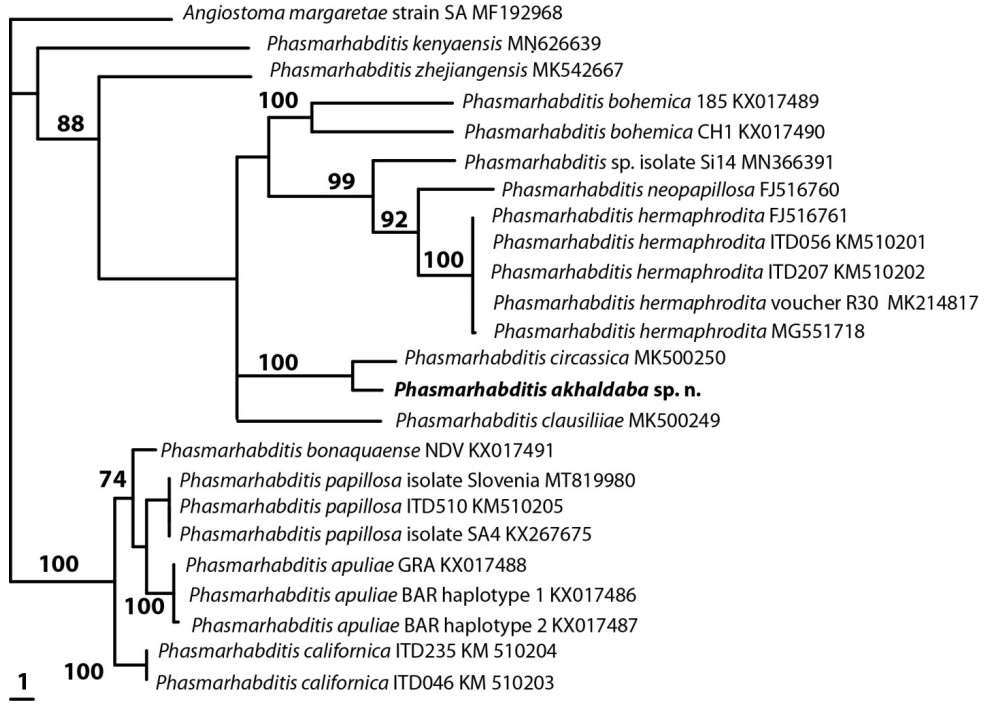

Fig. 6. Phylogenetic relationships of *Phasmarhabditis akhaldaba* sp. n. with other related *Phasmarhabditis* species, as inferred from ML (GTR + G model) and Bayesian analysis of the ITS region. *Angiostoma margaretae* was used as outgroup taxa. Bootstrap value and posterior probability (as a percentage) are assigned near the relevant nodes in ML/BA format.

Molecular analysis and phylogenetic analysis of *P. akhaldaba* sp. n. Molecular analysis of 191 bp long 28S rDNA sequences has shown the nearly complete identity for nematodes belonging to these two different strains with difference of only 1 bp. The difference in 1 bp was found between 960 bp long ITS rDNA sequences of these two strains. The analysis of 28S rDNA was uninformative to infer the phylogenetic links of *Phasmarhabditis akhaldaba* sp. n., which clustered with *P. circassica* under moderate bootstrap support (data not shown). The phylogenetic relationships of *P. akhaldaba* sp. n. inferred from ITS rDNA analysis are presented in Figure 6. Two close species, *P. akhaldaba* sp. n. and *P. circassica* formed the clade with maximal bootstrap support (100%) in this tree. Here, several *Phasmarhabditis* species formed two strongly supported clades: one formed by the group of *P. hermaphrodita* + *P. neopapillosa* and another clade, by two isolates of *P. bohemica*. West Caucasian species *P. clausiliae* also appeared related to *P. akhaldaba* sp. n. and *P. circassica* but without significant bootstrap support.

