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Redescription and phylogenetic analysis of the type species of the genus *Panagrellus* Thorne, 1938 (Rhabditida, Panagrolaimidae), *P. pycnus* Thorne, 1938, including the first SEM study

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Abstract

The identity of *Panagrellus pycnus*, the type species of the genus *Panagrellus*, is discussed after studying specimens from a cultured population collected in Italy that fits the original material of the species. A new characterization is consequently provided as follows: body 0.93–1.32 mm long, lip region continuous with the adjoining body, stoma with gymnostom very reduced, pharynx with not swollen metacorpus, neck 161–203 µm long, excretory pore at level of the metacorpus, post-vulval uterine sac 99–162 µm long or 2.6–3.8 times as long as the body diameter divided in a short tubular proximal part and a long swollen distal part, vulva post-equatorial (*V* = 63–69), female tail conical elongate with acute terminus (133–170 µm, *c* = 6.8–8.1, *c'* = 4.9–7.0), male tail conical elongate with acute terminus (104–137 µm, *c* = 7.8–10.9, *c'* = 3.6–5.1), and spicules 70–81 µm long having angular hook-like and very curved ventrad lamina ending in a spatulate tip with a refringent forked axis. The evolutionary relationships of this species and the genus *Panagrellus*, as derived from the analyses of 18S and 28S rDNA fragments, are discussed. Additionally, the phylogenetic relationships among the members of the infraorder Panagrolaimomorpha is studied, being the genus *Tarantobelus* transferred to the family Panagrolaimidae and the new subfamily Tarantobelinae n. subfam. is proposed to accommodate it.

Keywords

18S rDNA, 28S rDNA, Description, Molecular analysis, Morphology, Phylogeny, SEM, Tarantobelinae n. subfam., Taxonomy.

Panagrellus Thorne, 1938 is an infrequent genus belonging to the family Panagrolaimidae Thorne, 1937 (proposed as subfamily by Thorne (1937) and erected to family by Paramonov (1956)), and includes 15 species (Abolafia et al., 2016; Ivanova et al., 2018). This genus is characterized by having usually ventral curved spicules with hooked manubrium and bifurcate lamina tip. Its type species, *P. pycnus*, was described and succinctly illustrated by Thorne (1938) on the base of male and female specimens collected in slime secreted by wounds from the trunk of Great Plains cottonwood tree in Utah, USA. Later, Goodey (1943) transferred this species to the new erected genus *Turbator* Goodey, 1943 (currently junior synonym of *Panagrellus* together to *Tylorhabdus* Sukul, 1971). Andrássy (1958, 2005) and Varga (1958) reported this species from organic matter undergoing fermentation in Hungary. Hechler (1971a) revised the Thorne's material and redescribed *P. pycnus* providing some line illustrations of the stoma and of

the male and female posterior ends. This species is distinguished from its congeners by having spicules with angular hook-shaped manubrium and lamina very thin lacking dorsal hump and bearing spatulate terminus having forked refringent axis.

Currently, only five species of the genus [*P. ceylonensis* Hechler, 1971b, *P. dubius* Sanwal, 1960, *P. levitatus*, Ivanova, Perfilieva and Spiridonov, 2018, *P. redivivoides* Goodey, 1943 and *P. redivivus* (Linné, 1767) Goodey, 1945] have been characterized molecularly and used for phylogenetic analyses.

In the present paper, a cultured population of *P. pycnus* collected in Italy and used as food for carnivorous tardigrades is studied providing new LM and SEM illustrations and molecular data.

Materials and methods

Nematode extraction and culture

The nematodes were initially obtained from rotting pears on the ground in Borgata Città, Bologna, Italy (44°34′42.7″N 11°10′26.6″E; 27th October 2019; leg. Matteo Vecchi). Other than nematodes, the fruits were heavily colonized by *Drosophila* spp. and Nitidulidae beetles. Fruit pulp was spread on Yeast-Sucrose Agar (Yeast extract 1%, Sucrose 2%, Agar 1%) plates and after 1 week of incubation at 21°C a single gravid female was handpicked with a loop to start an isofemale line. Nematodes were mass cultured at 21°C in 0.3 L plastic containers on a substrate composed of 19 g of whole-grain wheat breakfast cereal (Weetabix®), 3 g of dry brewer's yeast and 60 mL of distilled water.

Nematode processing

The specimens were killed by heating, fixed in a 70% ethanol solution, transferred to pure glycerin following the Siddiqi's (1964) technique, and mounted on glass microscope slides with the glycerine-paraffin method (de Maeseneer and d'Herde, 1963) somewhat modified using hot liquid paraffin.

Light microscopy (LM)

Observations were made and measurements were taken using a Nikon Eclipse 80i (Nikon, Tokyo, Japan) microscope with a drawing tube (*camera lucida*) attached to it. Demanian indices and other ratios were calculated according to de Man (1881). Pictures were taken with a Nikon microscope equipped with differential interference contrast (DIC) optics and an associated Nikon Digital Sight DS-U1 camera. Micrographs were combined using Adobe® Photoshop® CS. The terminology used for the morphology of stoma and spicules follows De Ley et al. (1995) and Abolafia and Peña-Santiago (2017), respectively.

Scanning electron microscopy (SEM)

Specimens preserved in glycerine were selected and prepared for observation under SEM according to Abolafia (2015). They were cleaned in distilled water, dehydrated in a graded ethanol-acetone series, critical point dried, coated with gold, and observed with a Zeiss Merlin microscope (5 kV) (Zeiss, Oberkochen, Germany).

Molecular analyses

DNA extraction, PCR, and sequencing

Nematode DNA was extracted from single individuals using a modified DNA extraction and PCR assays described by Castillo et al. (2003) and Archidona-Yuste et al. (2016). The specimens were cut in small pieces using a sterilized dental needle on a clean slide with 18 ml of TE buffer (10 mM Tris-Cl + 0.5 mM EDTA; pH 9.0), transferred to a microtube and adding 2 µl proteinase K (700 µg/ml^{-1}) (Roche, Basel, Switzerland), and stored to –80°C within 15 min (for several days) until processing. The microtubes were incubated at 65°C (1 hr), then at 95°C (15 min). For DNA amplification, 3 µl of the extracted DNA was transferred to a microtube containing: 0.6 µl of each primer (10 mM), 3 µl Master Mix Taq DNA Polymerase (5x Hot FirePol Blend Master Mix) and ddH2O to a final volume of 20 µl. The primers used for amplification of the region of 18S rRNA gene were the forward primer SSU F_04 (5′-GCTTGTCTCCAAAGATTAAGCC-3′) and the reverse primer SSU R_26 (5′-CATTCTTGG CAAATGCTTTCG-3′) (Blaxter et al., 1998). The primers used for amplification of the D2-D3 region of 28S rRNA gene were the forward primer D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and the reverse primer D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (De Ley et al., 1999; Nunn, 1992). PCR cycle conditions were as follows: one cycle of 94°C for 15 min, followed by 35 cycles of 94°C for 45 sec + annealing temperature of 55°C for 45 sec + 72°C for 45 sec, and finally one cycle of 72°C for 5 min. After DNA amplification, 5 µl of product was loaded on a 1% agarose gel in 0.5% Tris-acetate-EDTA (40 mM Tris, 20 mM glacial acetic acid and 2 mM EDTA; pH = 8) to verify the amplification using an electrophoresis system (Labnet Gel XL Ultra V–2, Progen Scientific, London, UK). The bands were stained with 1.25 µl

RedSafe (20,000x) previously added to the agarose gel solution (25 ml). The sequencing reactions of the PCR products were performed at Sistemas Genómicos (Paterna, Valencia, Spain) according the Sanger et al. (1977) method. The DNA sequences obtained for *P. pycnus* (MZ656001 for the 18S rDNA and MZ656000 for the 28S rDNA) and *Tarantobelus arachnicida* Abolafia and Peña-Santiago, 2018 (MZ655998–MZ655999 for the 18S rDNA and MZ656002–MZ656003 for the 28S rDNA) were submitted to the GenBank database.

