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Morphological and molecular differentiation of two new species of *Pseudoacanthocephalus* Petrochenko, 1958 (Acanthocephala: Echinorhynchidae) from amphibians and reptiles in the Philippines, with identification key for the genus

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Abstract The genus *Pseudoacanthocephalus* Petrochenko, 1958 currently includes 14 species of acanthocephalans parasitic in amphibians and reptiles worldwide. This work describes two new species of *Pseudoacanthocephalus* from amphibians and reptiles collected in several localities on Luzon Island, Philippines. *Pseudoacanthocephalus nickoli* n. sp. was found in two species of frogs, *Rana luzonensis* Boulenger and *Rana similis* (Günther), and *Pseudoacanthocephalus smalesi* n. sp. was found in a scincid lizard, *Sphenomorphus abdictus* Brown & Alcala. Differential diagnoses of the two new species of *Pseudoacanthocephalus* from their congeners are provided. Comparative analysis of nuclear ribosomal rRNA sequences encompassing the 3′ end of 18S nuclear rDNA gene, internal transcribed spacer region (ITS1+5.8S+ITS2), and 5′ end of the 28S gene strongly corroborated the morphological evidence and demonstrated significant differences between the two new species as well as between these species and closely related species from continental China and Vietnam. No intraspecific sequence variability was detected among different individuals representing each of the examined species. This is the first report of *Pseudoacanthocephalus* in the Philippines. A key to known species of *Pseudoacanthocephalus* is provided.

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Introduction

The genus *Pseudoacanthocephalus* Petrochenko, 1958 currently includes 14 species of acanthocephalans parasitic in amphibians and reptiles worldwide (Petrochenko, 1958; Yamaguti, 1963; Amin et al., 2008; Bush et al., 2009; Smales, 2005, 2007). To the best of our knowledge, no members of *Pseudoacanthocephalus* or any other acanthocephalan species have been reported from amphibians or reptiles in the Philippines. As part of a survey of biodiversity of terrestrial vertebrates and their

parasites in the Philippines, we found acanthocephalans belonging to *Pseudoacanthocephalus* in two species of frogs, *Rana luzonensis* Boulenger and *Rana similis* (Günther), and one species of scincid lizard, *Sphenomorphus abdictus* Brown & Alcala. These acanthocephalans demonstrated substantial morphological and molecular differences from the known *Pseudoacanthocephalus* species described from Southeast Asia and elsewhere and are described herein as new to science. Keys to the identification of the known species of *Pseudoacanthocephalus* are provided.

Materials and methods

Specimens of *Pseudoacanthocephalus* were found in twelve *Rana similis*, one *Rana luzonensis* and six *Sphenomorphus abdictus* caught by hand during May–June 2009 from four localities in the Aurora Province, Luzon Island, Philippines (see taxonomic summaries for details). Live acanthocephalans were relaxed in water and fixed in 70 % ethanol. Morphology of the acanthocephalans was studied on temporary total mounts cleared in Berlese's medium using a compound Zeiss Axio Imager M1 microscope equipped with DIC optics. Drawings were made with aid of a drawing tube. All measurements in the text and tables are in micrometers unless otherwise stated.

Specimens used for scanning electron microscopy (SEM) were fixed in 70 % ethanol, dehydrated in a graded series of ethanol, and dried with hexamethyldisilazane (Ted Pella Inc., Redding, California) as transition fluid. The specimens were mounted on aluminum stubs using conductive double-sided tape and silver paste, coated with gold–palladium, and examined with the use of a Hitachi 4700 scanning electron microscope (Hitachi USA, Mountain View, California) at an accelerating voltage of 5–10 kV.

Type-material was deposited in the parasite collection of the Harold W. Manter Laboratory (HWML) of the University of Nebraska, Lincoln, Nebraska. Types were deposited at HWML with the understanding that representative types will ultimately be repatriated to collections in the Philippines. Hosts were deposited at the University of Kansas Natural History Museum, Lawrence, Kansas (KUMNH).

Genomic DNA for molecular analysis was extracted according to Tkach & Pawlowski (1999) from tissue taken from the middle of the body of each acanthocephalan specimen while the taxonomically important anterior and posterior regions were preserved as vouchers for morphological identification. DNA was extracted from six specimens of *P. nickoli* n. sp. obtained from four different individuals of *Rana similis* collected in three different localities (Table 1). DNA was also extracted from four specimens of *P. smalesi* n. sp. obtained from four different individuals of *Sphenomorphus abdictus* collected from two localities (Table 1).

For comparative sequence analysis DNA was also extracted from six specimens of *Pseudoacanthocephalus bufonis* (Shipley, 1903) Petrochenko, 1958 obtained from four different species of anuran amphibians collected in Jing Xin County Provincial Nature Reserve, China (Table 1), and from two specimens of *Pseudoacanthocephalus nguyenthileae* Amin, Ngyuen & Heckmann, 2008, obtained from *Duttaphrynus melanostictus* (Schneider) collected in Tam Dao National Park, northern Vietnam (Table 1).

Fragments of DNA spanning the 3′ end of 18S nuclear rDNA gene, internal transcribed spacer region (ITS1+5.8S+ITS2), and 5′ end of the 28S gene, were amplified by PCR on an Eppendorf Master Gradient thermal cycler. Forward primer ac58f (5′-GTC GTA ACA AGG TTT CCG T-3′) and reverse primer ac1500r1 (5′-CGA TTG ATT TGC ACG TC-3′) were used for PCR. PCR primers and additional internal primers ac300f (5′-GCG AAC AAG TAC CAT GAG GG-3′), ac300R (5′-CCC TCA TGG TAC TTG TTC GC-3′), ac900f (5′-CCG TCT TGA AAC ACG GAC TAA GG-3′), ac900R (5′-CCT TAG TCC GTG TTT CAA GAC GG-3′), ac58r (5′-TAT GCT TAA ATT CAG CGG GT-3′), ac28f (5′-ACC CGC TGA ATT TAA GCA TA-3′), and acITSend (5′-GTC GGT GTA CAG TGA ATC AC-3′) were used for sequencing. PCR products were purified directly using Qiagen Qiaquick™ (Valencia, CA) columns or USB® ExoSAP-IT® enzymatic clean-up (Cleveland, Ohio), cycle-sequenced using ABI BigDye™ chemistry, alcohol-precipitated, and run on an ABI Prism 3100™ automated capillary sequencer. The sequences were assembled using Sequencher™ (GeneCodes Corp., ver. 4.1.4) and submitted to GenBank (Table 1).

