JOURNAL OF NEMATOLOGY

VOLUME 45 DECEMBER 2013 NUMBER 4

Journal of Nematology 45(4):237–252. 2013.

Redescription of *Robustodorus megadorus* with Molecular Characterization and Analysis of Its Phylogenetic Position within the Family Aphelenchoididae

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Abstract: Based on a new record of the rare species *Robustodorus megadorus* from Utah, the generic diagnosis was amended to include the following characters: a labial disc surrounded by six pore-like sensilla; the absence of a cephalic disc; a lobed cephalic region devoid of annulation; a hexagonal inner cuticular structure of the pouch surrounding the stylet cone; large stylet knobs, rounded in outline and somewhat flattened on their lateral margins; a large spermatheca with an occluded lumen and lacking sperm; the excretory pore located between the median bulb and nerve ring. The stylet orifice consists of an open, ventral, elongate slit or groove. These characters distinguish the genus from the closely related genus *Aphelenchoides*. A lectotype and paralectotypes were designated. Results of phylogenetic analyses of the 18S and D2-D3 of 28S rRNA gene sequences revealed that *R. megadorus* occupies a basal position within one of the two main clades of the subfamily Aphelenchoidinae and shares close relationships with a species group of the genus *Aphelenchoides* that includes *A. blastophthorus*, *A. fragariae*, *A. saprophilus*, *A. xylocopae*, and *A. subtenuis*. Several specimens in our collection of *R. megadorus* were infected with *Pasteuria* sp. as were some of the paralectotypes.

Key words: Aphelenchoides megadorus, DNA, morphology, *Pasteuria*, phylogeny, *Robustodorus megadorus*, scanning electron microscopy.

In the early spring of 1940, the late Merlin W. Allen sampled an area of desert soil a few kilometers west of Utah Lake, near Mosida, UT. Among the nematodes he recovered was an aphelench with exceptionally robust stylet knobs and distinctive sclerotization of the stoma and cephalic region. It was described as a new species that he named *Aphelenchoides megadorus* (Allen, 1941). The species was subsequently moved to *Megadorus* (Goodey, 1960) and then to *Robustodorus* (Andrássy, 2007) to be regarded currently as "*Robustodorus megadorus* (Allen, 1941) Andrássy, 2007" (Hunt, 2008). No other species have been described. In the United States, *Robustodorus* has also been found in Idaho (Hafez et al., 2010) and Montana (Thorne and Malek, 1968). According to Andrássy (2007), *Robustodorus* has been reported from the Slovak Republic (Sabová, 1975, 1977; Sabová and Valocká, 1977; Lišková and Čerevková, 2011, the latter erroneously cited by Andrássy in 2007 as a report from the Czech Republic); Uzbekistan, Turkmenistan, and the Russian Far East (Strepkova, 1965 ; Kirjanova and Krall, 1971), but morphological data for these observations are lacking and there are no preserved specimens in the collection centers of the Slovak Republic, Russia, or other countries of the former Soviet Union.

Recently, one of us (C.N.) collected soil from a location near Eureka, UT, approximately 30 km southwest of Mosida. It contained numerous specimens of female and juvenile nematodes that, upon examination, proved to be *R. megadorus*. Because Allen's original description of *R. megadorus* did not designate a holotype, was based on drawings of poorly preserved specimens mounted in glycerol, and contained no photographs or genetic information of this monotypic genus, our purpose in this study is to provide a more detailed description of its anatomy, supplemented by light and scanning electron microscopy (SEM); sequences of its 18S, 28S, and ITS ribosomal RNA genes; and an analysis of its phylogenetic relationships.

Methods and Materials

Nematode samples: Soil and root samples were collected from an uncultivated area adjacent to an alfalfa (*Medicago sativa*) field south of Eureka, UT, located at N39° 52' 44.93" and W112° 07' 58.97" at an elevation of 1,796 m. Weeds and native vegetation in the area included mostly cheatgrass (*Bromus tectorum*) and some blue mustard (*Chorispora tenella*). Later, specimens were also found in the alfalfa field. The area is surrounded by juniper-sagebrush (*Juniperus* spp. and *Salvia* spp.) steppe. Nematodes were extracted from soil and roots under an intermittent mist for 72 hr (Ayoub, 1977).

Morphological study: Specimens for light microscopy were examined and photographed alive on glass microscope slides or hand-picked into cold 4% formalin

Received for publication June 27, 2013.
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This paper was edited by Erik J. Ragsdale.

and 1% glutaraldehyde in 0.01 M phosphate buffer at pH 7.3 and stored at 4°C for light microscopy and SEM. Nematodes for preservation also were fixed in TAF (7 ml 37% formaldehyde, 2 ml triethanolamine, 91 ml distilled water) at 60°C and stored at room temperature, then processed to glycerol and mounted on permanent collection slides by a modification of the Seinhorst (1959) technique proposed by Ryss (2003).

For SEM, nematodes fixed in phosphate-buffered aldehyde were transferred to special chambers (McClure and Stowell, 1978) in which they were rinsed for 15 min in distilled water, postfixed 2 hr in 1% aqueous osmium tetroxide, rinsed in distilled water and dehydrated in increasing concentrations of ethanol (10% to 100%) in 10% increments for 30 min each, followed by three changes of 100% ethanol. Alcohol was removed with a critical point dryer and the dried specimens stored under vacuum over silica gel. Dried specimens were mounted on double-sided adhesive tape placed on SEM stubs, sputter-coated with 30 nm of gold, and photographed on a Hitachi S-4800 SEM at 15.0 kV. Stylets for SEM were expressed from nematodes by placing living individual specimens in a 1 µl drop of 45% lactic acid on a 12-mm-round, glass cover slip. A small sliver of a broken cover slip, approximately 1 × 3 mm, was placed over the specimen and pressure applied to it with a needle until the nematode ruptured and the stylet and guiding apparatus were extruded. After sitting overnight, small triangles of filter paper were applied to the edge of the broken sliver to draw off the lactic acid, which was exchanged with 2% formalin, followed by three changes of 50% ethanol. The sliver was then floated on 50% ethanol and removed with forceps. Stylets adhering to the glass cover slips were air-dried and prepared for SEM as described above. Nine syntype specimens on six slides from Allen's (1941) collection were obtained from the USDA nematode collection in Beltsville, MD, and three of Allen's slides with eleven syntypes were obtained from the University of California, Davis nematode collection. These were also measured and photographed.

DNA extraction, PCR, and sequencing: Nematodes for DNA analysis were hand-picked into DESS (Yoder et al., 2006) and stored at 4°C. DNA was extracted and amplified by PCR as described previously (McClure et al., 2012). Individuals were cut in half in a 10-µl drop of sterile lysis buffer and lysed at 60°C for 20 min followed by 10 min at 98°C. Lysed nematodes were stored at -20°C for up to 2 wk before PCR. A Taq PCR Core kit (Qiagen, Valencia, CA) was used for PCR amplification of the D2-D3 region of the 28S rRNA gene, 18S rRNA gene, and the ITS rRNA region. The total reaction volume of 50 µl contained: 5 µl 10x PCR buffer, 5 µl Q solution, 1 µl dNTPs, 1 µl forward primer (10 µmol), 1 µl reverse primer (10 µmol), 0.25 µl Taq, 31.75 µl nuclease-free water, and 5 µl DNA. Primers D2A (5′-ACAAGTACCGT GAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACC AGCTACTA-3′) (De Ley et al., 1999) were used for the D2-D3 region. The ITS region was amplified with 5367 (5′-TTGATTACGTCCCTGCCCTTT-3′) and F195 (5′-TC CTCCGCTAAATGATATG-3′) primers (Schmitz et al., 1998) and the 18S region was amplified with two sets of primers. Set 1 was G18SU (5'-GCTTGTCTCAAAGAT TAAGCC-3') (Blaxter et al., 1998) and R18Tyl1 (5'-GG TCCAAGAATTTCACCTCTC-3'), and set 2 was F18Tyl1 (5'-CAGCCGCGGTAATTCCAGC-3') and R18Tyl2 (5'-CG GTGTGTACAAAGGGCAGG-3'), both sets of which were previously used for other members of the Aphelenchoididae (Chizhov et al., 2006). The thermocycler was programmed for 3 min at 94°C, followed by 40 cycles of 94°C for 1 min for denaturing, and an extension temperature of 72°C for 1 min, with a final extension at 72°C for 10 min. The annealing temperature for the D2A and D3B, F195 and 5367, and 18S primer set 1 was 52°C for 1 min, and for 18S primer set 2 the temperature was 58°C for 1 min. PCR products were separated on a 1.0% agarose gel, stained with ethidium bromide and viewed under UV light. For sequencing, the PCR product bands were cut from the gel and purified using a QIAquick Gel Extraction Kit Gel (Qiagen, Valencia, CA). Purified products were sequenced at the Genetics Core Facility at the University of Arizona. Original sequences were deposited in GenBank under accession numbers KC687094 (18S rRNA), KC687095 (28S rRNA), and KC865782 (ITS rRNA).