Phylogenetic analyses

For phylogenetic relationships, the analyses were based on 18S and 28S rDNA fragments. The newly obtained sequences were manually edited using BioEdit 7.2.6 (Hall, 1999) and aligned with other 18S and 28S rRNA gene sequences representative of Panagrolaimomorpha and closely related taxa available in GenBank (accession numbers available in Supplementary Table 1) with MAFFT ver. 7 (Katoh and Toh, 2008; Katoh et al., 2002) with the G-INS-i method (thread = 4, threadtb = 5, threadit = 0, reorder, adjustdirection, anysymbol, maxiterate = 1,000, retree 1, globalpair input). Alignments ends were trimmed using MEGA7 (Kumar et al., 2016) up to 1,851 and 1,195 bp for 18S and 28S respectively. The best-fit models of nucleotide substitution used for the phylogenetic analysis were selected using jModelTest 2.1.10 (Darriba et al., 2012). Sequences were concatenated with the R package 'concatipede' v1.0.0 (Vecchi and Bruneaux, 2021). The phylogenetic tree was generated with Bayesian inference method using MrBayes 3.2.6 (Ronquist et al., 2012). *Plectus aquatilis* was used as outgroup for the Rhabditida phylogenetic tree. The phylogenetic analyses were initiated with a random starting tree and run with the Markov Chain Monte Carlo (MCMC) (Larget and Simon, 1999) for 2×10^7 generations. The posterior tree distributions trace plots and ESS were checked with Tracer (Rambaut et al., 2018). The tree was visualized and saved with FigTree 1.4.4 (Rambaut, 2018).

Results

Panagrellus pycnus Thorne, 1938 (Figs. 1–4)
= *Turbator pycnus* (Thorne, 1938) Goodey, 1943
Material examined: 10 females and 10 males obtained from culture.

Measurements

Measurements are provided in Table 1.

Description

Adult

Moderately slender to slender nematodes of small size, 0.93–1.32 mm long. Upon fixation, habitus nearly straight in females or somewhat curved ventral, J-shaped, in males. Cuticle 1–2 µm thick, bearing minute transverse striations, with annuli 1–2 µm wide. Lateral fields occupying 10–20% of mid-body diameter, with four longitudinal incisures or three alae. Lip region continuous with the adjacent body: lips six, separate, slightly rounded, with protruding, rounded labial and cephalic sensilla; primary and secondary axils with similar morphology, primary ones slightly deeper. Oral opening large, with smooth margin. Amphids very small, oval, located at middle length of lateral lips. Stoma panagrolaimoid, 0.7–1.1 times the lip region diameter long: cheilostom large, slightly wider than long, with strongly refringent, bar-shaped rhabdia, posteriorly thicker; gymnostom very reduced, with small rhabdia; stegostom funnel-shaped with poorly refringent rhabdia, metastegostom bearing dorsal acute rhabdia (dorsal tooth). Pharynx also panagrolaimoid: pharyngeal corpus robust, subcylindrical, 2.2–4.0 times the isthmus length, with procorpus and metacorpus not well discernible; isthmus comparatively thin; basal bulb ovoid, with both valvular apparatus and posterior haustrulum well developed. Cardia small, surrounded by intestinal tissue. Nerve ring at 70–85% of neck length from the anterior end, surrounding the anterior part of isthmus. Excretory pore at 52–75% of neck length, at level of the posterior part of metacorpus. Hemizonid located at level of isthmus. Deirids 78–90% of neck length, at level of isthmus-bulb junction. Intestine without distinct specializations, but with slightly thinner walls at cardiac part; intestinal lumen with rest of diatom frustules.

Female

Reproductive system monodelphic-prodelphic. Ovary very long, lacking flexure at post-vulval region, having oocytes arranged in several rows at its distal part and then in only one rows at its proximal part. Oviduct short, slightly longer than the body diameter developing a scarcely discernible spermatheca at its proximal part. Uterus very long, 6.8–10.9 times as long as body diameter, tubular, frequently including uterine eggs (20–33 × 28–54 µm) inside in different stages of development. Post-vulval uterine sac well developed, 2.6–3.8 times as long as the body diameter, with very thin walls, frequently poorly discernible, with proximal part short, tubular, and distal part large,

