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Two New Species of *Rhabdias* (Nematoda: Rhabdiasidae) from the Marine Toad, *Bufo marinus* (L.) (Lissamphibia: Anura: Bufonidae), in Central America

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TWO NEW SPECIES OF *RHABDIAS* (NEMATODA: RHABDIASIDAE) FROM THE MARINE TOAD, *BUFO MARINUS* (L.) (LISSAMPHIBIA: ANURA: BUFONIDAE), IN CENTRAL AMERICA

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ABSTRACT: Two new *Rhabdias* species are described from the lungs of the cane toad *Bufo marinus* (L.) from Costa Rica and Nicaragua. *Rhabdias alabialis* n. sp. differs from other known species of the genus by the remarkable morphology of its head end, i.e., the absence of lips or pseudolabia, the slitlike oral opening, and the triangular shape of the buccal capsule in apical view. *Rhabdias pseudosphaerocephala* n. sp. is identified as a form previously known in Central and South America as *Rhabdias sphaerocephala* Goodey, 1924, a species initially described from toads in Europe. The new species is differentiated from *R. sphaerocephala* based on head-end morphology and sequences of nuclear rDNA.

Species of *Rhabdias* Stiles et Hassall, 1905, are a globally distributed group of nematodes parasitic in lungs of amphibians and reptiles and include more than 40 nominal species. Fifteen species have been reported from members of the Bufonidae; 11 of them seem to be specific to this host family. In Central and South America, 5 *Rhabdias* species have been reported from *Bufo* spp. Two of these, *Rhabdias sphaerocephala* Goodey, 1924 and *Rhabdias fulleborni* Travassos, 1926, are known as parasites of *Bufo marinus* (L.) (Kloss, 1971, 1974).

Beginning in 1996, the Area de Conservacion Guanacaste (ACG) (http://www.acguanacaste.ac.cr) in northwestern Costa Rica has supported an effort to perform an inventory of the eukaryotic parasites of all 940 species of vertebrates living within the ACG (http://www.parasitesrus.com). As part of that project, we collected parasites inhabiting specimens of *Bufo marinus*. Additionally, *Rhabdias* spp. have been collected from *B. marinus* from several localities in western Nicaragua. Examination of that material revealed the presence of 2 distinct *Rhabdias* species. One of these was identical to nematodes previously reported in Central and South America as *R. sphaerocephala* (Bravo-Hollis and Caballero, 1940; Brenes and Bravo-Hollis, 1959; Kloss, 1971, 1974). Morphological comparison of the worms studied with the original description of *R. sphaerocephala* Goodey, 1924 from *Bufo bufo* from Great Britain and the material from the same host species from Ukraine, as well as analysis of the DNA sequences, demonstrated substantial differences between South and Central American forms and European forms. The second new species possesses morphological features that readily differentiate it from all known species of the genus. Two new species of *Rhabdias* are, therefore, described herein.

MATERIALS AND METHODS

All nematodes were taken from freshly killed hosts. Some of them were fixed in glacial acetic acid and stored in 70% ethanol, while others were heat-killed in saline and stored in 70% ethanol. Specimens for molecular analysis were placed directly into 95% ethanol. Museum specimens of *R. fulleborni* were examined. Those included permanent total mounts of neotypes on slides (Museu de Zoologia da Universidade de São Paulo, no. 1650), and material from *B. marinus* from Bermudas (U.S. National Parasite Collection, nos. 83196 and 87195), stored in alcohol. Specimens of *R. sphaerocephala* for morphological and molecular study were collected from *Bufo bufo* near Kyiv, Ukraine.

For light microscopy, specimens were cleared in glycerol by gradual evaporation from a 5% solution of glycerol in 70% ethanol, or in glycerol–phenol (3:1) solution. Measurements are in micrometers unless otherwise noted. Drawings were made with the aid of a drawing tube.

Nematodes used for SEM were initially fixed in ethanol, then dehydrated in a graded series of ethanol and acetone and dried using hexamethyldisilazane (HMDS) (Ted Pella, Inc., Redding, California) as transition fluid. Dry specimens were mounted on stubs, coated with gold-palladium, and examined using a Hitachi 4700 scanning electron microscope (Hitachi USA, Mountain View, California) at an accelerating voltage of 10–15 kV.

For molecular analysis, live worms recovered from the host were rinsed thoroughly in saline and fixed in 95% ethanol. Genomic DNA was extracted from single specimens of worms according to Tkach and Pawlowski (1999). DNA fragments spanning the 3′ end of 18S nuclear rDNA gene, ITS region (ITS1 + 5.8S + ITS2), and 5′ end of the 28S (including variable domains D1–D3) were amplified by PCR on an Eppendorf Master Gradient thermal cycler using forward primer ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and reverse primer 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′).