Sequences of all four *Pseudoacanthocephalus* species were initially aligned using Clustal W as implemented in the BioEdit program, version 7.0.1 (Hall, 1999) and manually refined using BioEdit. Since multiple sequences within each species were identical, only one representative sequence was used for the pairwise comparisons.

Table 1 *Pseudoacanthocephalus* specimens used for DNA extracts, their hosts, localities, and GenBank accession numbers. All Philippine localities are in Aurora province, Luzon Island

Acanthocephalan species	Host species	Locality	GenBank No.
P. nickoli n. sp.	*Rana similis*	Barangay Zabali, Municipality of Baler, Philippines	KC491884
P. nickoli n. sp.	*Rana similis*	Aurora Memorial National Park, Sitio Dimani, Barangay Villa Aurora, Municipality of Maria Aurora, Philippines	KC491885
P. nickoli n. sp. (n = 4)	*Rana similis*	Sitio Minoli, Municipality of San Luis, Philippines	KC491886– KC491889
P. smalesi n. sp.	*Sphenomorphus abdictus*	Barangay Zabali, Municipality of Baler, Philippines	KC491892
P. smalesi n. sp. (n = 3)	*Sphenomorphus abdictus*	Sitio Minoli, Municipality of San Luis, Philippines	KC491893–KC491895
P. bufonis (n = 2)	*Polypedates megacephalus*	Jing Xin County Provincial Nature Reserve, Guangxi Province, China	KC491878–KC491879
P. bufonis	*Fejervarya limnocharis*	Jing Xin County Provincial Nature Reserve, Guangxi Province, China	KC491880
P. bufonis	*Odorrana livida*	Jing Xin County Provincial Nature Reserve, Guangxi Province, China	KC491881
P. bufonis	*Polypedates mutus*	Jing Xin County Provincial Nature Reserve, Guangxi Province, China	KC491882
P. bufonis	*Polypedates* sp.	Jing Xin County Provincial Nature Reserve, Guangxi Province, China	KC491883
P. nguyenthileae (n = 2)	*Duttaphrynus melanostictus*	Tam Dao National Park, Vinh Phuc Province, Vietnam	KC491890–KC491891

Morphological data

Pseudoacanthocephalus nickoli n. sp.

Type-host: *Rana similis* (Günther) (Amphibia: Anura: Ranidae).
Other host: Rana luzonensis Boulenger (Amphibia: Anura: Ranidae).
Type-locality: Barangay Zabali, Municipality of Baler, Aurora Province, Luzon Island, Philippines (75 m a.s.l.; 15°44′31″N, 121°34′34″E).
Other localities: Aurora Memorial National Park, Sitio Dimani, Barangay Villa Aurora, Municipality of Maria Aurora, Aurora Province (500 m a.s.l, 15°41′6″N, 121°20′28″E); Sitio Minoli, Municipality of San Luis, Aurora Province (600 m a. s. l., 15°40′48″N, 121°31′44″E); Barangay Lipimental, Municipality of San Luis, Aurora Province (543 m a.s.l.; 15°39′14″N, 121°30′25″E). All collecting sites were on Luzon Island, Philippines.
Site: Intestine.
Type-material: Male holotype and female allotype: HWML 67138 (labelled: ex. *Rana similis*, Barangay Zabali, Municipality of Baler, Aurora Province, Luzon Island, Philippines, 10 June 2009, coll. V. Tkach); host KUMNH#322671. Paratypes: HWML 67139 (labelled: ex. *Rana similis*, Aurora Memorial National Park, Sitio Dimani, Barangay Villa Aurora, Municipality of Maria Aurora, Aurora Province, Luzon Island, Philippines, 22 May 2009, coll. V. Tkach); host: KUMNH#322655.
Voucher specimens deposited: HWML 67140–67149; hosts: KUMNH#322673–322719, 322656–322665, 322728–322736.
Etymology: This species is named in honour of Dr. Brent Nickol (University of Nebraska, Lincoln) in recognition of his fundamental contributions to the knowledge of the acanthocephalans.

Description (Figs. 1–3; Table 2)

[Measurements based on 8 adult males and 12 adult females from the type-series.]

General. Trunk medium-sized, smooth, widest in anterior third, narrowing in the middle and somewhat widening again towards posterior end. Females larger