Phylogenetic analysis: Original sequences for the 18S rRNA and the D2 and D3 of 28S rRNA gene for *R. megadorus* were aligned with published homologous gene sequences (Chizhov et al., 2006; Ye et al., 2007; Kanzaki et al., 2008; Zhao et al., 2008; van Megen et al., 2009) using ClustalX 1.83 (Thompson et al., 1997) according to default parameters. Four alignments, three of which with reduced sequence numbers for each gene, were generated for this study. Sequences included in the reduced datasets are marked in phylogenetic trees obtained from the full datasets. Outgroup taxa for each dataset were chosen according to the results of previously published analyses (van Megen et al., 2009). The full sequence datasets for each gene were analyzed by Bayesian inference (BI) as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), whereas the six datasets with reduced numbers of taxa were analyzed under the maximum likelihood (ML) criterion in PAUP* 4b10 (Swofford, 2003). The best-fit models of DNA evolution by BI and ML were obtained according to the Akaike Information Criterion using the program jModelTest 0.1.1 (Posada, 2008). The BI analysis under the GTR + I + G model for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilized after approximately 10^3 generations. After discarding burn-in

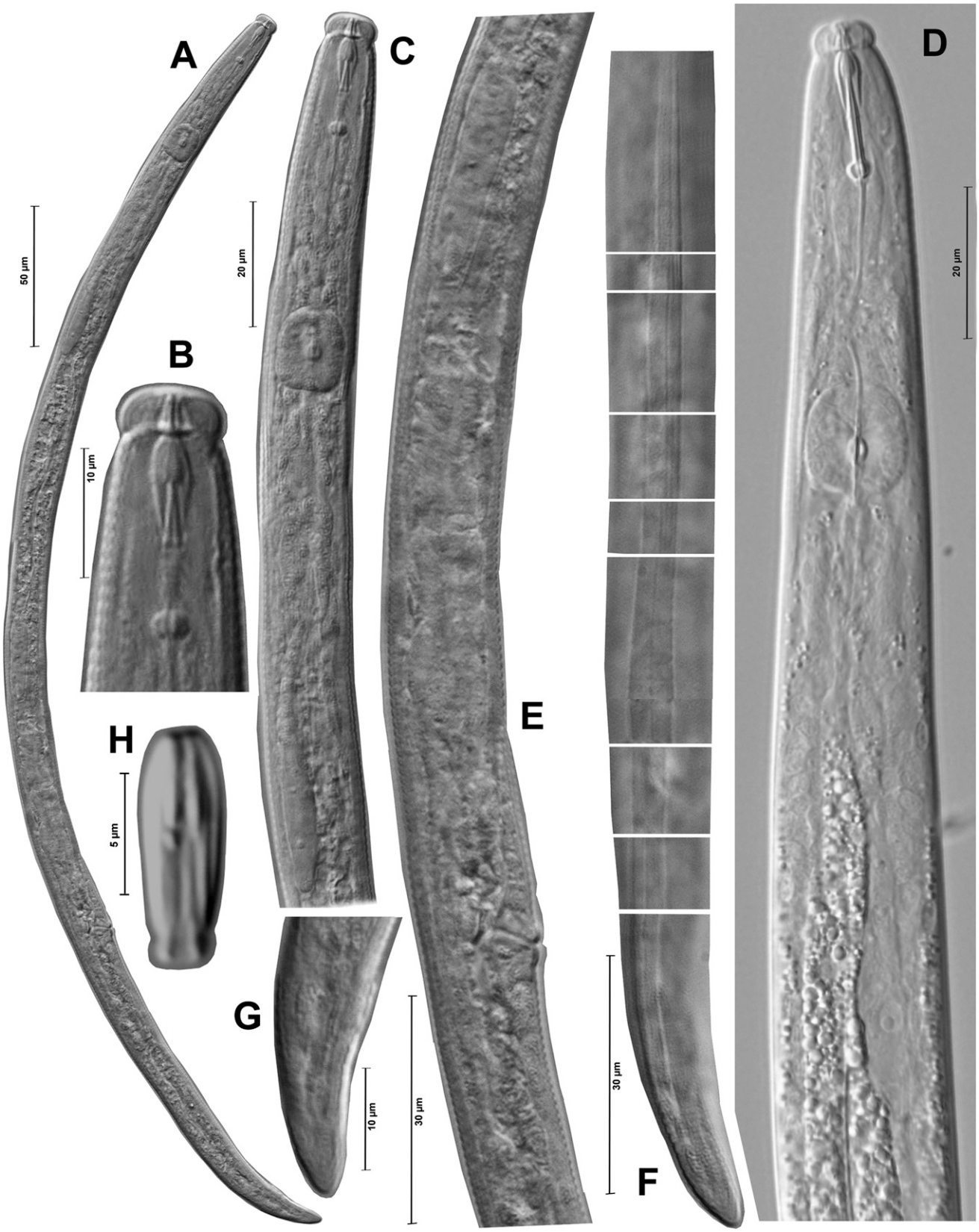

Fig. 1. *Robustodorus megadorus*. A. Adult female. B. Head. C. Anterior portion of digestive system. D. Anterior region of live specimen. E. Reproductive system. F. Lateral field along body length. G. Tail. H. Stylet guiding apparatus extracted from female. Except for D and H, all specimens were fixed in TAF and mounted in glycerol.

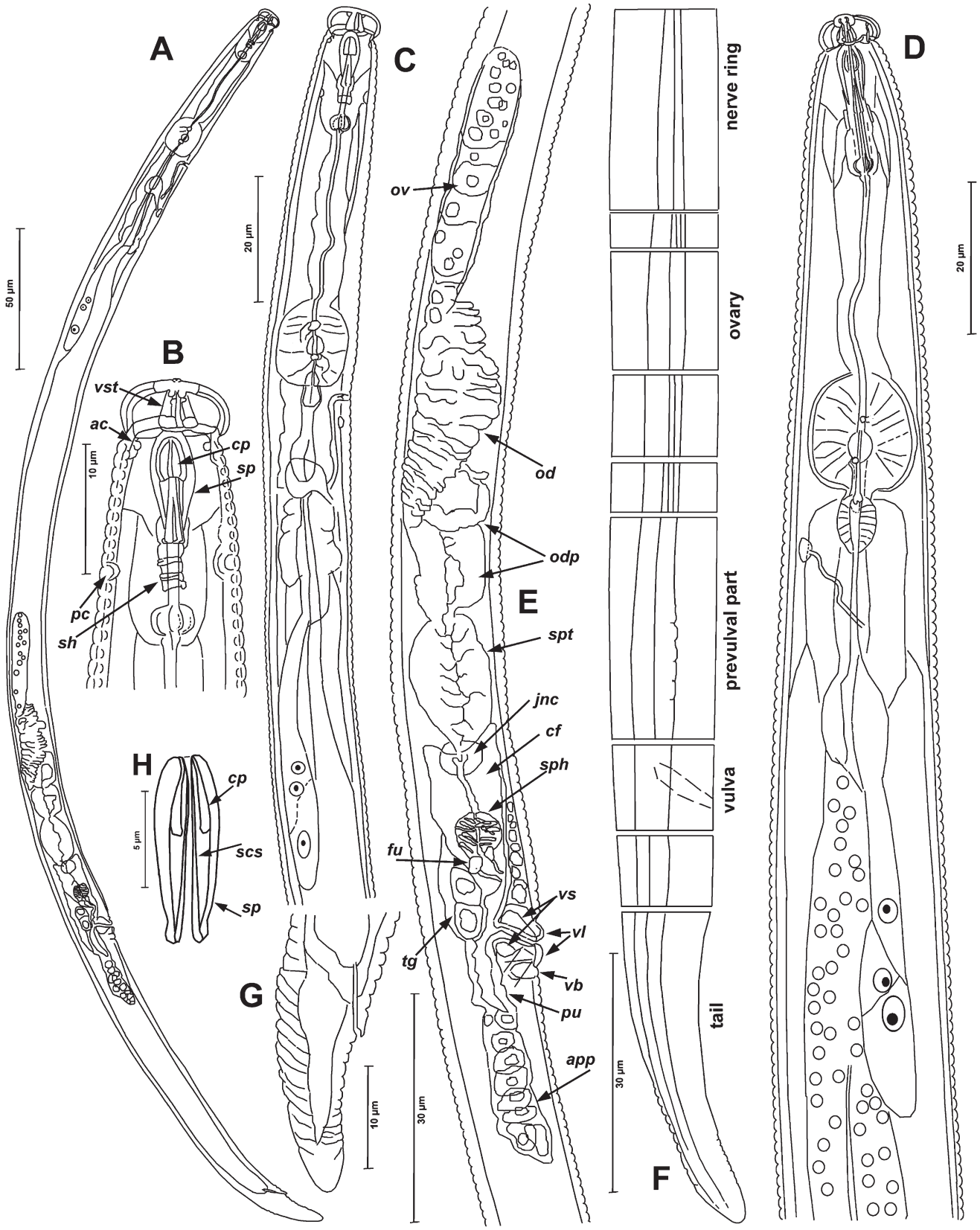

FIG. 2. *Robustodorus megadorus*. A. Adult female body outline. B. Head. C. Anterior region. D. Anterior region of live specimen; others fixed in TAF and mounted in glycerol. E. Reproductive system. F. Lateral field along body length. G. Tail. H. Stylet guiding apparatus extracted from female. *ac* = anterior cephalid; *app* = cellular appendix in the posterior genital branch; *cf* = crustaformeria; *cp* = stylet conus hexagonal protector; *fu* = cuticular funnel between uterus and crustaformeria; *jnc* = crustaformeria-spermatheca junction; *od* = oviduct; *odp* = oviduct posterior pouch; *pc* = posterior cephalid; *pu* = posterior uterus; *scs* = stylet conus sheath; *sh* = stylet sheath; *sp* = stylet conus pouch; *sph* = sphincter between anterior uterus and crustaformeria; *spt* = spermatheca; *tg* = four-celled uterine "ganglion"; *vb* = posterior vulval band; *vl* = vulval lips; *vs* = vaginal sphincter; *vst* = vestibule of cephalic framework.

samples and evaluating convergence the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. A Shimodaira-Hasegawa (SH) test, as implemented in PAUP with RELL bootstrapping for 1,000 replicates, was applied to test alternative ML topologies generated from the six reduced datasets. A chi-square test of homogeneity of base frequencies across taxa was performed using PAUP.