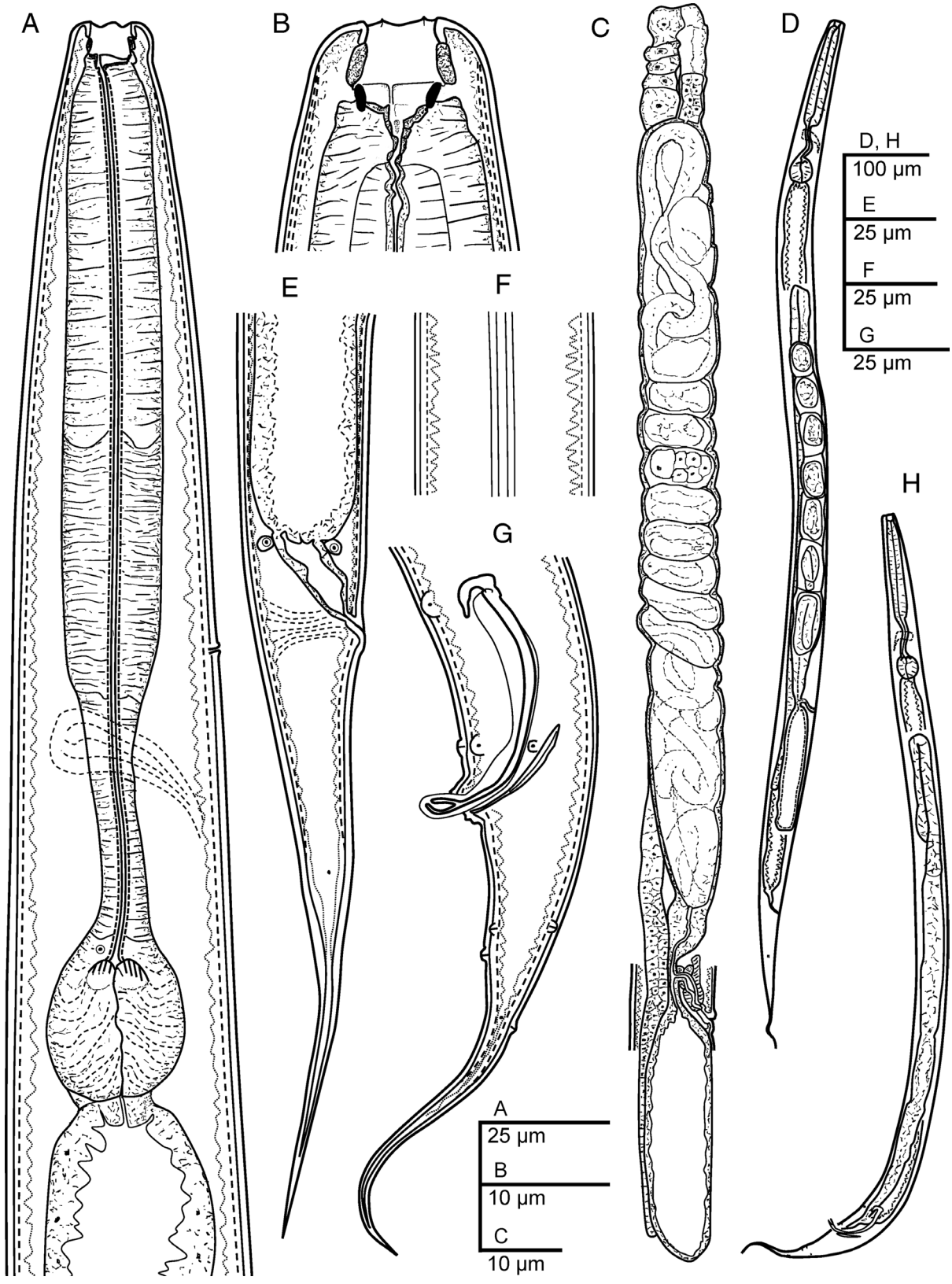

Figure 1: *Panagrellus pycnus* Thorne, 1938 (line drawing). (A) Neck; (B) Anterior end; (C) Female reproductive system; (D) Entire female; (E) Female posterior end; (F) Lateral field; (G) Male posterior end; (H) Entire male.

Figure 2: *Panagrellus pycnus* Thorne, 1938 (light microscopy). (A) Neck (anterior white arrow pointing the excretory pore, posterior white arrow pointing the hemizonid, black arrow pointing the deirid); (B) Anterior end; (C) Vagina; (D) Female reproductive system; (E) Female posterior end (arrow pointing the phasmid); (F) Lateral field; (G) Entire female; (H) Male posterior end (black arrows pointing genital papillae, GP; mid-ventral papillae, MP; white arrow pointing the phasmid, ph); (I) Entire male.

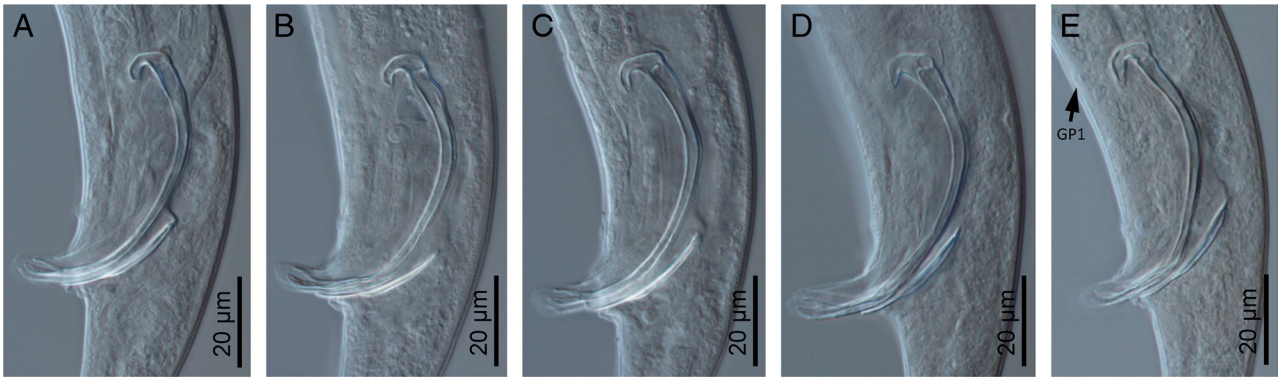

Figure 3: *Panagrellus pycnus* Thorne, 1938 (light microscopy). (A–E) Morphological variability of spicules and gubernaculum.

swollen. Vagina extending inwards to 31–43% of body diameter, sigmoid. Vulva slightly protruding. Rectum short, 0.9–1.3 times the anal body width; three small gland-like cells are distinguishable around the intestine-rectum junction. Tail conical-elongate with acute terminus. Phasmids located at 34–40% of tail length from anus.

Male

Reproductive system monorchic, with testis reflexed ventrad anteriorly. Spicules paired and symmetrical: manubrium ventrally bent, angular hook-shaped, reduced calamus, and very curved ventrad lamina lacking dorsal hump, with well-developed ventral velum, narrower at its middle length, and spatulate tip in lateral view having a refringent forked axis. Gubernaculum well developed, slightly curved, about 0.4 times of the spicules length, with thin corpus. Three small gland-like cells are distinguishable around the beginning of the cloaca. Tail conical, slightly curved ventrad, conoid anterior to phasmid and them filiform. Genital papillae seven pairs, tree pre-cloacal (GP1 and GP2 subventral, GP3 lateral) and four post-cloacal: two pairs subventral (GP5, GP6) and one subdorsal (GP4) at the middle of tail length and one subdorsal (GP7) at beginning of the filiform part. One mid-ventral adcloacal papilla (MP) present. Phasmids at 32 to 50% of tail length from the cloacal aperture, close to genital papillae GP7.

Remarks

The material examined in this study agrees well with the type population of *P. pycnus* described by Thorne (1938), especially in the morphology of the spicules although, unfortunately, Thorne (op. cit.) did not provide their measurements. Apparently, according to the drawing illustrations, the author did not observe the post-vulval uterine sac which is now know to be very large but (in the specimens examined in this study) its walls are very thin and sometimes not very well discernible. Also, the vagina structure was not illustrated with precision. Later, Hechler (1971a) described lectotype specimens of this species and observed the presence of post-vulval uterine sac but it was not described or illustrated. The main characters to identify this species, the spicules morphology (with manubrium having dorsal angular side and ventral hook-like side; Fig. 4) and morphometry (56–61 vs 54–70 µm, measured as a straight line, or chord, connecting the spicule manubrium with the lamina tip) agree perfectly with *P. pycnus*, while the gubernaculum is slightly longer (29–32 vs 25–27 µm). Unfortunately, most of the measurements were not provided in the previous records of the species (Table 2).

On the other hand, the most similar species to *P. pycnus* is *P. leperisini* Massey, 1974, with which it could be confused by having similar spicules morphology. However, *P. pycnus* presents larger body (0.80–1.4 mm in Thorne's description, 0.90–1.41 in Hechler's description and 0.93–1.32 mm in the present paper vs 0.74–0.97 mm), lip region wider (wider than the adjacent part of body vs narrower) and longer spicules (70–81 vs 60 µm).