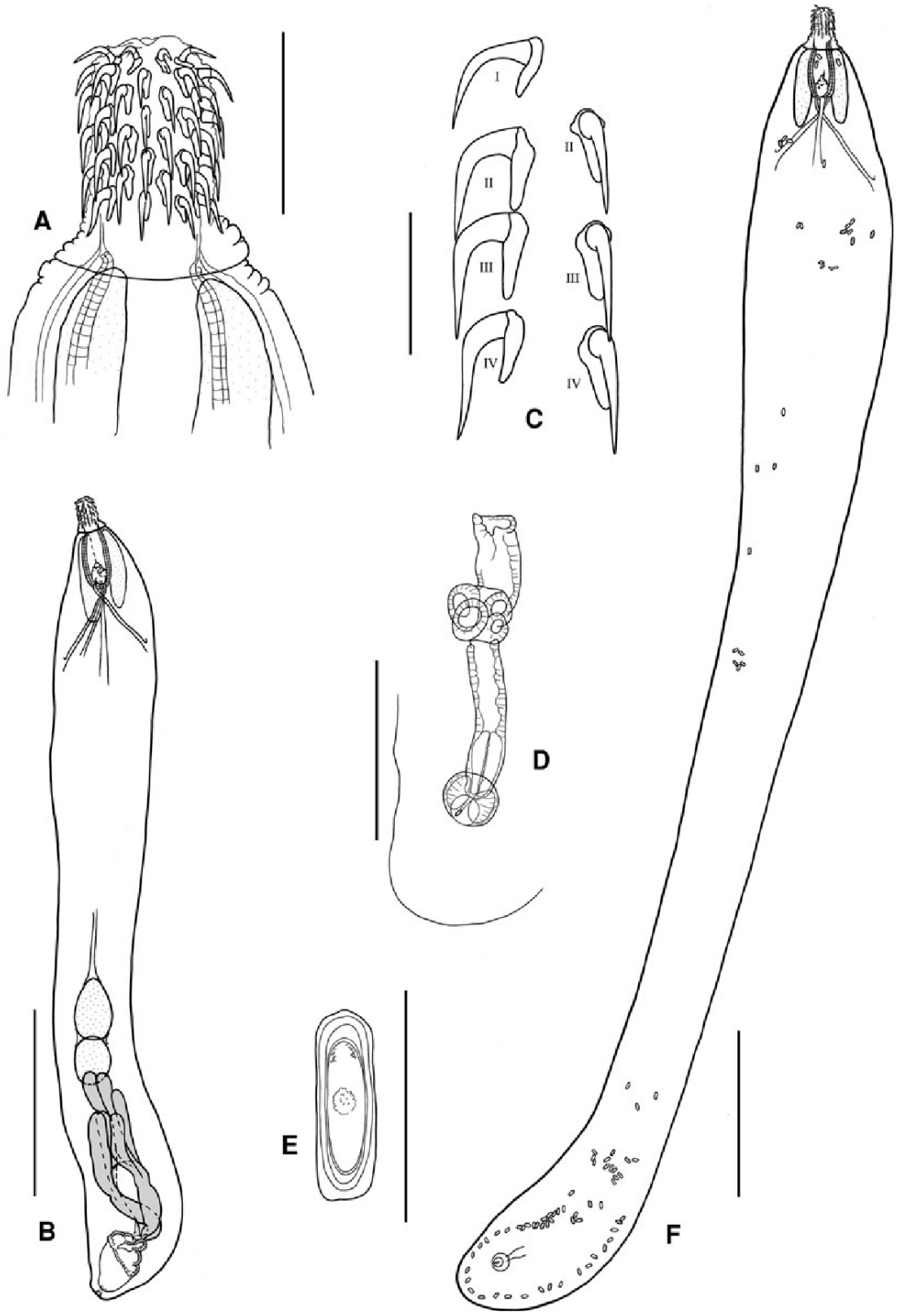

Fig. 1 *Pseudoacanthocephalus nickoli* n. sp. A, Proboscis of holotype male; B, Total view of holotype male; C, Hooks of a longitudinal row of holotype male; D, Terminal part of female reproductive system; E, Egg; F, Total view of allotype female. *Scale-bars*: A, 300 μm; B, F, 2,000 μm; C, E, 100 μm; D, 500 μm

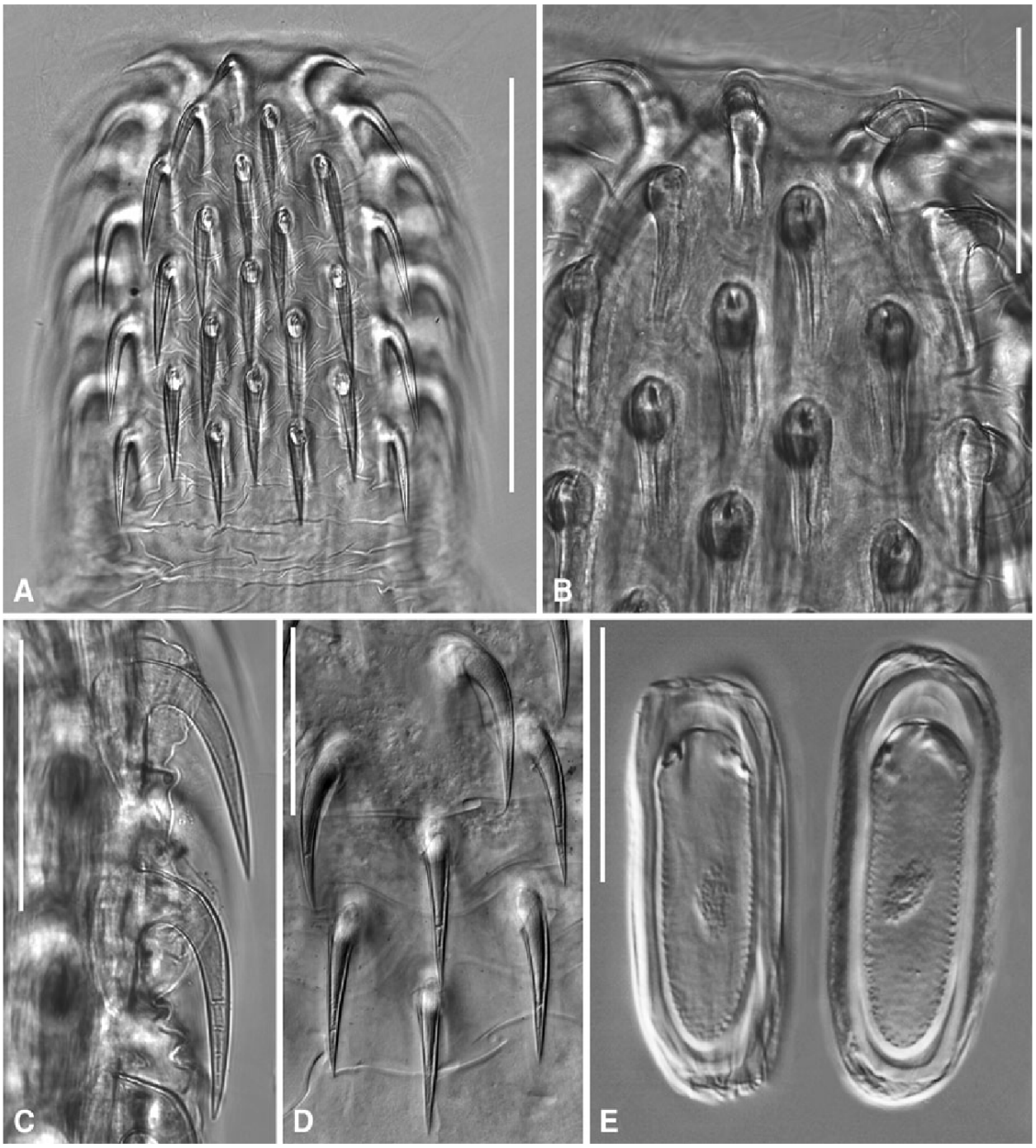

Fig. 2 Light microscopical photographs of *Pseudoacanthocephalus nickoli* n. sp. A, Proboscis of holotype male at different optical levels; B–D, Proboscis hooks. Note the shape of hook roots on images B and C; E, Egg. *Scale-bars*: A, 300 μm; B–D, 100 μm; E, 50 μm