Classification of the phylum Nematoda as proposed by De Ley and Blaxter (2002) and further developed for aphelenchs by Hunt (2008) is used in the present article.

Results

Morphological description (Figs. 1–6; Table 1): The female body is short and C-shaped when fixed. Lateral fields are marked with three incisures (two ridges), with the dorsal ridge distinctly wider anterior to vulva and the ventral ridge wider posterior to the vulva. Anteriorly the ventral ridge may have one or two short additional incisures. Neither deirids, phasmids, nor paravulval papillae were detected. Body annules are 1 to 1.5 μm wide at midbody.

The cephalic region, set-off by deep constriction, is hemispherical and smooth, and its diam. twice its height (Figs. 1B; 2B; 4A,B). The small oral disc is slightly elevated and surrounded by six sensillar pores. Amphids are small pores, subdorsal, and in the same latitude as four cephalic papillae (Fig. 4A,B). Cephalic papillae form the outline of a square; two subdorsal papillae are more dorsal than the amphids. By SEM, the cephalic region is smoothly six-lobed, with two equatorial lobes (with subdorsal amphids), two subdorsal lobes, and two subventral lobes (Fig. 4B). The subdorsal and subventral lobes bear one cephalic papilla each. Amphids are located on the dorsal sublobes of the equatorial lobes. Cephalic papillae are located on sublobes of corresponding lobes, which are close to the equatorial line. Cephalic papillae of subdorsal lobes are situated on lower sublobes, whereas the cephalic papillae of subventral lobes are situated on upper sublobes (Fig. 4B). The stylet is 17 to 20 μm long, robust, with the conical portion slightly longer than the cylindrical shaft, (Figs. 1B,D; 5A,B). The stylet orifice is an elongate slit on the ventral surface (Fig. 5A,B). The stylet knobs are 2 to 3 μm in diameter. Each is c-shaped in outline, but somewhat flattened on its lateral margins anteriorly (Fig. 5A,B).

The anterior end of the stylet is surrounded by a cylindrical, tear-drop-shaped guiding apparatus bearing a thickened ellipsoidal pouch at its anterior end and having a distinct hexagonal inner cuticular structure (Fig. 2B,H). A cephalic disc outside of the labial disc, such as present in *Schistonchus* spp., is absent. The cephalic framework is heavily sclerotized with a massive basal plate forming a hexagonal cuticular vestibule around the stylet tip (Fig. 2B, *vst*). The cephalic framework has six radial cuticular partitions: ventral, dorsal, two subdorsal, and two subventral are attached to the vestibule. Partitions are located between the cephalic region lobes and divide the inner space of cephalic framework, thus supporting the whole cupola of the region (Fig. 4B). Outer cephalic radial ridges, such as those in *Laimaphelenchus*, were not detected. Anterior cephalids are located just posterior to the cephalic framework; the posterior cephalids are in the central region of the stylet shaft (Fig. 2B).

The metacorpus is rounded-ovoid with a ratio of length to width of 1.4:1 (Figs. 1C,D; 2C). The gland duct of the dorsal pharyngeal gland opens into the lumen of the metacorpus just anterior to the valve; the two subventral glands ducts open immediately after the valve. The excretory pore is located on the ventral surface between the metacorpus and nerve ring. The hemizonid is two to three annules posterior to the round excretory pore. The pharyngo-intestinal junction, just posterior to the metacorpus, includes a small cuticular valve surrounded by muscle fibers (Fig. 2D). The pharyngeal glands extend in a lobe, 45 to 80 μm in length, overlapping the intestine dorsally (Figs. 1C,D; 2A,C,D). The large dorsal-gland nucleus is in the posterior lobe; the two smaller nuclei of the subventral glands are anterior to the nucleus of the dorsal gland (Figs. 1C,D; 2C,D).

The female reproductive system is monodelphic, with a postuterine branch 1.3 to 2.3 times the body diameter at the vulva. The postuterine branch consists of a hollow sack with a length less than the body diameter at the vulva and a cellular appendix. The vulva is a transverse, thick-lipped slit, devoid of papillae, situated in the anterior part of a distinct depression that is visible in lateral view in living specimens (Figs. 2E; 4C,D). A vulval flap is lacking. The anterior outer lip of the vulva projects a micron or more from the ventral surface (Fig. 4C,D). Small, curved, inner lips are visible by SEM (Fig. 4C). The vagina is cuticular, sloping anteriad. A pear-shaped sphincter surrounds the vagina. Opposite the vagina, at the dorsal side of the uterus, there is a four-celled structure (two pairs of large cells) referred to herein as a "ganglion" (Fig. 2E, *tg*). The pear-shaped crustaformeria is anterior to the uterus from which it is separated by a sphincter of six to seven pairs of muscle cells. An oval spermatheca, anterior to the crustaformeria, is devoid of sperm or large inner cavity, with the lumen occluded (Fig. 2E). A spherical fold separates the crustaformeria and spermatheca. Anterior to the spermatheca is a two-chambered pouch (with two hollow cavities), which is considered in this study as an expanded posterior part of the oviduct. The anterior part of the oviduct is long and wide, multifolded, and wrinkled, continuing to a short ovary with a terminal apical cell (Fig. 2E).

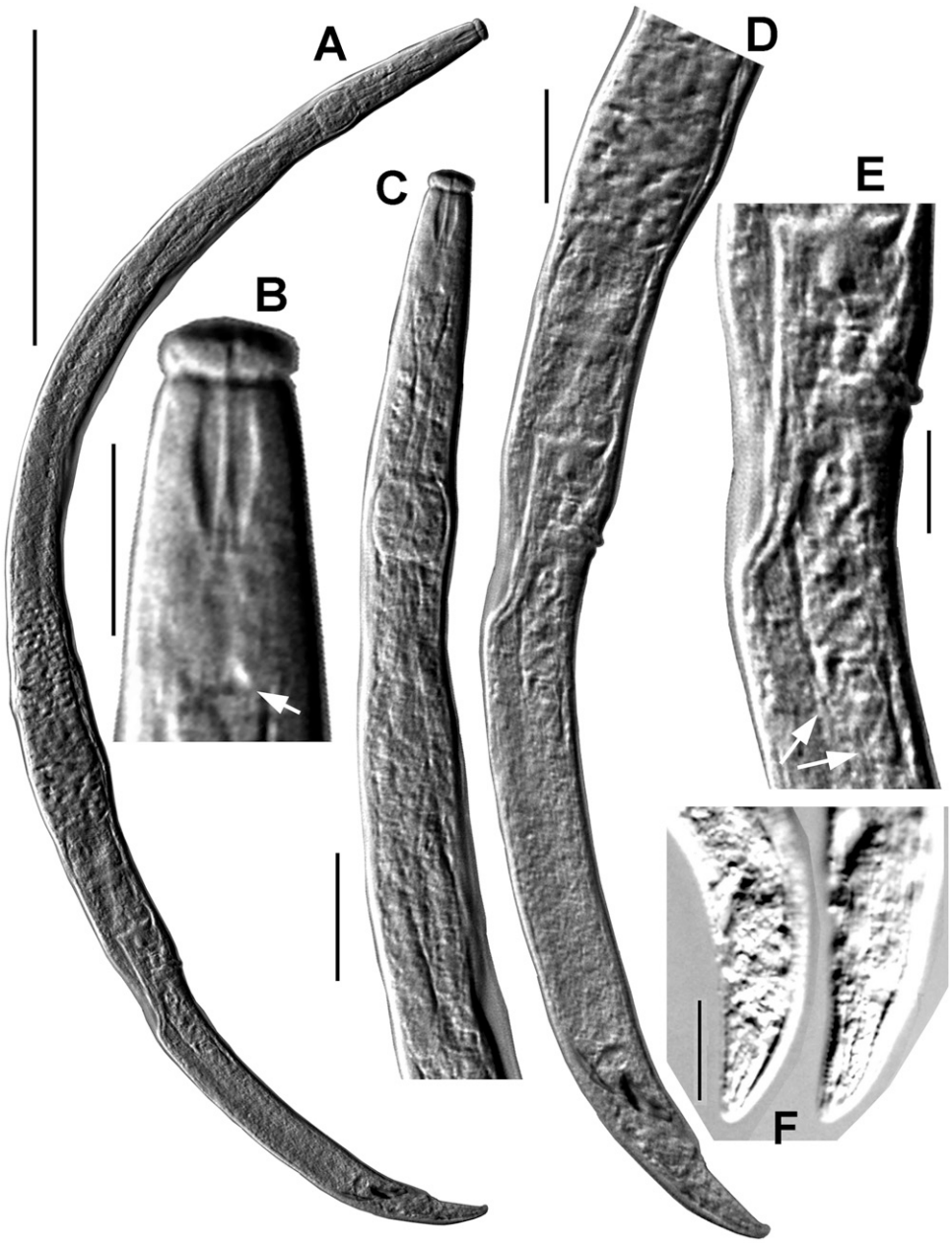

Fig. 3. *Robustodorus megadorus*. Female lectotype A–E and paralectotypes F from Allen's 1941 collection. A. Body shape. B. Head. C. Anterior. D. Posterior. E. Vulval region and the posterior genital branch. F. Tail shape. Scale 100 μm for A, 20 μm for C and D, and 10 μm for others. Arrows: stylet knobs (B) and cellular appendix in the posterior genital branch (E).

The tail, slightly curved ventrally, is conically rounded, with 9 to 15 ventral annuli, its length twice as long as the anal body diam. (Figs. 1; 2F,G; 4E,F). The anus is a transverse, arched slit, with the anterior anal lip more prominent (Fig. 4F). The tail tip is smooth, its smooth zone not more than the width of two tail annules. Lateral fields extend to the terminus.