Diagnosis

Panagrellus pycnus is characterized by having 0.93–1.32 mm long body, lip region continuous with the adjoining body, lips separated six having rounded sensilla, amphids small, stoma with gymnostom very reduced, pharynx with not swollen metacorpus, neck 161–203 µm long, excretory pore at level of the metacorpus, female reproductive system

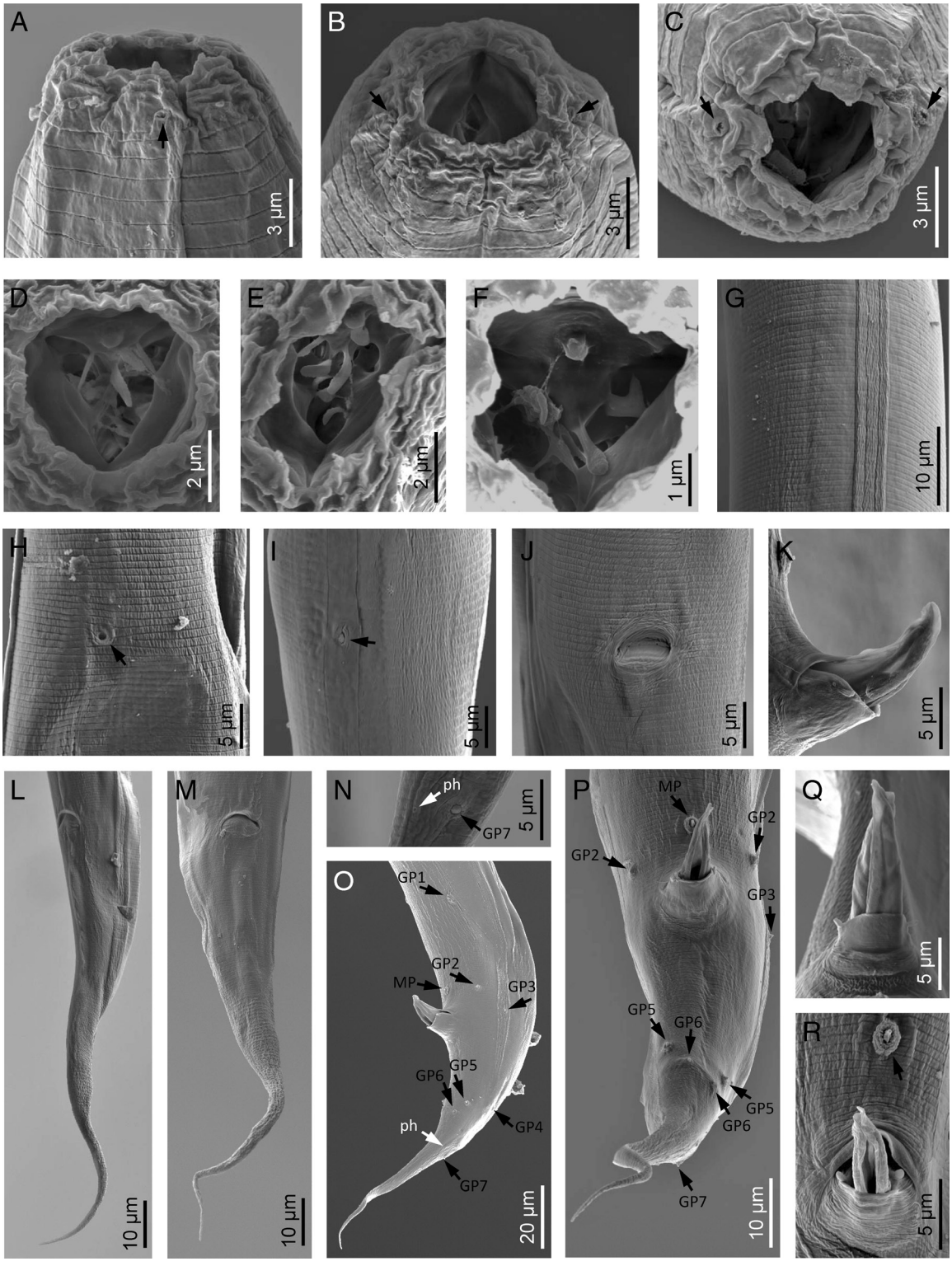

Figure 4: *Panagrellus pycnus* Thorne, 1938 (scanning electron microscopy). A–C: Lip region in lateral, ventral and frontal views, respectively (arrows pointing the amphids); D–F: Stomatal denticles; G: Lateral field; H: Excretory pore; I: Deirid; J: Vagina; K, Q: Spicules tip in lateral and dorsal views, respectively; L, M: Female posterior end in lateral and ventral views, respectively; N: Male phasmid; O, P: Male posterior end in lateral and ventral views, respectively (black arrows pointing the genital papillae, white arrow pointing the phasmid); R: Mid-ventral papillae (arrow).

Table 1. Morphometrics of *Panagrellus pycnus* Thorne, 1938 obtained from culture.

Sex	Female	Male
n	10	10
Body length (*L*)	1,114.2 ± 103.5 (993–1258)	1,022.0 ± 117.5 (931–1,322)
a	26 ± 2.0 (23.6–29.6)	27 ± 1.6 (24.6–29.9)
b	6.9 ± 3.4 (5.3–16.5)	5.4 ± 0.5 (4.6–6.5)
c	7.5 ± 0.4 (6.8–8.1)	8.6 ± 0.9 (7.8–10.9)
c'	6.0 ± 0.6 (4.9–7.0)	4.4 ± 0.5 (3.6–5.1)
V	66.5 ± 1.8 (63–69)	–
Lip region width	14.8 ± 1.2 (12–16)	14.1 ± 0.7 (13–15)
Stoma length	13.8 ± 1.4 (12–16)	12.7 ± 1.2 (10–14)
Pharyngeal corpus length	109.4 ± 4.9 (104–120)	107.4 ± 6.8 (93–114)
Isthmus length	39.6 ± 5.7 (30–50)	40.0 ± 5.7 (32–53)
Bulbus length	27.9 ± 2.0 (24–31)	27.8 ± 2.5 (24–33)
Pharynx length	176.1 ± 6.6 (167–190)	175.2 ± 11.8 (151–189)
Nerve ring – anterior end	148.3 ± 8.8 (137–160)	139.7 ± 11.0 (122–161)
Excretory pore – anterior end	128.9 ± 7.5 (120–139)	115.8 ± 11.0 (104–139)
Deirid-anterior end	153.3 ± 9.9 (142–160)	163.0 ± 6.1 (156–167)
Neck length	189.9 ± 6.8 (181–203)	187.9 ± 12.5 (161–202)
Body diameter at neck base	37.9 ± 3.1 (34–44)	35.1 ± 2.7 (33–42)
Body diameter at midbody	42.4 ± 4.6 (37–52)	37.6 ± 4.1 (32–47)
Lateral field width	5.7 ± 2.1 (4–8)	6.3 ± 1.0 (5–8)
Anterior ovary or testis length	551.4 ± 49.5 (463–650)	233.0 ± 41.7 (188–289)
Anterior oviduct length	56.1 ± 15.8 (40–91)	–
Anterior uterus length	377.4 ± 63.8 (287–496)	–
Post-vulval uterine sac length	133.4 ± 22.2 (99–162)	–
Vagina length	42.0 ± 8.5 (36–48)	–
Vulva – anterior end	740.9 ± 74.0 (668–863)	–
Rectum or cloaca length	28.2 ± 3.8 (20–32)	9.8 ± 0.7 (9–11)
Anal body diameter	25.1 ± 2.8 (22–30)	27.2 ± 3.4 (23–34)
Tail length	148.9 ± 11.9 (133–170)	119.6 ± 10.1 (104–137)
Phasmid - anus distance	55.6 ± 6.1 (46–67)	47.7 ± 6.7 (36–57)
Spicules length (arc)	–	77.4 ± 3.3 (70–81)
Gubernaculum length	–	30.0 ± 1.2 (29–32)

Notes: Measurements in μm and in the form: mean ± standard deviation (range) where appropriate. Demanian indices (de Man, 1881): a = body length/body diameter; b = body length/pharynx length; c = body length/tail length; c' = tail length/anal body diameter; V = (distance from anterior region to vulva/body length)x100.