PCR reactions were performed in a total volume of 51 μl containing 42 μl H_2O, 5 μl *Taq* buffer, 1 μl dNTP at a concentration of 10 pM/μl, 1 μl of each primer at a concentration of 10 pM/μl, 0.25 μl of Eppendorf *Taq* polymerase at a concentration of 5 units/μl, and 1–1.5 μl of template gDNA extract. The thermocycling profile was as follows: 2 min denaturation at 94 C; then 40 cycles of 30 sec at 94 C, 30 sec at 52–56 C, 2 min at 72 C; and a final 7-min extension at 72 C.

PCR products were purified using Qiagen Qiaquick™ (Valencia, California) columns and sequenced directly on an ABI Prism 3100™ automated capillary sequencer using ABI BigDye™ chemistry (Foster City, California) according to manufacturer's protocols. DNA products were sequenced in both directions using the 2 PCR primers and, additionally, internal primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), 300R (5′-CAACTTTCCCTCACGGTACTTG-3′), 300F (5′-CAAGTACCGTGAGGGAAAGTTG-3′), and ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′). Contiguous sequences were assembled and edited using Sequencher™ version 4.1.1 (GeneCodes Corp., Ann Arbor, Michigan) and submitted to GenBank under accession numbers DQ845734–DQ845738 (*R. pseudosphaerocephala*) and DQ845739–DQ845741 (*R. sphaerocephala*) Sequences were manually aligned and compared using the BioEdit program, version 7.0.1 (Hall, 1999).

DESCRIPTIONS

Rhabdias alabialis n. sp.
(Figs. 1, 3A)

General (Based on 50 specimens; measurements are for holotype, followed by limits for 25 paratypes in parentheses. Measurements are presented in micrometers unless otherwise stated. Calculations for the

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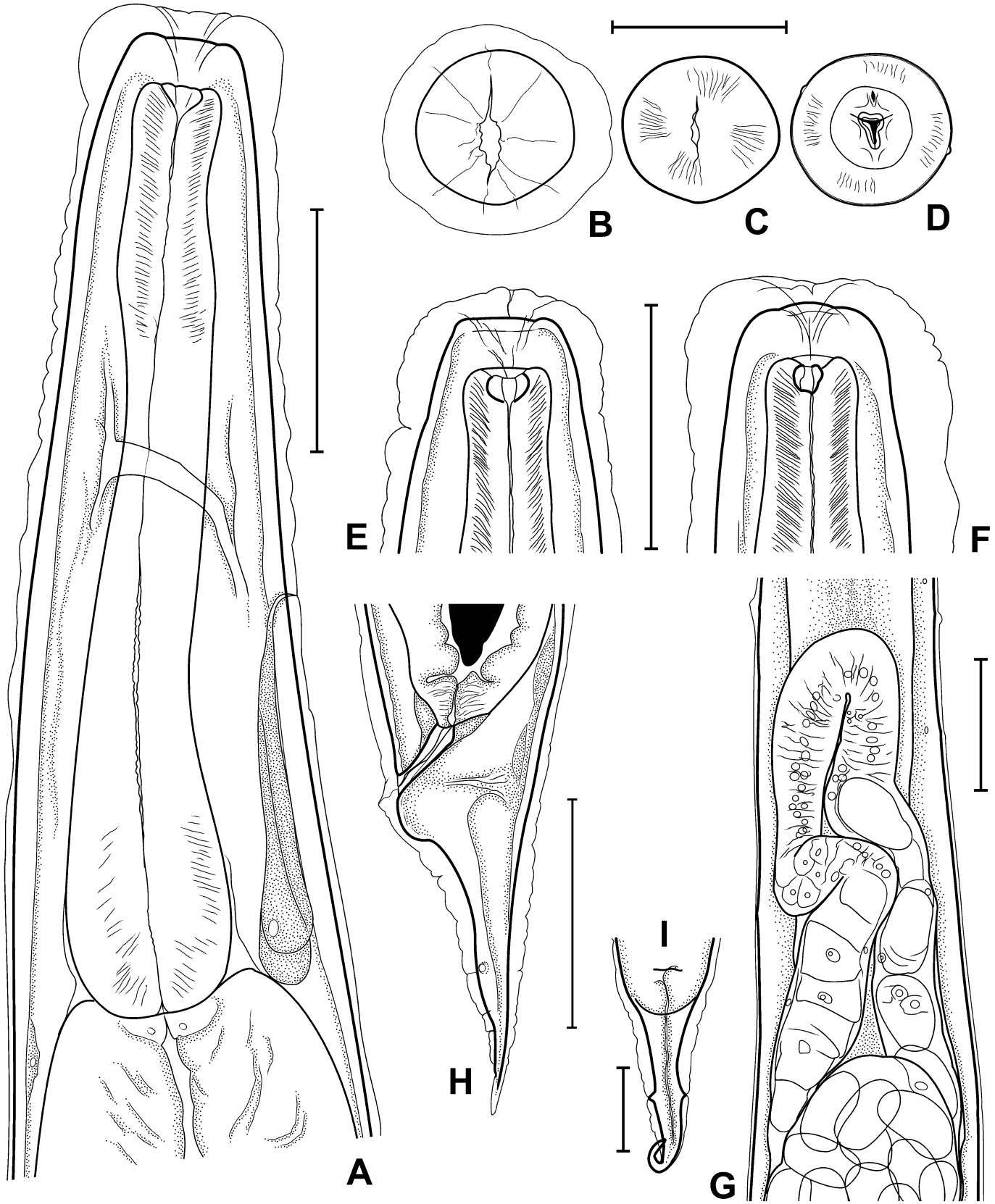