Our population of *R. megadorus* was infected with a bacterium, *Pasteuria* sp. (Fig. 6A,B,D). Mature bacterial endospores were found attached to the nematode's cuticle (Fig. 6D) and inside the body cavity. In some cases, spores inside the body were confined to the intestinal lumen where they were distributed from the metacorpus to the rectum (Fig. 6A,B). In other specimens, endospores filled the entire body in a manner similar to those infected specimens that we found in Allen's (1941) slides (Fig. 6C).

Compared to the specimens collected by Allen in 1940 (Fig. 3), the specimens in our collection showed some differences in morphometrics, probably caused previously by slight flattening because of pressure of the cover slip (see increased body width and a-value in Allen's specimens in Table 1). Small differences in the c ratio may have been caused by the method of fixation: the part of the body between pharynx and anus is more shortened by cold formalin fixation than in nematodes

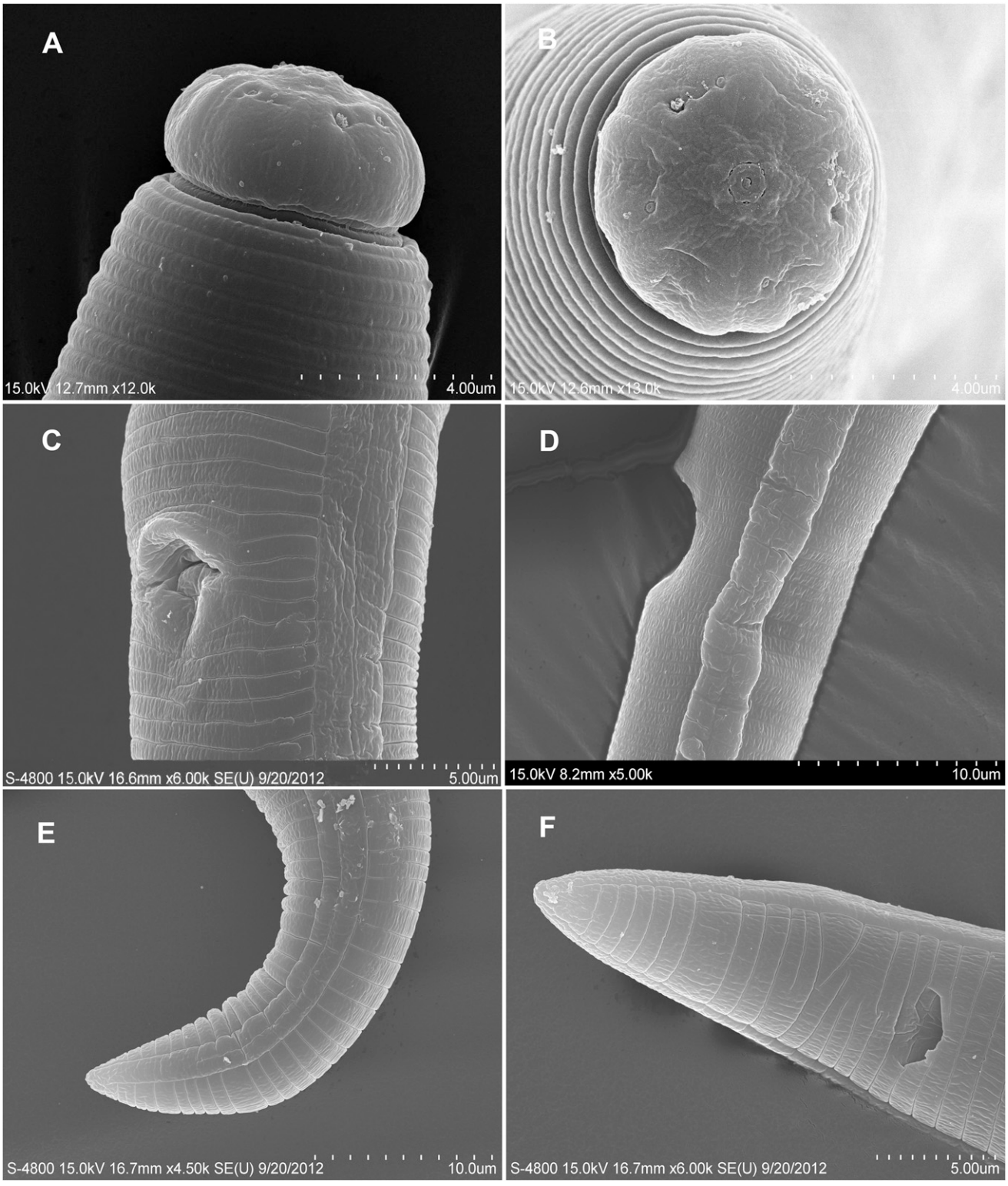

Fig. 4. *Robustodorus megadorus*, adult female. Scanning electron micrographs. A. Cephalic region. B. En face view of head. C. Vulva, subventral view. D. Vulva, lateral view. E. Tail, lateral view. F. Tail, ventral view.

newly fixed in TAF. However, these differences are not significant and they did not impede the identification of *R. megadorus* in our samples from Utah. None of the specimens collected by Allen in 1940 were labeled as the holotype. Because all of Allen's specimens of *R. megadorus* (= *A. megadorus*) are consequently syntypes, a lectotype was selected from the syntypes according to Article 74 of the International Code of the Zoological Nomenclature. The remaining specimens have been designated as paralectotypes (Table 1).

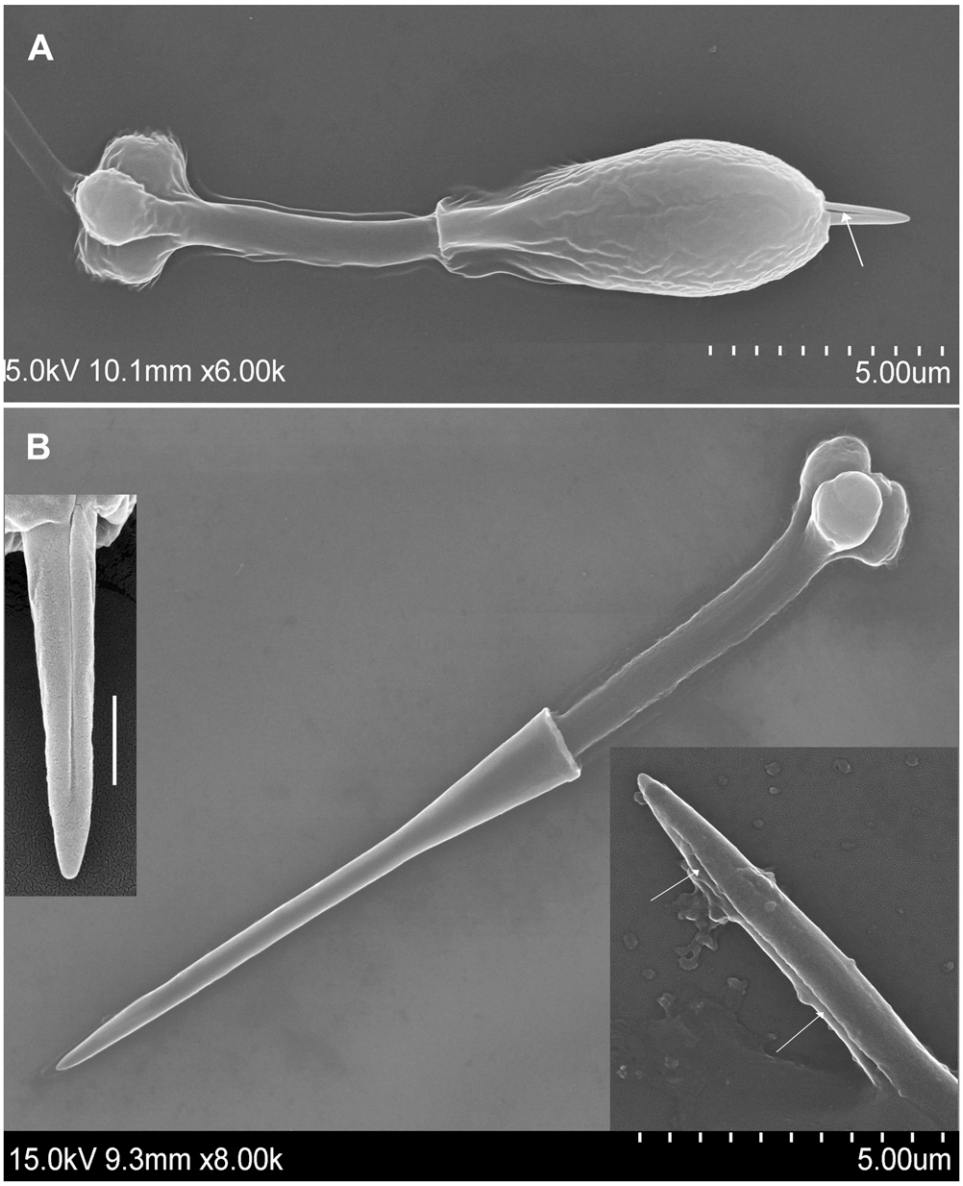

FIG. 5. *Robustodorus megadorus*, adult female stylet. A. Stylet enclosed in guiding apparatus. Arrow points to ventral orifice. B. Stylet without guiding apparatus. Upper inset: Ventral view of stylet tip. Lower inset: subventral view of stylet tip. Arrows point to orifice. Scale for both insets 0.5 μm.

Lectotype and paralectotypes: The lectotype slide, "N 2058_6" (collected by Allen in Utah, near Mosida, 1940), was deposited in the Nematode Collection at the University of California, Davis, CA. Paralectotypes (10 females) were deposited in the same collection, and at the USDA Nematology Laboratory at Beltsville, MD (nine females). Specimens collected by authors, and used for this study, were deposited at the USDA Nematology Laboratory (five females), the University of California Nematode Collection (five females), and the Nematode Collection of the Zoological Institute, RAS, St. Petersburg, Russia (five females).