DISCUSSION

Caucasus region is one of the centres of genetic diversity and in particular, has very rich gastropod fauna with high level of endemism (Sysoev & Schileyko, 2009; Mumladze *et al.*, 2014). Two *Phasmarhabditis* species, *P. circassica* and *P. clausiliiae*, described recently from the area close to Republic of Georgia, the foothills of West Caucasus, were found morphologically and phylogenetically related to each other. Phylogenetic analysis has shown the greater affinity of the new species to *P. circassica* from Republic of Adygea, which is located by the other side of Caucausus mountains. From both West Caucasian species *P. akhaldaba* sp. n. differs by 32 bp, which is the average distance between species or group of species. Morphological differences between Georgian and West Caucasian species are quite scant and include generally greater body size, and slightly longer spicules and smaller phasmids in males. All three species from the Caucasus form a distinct group distanced from *P. hermaphrodita*, *P. papillosa* and *P. neopapilosa* with the world-wide distribution, as well as exotic species from African continent and South-Eastern Asia (*P. kenyaensis*, *P. meridionalis*, *P. zhejangensis*) but showing affinity to species found in the South of Europe (*P. bohemica* and undescribed *Phasmarhabditis* from Sicily – Ivanova *et al.*, 2019).

The gastropod host of strain 'quick' of *P. akhaldaba* sp. n. is *Deroceras reticulatum* (Pulmonata, Agriolimacidae) with its European origin and worldwide distribution. This slug species was registered as a host for a number of *Phasmarhabditis* species in Europe, USA and South Africa: *P. bohemica*, *P. californica*, *P. hermaphrodita*, *P. papillosa*, *P. safricana* (Tandigan de Ley *et al.*, 2014, 2016; Pieterse *et al.*, 2017; Ross *et al.*, 2018; Nermut' *et al.*, 2017). The ability of *D. reticulatum* to spread and acclimatise in different climatic conditions and tolerate diverse *Phasmarhabditis* species makes this voracious species one of the major slug pests.

Shortage of reliable diagnostic characters in the morphology of *Phasmarhabditis* highlights the importance of a phylogenetic analysis based on suitable molecular markers to resolve relationships within the genus. The presence of strains differing markedly in metrical characteristics also does not facilitate separation of species. The phenomenon of co-occurring of two isolates of the same species at the same plot probably could be explained by the analysis of different molecular marker but will be a subject of a different study.

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REFERENCES

ANDRÁSSY, I. 1976. *Evolution as a Basis for the Systematization of Nematodes*. UK, Pitman Publishing. 288 pp.

ANDRÁSSY, I. 1983. *A Taxonomic Review of the Suborder Rhabditina (Nematoda: Secernentia)*. France, ORSTOM. 241 pp.

AZZAM, K.M. 2003. Description of nematode *Phasmarhabditis tawfiki* n. sp. isolated from Egyptian terrestrial snails and slugs. *Journal of the Egyptian German Society of Zoology* 42: 79-87.

CURRAN, J. & DRIVER, F. 1994. Molecular taxonomy of *Heterorhabditis*. In: *COST 812: Biotechnology: Genetics of Entomopathogenic Nematode-Bacterium Complexes. Proceedings of Symposium and Workshop, St Patrick's College, Maynooth, Kildare County, Ireland* (A.M. Burnell, R.-U. Ehlers & J.-P.

Masson Eds). pp. 41-48. Luxembourg, European Commission.

HOLTERMAN, M., VAN DER WURFF, A., VAN DEN ELSEN, S., VAN MEGEN, H., BONGERS, T., HOLOVACHOV, O., BAKKER, J. & HELDER, J. 2006. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution towards crown clades. *Molecular Biology and Evolution* 23: 1792-1800. DOI: 10.1093/molbev/msl044

HOMINICK, W.M., BRISCOE, B.R., DEL PINO, F.G., JIAN HENG, HUNT, D.J., KOZODOY, E., MRÁČEK, Z., NGUYEN, K.B., REID, A.P., SPIRIDONOV, S., STOCK, P., STURHAN, D., WATURU, C. & YOSHIDA, M. 1997. Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *Journal of Helminthology* 71: 271-298. DOI: 10.1017/s0022149x00016096

HOOPER, D.J., WILSON, M.J., ROWE, J.A. & GLEN, D.M. 1999. Some observations on the morphology and protein profiles of the slug-parasitic nematodes *Phasmarhabditis hermaphrodita* and *P. neopapillosa* (Nematoda: Rhabditidae). *Nematology* 1: 173-182. DOI: 10.1163/156854199508144