FIGURE 1. *Rhabdias alabialis* n. sp. (**A**) Anterior part of the body, lateral view. (**B**) Head end, apical view; (**C**) optical section of the head end at level anterior to the buccal capsule; (**D**) optical section of the head end at level of the buccal capsule; (**E**) head end, lateral view; (**F**) head end, dorsoventral view; (**G**) loop of anterior genital tube; (**H**) tail end, lateral view; (**I**) tail, ventral view, showing phasmids. Scale bars: A, E, F, I = 0.1 mm; B, C, D = 0.05 mm; G, H = 0.2 mm.

TABLE I. Metric characters of *Rhabdias alabialis* (type series, n = 26). Measurements are in micrometers unless otherwise noted. SD, standard deviation; CV, coefficient of variation.

Characters	Mean	Minimum	Maximum	SD	CV
Body length, mm	8.67	7.67	9.27	0.44	5.1
Body width	302.8	260	328	16.6	5.5
Buccal capsule depth	10	10	10	0	0
Buccal capsule maximum width	12.1	10	15	1.4	11.2
Esophagus length	395.8	340	445	20.0	5.0
Esophagus length as percentage of body length	4.6	4.2	5.2	0.2	5.2
Width of esophagus:					
At anterior end	33.9	32	37	1.4	4.3
At middle of muscular part	40.2	37	45	1.9	4.6
At middle of glandular part	33.2	32	35	1.1	3.4
Width of esophageal posterior bulb	65.2	57	70	3.0	4.6
Distance from anterior end of esophagus to nerve ring	146.2	136	167	8.0	5.5
Distance from anterior end of esophagus to nerve ring as percentage of esophagus length	37.0	31.9	43.6	2.8	7.6
Distance from anterior end to vulva, mm	4.29	3.62	4.80	277.3	6.5
Distance from anterior end to vulva as percentage of body length	49.4	47.2	51.8	1.3	2.7
Tail length	300.7	278	328	13.5	4.5
Tail length as percentage of body length	3.5	3.1	3.8	0.2	5.3
Egg length (n = 20)	98.2	92	105	3.3	3.4
Egg width (n = 20)	54.6	52	57	1.7	3.1

whole sample are given in Table I): Body 7.97 (7.67–9.27) mm long, 315 (260–328) wide at midbody. Anterior end rounded, posterior end tapering. Outer layers of body cuticle prominently swollen, especially at anterior end, with irregular transverse folds. Oral opening slitlike, orientated dorsoventrally. Eight radial folds of epicuticle surrounding oral opening. Vestibulum 20 (15–25) deep, slitlike in apical view, funnel-shaped in lateral view, lined with folded cuticle. Lips or pseudolabia absent. Buccal capsule (esophastome) triangular in apical view, infundibuliform in lateral view, 10 (10–10) deep, 12 (10–15) wide. Buccal capsule walls (esophorhabdion) dense and thick. Esophagus typical for species of the genus, club-shaped, 389 (340–445) long, with prominent dilation at midlength of anterior muscular part and egg-shaped posterior bulb. Anterior end of esophagus 35 (32–37) wide; maximum width of muscular dilation 37 (37–45); minimum width of glandular part 32 (32–35); posterior bulb 65 (57–70) wide. Nerve ring encircling esophagus posterior to its muscular swelling, 148 (136–167) from anterior end of esophagus. Excretory pore posterior to nerve ring. Excretory glands relatively short, approximately half as long as esophagus. Anterior part of intestine wide, thick-walled, with narrow lumen. Intestinal lumen behind posterior limb of genital system wide, filled with black contents. Muscular prerectal sphincter of intestine obvious. Rectum short, straight, lined with thick cuticle. Genital system amphidelphic. Vulva transverse, near midbody, 3.95 (3.62–4.80) mm from anterior end. Vagina reduced. Uteri long, straight, thin-walled, filled with numerous eggs; most eggs containing larvae. Both limbs of genital system bending at region of seminal receptacles. Ovaries (syngonia) situated beside intestine; each ovary far overlapping level of vulva. Tail conical, 303 (278–328) long, with prominent ventral postanal inflation of body wall. Phasmids comparatively large, conical, situated at middle of tail length. Tail tip lacking cuticular swelling.