Diagnosis and relationships: Robustodorus differs from other taxa of the subfamily Aphelenchoidinae by virtue of its extremely strong stylet with a narrow lumen, strong knobs, and a guiding apparatus that bears a thickened, ellipsoidal pouch with an inner hexagonal cuticular structure at its anterior end. The stylet shape and guiding apparatus are unique in the family Aphelenchoididae whose members for the most part have a weak, short stylet devoid of prominent knobs. Species of *Schistonchus* may have long, robust stylets with stout knobs, but they do not possess the drop-shaped cuticular structure in the anterior part of the guiding apparatus. Additional characters distinguishing *Robustodorus* from other genera of Aphelenchoididae are a short tail with rounded tip devoid of mucrons and the position of excretory pore being between the median bulb and nerve ring. The closest genus is *Aphelenchoides*, especially a group of its species with the same position of the excretory pore: *A. blastophthorus*, *A. fragariae*, *A. saprophilus*, *A. xylocopae*, and *A. subtenuis*. *Robustodorus*

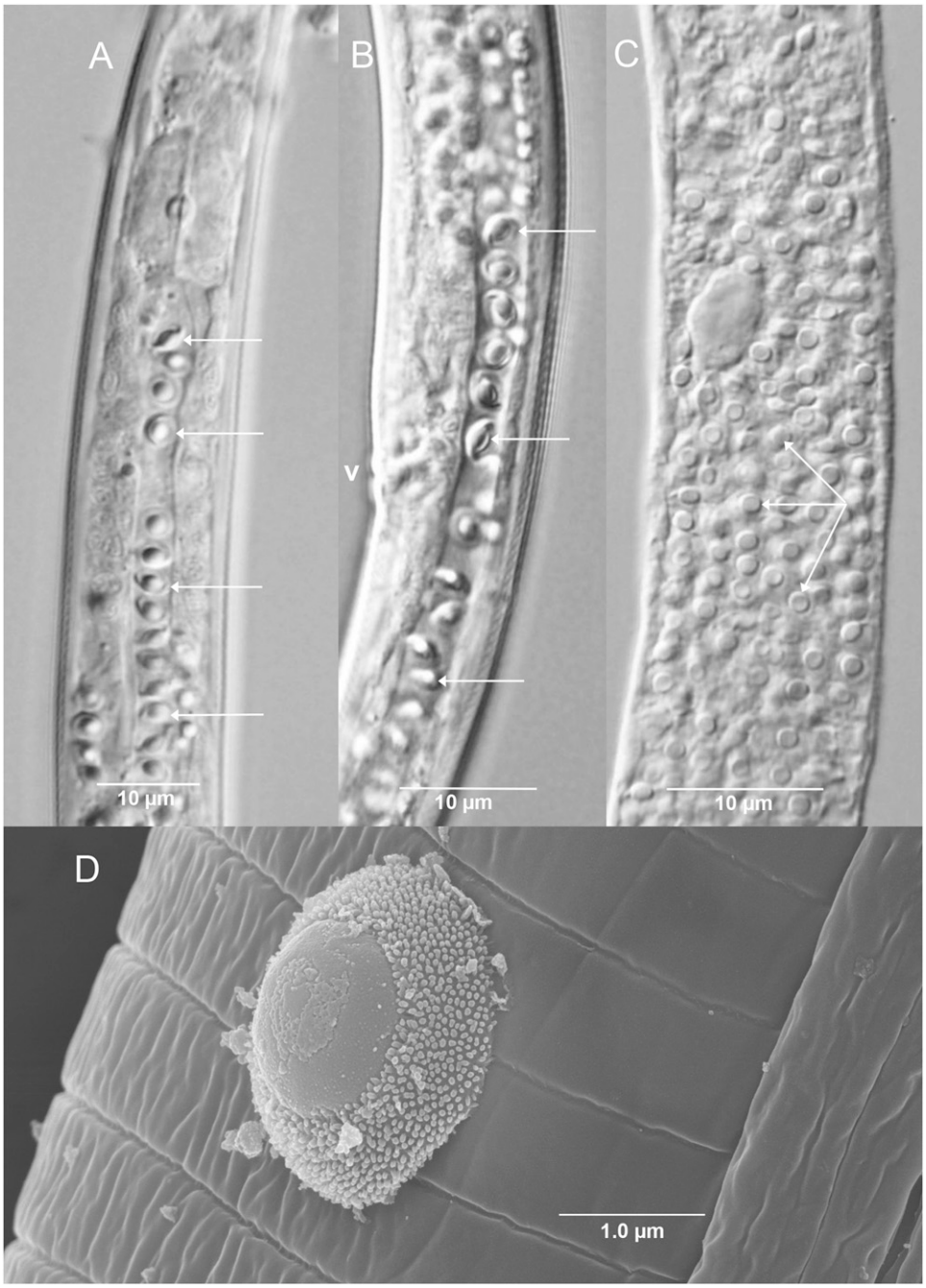

Fig. 6. *Pasteuria* sp. infecting *Robustodorus megadorus*. A. Mature endospores (arrows) in the intestinal lumen at its junction with the metacorpus. B. Mature endospores (arrows) in the intestinal lumen, anterior and posterior to the vulva (v). C. Mature endospores in the body cavity of a *R. megadorus* female collected by M. W. Allen (1941). D. Scanning electron micrograph of an endospore attached to the cuticle of *R. megadorus*.

differs from these species by possessing three prominent incisures in the lateral field rather than four or two weak incisures, and the absence of a mucron, whereas the *Aphelenchoides* species mentioned above have a distinct and a long, needle-like ventral mucron at the tip of the tail.

Phylogenetic position and relationships with other aphelenchids: The full, aligned dataset 1, which comprised the D2-D3 region of the 28S rRNA gene, included sequences for 101 taxa and was 874 bp in length. The chi-square test of homogeneity of base frequencies across taxa resulted in significant *P*-values (chi-square = 1,115.12, df = 300, $P = 0.00$). Different A-T contents were observed for *Schistonchus*: species from subclade 1 had an A-T content of 45% and the content of subclade 2 was 68%, whereas the average for the D2-D3 dataset was 50%. Phylogenetic relationships within Aphelenchoididae, as inferred in the BI analysis of the full dataset 1, are presented in Fig. 7. The BI tree contains four major clades: (1) *Tylaphelenchus* and *Pseudaphelenchus*;

TABLE 1. Morphometrics of *Robustodorus megadorus*. All measurements in μm and in the format: mean ± s.d. (range).

	Utah collection, 2012. n = 15	Lectotype selected from Allen syntypes, UCD collection, slide 2059_06; new measurements	Allen syntypes (paralectotypes and a lectotype), UCD collection, new measurements, n = 11	Allen paralectotypes, Beltsville collection, new measurements, n = 9	Allen, 1941 publication, n = not stated.
L	496.3 ± 38.9 (455-546)	507	496 ± 37.1 (441-549)	452.6 ± 79.1 (348-568)	500
a	30.4 ± 1.6 (28.4-32.1)	21.1	20.0 ± 4.1 (14.6-26.7)	22.8 ± 2.6 (18.7-27.0)	31
b	7.7 ± 0.4 (7.3-8.3)	7.7	7.3 ± 0.6 (5.8-7.8)	7.0 ± 1.0 (5.7-8.6)	8.3
b'	3.7 ± 0.3 (3.5-4.1)	3.7	3.8 ± 0.4 (3.2-4.6)	3.6 ± 0.5 (3.0-4.8)	-
c	26.3 ± 2.6 (22.8-28.7)	22.0	19.4 ± 2.0 (16.3-22.1)	19.7 ± 3.0 (16.7-25.8)	22.7
c'	2.0 ± 0.1 (1.8-2.1)	2.6	2.2 ± 0.4 (1.7-3.0)	2.1 ± 0.3 (1.8-2.6)	2
V	73 ± 1.2 (72-75)	73	73 ± 1.4 (71-75)	72 ± 2.2 (68-76)	72
Stylet	18 ± 0.8 (17-19)	19	18.7 ± 0.5 (18-19)	18.9 ± 1.2 (17-20)	17
Stylet cone	12 ± 1.1 (11-13)	12	10.8 ± 0.8 (10-12)	11 ± 0.8 (10-12)	-
Cephalic region diameter	8.0	7	7.7 ± 1.3 (6-9)	7.9 ± 0.7 (7-9)	-
Cephalic region height	3.5-4	3	3.5 (3-4)	3.7 (3-4)	-
Stylet base width	3	3	2.5-3	3.5 (2.6-4)	-
Stylet base height	2.4 (2-3)	2.5	2.5	2.8 (2.5-3.2)	-
Stylet knob radius	1.4 (1.3-1.5)	1.5	1-1.5	1.4-1.5	-
Median bulb posterior end from head end	60.3 ± 0.5 (60-61)	60	61.1 ± 3.6 (56-68)	56.3 ± 1.6 (54-59)	-
Median bulb length	13.7 ± 0.5 (13-14)	14	15.9 ± 2.4 (13-21)	14.1 ± 1.1 (13-16)	-
Median bulb diam.	10.7 ± 0.5 (10-11)	12	14.3 ± 2.8 (11-20)	12.4 ± 1.5 (10-15)	-
Median bulb ratio (L/W)	1.3 ± (1.2-1.4)	1.2	1.2 ± 0.2 (0.9-1.5)	1.2 ± 0.1 (1-1.3)	-
Median bulb valve diam.	3-3.5	3.5	3-4	3.5 (3-4)	-
Excretory pore from head end	67.3 ± 4.0 (62-71)	73	77 ± 7.1 (63-87)	70.4 ± 3.2 (67-75)	-
Nerve ring posterior border from head end	75.3 ± 2.1 (73-78)	79	82.6 ± 7.0 (73-98)	77.1 ± 3.3 (73-81)	-
Pharynx to pharynx-intestinal valve end	64.3 ± 1.8 (62-66)	66	68.7 ± 8.7 (63-94)	64 ± 3.4 (60-71)	-
Pharynx to gland lobe end	133.3 ± 5.2 (128-140)	138	133 ± 15.2 (111-170)	124.4 ± 12.6 (110-145)	-
Gland lobe	69 ± 4.4 (66-75)	72	64.4 ± 9.0 (45-76)	60.4 ± 11.4 (45-80)	-
Gland lobe/body diam.	4.2 ± 0.3 (3.9-4.7)	3.0	2.7 ± 0.7 (1.4-3.4)	3.1 ± 0.7 (1.7-3.8)	3-5
Body diam. (max.)	16.3 ± 0.5 (16-17)	24	26 ± 6.8 (18-36)	20 ± 3.9 (16-26)	-
Vulval body diam.	15.0 ± 0.8 (14-16)	18	20.3 ± 4.2 (15-28)	17.3 ± 3.4 (13-22)	-
Posterior genital branch	27.0 ± 3.9 (22-31)	42	35.4 ± 5.0 (27-42)	30 ± 9.0 (18-40)	-
Posterior genital branch without celled appendix	10.7 ± 1.0 (10-12)	15	17.1 ± 3.2 (14-25)	14.1 ± 4.1 (7-21)	-
Posterior genital branch/vulval diam.	1.8 ± 0.2 (1.6-1.9)	2.3	1.8 ± 0.4 (1.3-2.3)	1.7 ± 0.3 (1.4-2.2)	2-3
Posterior genital branch sac without rudimentary ovary/vulval diam.	0.7 ± 0.04 (0.7-0.8)	0.8	0.9 ± 0.2 (0.5-1.1)	0.8 ± 0.2 (0.5-1.1)	-