HUANG, R.-E., YE, W., REN, X. & ZHAO, Z. 2015. Morphological and molecular characterization of *Phasmarhabditis huizhouensis* sp. nov. (Nematoda: Rhabditidae), a new rhabditid nematode from south China. *PLoS ONE* 10: e0144386. DOI: 10.1371/journal.pone.0144386

IVANOVA, E.S. & SPIRIDONOV, S.E. 2017. *Phasmarhabditis meridionalis* sp. n. (Nematoda: Rhabditidae) from a land snail *Quantula striata* (Gastropoda: Dyakiidae) from south of Vietnam. *Russian Journal of Nematology* 25: 129-140.

IVANOVA, E., CLAUSI, M., SPARACIO, I. & SPIRIDONOV, S.E. 2019. Preliminary data on the parasite survey of terrestrial gastropods of Sicily. *Russian Journal of Nematology* 27: 37-45. DOI: 10.24411/0869-6918-2019-10005

IVANOVA, E.S., GERASKINA, A.P. & SPIRIDONOV, S.E. 2020. Two new species of *Phasmarhabditis* Andrássy, 1976 (Nematoda: Rhabditidae) associated with land snails in Northwest Caucasus, Russian Federation: description and molecular affiliation. *Nematology* 22: 179-197. DOI: 10.1163/15685411-00003299

MENGERT, H. 1953. Nematoden und Schneken. *Zeitschrift fur Morphologie und Ökologie der Tiere* 41: 311-349.

MUMLADZE, L., CAMERON, R.D. & POKRYSZKO, B.M. 2014. Endemic land molluscs in Georgia (Caucasus): How well are they protected by existing reserves and national parks? *Journal of Molluscan Studies* 80: 67-73. DOI: 10.1093/mollus/eyt047

NADLER, S.A., D'AMELIO, S., FAGERHOLM, H.-P., BERLAND, B. & PAGGI, L. 2000. Phylogenetic relationships among species of *Contracaecum* Railliet & Henry, 1912 and *Phocascaris* Høst, 1932 (Nematoda: Ascaridoidea) based on nuclear rDNA sequence data. *Parasitology* 121: 455-463. DOI: 10.1017/S0031182099006423

NERMUT', J., PŮŽA, V. & MRÁČEK, Z. 2016A. *Phasmarhabditis apuliae* n. sp. (Nematoda: habditidae), a new rhabditid nematode from milacid slugs. *Nematology* 18: 1095-1112. DOI: 10.1163/15685411-00003017

NERMUT', J., PŮŽA, V., MEKETE, T. & MRÁČEK, Z. 2016B. *Phasmarhabditis bonaquaense* n. sp. (Nematoda: Rhabditidae), a new slug-parasitic nematode from the Czech Republic. *Zootaxa* 4179: 530-546. DOI: 10.11646/zootaxa.4179.3.8

NERMUT', J., PŮŽA, V., MEKETE, T. & MRÁČEK, Z. 2017. *Phasmarhabditis bohemica* n. sp. (Nematoda: Rhabditidae), a slug-parasitic nematode from the Czech Republic. *Nematology* 19: 93-107. DOI: 10.1163/15685411-00003034

NICHOLAS, K.B., NICHOLAS, H.B. JR. & DEERFIELD, D.W. 1997. *Multiple Sequence Alignment Editor and Shading Utility, Version 2.7.000*. URL: http://www.psc.edu/biomed/genedoc (accessed: October 2, 2017).

PIETERSE, A., TIEDT, L.R., MALAN, A.P. & ROSS, J.L. 2017. First record of *Phasmarhabditis papillosa* (Nematoda: Rhabditidae) in South Africa, and its virulence against the invasive slug, *Deroceras panormitanum*. *Nematology* 19: 1035-1050. DOI: 10.1163/15685411-00003105

PIETERSE, A., ROWSON, B., TIEDT, L.R., MALAN, A.P., HAUKELAND, S. & ROSS, J.L. 2020. *Phasmarhabditis kenyaensis* n. sp. (Nematoda: Rhabditidae) from the slug, *Polytoxon robustum*, in Kenya. *Nematology* 23: 229-245. DOI: 10.1163/15685411-bja10040