Taxonomic summary

Type host: marine toad or cane toad *Bufo marinus* (L.) (Anura: Bufonidae).

Site of infection: Lungs.

Type locality: Puesto Rio Murcielago, Sector Murcielago, Area de Conservacion Guanacaste, Guanacaste Province, Costa Rica.

Other localities: Sector Santa Rosa: Rio Cuajiniquil, at crossing of Cafetal Road (10°52.51′N, 85°36.42′W, elevation 281 m); Quebrada Costa Rica, Camino Playa Naranjo (10°50.04′N, 85°37.29′W, elevation 287 m); Area Administrativa, Estacion Santa Rosa, Quebrada Guapote, Camino Rosa Maria (10°49.22′N, 85°36.51′W, elevation 275 m); Sector San Gerardo: Estacion San Gerardo (10°52.50′N, 85°23.21′W, elevation 605 m); Rio Pizote between Brasilia and Dos Rios (10°56.02′N, 85°24.05′W), Area de Conservacion Guanacaste, Guanacaste Province, Costa Rica.

Type specimens deposited: Holotype: USNPC 98143; paratypes: USNPC 98144.

Etymology: The species name is given in reference to the absence of lips characteristic of this species.

Remarks

Rhabdias alabialis is readily distinguishable from all other species of the genus by its unique head-end morphology, i.e., its buccal capsule shape (triangular in apical view), absence of lips, and slitlike oral opening. With regard to the latter character, *R. alabialis* is similar to *Rhabdias bicornis* Lu, 1934, described from *Bufo* spp. in China. *Rhabdias bicornis*, however, possesses 2 prominent lateral pseudolabia (Lu, 1934; Kung and Wu, 1945; Hsu, 1960), which are absent in *R. alabialis*. *Rhabdias bicornis* also lacks the dilation of the esophagus in the midregion of its muscular part, which is present in *R. alabialis*.

***Rhabdias pseudosphaerocephala* n. sp.**

(Figs. 2, 3B)

General (Based on 26 specimens; measurements are for holotype, followed by limits for 25 paratypes in parentheses. Measurements are presented in micrometers unless otherwise stated. Calculations for the whole sample are given in Table II): Body 7.90 (6.17–9.60) mm long and 330 (290–380) wide at midlength. Anterior end rounded, posterior end tapered. Body cuticle swollen, especially on head end, and covered with irregular transverse folds. Oral opening round, surrounded with 4 submedian lips overhanging edge of oral opening. Two lateral pseudolabia rounded, comparatively small. Each submedian lip and pseudolabium bearing small papilla on the top. Amphids porelike, situated on pseudolabia. Buccal capsule funnel-shaped, thick-walled, 10 (7–12) deep and 17 (15–17) wide. Esophagus club-shaped, dilation of anterior muscular part present. Esophagus length 410 (400–460) or 5.2% (4.4–6.5%) of body length. Anterior end of esophagus 37 (35–40) wide; maximum width of muscular dilation 42 (40–50) wide; minimum width of glandular part 37 (35–42); posterior bulb 72 (67–85) wide. Nerve ring encircling esophagus posterior to its muscular swelling, 160 (140–190) from anterior end of esophagus (39.0% [33.3–43.2%] of esophagus length). Excretory pore posterior to nerve ring. Shape of intestine and