than males. Proboscis short, cylindrical. Proboscis receptacle attached to the base of proboscis and extends into neck and trunk. Proboscis receptacle wall bi-layered, with internal layer thicker than external. Neck short, conical. Cerebral ganglion irregularly shaped, situated near bottom of proboscis receptacle.

Lemnisci attached at border between neck and trunk, extend beyond bottom of proboscis receptacle. First hooks in neighboring rows arranged unevenly.

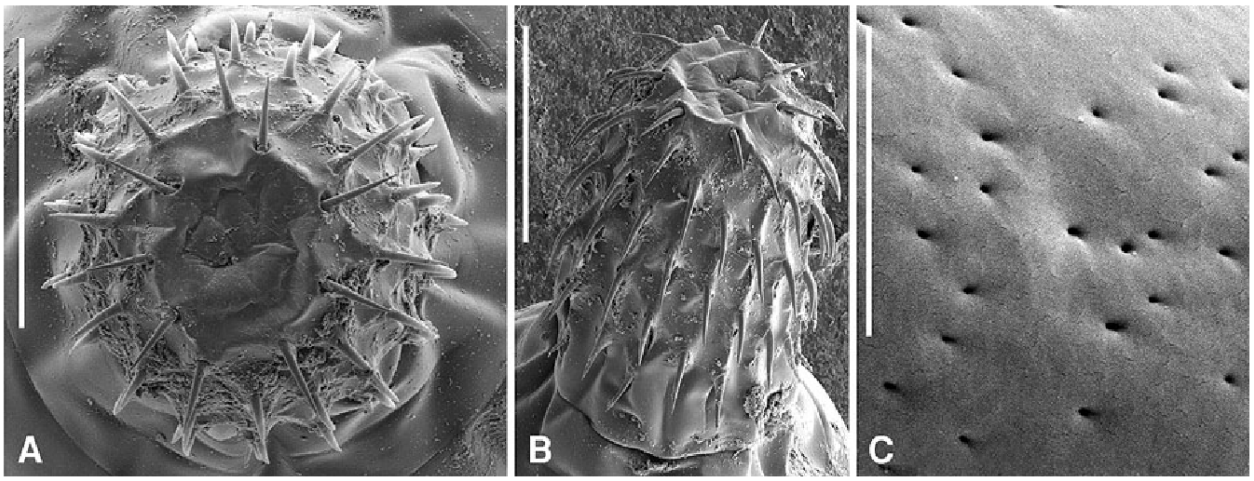

Fig. 3 Scanning electron microscopical photographs of *Pseudoacanthocephalus nickoli* n. sp. A, Apical view of proboscis; B, Latero-apical view of proboscis; C, High power image of the proboscis hook surface. *Scale-bars*: A, B, 200 μm; C, 2 μm

Table 2 Measurements of *Pseudoacanthocephalus nickoli* n. sp. from *Rana similis*

Character	Males (n = 56) Range (Mean)	Females (n = 77) Range (Mean)
Trunk length (mm)	5.77–10.86 (8.12)	11.20–25.00 (17.38)
Trunk width (anterior part) (mm)	0.92–1.60 (1.22)	1.25–2.4 (1.81)
Trunk width (middle part) (mm)	0.82–1.5 (1.05)	0.61–1.38 (1.06)
Trunk width (posterior part) (mm)	0.80–1.5 (1.14)	0.85–1.38 (1.28)
Proboscis	280–400 (335) × 220–310 (267)	300–450 (360) × 250–330 (290)
Number of hook rows	14–18 (15.4)	14–18 (15.8)
Number of hooks per row	4–5 (4.1)	4–6 (4.5)
Hook blade length*:		
# 1	63–82 (73)/70–85 (77)	65–85 (76)/64–93 (79)
# 2	73–97 (84)/75–93 (80)	72–96 (85)/76–96 (88)
# 3	78–97 (89)/75–95 (88)	75–98 (91)/79–100 (93)
# 4	83–98 (90)/70–95 (86)	85–100 (92)/75–102 (93)
# 5	78–92 (87)/90	78–103 (89)/90–98 (89)
# 6	–	90–102 (96)
Neck length	50–160 (94)	80–200 (130)
Proboscis receptacle	560–760 (677) × 200–310 (258)	600–980 (770) × 250–430 (300)
Lemnisci length	660–1,100 (894)	760–1,420 (1,070)
Anterior testis	500–890 (676) × 330–580 (478)	–
Posterior testis	430–890 (587) × 320-660 (450)	–
Female genital apparatus length	–	920–1,230 (1080)
Cement gland length (mm)	1.32–2.11 (1.75)	–
Saefftigen's pouch length (mm)	0.65–1.2 (0.89)	–
Penis length	190–360 (283)	–
Eggs	–	75–89 (82) × 26–30 (27)

* Blade length of the hooks in the rows beginning more anteriorly is separated with slash from the blade length of the hooks in the rows beginning more posteriorly

Rows that begin more anteriorly alternating with the rows beginning more posteriorly. Blade length of homologous hooks in neighboring rows differ substantially; therefore, both hook lengths are provided and separated with a slash mark "/." In rows beginning more anteriorly, hook blade length increases from apical to basal hook. In the remaining rows hook blade length increases from first to penultimate hook, while last hooks have blades that are shorter than those of the penultimate hook. Hook roots simple, widened in proximal part.