(*Continued*)

Table 1. Continued.

	Utah collection, 2012. n = 15	Lectotype selected from Allen syntypes, UCD collection, slide 2059_06; new measurements	Allen syntypes (paralectotypes and a lectotype), UCD collection, new measurements, n = 11	Allen paralectotypes, Beltsville collection, new measurements, n = 9	Allen, 1941 publication, n = not stated.
Posterior genital branch/V-anus distance, %	24 ± 2.0 (22-26)	37	32 ± 4.7 (23-38)	29 ± 6.5 (18-39)	-
Posterior genital branch without celled appendix/V-anus distance, %	9 ± 0.8 (8-10)	13	16 ± 2.6 (13-20)	13 ± 2.7 (8-17)	-
Tail	19 ± 1.5 (17-20)	23	25.8 ± 3.1 (20-31)	23.3 ± 4.6 (17-29)	-
Tail diam.	9.7 ± 1.3 (8-11)	9	11.9 ± 2.1 (8-15)	10.9 ± 1.9 (8-13)	-
Annuli (width of 10 at midbody)	11.7 ± 0.49 (11-12)	14	15 ± 5.0 (10-23)	12.2 ± 2.7 (9-17)	11-17

(2) *R. megadorus*, all *Schistonchus*, *Laimaphelenchus*, and *Aphelenchoides* species, except for *A. stammeri*; (3) Ektaphelenchinae and Acugutturinae; (4) *Bursaphelenchus* species, except *B. abruptus*. Two lineages, namely those of *A. stammeri* and *B. abruptus*, have unresolved relationships to other aphelenchids. Clade 2, which contains Aphelenchoididae, is divided into two subclades. *Robustodorus megadorus* occupies a basal position in the subclade 2a.

The full dataset 2, containing the 18S rRNA gene, included sequences for 72 taxa and was 1,800 bp in length. The chi-square test of homogeneity of base frequencies across taxa resulted in no significant *p*-values (chi-square = 232.17, df = 213, $P = 0.18$). The BI tree reconstructed based on the full dataset 2 is given in Fig. 8. *Pseudaphelenchus* and *Tylaphelenchus* occupy basal positions within the family Aphelenchoididae (clade 1). The tree recovered three major clades: (2) the majority of the Aphelenchoididae taxa; (3) Ektaphelenchinae taxa, *Seinura demani, Noctuidonema* sp., and *Anomyctus xenurus;* (4) *Bursaphelenchus* spp., *Ruehmaphelenchus* spp., and *Aphelenchoides stammeri*. *Robustodorus megadorus* and *A. subtenuis* are sister taxa at the basal position of the subclade 2a.

The "constrain" option in PAUP was used to infer several trees from six reduced datasets (Table 2). The trees supporting some traditional views on aphelenchid relationships were tested with SH test. This test showed that the D2-D3 28S rRNA and 18S rRNA gene datasets strongly rejected the monophyly of each of the genera *Aphelenchoides*, *Laimaphelenchus*, *Schistonchus*, and *Ektaphelenchus*, and they confirmed the monophyly of *Bursaphelenchus*. Tests of the D2-D3 28S rRNA dataset also rejected the monophyly of each of the genera *Deviburs aphelenchus* and *Ektaphelenchoides*. The SH test of the 18S rRNA dataset rejected the placement of *Anomyctus* within the subfamily Aphelenchoidinae and monophyly of the subfamily Ektaphelenchinae. The results of SH test for other alternative hypotheses of the phylogeny of the family Aphelenchoididae are also given in Table 2.

Because the ITS rRNA gene sequence of *R. megadorus* showed low similarity and could not be unambiguously aligned with ITS sequences of other Aphelenchoididae, it was not included in our phylogenetic analyses.

Discussion

Results of our observations supplement the diagnosis of *R. megadorus* with the following characters: a labial disc surrounded by six pore-like sensilla; the absence of a cephalic disc; a lobed cephalic region devoid of annulation; a hexagonal inner cuticular structure of the pouch surrounding the stylet cone; large stylet knobs, rounded in outline and somewhat flattened on their lateral sides; the position of the excretory pore between the median bulb and nerve ring; a massive spermatheca with an occluded lumen, without an inner cavity, and devoid of sperm; division of the female genital system into an ovary, oviduct, oviduct pouch, a spermatheca, a crustaformeria with muscular sphincters, a uterus with a four-celled "ganglion," and a cuticular vagina supplied with pear-shaped muscles. A division of the posterior branch of the genital system into a posterior uterus and an appendix with cellular structure has been revealed. These characters distinguish the genus from the closely related genus *Aphelenchoides*. The stylet orifice consists of an open, ventral, elongate slit or groove, comparable with that described for *Aphelenchus avenae* (Ragsdale et al., 2008). Only its anterior portion was visible in our SEM preparations, so the appearance of the slit in the basal portion of the cone and its union with the lumen of the stylet shaft could not be ascertained. The function of the cellular appendix in the posterior genital branch is not known. Because males are absent and the spermatheca empty, the cells within the appendix are not likely to be sperm. Nor is it likely that the structure is a rudimentary ovary, because in aphelenchs the germinal cells of the genital primordium in juveniles move only to the anterior genital tube during maturation (Ryss and Chernetskaya, 2009).