Table 2. Comparative morphometrics of *Panagrellus pycnus* Thorne, 1938.

Reference	Present paper		Thorne (1938)		Hechler (1971a)		Andrássy (2005)**	
Country	Italy		USA		USA		Hungary	
Sex	Female	Male	Female	Male	Female	Male	Female	Male
n	10	10	?	?	12	15	?	?
Body length (*L*)	993–1,258	931–1,322	1,000–1,400	800–1,200	1,170–1,410	900–1,222	1,170–1,410	800–1,200
a	23.6–29.6	24.6–29.9	18.0	21.0	16.0–21.5	20.6–26.9	16.0–22.0	21.0–27.0
b	5.3–16.5	4.6–6.5	8.0	6.8	6.1–8.0	5.7–7.4	6.1–8.0	5.7–7.4
c	6.8–8.1	7.8–10.9	8.5	9.0	7.6–10.4	7.9–10.4	8.0–10.0	8.0–10.0
c'	4.9–7.0	3.6–5.1	4.7*	4.2*	5.8*	4.7*	6.0	3.5–4.0
V	63–69	–	73	–	71–77	–	71–77	–
Stoma length	12–16	10–14	?	?	11–14	10–13	12–14	12–14
Spicules length (arc)	–	70–81	–	?	–	71–81*	–	?
Spicules length (chord)	–	56–61	–	?	–	54–70	–	50–70
Gubernaculum length	–	28–29	–	?	–	25–27	–	25–27

Notes: Measurements in µm. *Measurement from drawings. **Measurements adapted from Hechler (1971). ? Unknown measurement. – Character absent.

monodelphic-prodelphic with post-vulval uterine sac 99–162 µm long or 2.6–3.8 times as long as the body diameter divided in a short tubular proximal part and a long swollen distal part, vulva post-equatorial (*V* = 63–69), female tail conical elongate with acute terminus (133–170 µm, *c* = 6.8–8.1, *c′* = 4.9–7.0), male tail conical elongate with acute terminus (104–137 µm, *c* = 7.8–10.9, *c′* = 3.6–5.1), spicules 70–81 µm long having angular hook-shaped and very curved ventrad lamina ending in a spatulate tip with a refringent forked axis, and gubernaculum 29–32 µm long.

Differential diagnosis

The *Panagrellus* species are easily differentiated by the size (Table 3) and morphology (Fig. 5) of the spicules (Abolafia et al., 2016). Three main groups are distinguished according the morphology of the spicules. The first group, (Fig. 5B–D) with more simple spicules, includes three species [*P. dorsobidentatus* (Rühm, 1956) Baker, 1962, *P. ludwigi* (de Man, 1910) Goodey, 1945 and *P. ventrodentatus* (Heindl-Mengert, 1956) Baker, 1962] having robust spicules with irregular truncated manubrium, probably a plesiomorphic character. The second group (Fig. 5E–I), with five species [*P. japonicus* (Yokoo and Ota, 1961) Andrássy, 1984 *P. levitatus P. nepenthicola* (Menzel, 1922) Goodey, 1945, *P. redivivoides* (Goodey, 1943) Goodey, 1945 and, probably, *P. ulmi* Abolafia, Alizadeh and Khakver, 2016], presents ventrally curved spicules with rounded manubrium, straight or slightly ventral bent, and lamina with variable bifurcated tip. The third group (Fig. 5J–P), composed by seven species [*P. ceylonensis*, *P. dubius*, *P. filiformis* (Sukul, 1971) Andrássy, 1984 *P. leperisini*, *P. pycnus*, *P. redivivus* and *P. silusioides* Tsalolikhin, 1965], have ventrally curved spicules with ventral bent manubrium and lamina with well-developed bifurcated tip, probably an apomorphic character. Between this last group, the curvature degree of the manubrium is a diagnostic character. Thus, spicules with spirally curved manubrium appear in *P. ceylonensis* (with scarcely sigmoid lamina and short bifurcated terminus), *P. dubius* (with very curved lamina and long bifurcated terminus) and *P. filiformis* (with curved lamina while the terminus was not well illustrated). Manubrium poorly ventrally bent appears in *P. redivivus* and *P. silusioides*, having scarcely to very bent manubrium respectively, and slightly developed dorsal hump at lamina, while hook-shaped manubrium appears in some *P. ceylonensis* (narrow manubrium and poorly curved lamina), *P. leperisini* and *P. pycnus* (both species with wide manubrium and 'C'-like lamina), more ventrally curved in *P. ceylonensis*, while *P. pycnus* is more anteriorly curved than *P. leperisini*.

Molecular characterization and phylogenetic position

One 928 bp 18S rDNA sequence (GenBank accession number MZ656001) and one 766 bp 28S rDNA sequence (GenBank accession number MZ656000) were obtained for *P. pycnus*. After the molecular analysis, *P. pycnus* exhibits a high level of rDNA similarity with other *Panagrellus* species (Fig. 6).

Discussion

On the identity of *Panagrellus pycnus* and other related species

The morphology and morphometry of the material examined now agree with the type population of *Panagrellus pycnus* described by Thorne (1938) and redescribed by Hechler (1971a, b). The main characters to distinguish this material from other species are the size of the spicules (70–81 µm length and reaching the GP1) and the presence of a manubrium with angular dorsal side and hook-like ventral side. Two species, *P. ceylonensis* and *P. leperisini*, are similar to *P. pycnus*, but the former species differs in size (70–81 µm reaching the GP1 vs 81–89 µm length reaching the GP1 in *P. ceylonensis* and 56–62 µm length not reaching the GP1 in *P. leperisini*) and morphology (lamina ventrally more curved at first third *vs* homogeneously curved but ventrally convex at its mid-length in *P. ceylonensis* and slightly ventrally more curved anterior and posteriorly in *P. leperisini*).

Other species have very similar spicules and could be confused with *P. pycnus*. For example, some populations of *P. pycnus* could have been confused with *P. redivivus*. Thus, de Man (1914, as Anguillula silusiae), Goodey (1943), Hechler (1970, 1971a, Zullini (1982) and Ferris (2009) described several populations of *Panagrellus* agreeing with *P. redivivus*, showing specimens with spicules that do not reach the pre-cloacal GP1 and possessing a dorsal hump with a slight ventral bend to the calamus. However, the material examined by Corrêa de Carvalho and Álvares Corrêa (1953) and Rühm (1956) does not agree well with *R. redivivus* as observed Hechler (1971a); thus, the material described by Corrêa de Carvalho and Álvares Corrêa (op. cit.) shows spicules reaching the GP1 (*vs* not reaching the GP1 in *P. redivivus*, see de Man, 1914 as *P. silusiae* (Aubertot, 1925; Goodey, 1922; Hechler, 1971a) having almost

Table 3. Length of the spicules (measured in arc) of the *Panagrellus* species with hooked manubrium.