Lacunar system formed by 2 large longitudinal lateral canals which give rise to numerous small, branching canals forming a network. Genital pore terminal in males, subventral in females.

Male [Proboscis was evaginated and could be examined in only 5 specimens.] Trunk 5.66–8.40 mm long. Trunk widens in its first third to 0.90–1.20 mm, narrows to 0.88–1.01 mm at level of testes, and widens again in posterior part to 0.90–1.13 mm. Proboscis 280–320 × 240–250. Neck length 80–140. Proboscis receptacle 580–730 × 200–300. Lemnisci 660–930 × 130–250. Proboscis armed with 15–18 longitudinal rows of 4 hooks in a row. Hook blade length: (1) 63–75/72–78; (2) 75–88/83–85; (3) 82–94/83–93; (4) 85–98/75–90. Hook root length: (1) 40–48/38–50; (2) 48–57/49 –58; (3) 50–57/49–55; (4) 50–55/35–55. Reproductive system occupies posterior part of trunk. Testes subequatorial, tandem, somewhat overlapping each other; anterior testis 480–700 × 340–440, posterior testis 480–560 × 330–400. Cement glands normally 6, arranged in 2 groups. Ducts from 3 glands of either group merge into single duct; these ducts then merge into short common duct that opens outside. Number of cement glands may vary. One male had 5 cement glands grouped in 2 + 3 pattern. Another male had 7 cement glands grouped in 3 + 4 pattern. Two or 3 cement glands reach posterior margin of posterior testis and may pass it. Other glands shorter. Total length of cement gland complex 1.56–2.00 mm. Saefftigen's pouch club-shaped, 620–1,000. Penis 290–330 long.

Female [Proboscis was evaginated and could be examined in only 6 specimens.] Trunk length 11.20–16.90 mm, maximum width in anterior third 1.30–1.90 mm. Trunk narrows in middle region to 0.85–1.00 mm and widens in posterior region to 0.95–1.35 mm. Proboscis 300–360 × 250–300. Neck length 110–190. Proboscis receptacle 600–800 × 250–360. Lemnisci 920–1,100 × 180–360. Proboscis armed with 15–16 longitudinal rows of 4–5 hooks in a row. Hook blade length: (1) 73–85/68–93; (2) 78–95/83–95; (3) 88–97/92–100; (4) 88–100/75–98; (5) 78–96/92–98. Hook root length: (1) 40–48/48–53; (2) 50–63/50–65; (3) 54–65/54–67; (4) 50–65/45–58; (5) 43–50/50. Reproductive system length from edge of uterine bell to genital pore 1.00–1.23 mm. Vagina with single sphincter. Eggs 80–85 × 27–30, without polar prolongation of middle membrane. Acanthor 53–58 × 18–19.

Remarks

Pseudoacanthocephalus nickoli n. sp. differs from all known species of the genus in the unusual difference in the length of hook blades in alternating rows. In rows starting closer to the apex of the proboscis the hook blade length increases from the apical to basal hooks and the basal hook has the longest blade. In the alternating rows the hook blade length increases from apical to the penultimate hook, but the last (basal) hook has a shorter blade than the penultimate hook.

The arrangement of proboscis hooks in *P. nickoli* n. sp. is similar to that in *P. bufonis*, *P. reesei* Bush, Duszynski & Nickol, 2009, and *P. nguyenthileae*.

The original description of *P. bufonis* (the type-species of the genus) from amphibians in Thailand (Shipley, 1903) was rather superficial and the type-material is not available (Kennedy, 1982). Petrochenko (1958) provided an amended description of *P. bufonis* based on material from Central Asia. However, Petrochenko's material originated from a geographical region distant from the type-locality. Moreover, there were some morphological differences between Petrochenko's description and the original description; so that Kennedy (1982) suggested that Petrochenko's material belongs to a different species of *Pseudacanthocephalus*. On the other hand, Kennedy's (1982) redescription was based on specimens collected from Indonesia, which also raises questions about the identity of his material. Therefore, in our differentiation, we used the original description by Shipley (1903) and the redescription of *P. bufonis* by Bush et al. (2009) based on the recent material collected in China, which is close to the type-locality.