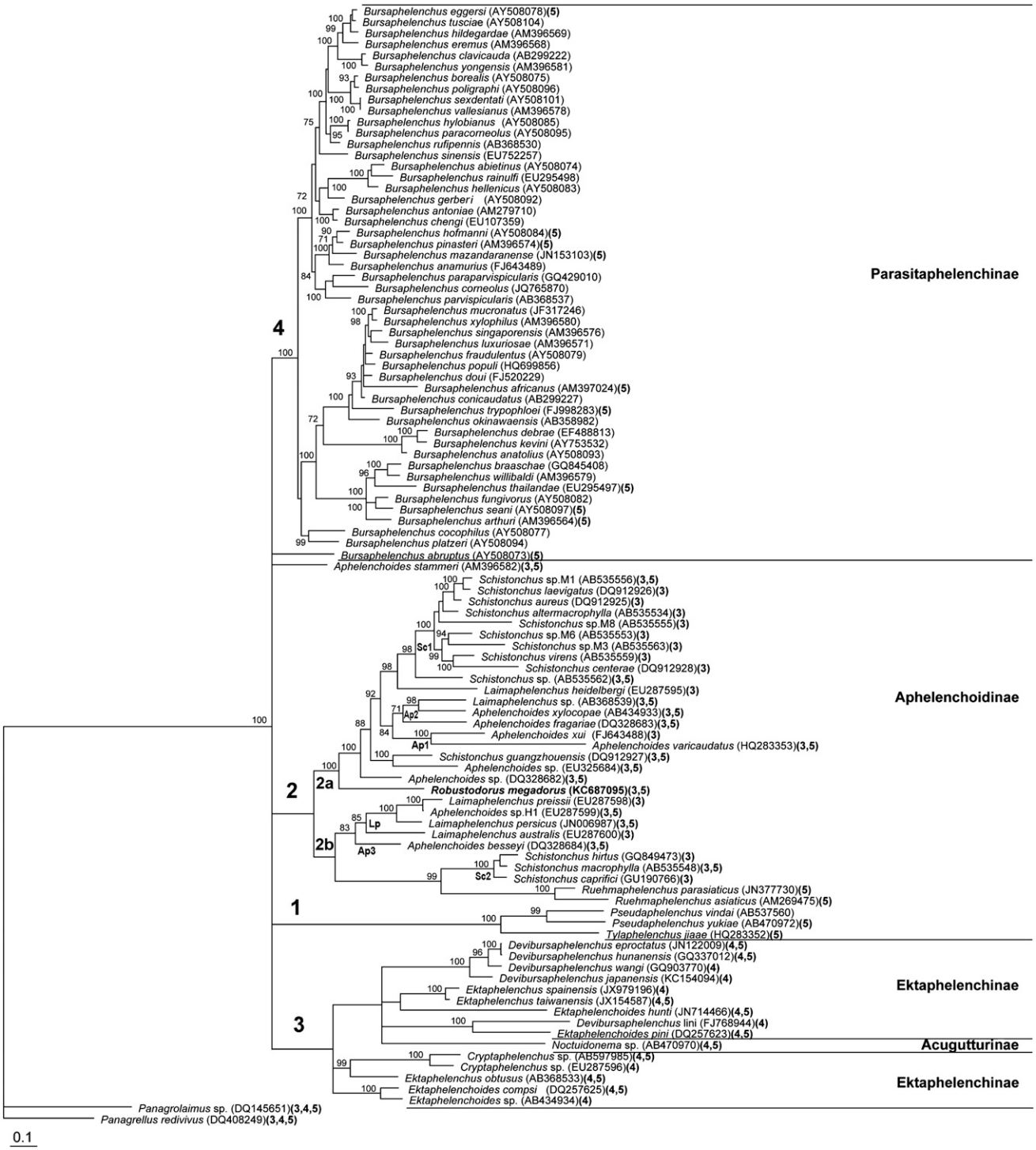

FIG. 7. Phylogenetic relationships within nematodes of the family Aphelenchoididae (Nematoda: Tylenchina) as inferred by Bayesian analysis of D2-D3 28S rRNA gene sequences. Posterior probability values more than 70% are given on appropriate clades. Original sequences are indicated in bold. Sequences used in the reduced datasets are marked by the brackets with the dataset number. Numeration of clades according to Kanzaki and Giblin-Davis (2012). Additional subclades indicate species groups within lineages: *Aphelenchoides*-1 (Ap1); *Aphelenchoides*-2 (Ap2); *Aphelenchoides*-3 (Ap3); *Schistonchus*-1 (Sc1); *Schistonchus*-2 (Sc2); *Laimaphelenchus penardi* group (Lp).

In the inferred phylogenetic trees (Figs. 7,8), some clades join species belonging to different genera. Therefore, in a discussion of the phylogenetic position of *Robustodorus*, it is important to find morphological characters that might be markers for different inferred branches of lineages on the phylogeny, which was well reconstructed and well tested. However, we regard the phylogeny as only an independent way of verifying phylogenetically informative morphological characters and not necessarily an infallible infrastructure for assigning

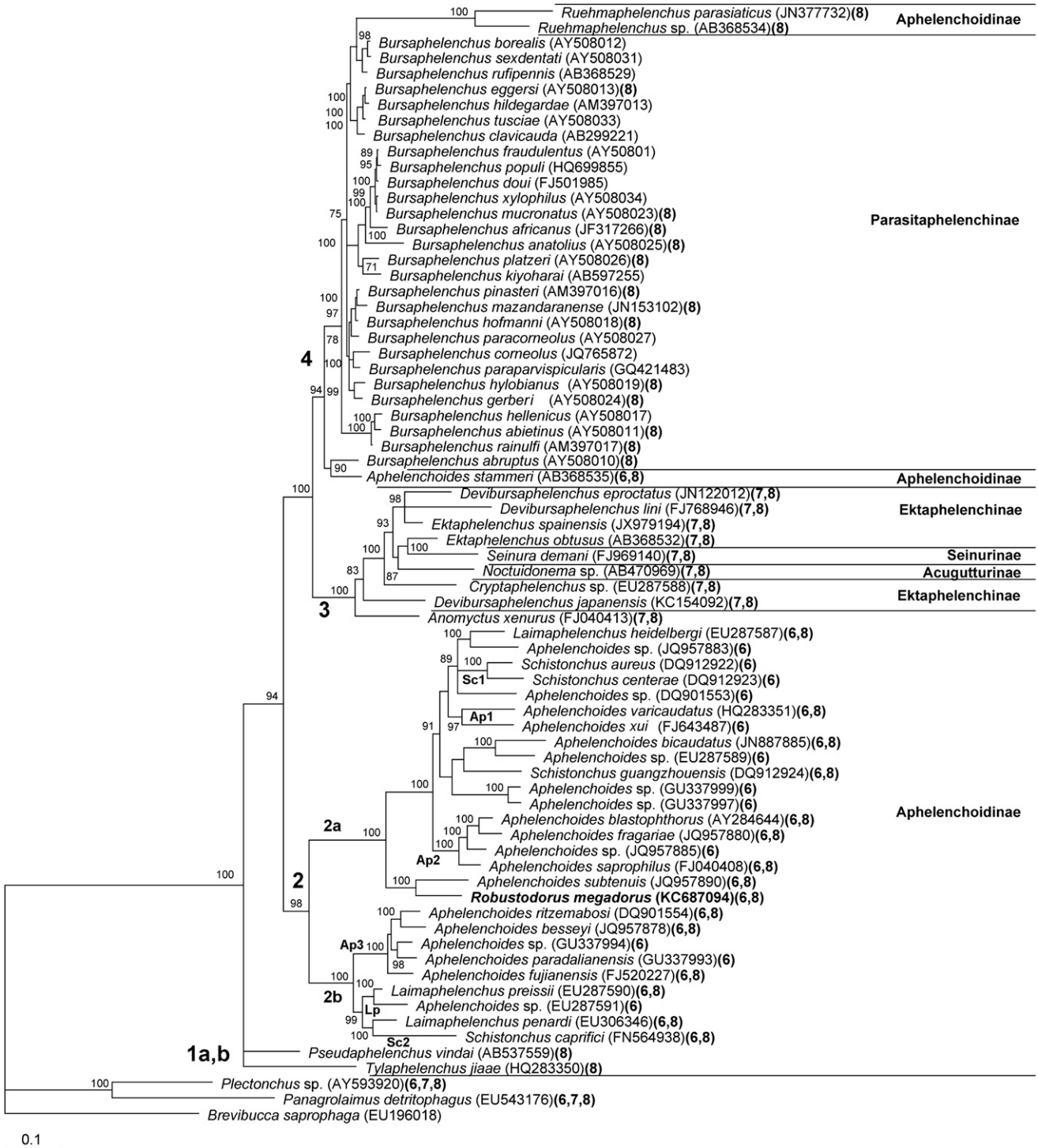

FIG. 8. Phylogenetic relationships within nematodes of the family Aphelenchoididae (Nematoda: Tylenchina) as inferred by Bayesian analysis of 18S rRNA gene sequences. Posterior probability values more than 70% are given on appropriate clades. Original sequence is indicated in bold. Sequences used in the reduced datasets are marked by brackets enclosing the dataset number. Numeration of clades is according to Kanzaki and Giblin-Davis (2012). Additional subclades indicate species groups within lineages: *Aphelenchoides*-1 (Ap1); *Aphelenchoides*-2 (Ap2); *Aphelenchoides*-3 (Ap3); *Schistonchus*-1 (Sc1); *Schistonchus*-2 (Sc2); *Laimaphelenchus penardi* group (Lp).

them. The morphological structures do not reflect the genes directly nor do the genes transcribe directly the characters of taxonomic importance. Thus, the molecular tree may show only the relatively high probability of the appearance of the common morphological presumptive "markers" synapomorphic for a given phylogenetic clade. In the discussion below, the numeration of clades according to Kanzaki and Giblin-Davis (2012) is used.

Both trees (18S and D2-D3 of 28S rRNA) have a similar arrangement of species in clades 2a and 2b.

TABLE 2. Results of Shimodaira-Hasegawa test of tree topologies and alternative phylogenetic hypotheses of the Aphelenchoididae.

Gene Hypothesis	D2 and D3 of 28S rRNA			18S rRNA		
	-*ln* L	Δ *ln* L	*P*	-*ln* L	Δ *ln* L	*P*
	Dataset 3			Dataset 6		
ML tree	12551.41244	best	-	14952.55519	best	-
Aphelenchoides is a monophyletic genus	12856.77489	305.36244	0.000*	15520.20437	567.64919	0.000*
Aphelenchoides species from the subclade 1 is a monophyletic group	12610.81836	59.40592	0.038*	15138.20354	185.64835	0.000*
Aphelenchoides species from the subclade 2 is a monophyletic group	12613.93398	62.52154	0.024*	14975.06428	22.50909	0.528
Aphelenchoides species excluding *A. stammeri* is a monophyletic group	12855.23425	303.82181	0.000*	15499.96096	547.40577	0.000*
Laimaphelenchus is a monophyletic genus	12816.34670	264.93426	0.000*	15315.57783	363.02264	0.000*
Schistonchus is a monophyletic genus	12613.31163	61.89919	0.030*	15228.50985	275.95466	0.000*
Schistonchus species from the subclade 1 is a monophyletic group	12567.50831	16.09587	0.553	14983.34492	30.78973	0.400
Robustodorus megadorus is a separate basal lineage and does not cluster with *A. subtenuis*	-	-	-	14975.87944	23.32425	0.514
Aphelenchoides blastophthorus + *A. fragariae* + *A. saprophilus* + *A. subtenuis* + *Aphelenchoides* sp.	-	-	-	15071.61720	119.06202	0.001*
	Dataset 4			Dataset 7		
ML tree	7507.30217	best	-	8108.81197	best	-
Deviblursaphelenchus is a monophyletic genus	7546.57766	39.27549	0.003*	8114.82551	6.01354	0.640
Ektaphelenchus is a monophyletic genus	7546.57766	39.27549	0.003*	8152.76125	43.94929	0.015*
Ektaphelenchoides is a monophyletic genus	7548.29873	40.99656	0.001*	-	-	-
Ektaphelenchidae is a monophyletic family	7509.99140	2.68923	0.610	8147.20616	38.39420	0.017*
	Dataset 5			Dataset 8		
ML tree	17530.50886	best	-	21419.40884	best	-
Bursaphelenchus is a monophyletic genus	17531.30216	0.79330	0.776	21429.62324	10.21439	0.724
Anomyctus within Aphelenchoididae	-	-	-	21499.45649	80.04765	0.005*
Pseudaphelenchus and *Tylaphelenchus* within Aphelenchoididae	17530.50886	0.00000	1.00	21419.40884	0.00000	1.00
Aphelenchoides stammeri within Aphelenchoididae	17543.27156	12.76271	0.259	21527.75749	108.34864	0.000
Ruehmaphelenchus within Aphelenchoididae	17530.50886	0.00000	1.00	21425.45166	6.04282	0.762
Robustodorus and *Ruehmaphelenchus* are sister taxa	17538.21205	7.70320	0.395	21527.75749	108.34864	0.000*
Bursaphelenchus and *Ruehmaphelenchus* are sister taxa	17536.42240	5.91354	0.776	21435.42177	16.01293	0.570

* Trees significantly worse than the best tree at $P < 0.05$.