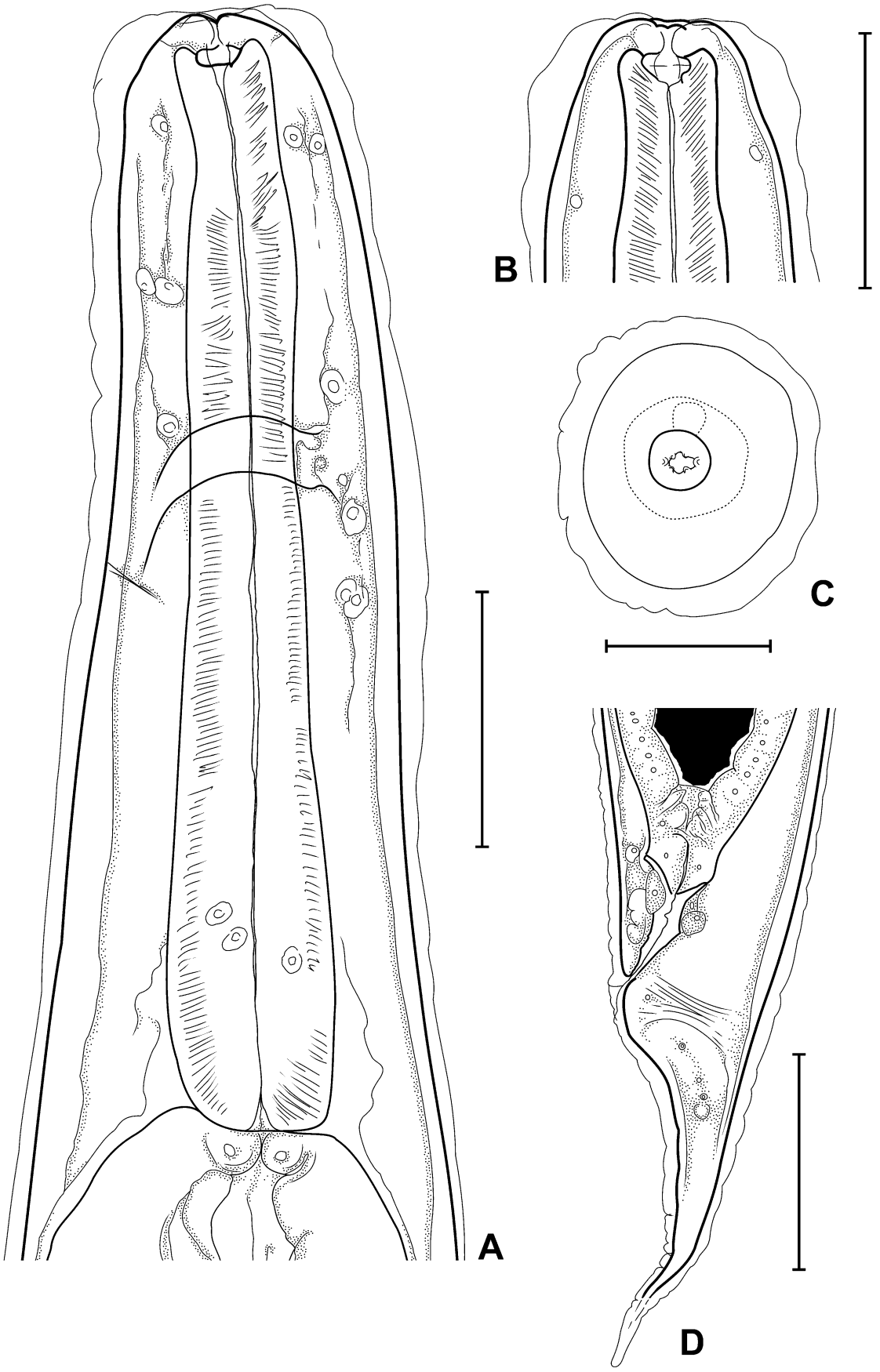

FIGURE 2. *Rhabdias pseudosphaerocephala* n. sp. (**A**) Anterior part of the body, lateral view. (**B**) Head end, dorsoventral view; (**C**) head end, apical view; (**D**) tail end, lateral view. Scale bars: A, B = 0.1 mm; C = 0.05 mm; D = 0.2 mm.