References	*Ceylonensis*	*Dubius*	*Filiformis*	*Leperisini*	*Pycnus*	*Redivivus*	*Redivivus* as *leucocephalus*	*Redivivus* as *parasiticus*	*Silusioides*
Present paper	–	–	–	–	70–81	–	–	–	–
Goodey (1922)	–	–	–	–	–	70*	–	–	–
Steiner (1936)	–	–	–	–	–	–	?	–	–
Thorne (1938)	–	–	–	–	74***	–	–	–	–
Sandground (1939)	–	–	–	–	–	–	–	65–68*	–
Rühm (1956)	–	–	–	–	–	70–90* *ceylonensis*?	–	–	–
Tsalolikhin (1965)	–	–	–	–	–	–	–	–	75–90**
Sanwal (1960)	–	60–62*	–	–	–	–	–	–	–
Hechler (1970)	–	–	–	–	–	77*	–	–	–
Hechler (1971a)	–	56–60*	–	–	71–81*	64–74*	45*	–	–
Hechler (1971b)	81–89*	–	–	–	77–83*	–	–	–	–
Sukul (1971)	–	–	27*	–	–	–	–	–	–
Massey (1974)	–	–	–	60*	–	–	–	–	–
Stock and Nadler (2006)	83* *redivivus*? *silusioides*?	64* *dubius*? *leperisini*?	–	–	–	65* *ceylonensis*? *parasiticus*?	–	–	–

Notes: *Obtained from drawings. **Obtained from other measurements. ***Obtained from drawings in Goodey (1943). ?Presumable identity of the material examined by the author referenced.

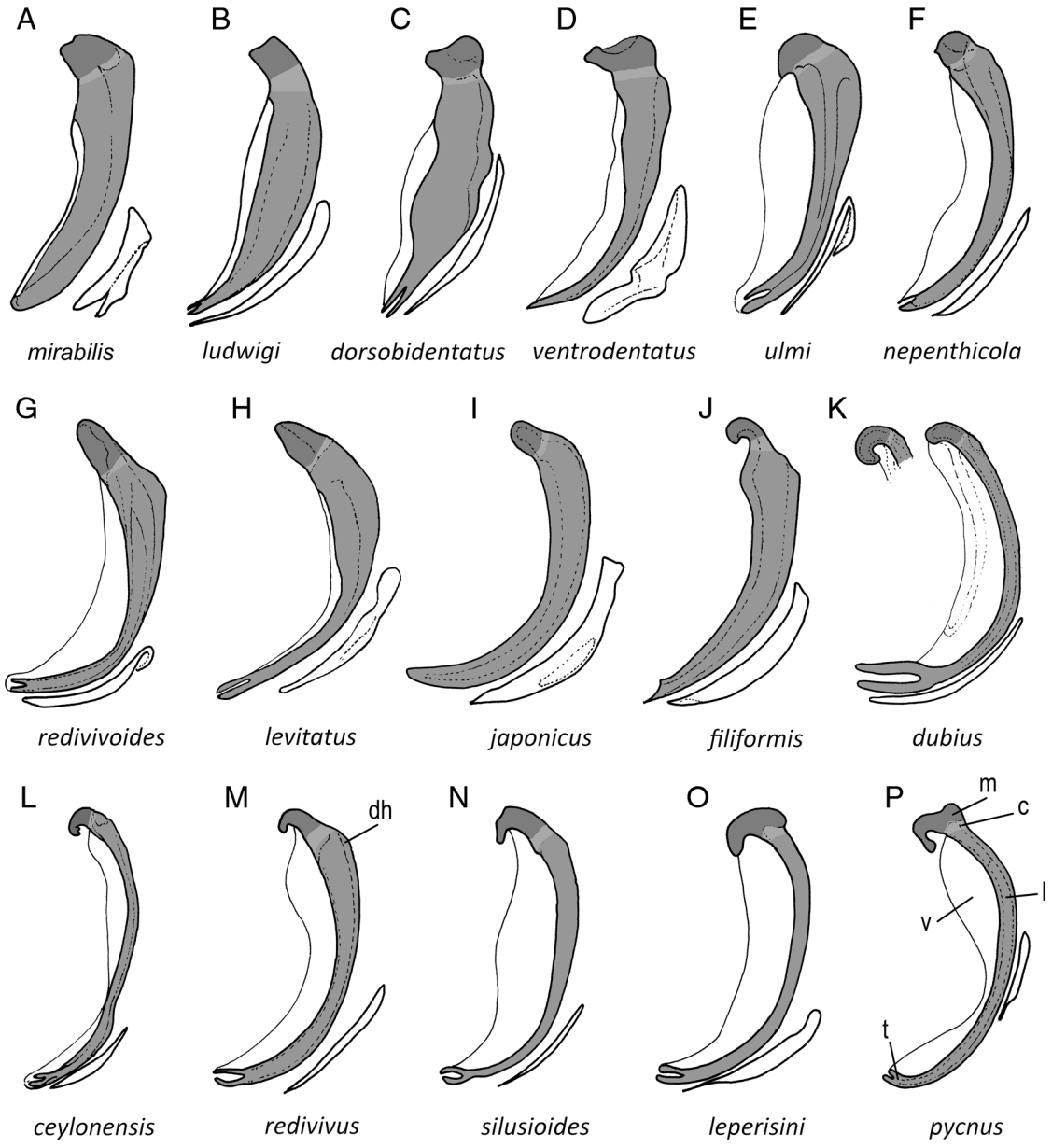

Figure 5: Morphology of the spicules and gubernacula of the species of the genera *Baujardia* Bert, Tandingan De Ley, Van Driessche, Segers and De Ley, 2003 and *Panagrellus* Thorne, 1938. (A) *Baujardia*; (B–D) *Panagrellus* species with truncated manubrium; (E–I) *Panagrellus* species with rounded or conoid manubrium; (E–P) *Panagrellus* species with curved or hook-shaped manubrium (c = calamus, dh = dorsal hump, l = lamina, m = manubrium, v = velum, t = tip).

angular hook-like manubrium; on the other hand, the material described by Rühm (op. cit.) is very similar to *P. ceylonensis* agreeing in measurements, excretory pore at basal bulb level and spicules slightly sigmoid with narrow hooked manubrium.