Robustodorus, together with *Aphelenchoides subtenuis*, occupies a basal branch in the clade 2a in the 18S tree (Fig. 8). The taxa of clade 2a have the excretory pore at level of or anterior to the nerve ring, whereas in the taxa of clade 2b the excretory pore is posterior to nerve ring.

The most advanced part of the clade 2a includes the species of the *Schistonchus*-1 group (*S. laevigatus*, *S. aureus*, *S. altermacrophylla*, *S. virens*, *S. centerae*, and *S. aculeata*). The *Schistonchus*-1 group differs from neighboring taxa of clade 2a in having the position of excretory pore near the head (as in most species of the *Schistonchus*-1 group) or on the head, near the lips (as in *S. aculeata*). The *Schistonchus*-1 group further differs from the latter taxa in the absence or strong reduction of the posterior genital branch in females and in the division of the pharyngeal glands into two lobes, the dorsal lobe that is longer and the ventral lobe that is shorter. An additional difference is the peculiar shape of the male spicules with massive condylus and narrow, strongly curved and short conical part. More basal taxa of clade 2a have the excretory pore positioned more posteriorly (near the median bulb or closer to the nerve ring), a single dorsal lobe of pharyngeal glands, and the posterior female genital branch with a length of two or more vulval diameters.

The closest branch to *Schistonchus*-1 is the species *Laimaphelenchus heidelbergi* (Fig. 8). Unlike species in *Schistonchus*-1, which are parasites of the Moraceae, *Laimaphelenchus* species inhabit trees of Pinaceae and possess branching tail-tip mucrons. However, the mucron of *Laimaphelenchus heidelbergi* consists of a single terminal tubercule covered by tiny 20 to 30 knob-like appendages, and its excretory pore is located opposite the nerve ring. Moreover, its cephalic region is not crossed with the longitudinal radial double ridges, which are typical for *Laimaphelenchus*. Therefore, it would be logical to consider *Schistonchus*-1 a member of the neighboring species group (more basal in the clade) *Aphelenchoides*-1, including *Aphelenchoides bicaudatus* and *A. variacaudatus* (Figs. 7,8). These species also have bifurcate tails, i.e., with two mucrons, the excretory pore at the level of the median bulb, and a posterior female genital branch that is three or more vulval diameters long. This group includes *Aphelenchoides xui* from pinewood in South Africa (Wang et al., 2013; Figs. 7,8). *Aphelenchoides xui* has a multipapillate female tail terminus

(as do *L. heidelbergi* and some of specimens of *A. variacaudatus*) and a posterior female genital branch that is three or more vulval diameters long. Another species close to the *Aphelenchoides*-1 group is *Schistonchus guangzhouensis*, with its excretory pore located at the level of the median bulb and the female posterior genital branch 6 to 7 vulval diam. long. *Aphelenchoides*-1, *L. heidelbergi*, and *S. guangzhouensis* are recommended to be included in *Aphelenchoides*; they all inhabit the Southern Hemisphere (Australia, South Africa) or southern portions of the Northern Hemisphere (China).

Based on the position of the excretory pore between the nerve ring and median bulb, and on the rounded tail terminus, as well as on distribution of many species of the group in North America, on Poaceae hosts, species of the *Aphelenchoides*-2 group (*A. blastophthorus*, *A. fragariae*, *A. saprophilus*, *A. xylocopae*, *A. subtenuis*) are the closest to *R. megadorus*. However, *Robustodorus* differs from this group in the presence of a massive, knobbed stylet, and three prominent incisures of the lateral field (in the *Aphelenchoides*-2 there are four, or sometimes two, weak incisures). The species of *Aphelenchoides*-2 have one distinct and long needle-like ventral mucron. In contrast, *R. megadorus* lacks a mucron on its rounded tail tip.

In clade 2b, subclades separated from other clades by the most phylogenetic distance are *Ruehmaphelenchus* spp. (with generic morphological characters) and the *Schistonchus*-2 group (*S. hirtus*, *S. macrophylla*, *S. caprifici*) (Fig. 7). The *Schistonchus*-2 group is unique among aphelenchs in that its members possess a large cephalic disc as well as a small labial disc (the cephalic disc is absent in all other taxa in clade 2), a lateral field with eight incisures as well as additional incisures that are indistinct under LM (other members of the clade 2b have three or four incisures), male spicules with a large condylus and with narrow, sharply conical lamina (other taxa of clade 2b have ordinary aphelenchoid spicules), and the presence of the small blunt vulval flap in females (the flap is absent or large and sharp in other taxa of the clade 2b).

The other subclade in clade 2b includes species of *Aphelenchoides*-3 (*A. ritzemabosi*, *A. besseyi*, *A. paradalianensis*, *A. fujianensis)* and the *Laimaphelenchus penardi* group *(L. preissii*, *L. penardi*, *L. persicus*, *L. australis)* (Figs. 7,8). They resemble each other in having the position of the excretory pore posterior to the nerve ring (this location is typical for the entire clade 2b) and by the shared presence of a stellate mucron at the tail tip: the species of *Aphelenchoides*-3 have four (or three to five) star-like arranged mucrons, the species of *Laimaphelenchus penardi* group have a tail tip with peduncle (stub) bearing usually four tubercules or sometimes up to 10 projections (in *L. preissii*). The *Laimaphelenchus penardi* group differs from the *Aphelenchoides*-3 group by the presence of six distinct double ridges dividing the head into six lobes (*Aphelenchoides* species do not have such ridges) and by sperm morphology. In the *Laimaphelenchus penardi* group, sperm are cytoplasmic-ameboid and, sometimes in the female genital tract, disc-like and assembled in columns, whereas in the *Aphelenchoides*-3 species group, sperm are small and nucleic (non-cytoplasmic). Species of the *Laimaphelenchus penardi* group are found in Pinaceae woody hosts, whereas members of the *Aphelenchoides*-3 group mainly infect members of the Rosaceae, Asteraceae, and other herbaceous plants.

Division of the species of *Aphelenchoides*, *Schistonchus*, and *Laimaphelenchus* into several groups according to their positions in the clades (Figs. 7,8) are original. This grouping is used here for better description of the morphological markers of the molecular clades combining species groups from different genera. The *Aphelenchoides*-1 group (*A. variacaudatus, A. xui*) and *Aphelenchoides*-2 group (*A. blastophthorus*, *A. fragariae*, *A. saprophilus*, *A. xylocopae*, *A. subtenuis*) belong to the subclade 2a, *Aphelenchoides*-3 (*A. ritzemabosi, A. besseyi*, *A. paradalianensis*, *A. fujianensis*) belongs to the subclade 2b. The *Schistonchus*-1 group (*S. laevigatus*, *S. aureus*, *S. altermacrophylla*, *S. virens*, *S. centerae*, and *S. aculeata*) and *S. guangzhouensis* belongs to the subclade 2a; *Schistonchus*-2 group (*S. hirtus*, *S. macrophylla*, *S. caprifici*) belongs to the subclade 2b. The *Laimaphelenchus penardi* group (*L. preissii*, *L. penardi*, *L. persicus*, *L. australis*) belongs to the clade 2b, whereas *L. heidelbergi* is in subclade 2a.

Independent phylogenetic analysis of molecular data verifies the relatively high taxonomic value of the following characters in Aphelenchoididae: excretory pore position, the structure of appendages of the female tail tip, the presence of ridges and cephalic disc in the cephalic region, the length and structure of the female posterior genital branch, the number of incisures in the lateral field, and the structure of the male spicule condylus and female anterior vulval lip. The large stylet of *Robustodorus*, with its developed knobs and the hexagonal inner cuticular structure in the guiding apparatus pouch surrounding the stylet cone, is unique among the Aphelenchoididae, but these characters have less value for the taxonomy and phylogeny of the family. *Robustodorus* is basal to three different lineages of *Schistonchus* in clade 2: *Schistonchus*-1, *Schistonchus guangzhouensis*, and *Schistonchus*-2, all of which have a strong and long stylet, as in *Robustodorus*. This finding hints at the evolutionary plasticity of the aphelenchid stylet under different selective pressures.

Acknowledgments. Authors are grateful to Dr. Zafar Handoo, Beltsville, and Dr. Steven Nadler, UCD, for the loan of valuable specimens of *R. megadorus*; Dr. Vladimir Gaar, Czech Republic, and Dr. Andrea Čerevková, Slovak Republic, for literature on nematode records and help with information on available collection materials.

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