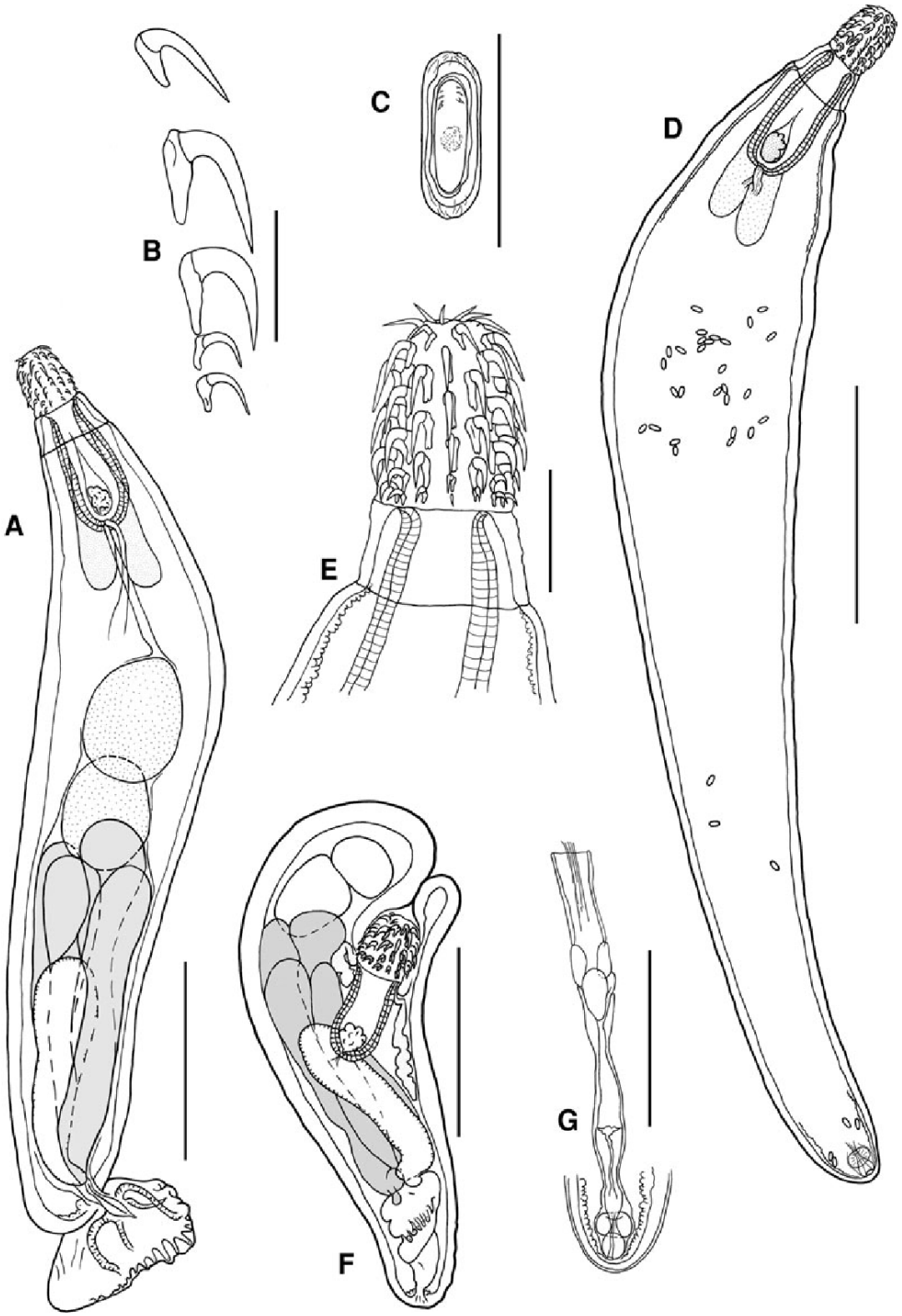

Fig. 4 *Pseudoacanthocephalus smalesi* n. sp. A, Total view of holotype male; B, Hooks of a longitudinal row of holotype male; C, Egg; D, Total view of allotype female; E, Proboscis of holotype male; F, Juvenile male; G, Terminal part of female reproductive system. *Scale-bars*: A, F, 1,000 μm; B, C, 100 μm; D, 2000 μm; E, 300 μm; G, 500 μm

Pseudoacanthocephalus nickoli n. sp. differs from *P. bufonis* in body shape. The trunk of the new species is narrow in the middle, while in *P. bufonis* it is spindle-shaped without such narrowing. In addition, *P. nickoli* n. sp. has a shorter proboscis (280–450 *vs* 408–542 in *P. bufonis*) and neck (50–200 *vs* 240–415 in *P. bufonis*). The new species has larger eggs (75–89, mean 82) than *P. bufonis* (55–72, mean 65). The reproductive system in the males of *P. nickoli* n. sp. occupies much smaller proportion of the body length than in the males of *P. bufonis*.

Pseudoacanthocephalus nickoli n. sp. differs from *P. reesei* in having shorter proboscis (280–450 in *P. nickoli* n. sp. *vs* 432–490 in *P. reesei*) and neck (50–200 in *P. nickoli* n. sp. *vs* 250–396 in *P. reesei*), somewhat shorter blades of largest hooks (78–102 in *P. nickoli* n. sp. *vs* 72–139 in *P. reesei*) and more numerous hook rows (14–18 in *P. nickoli* n. sp. *vs* 12–15 in *P. reesei*).

The new species differs from *P. nguyenthileae* in the number of cement glands (6 in *P. nickoli* n. sp. *vs* 8 in *P. nguyenthileae*) and egg size (75–89 × 26–30 in *P. nickoli* n. sp. *vs* 70–80 × 20–26 in *P. nguyenthileae*).

Pseudoacanthocephalus smalesi n. sp.

Type-host: *Sphenomorphus abdictus* Brown & Alcala (Reptilia: Squamata: Scincidae)
Type-locality: Sitio Minoli, Barangay Real, Municipality of San Luis, Aurora Province, Luzon Island, Philippines (600 m a. s. l.; 15°40′48″N, 121°31′44″E).
Other localities: Barangay Zabali, Municipality of Baler, Aurora Province, Luzon Island, Philippines (75 m a.s.l., 15°44′31″N, 121°34′34″E).
Site: Intestine.
Type-material: Male holotype and female allotype: HWML 67131 (labeled: ex. *Sphenomorphus abdictus*, Sitio Minoli, Barangay Real, Municipality of San Luis, Aurora Province, Luzon Island, Philippines, 13.vi.2009, coll. V. Tkach; host KUMNH#323279). Paratypes: HWML 67132 (labelled: ex. *Sphenomorphus abdictus*, Barangay Zabali, Municipality of Baler, Aurora Province, Luzon Island, Philippines, 5 June 2009, coll. V. Tkach 75; host KUMNH#323262).
Voucher specimens deposited: HWML 67133–67137; hosts: KUMNH#323271–323274, 323266– 323267.

Etymology: This species is named in honour of Dr. Lesley Smales in recognition of her significant contributions to the knowledge of acanthocephalans.

Description (Figs. 4–6; Table 3)

[Measurements based on 4 adult males and 4 adult females from the type-series].

General. Trunk close to spindle-shaped, medium-sized, smooth, with thick tegument. Females larger than males. Proboscis short, widening posteriorly. Neck well defined. Proboscis receptacle attached to the base of proboscis and extends into neck and trunk; wall of proboscis receptacle bi-layered, internal layer thicker than external. Neck short, conical. Cerebral ganglion oval, 160–240 × 120–160, situated near bottom of proboscis receptacle. Lemnisci attached at border between neck and trunk and extend far beyond bottom of proboscis receptacle. All hooks simple, with well-developed roots. Roots of first 3–4 hooks in each row have a small, but distinct proximal widening. Blades of last 1–2 hooks in each row substantially shorter and thinner than blades of more anterior hooks. Length of homologous hooks in neighboring rows differ substantially; therefore, both lengths are provided and separated with a slash mark "/." Lacunar system formed by 2 large longitudinal lateral canals which give rise to numerous small, branching canals that form a network. Genital pore subterminal in both sexes.