On the other hand, Stock and Nadler (2006) characterized morphological and molecularly three species of the genus *Panagrellus*. However, there are some discrepancies about their identity. With respect to the morphology of the illustrated spicules, the spicule named as '*P. ceylonensis*' (see Fig. 3B) is more similar to *P. redivivus* and *P. silusioides* by having ventral bent manubrium and wide velum; the spicule named as '*P. redivivus*' (see Fig. 3D) agrees

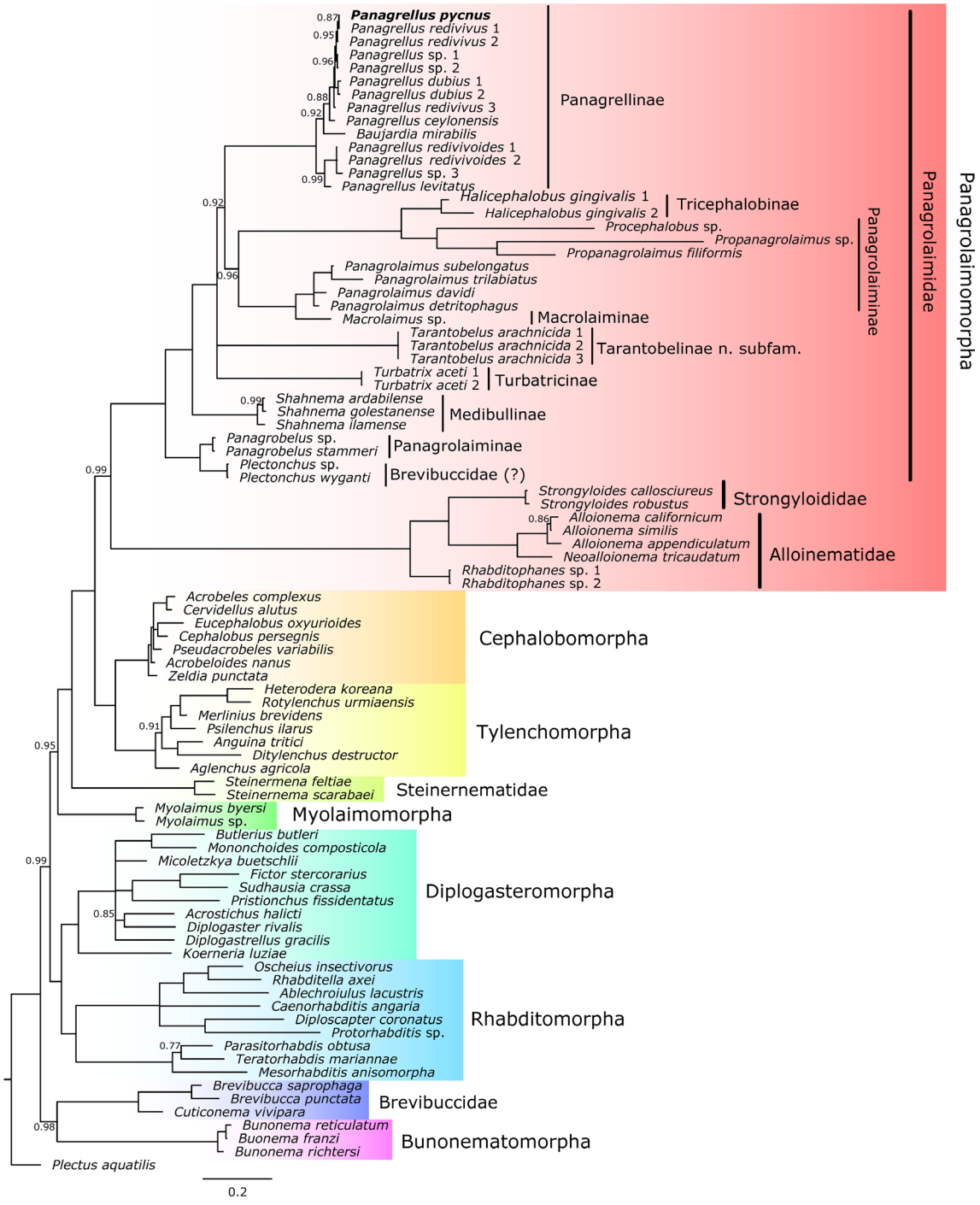

Figure 6: Bayesian Inference tree showing the phylogenetic position of *Panagrellus pycnus* and its related taxa based on a concatenated 18S and 28S rDNA regions. Bayesian posterior probabilities (pp) are given for each clade. Nodes with pp < 0.70 were collapsed. When pp = 1, its value is not shown. Scale bar shows the number of substitutions per site.

with *P. ceylonensis* (although it is unusually short, 65 µm long) and *P. parasiticus* (junior synonym of *P. redivivus*) by having spirally curved manubrium and narrow velum; and the spicule named as '*P. dubius*' (see Fig. 3E), agreeing with this species by having long bifurcated terminus but also it is similar to *P. leperisini* by the size and by having angular hook-like manubrium. However, other morphological and morphometrical characters are necessary to correctly identify these species.

On the phylogenetic position of *Panagrellus pycnus* in the genus *Panagrellus*

The material examined now of *Panagrellus pycnus* is phylogenetically related with other sequenced species of the genus *Panagrellus*. The species included in this genus are characterized by having very short gymnostom with minute rhabdia, post-vulval uterine sac differentiated in a short tubular proximal part and a large swollen distal part, and female and male tails conoid-elongate. An important character of the spicules is the ventral curvature and hook-shaped manubrium. There are two primary clades in the rDNA phylogenetic tree of *Panagrellus* (Fig. 6). One includes the species *P. levitatus* and *P. redivivoides* and is characterized by the more robust spicules with rounded manubrium, like other genera of the family Panagrolaimidae. The second clade includes *P. ceylonensis*, *P. dubius*, *P. pycnus* and *P. redivivus* and is characterized by thinner and elongate spicules, increasing the curvature of the manubrium from slightly ventrally curved to hook-like.

Phylogenetic position of the genus *Panagrellus* and its related genera

Panagrellus is recovered as paraphyletic (Fig. 6) with *Baujardia* Bert, Tandingan De Ley, Van Driessche, Segers and De Ley, 2003 located inside the genus. Indeed, *Baujardia* is very similar to *Panagrellus*, only differentiated from it by the morphology of the stoma, slightly longer in *Baujardia*. Unfortunately, only a sequence based on 18S rDNA was obtained of *Baujardia mirabilis* Bert, Tandingan De Ley, Van Driessche, Segers and De Ley, 2003, the only species of the genus, leading to the possible consideration of the synonymy of *Baujardia*.

With respect to the subfamily Panagrellinae Andrássy, 1976 containing the genera *Baujardia* and *Panagrellus*, is shown as monophyletic. This subfamily was synonymized with Baujardinae Andrássy, 2005 by Abolafia et al. (2016) and the present molecular analysis agree with these authors.

Other subfamilies of the family Panagrolaimidae as Medibullinae Siddiqi, 1993 [tranferred to Panagrolaimidae by Abolafia and Peña-Santiago (2018), including *Shahnema* Siddiqi, 2014] Tricephalobinae Andrássy, 1976 (including *Halicephalobus* Timm, 1956 and Turbatricinae Goodey, 1943 (including *Turbatrix* Peters, 1927) are shown as monophyletic.

Conversely, the subfamily Panagrolaiminae is shown as polyphyletic. Four genera belonging to this subfamily, *Panagrobelus* Thorne, 1939, *Panagrolaimus* Fuchs, 1930 *Procephalobus* Steiner, 1934 and *Propanagrolaimus* Andrássy, 2005 appear not directly related, unlike what was proposed by Andrássy (1984, 2005). However, additional morphological and molecular studies are necessary to confirm the relationships between these genera and, after that, it could be suitable to reestablish the subfamily Procephalobinae Paramonov, 1956 to include *Procephalobus* and *Propanagrolaimus*, among others.