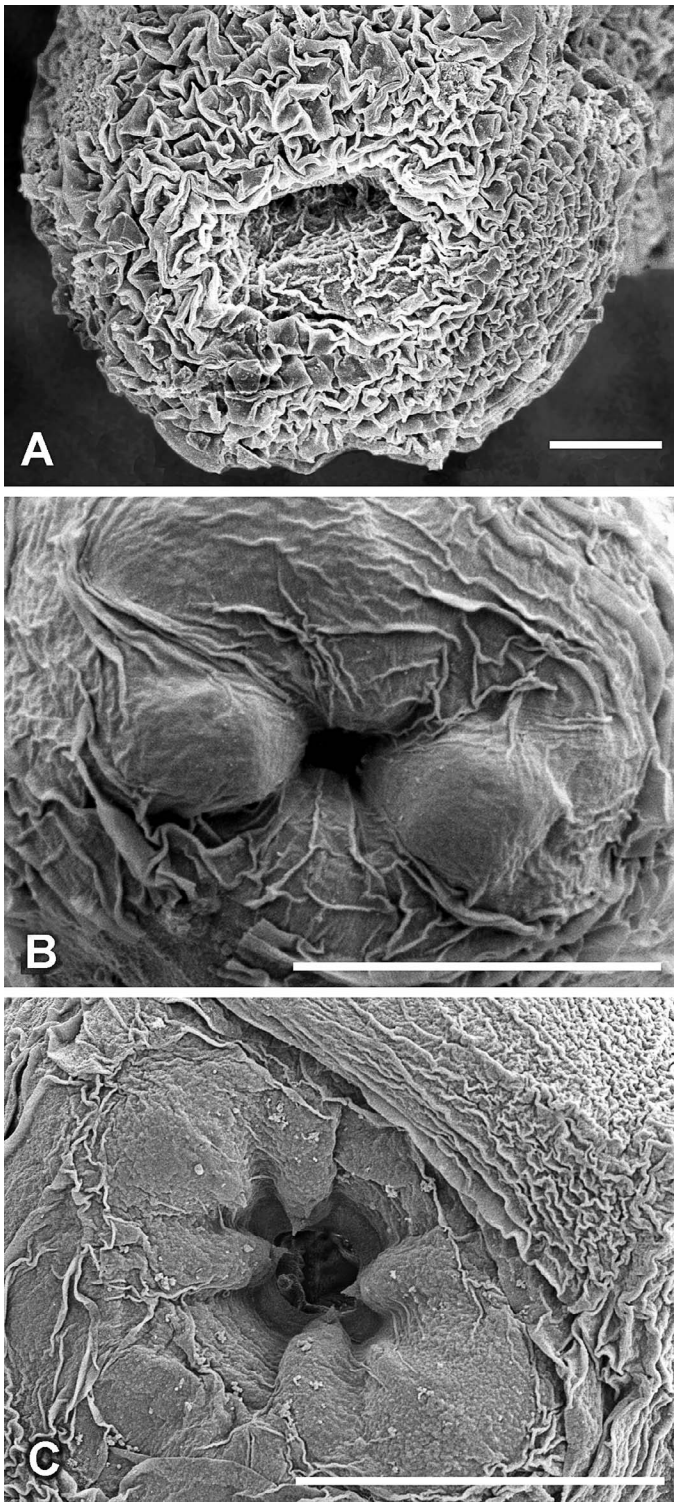

FIGURE 3. (**A**) *Rhabdias alabialis* n. sp. (**B**) *Rhabdias pseudosphaerocephala* n. sp. (**C**) *Rhabdias sphaerocephala*. Scale bars: A = 10 μm, B = 30 μm, C = 30 μm.

rectum same as in previous species, as well as genital system structure. Vulva almost equatorial, distance from anterior end to vulva 3.92 (3.30–4.95) mm, or 49.7% (47.5–53.4%) of body length. Tail comparatively short, conical. Tail length 350 (310–410), or 4.4% (3.7–5.0%) of body length.

Taxonomic summary

Type host: Bufo marinus (L.) (Anura: Bufonidae), marine toad or cane toad.

Site of infection: Lungs.

Type locality: City of Leon, Leon Province, Nicaragua (12°25.57′N, 86°52.53′W).

Other localities: City of Chinandega, Chinandega Province, Nicaragua; Sector Santa Rosa: Quebrada Costa Rica, Camino Playa Naranjo (10°50.04′N, 85°37.29′W); Area Administrativa, Estacion Santa Rosa; Rio Cuajiniquil, at crossing of Cafetal Road (10°52.51′N, 85°36.42′W); Sector San Gerardo: Estacion San Gerardo (10°52.50′N, 85°23.21′W); Sector Santa Elena: Santa Elena, Laguna los Jicaros; Sector Maritza: Maritza station (10°57.40′N, 85°29.30′W), Area de Conservacion Guanacaste, Guanacaste Province, Costa Rica.

Type specimens deposited: Holotype: USNPC 98145; paratypes: USNPC 98146.