Male. Body spindle-shaped, gradually widening in middle region, widest at level of anterior testis, and then gradually narrowing towards posterior end. Trunk 3.98–4.65 mm in length, 0.97–1.02 mm in maximum width. Proboscis length 290–330, width at base 270–290. Neck length 230–260. Proboscis receptacle length 640–700, maximum width in posterior third 320–340. Lemnisci 830–960. Proboscis armed with 12 longitudinal rows of 4–5 hooks in a row. Number of hooks in rows alternating regularly or irregularly. First 3 hooks substantially larger than remaining 1–2 hooks. Hook blade length: (1) 78–85/88–100; (2) 105/100–108; (3) 93–100/68–88; (4) 43–58/35–43; (5) 35–43. Blade length of basal hook (4th or 5th depending on number of hooks in row) 35–43. Hook root length: (1) 43–50/50–65; (2) 65–78/70–80; (3) 65–80/58–65; (4) 28–50/28–33; (5) 25–30. Root length of basal hook 28–33.

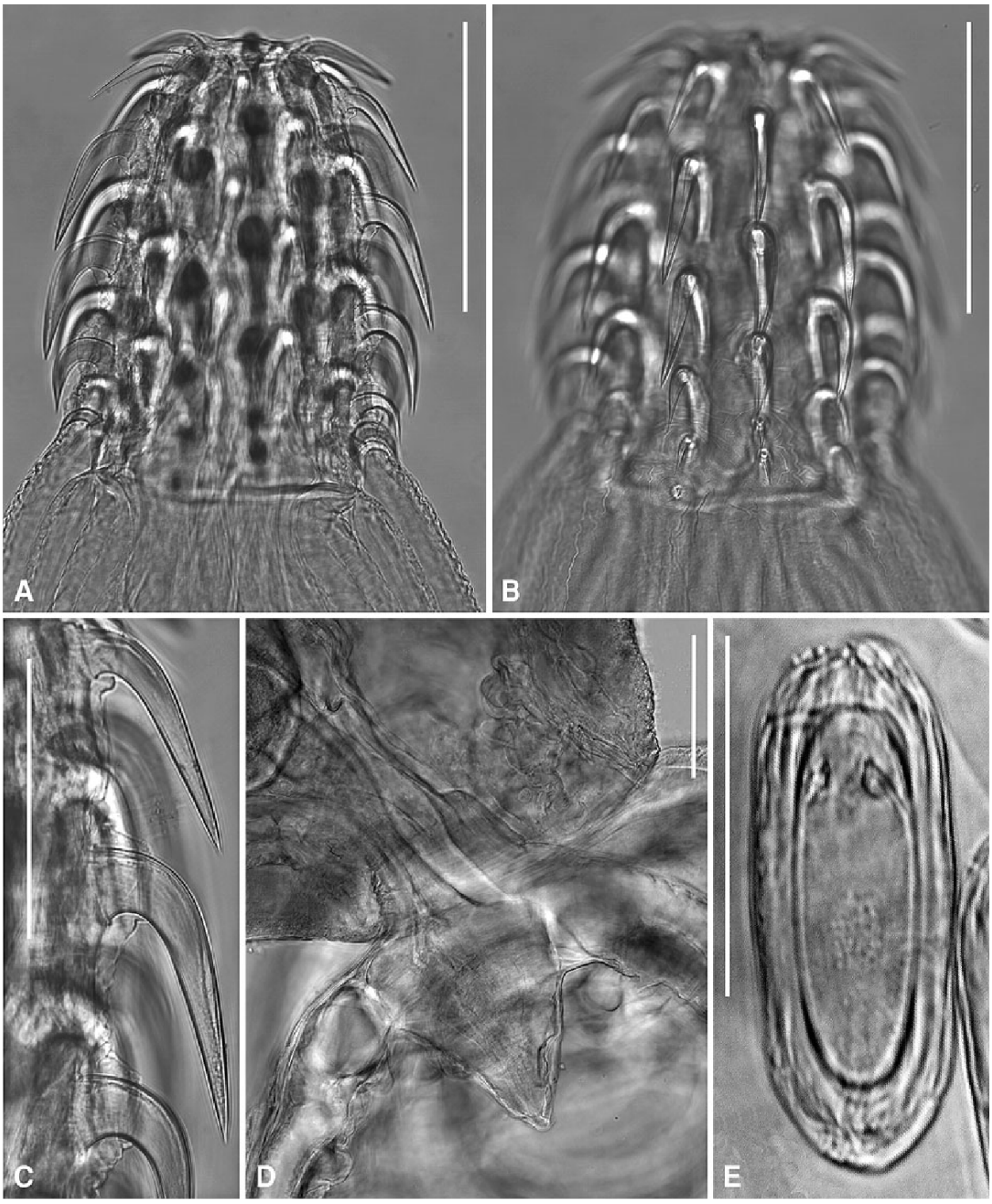

Fig. 5 Light microscopical photographs of *Pseudoacanthocephalus smalesi* n. sp. A, B, Proboscis of holotype male at different optical levels. Note the smaller posterior hooks; C, Higher power lateral view of middle hooks; D, Fragment of male reproductive system with penis; E, Egg. *Scale-bars*: A, B, 300 μm; C, D, 100 μm; E, 50 μm