Nevertheless, the genus *Macrolaimus* Maupas, 1900 currently belonging to the family Chambersiellidae Thorne, 1937 subfamily Macrolaiminae Sanwal, 1971, appears related with *Panagrolaimus*. Unfortunately, not enough species have been sequenced to clarify its phylogenetic position. To this respect, Kitagami et al. (2019) sequenced a species identified as *Panagrolaimus* sp. (LC382049), however, it agrees with some species of the genus *Macrolaimus* according the stoma morphology being showed in the present phylogenetic tree belonging to this last genus (see Supplementary Fig. S1 in Kitagami et al., op. cit.).

On the other hand, the genera *Plectonchus* Fuchs, 1930 and *Tarantobelus* Abolafia and Peña-Santiago, 2018 proposed as belonging to the family Brevibuccidae Paramonov, 1956 by Andrássy (1976, 1984, 2005) and Abolafia and Peña-Santiago (2018), respectively, appear not closely related with this family in this new study. According to this, both genera are transferred to the family Panagrolaimidae. Regarding the genus *Plectonchus* (with post-equatorial vulva and without post-vulval uterine sac) appears related to the panagrolaimid genus *Panagrobelus* (with equatorial vulva and very short post-vulval uterine sac); however with only limited 18S rDNA sequence in support of the genus, there is a clear need to obtain detailed morphological and molecular data, without which the genus should be considered *incertae sedis* until to obtain a detailed morphological-molecular study of its species. Secondly, the genus *Tarantobelus* appears forming part of a well-supported, separate clade which is proposed now as a new subfamily, Tarantobelinae n. subfam., clearly differentiated from other subfamilies, especially by having short and robust pharyngeal isthmus and vulva post-equatorial. This new subfamily is composed by the only genus *Tarantobelus*.

Taxonomic account

Tarantobelinae n. subfam.

Diagnosis: small body with cuticle nearly smooth, lateral field inconspicuous, lip region with six separated lips, each bearing a small cuticular flap topping it, panagrolaimoid stoma with well-developed gym-

nostom having broad and refringent rhabdia, panagrolaimoid pharynx with isthmus robust and slightly longer than basal bulb, excretory pore situated at level of isthmus, female reproductive system monodelphic-prodelphic, length of post-vulval sac less than the corresponding body diameter, vulva post-equatorial and distinctly protruding, female tail conical with acute tip, male tail conical with a long and thin mucro, spicules curved ventrad with rounded manubrium, and thick gubernaculum.

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Table S1. GenBank accession numbers of the species used in the phylogenetic tree.

Species	18S rDNA	28S rDNA
Ablechroiulus lacustris	EU196013	EU195976
Acrobeles complexus	KU180671	DQ145620
Acrobeloides nanus	DQ102707	DQ903076
Acrostichus halicti	HQ130163	HQ130212
Aglenchus agricola	KJ869356	KP835679
Alloionema appendiculatum	FJ665982	KP204846
Alloionema californicum	KX017492	NA
Alloionema similis	KX185606	NA
Anguina tritici	AY593913	KC818620
Baujardia mirabilis	AF547385	NA
Brevibucca punctata	NA	DQ077787
Brevibucca saprophaga	EU196018	KU180677
Bunonema reticulatum	EU196017	EU195989
Bunonema richtersi	FJ040451	NA
Buonema franzi	AJ966477	NA
Butlerius butleri	KP453998	NA
Caenorhabditis angaria	JN636068	JN636068
Cephalobus persegnis	AY284663	AF143368
Cervidellus alutus	AF202152	KU180683
Cuticonema vivipara	EU196019	EU195991
Diplogaster rivalis	KJ636326	NA
Diplogastrellus gracilis	KJ877216	KJ877249
Diploscapter coronatus	AY593921	NA
Ditylenchus destructor	KJ636422	MN307126
Eucephalobus oxyurioides	AY284665	HM439768
Fictor stercorarius	KJ877235	KJ877282
Halicephalobus gingivalis 1	JX674039	JX194163
Halicephalobus gingivalis 2	NA	KU180686
Heterodera koreana	MZ027493	MZ027488
Koerneria luziae	AB597232	KJ877284
Macrolaimus sp.	LC382049	NA
Merlinius brevidens	KX789708	NA
Mesorhabditis anisomorpha	AF083013	NA
Micoletzkya buetschlii	JX163973	NA
Mononchoides composticola	KP067833	NA
Myolaimus byersi	KU180665	KU180676
Myolaimus sp.	NA	DQ145643
Neoalloionema tricaudatum	KR817916	KR817917

Oscheius insectivorus	AF083019	EU195968
Panagrellus ceylonensis	NA	DQ408251
Panagrellus dubius 1	NA	DQ145648
Panagrellus dubius 2	NA	DQ408252
Panagrellus levitatus	KY126845	NA
Panagrellus pycnus	MZ656001	MZ656000
Panagrellus redivivoides 1	MH608262	MH608297
Panagrellus redivivoides 2	MH608263	MH608298
Panagrellus redivivus 1	AF083007	DQ408250
Panagrellus redivivus 2	MK541674	MK541658
Panagrellus redivivus 3	NA	AF331910
Panagrellus sp. 1	MN082326	NA
Panagrellus sp. 2	MH608264	MH608299
Panagrellus sp. 3	KP876562	KM489128
Panagrobelus sp.	LC382079	NA
Panagrobelus stammeri 1	FJ969134	NA
Panagrolaimus davidi	AJ567385	AY878385
Panagrolaimus detritophagus	EU543176	GU014547
Panagrolaimus subelongatus	KY119431	NA
Panagrolaimus trilabiatus	KF011487	NA
Parasitorhabdis obtusa	EU003189	EF990724
Plectonchus sp.	AY593920	NA
Plectonchus wyganti	KJ636307	NA
Plectus aquatilis	AY284700	EF417147
Pristionchus fissidentatus	KJ877237	KJ877273
Procephalobus sp.	EU543179	NA
Propanagrolaimus filiformis	KJ636392	NA
Propanagrolaimus sp.	KJ434175	NA
Protorhabditis sp.	AF083001	AY602168
Pseudacrobeles variabilis	AF202150	NA
Psilenchus ilarus	MK639403	MW716284
Rhabditella axei	NA	AY602177
Rhabditophanes sp. 1	JX674037	JX674036
Rhabditophanes sp. 2	AF202151	AY294185
Rotylenchus urmiaensis	KP718970	KP718967
Shahnema ardabilense	KM454872	KM454873
Shahnema golestanense	KM454874	KM454875
Shahnema ilamense	KM454870	KM454871
Steinermena feltiae	FJ040419	NA
Steinernema scarabaei	FJ040424	AY172023
Strongyloides callosciureus	AB272231	AB272231
Strongyloides robustus	AB272232	NA
Sudhausia crassa	KJ877232	KJ877279
Tarantobelus arachnicida 1	MG669658	MF177710

Tarantobelus arachnicida 2	MZ655998	MZ656002
Tarantobelus arachnicida 3	MZ655999	MZ656003
Teratorhabdis mariannae	EF990716	EF990721
Turbatrix aceti 1	AF202165	AY294184
Turbatrix aceti 2	KU180673	KU180690
Zeldia punctata	NA	DQ145662