Remarks

Rhabdias pseudosphaerocephala n. sp. is most similar to *R. fulleborni* Travassos, 1926 by general morphology, host specificity, and geographic distribution. Detailed differentiation between the 2 species was provided by Kloss (1971, 1974) who identified *R. pseudosphaerocephala* n. sp. as *R. sphaerocephala* Goodey, 1924. Examination of type material of *R. fulleborni* and material from the USNP confirmed that the species differs from *R. pseudosphaerocephala* by a more slender body, especially at the anterior end, and a narrower anterior-most part of the esophagus. In addition, *R. pseudosphaerocephala* n. sp. differs from *R. fulleborni* by having a relatively shorter esophagus; Figure 4 depicts the results of comparing the relative length of the esophagus in *R. fulleborni* and *R. pseudosphaerocephala* based on measurements published by Kloss (1971) and our data. Both samples of *R. pseudosphaerocephala* measured in present study appeared to be almost identical to "*R. sphaerocephala*" studied by Kloss (1971); all 3 samples were clearly different from *R. fulleborni*.

The specimens described here as *R. pseudosphaerocephala* n. sp. were first identified in Central and South America (Bravo-Hollis and Caballero, 1940; Brenes and Bravo-Hollis, 1959; Kloss, 1971, 1974) as *R. sphaerocephala* Goodey, 1924, originally described from the European common toad (*B. bufo*) (Goodey, 1924). The 2 species resemble each other by the presence of cuticular swelling at the head end. In *R. sphaerocephala*, however, the cuticular swelling on the head end is distinctly separated from cuticle covering the rest of the body (Goddey, 1924; Kuzmin, 1997). The 2 species also substantially differ by the head-end morphology. *Rhabdias sphaerocephala* possesses 6 lips surrounding an oral opening at some distance from its edge (Fig. 3C), whereas in *R. pseudosphaerocephala*, the oral opening is surrounded by 4 submedian lips overhanging the edge of oral opening and 2 lateral pseudolabia (Fig. 3B). In *R. sphaerocephala*, the buccal capsule is not surrounded by esophageal tissue (Kuzmin, 1997), whereas in *R. pseudosphaerocephala*, the buccal capsule walls are completely surrounded by esophageal tissue (Figs. 2A, B). In addition, *R. sphaerocephala* possesses a relatively longer esophagus, as shown in Figure 5.

The amplified and sequenced fragment of nuclear rDNA included the complete ITS region (ITS1, 5.8S, ITS2) and fragment at the 5′ end of the 28S gene, including variable domains D1–D3. Alignment required only 2 gaps because the species under investigation were obviously closely related. Alignment of sequenced fragments was 1,607 bases long; sequences of both *R. pseudosphaerocephala* n. sp. and *R. sphaerocephala* were 1,606 bases long. Twenty of 1,607 alignment sites (1.44%) were variable. This level of variability appears meaningful considering that sequences obtained from 7 specimens of *R. pseudosphaerocephala* n. sp., collected at different times from 2 different localities in Nicaragua and 3 in Costa Rica, showed no intraspecific variation. Similarly, sequences obtained from 3 specimens of *R. sphaerocephala* from Ukraine were identical. Variable sites were scattered along alignment with the majority in the ITS1 region. Thus, molecular data strongly support observed morphological differences between *R. pseudosphaerocephala* n. sp. and *R. sphaerocephala*.

DISCUSSION

Bilateral symmetry of cephalic structures has been reported for several *Rhabdias* species. Baker (1978) described lateral

TABLE II. Metric characters of *Rhabdias pseudosphaerocephala* (type series, n = 26). Measurements are in micrometers unless otherwise noted. SD, standard deviation; CV, coefficient of variation.

Characters	Mean	Minimum	Maximum	SD	CV
Body length, mm	8.16	6.17	9.60	0.84	10.3
Body width	341.5	290	380	23.6	6.9
Buccal capsule depth	10	7	12	0.7	7.1
Buccal capsule maximum width	17.2	15	17	0.8	4.7
Esophagus length	416.9	400	460	15.7	3.8
Esophagus length as percentage of body length	5.1	4.4	6.5	0.5	9.1
Width of esophagus:					
At anterior end	37.9	35	40	1.7	4.5
At middle of muscular part	44.1	40	50	2.5	5.8
At middle of glandular part	38.9	35	42	1.8	4.5
Width of esophageal posterior bulb	76.1	67	85	4.5	6.0
Distance from anterior end of esophagus to nerve ring	161.9	140	190	11.0	6.8
Distance from anterior end of esophagus to nerve ring as percentage of esophagus length	38.9	33.3	43.2	2.4	6.2
Distance from anterior end to vulva, mm	4.11	3.30	4.95	0.41	10.0
Distance from anterior end to vulva as percentage of body length	50.2	47.5	53.4	1.6	3.1
Tail length	351.5	310	410	24.1	6.9
Tail length as percentage of body length	4.3	3.7	5.0	0.4	8.3