ROSS, J.L., PIETERSE, A., MALAN, A.P. & IVANOVA, E. 2018. *Phasmarhabditis safricana* n. sp. (Nematoda: Rhabditidae), a parasite of the slug *Deroceras reticulatum* from South Africa. *Zootaxa* 4420: 391-404. DOI: 10.11646/zootaxa.4420.3.5

SEINHORST, J.W. 1959. A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica* 4: 67-69. DOI: 10.1163/187529259X00381

SCHNEIDER, A. 1859. Über eine Nematoden Larve und gewisse Verschiedenheiten in den Geschlechtsorganen der Nematoden. *Zeitschrift für wissenschaftliche Zoologie* 10: 176-178.

SUDHAUS, W. 2014. Order Rhabditina: "Rhabditidae". In: *Handbook of Zoology. Volume 2: Nematoda* (A. Schmidt-Rhaesa Ed.). pp. 537-555. Berlin, Germany, Walter de Gruyter GmbH. DOI: 10.1515/9783110274257.537

SYSOEV, A. & SCHILEYKO, A. 2009. *Land Snails and Slugs of Russia and Adjacent Countries*. Bulgaria-Russia, Pensoft Publishers. 312 pp.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731-2739. DOI: 10.1093/molbev/msr121

TANDINGAN DE LEY, I.T., MCDONNELL, R.J., LOPEZ, S., PAINE, T.D. & DE LEY, P. 2014. *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditidae), a potential biocontrol agent isolated for the first time from invasive slugs in North America. *Nematology* 16: 1129-1138. DOI: 10.1163/15685411-00002838

TANDIGAN DE LEY, I.T., HOLOVACHOV, O., MCDONNELL, R.J., BERT, W., PAINE, T.D. & DE LEY, P. 2016. Description of *Phasmarhabditis californica* n. sp. and first report of *P. papillosa* (Nematoda: Rhabditidae) from invasive slugs in the USA. *Nematology* 18: 175-193. DOI: 10.1163/15685411-00002952

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4882. DOI: 10.1093/nar/25.24.4876

VRAIN, T., WAKARCHUK, D.A., LEVESQUE, A.C. & HAMILTON, R.I. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15: 563-573.

ZHANG, C.-N. & LIU, Q.-Z. 2020. *Phasmarhabditis zhejiangensis* sp. nov. (Nematoda: Rhabditidae), a new rhabditid nematode from Zhejiang, China. *PLoS ONE* 15: e0241413. DOI: 10.1371/journal.pone. 0241413

E.S. Ivanova, O.A. Gorgadze, M.A. Lortkhipanidze and S.E. Spiridonov. *Phasmarhabditis akhaldaba* sp. n., ассоциированный со слизнем *Deroceras reticulatum* в горах Малого Кавказа в Республике Грузия.

Резюме. Новый вид нематод *Phasmarhabditis akhaldaba* sp. n. был обнаружен в Боржомском ущелье в Республике Грузия. Обнаружено два штамма, первый выделен из слизня *Deroceras reticulatum*, а второй из трупа личинки вощинной моли *Galleria mellonella*, использованной в качестве ловчего насекомого для энтомопатогенных нематод. Штаммы, обозначенные как «быстрый» и «тихий», различались по длине инвазионных личинок и определяемой визуально скорости их движения во время миграции. Морфологически, взрослые особи нового вида характеризуются головой с шестью невысокими закругленными губами, несущими круг из шести губных папилл, четыре головные папиллы и поровидные амфиды, расположенные на вершине латеральных губ, самцами с довольно широкой бурсой и спикулами 65-67 мкм длиной без отверстий на концах. Средняя длина инвазионных личинок (dauer juvenile) в двойной кутикуле *Phasmarhabditis akhaldaba* sp. n. штамма «быстрый» составляла 862 мкм и штамма «тихий» 702 мкм. Молекулярный анализ частичных последовательностей ITS участков рибосомальной ДНК показали их идентичность у этих штаммов. Морфологически и генетически новый вид продемонстрировал родство с другим видом с Кавказа – *P. circassica* Ivanova, Geraskina & Spiridonov, 2020.