pseudolabia in 2 North American species, i.e., *Rhabdias americanus* Baker, 1978 and *Rhabdias ranae* Walton, 1929 (the specimens of *R. ranae* described by Baker, 1978, are considered here as belonging to *Rhabdias bakeri* nov. sp. [Tkach et al., 2006]). Similar structures were described in *Rhabdias africanus* Kuzmin, 2001 (Kuzmin, 2001). However, in all these species, the submedian lips are present, at least in the shape of ''protuberances'' (sensu Baker, 1978). In *R. bicornis*, lips are completely replaced with lateral pseudolabia extending anteriorly (Lu, 1934). Subadults of *R. americanus* and *R. ranae* lack lateral pseudolabia, and their formation occurs during the maturation of adult stage (Baker, 1979). In the present study, we dealt only with gravid specimens of *R. alabialis* and all of them lacked any sign of lateral pseudolabia.

The identification of some *Rhabdias* from *B. marinus* as *R. sphaerocephala* was previously questioned by Chabaud et al. (1961), Hartwich (1975), and Baker (1978). In the present study, we found both morphological and molecular differences among the European *R. sphaerocephala* and the American *R. pseudosphaerocephala* n. sp. This differentiation is in agreement with the fact that almost all known *Rhabdias* species are distributed on a single continent. The only remaining exception is *Rhabdias fuscovenosa* (Railliet, 1899), a parasite of snakes occurring in Europe, Asia, and North America. We believe, however, that a thorough study of the forms of the species from distant parts of its area, involving both morphological and molecular analysis, might reveal several sibling species within the *R. fuscovenosa* complex.

Differences between *R. pseudosphaerocephala* n. sp. and *R. fulleborni* are not very obvious. Bravo-Hollis and Caballero (1940) reported that *R. sphaerocephala* from Mexico (=*R. pseudosphaerocephala*) was shorter and had a postequatorial vulva. Kloss (1971, 1974) stated that ''*R. fulleborni* differs from *R. sphaerocephala* by its slenderness, and by attaining a longer

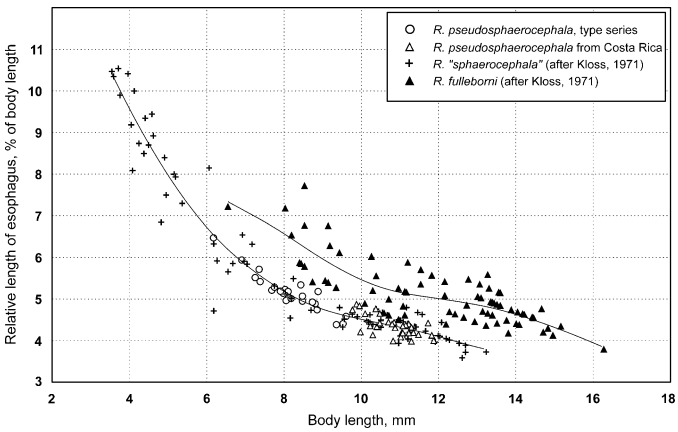

FIGURE 4. Relationship of the relative length of esophagus (percentage of total length) to the total length in *R. pseudosphaerocephala* sp. nov. and *R. fulleborni*. Lines = the least-squares lines.

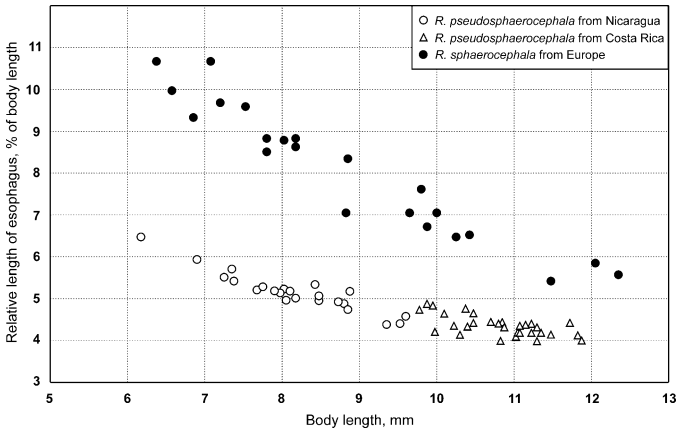

FIGURE 5. Relationship of the relative length of esophagus (percentage of total length) to the total length in *R. pseudosphaerocephala* sp. nov. and *R. sphaerocephala*.

length'' (Kloss, 1971). Apparently, the slenderness of any *Rhabdias* sp. is quite a subjective character, and the body length in both species is very similar. We add the relative length of esophagus as additional character for the separation of these species.

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