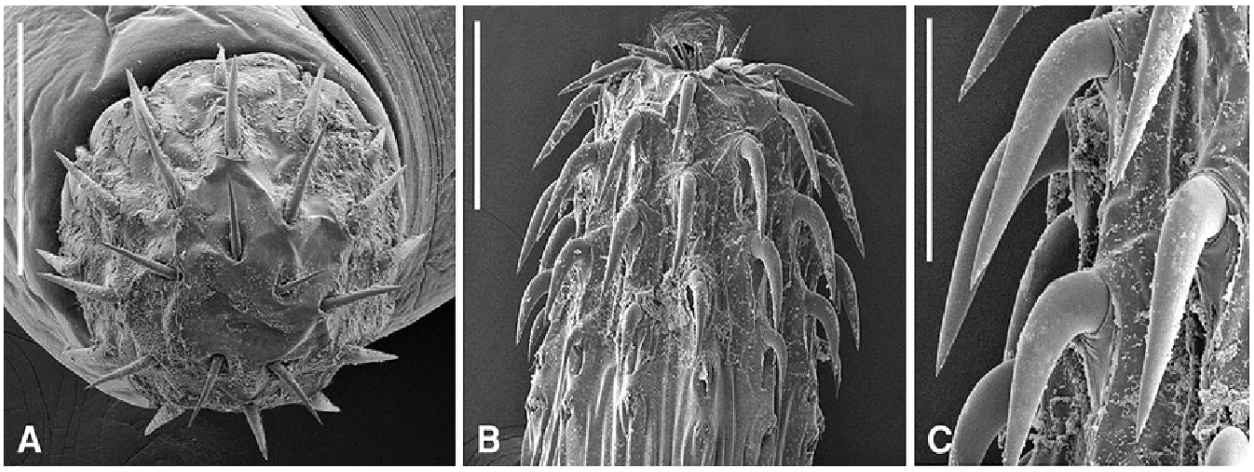

Fig. 6 Scanning electron microscopical photographs of *Pseudoacanthocephalus smalesi* n. sp. A, Apical view of proboscis; B, Lateral view of proboscis; C, High power image of the proboscis hooks surface. *Scale-bars*: A, B, 200 μm; C, 100 μm

Table 3 Measurements of *Pseudoacanthocephalus smalesi* n. sp. from *Sphenomorphus abdictus*

Character	Male (n = 14) Range (Mean)	Female (n = 6) Range (Mean)
Trunk (mm)	3.98–5.06 (4.41) × 0.90–1.02 (0.96)	7.30–11.40 (9.25) × 1.26–1.73 (1.50)
Proboscis	260–340 (310) × 250–330 (280)	430–510 (460) × 320–370 (340)
Number of hook rows	11–13 (11.88)	12–13 (12.67)
Number of hooks per row	3–5 (4.25)	5–6 (5.33)
Hook blade length*:		
# 1	75–85 (82)/85–100 (95)	90–132 (110)/100–128 (110)
# 2	95–110 (108)/88–108 (103)	118–138 (127)/125–143 (133)
# 3	63–100 (83)/62–95 (79)	113–130 (126)/110–133 (119)
# 4	28–58 (44)/35–55 (44)	65–116 (91)/50–83 (65)
# 5	35–43 (38)	43–63 (54)/40–50 (44)
# 6	–	38–53 (46)
Basal hook blade length	25–50 (40)	38–50 (44)
Neck length	220–260 (234)	220–400 (395)
Proboscis receptacle	620–730 (673) × 300–350 (321)	810–1,070 (923) × 260–410 (330)
Lemnisci length	750–960 (846)	1,000–1,240 (1,193)
Anterior testis	580–980 (710) × 380–580 (497)	–
Posterior testis	410–720 (590) × 340–510 (428)	–
Female genital apparatus length (mm)	–	0.94–1.82 (1.34)
Penis length	290–360 (323)	–
Eggs	–	78–83 (81) × 28–31(29)

* Blade length of the hooks in the rows beginning more anteriorly is separated with slash from the blade length of the hooks in the rows beginning more posteriorly

Reproductive system occupies approximately two thirds of trunk length. Testes oval, tandem, frequently overlapping each other. Anterior testis 650–720 × 500–550, somewhat larger than posterior testis, 580–670 × 420–510. Cement glands 4, large, 1.7–2.1 (1.88) mm, arranged in two pairs, extending from posterior end of

trunk to posterior testis. Saefftigen's pouch club-shaped, with muscular walls, 1,030–1,200 × 280–380. Penis 340–350 long. Genital pore subterminal, dorsal.

Female. Trunk irregularly spindle-shaped, widening in second quarter of its length and tapering towards posterior end. Widening asymmetrical, more pronounced on dorsal side. Trunk length 7.30–11.40 mm, maximum width 1.35–1.73 mm. Proboscis 430–510, width at base 320–370. Neck 220–400. Proboscis receptacle 810–1,070 × 810–1,070. Lemnisci 1.00–1.24 mm. Proboscis armed with 12–13 (more frequently 13) longitudinal rows of 5–6 hooks in a row. Hook number in rows alternating regularly. First 3–4 hooks substantially larger than remaining hooks. Hook blade length: (1) 87–90/100–120; (2) 118–128/125–145; (3) 130–138/110–128; (4) 115–125/55–95; (5) 63–75/43–50; (6) 38–53. Blade length of basal hook 38–53. Hook root length: (1) 38–50/65–83; (2) 78–95/90–98; (3) 80–100/83–105; (4) 65–97/58–78; (5) 53–55/30–43; (6) 25–35. Root length of basal hook 25–37.

Reproductive system length 1.29–1.32 mm. Vagina 270–470, with single sphincter, 70–90 × 130; uterus 440–600. Genital pore subterminal, ventral. Eggs oval, 78–83 × 28–30, without polar prolongations of middle membrane. Well-defined embryonic hooks present on both sides of acanthor body.

Juvenile males. [Based on 5 specimens found in the intestines of three *S. abdictus*]. Proboscis retracted into anterior part of body, but not invaginated. Trunk 2.2–3.4 × 0.86–1.12 mm. Proboscis 300–360 × 260–300. Proboscis armament and hook morphology similar to those of adult worms. Proboscis armed with 12–13 longitudinal rows of 4–5 hooks. Hook blade length: (1) 83–98/88–110; (2) 98–108; (3) 50–93/68–78; (4) 43–55/40–47; (5) 33–43. Hook root length: (1) 43–48/48–55; (2) 60–70/65–73; (3) 45–68/50–60; (6) 28. Neck retracted and could not be seen. Proboscis receptacle 440–670 × 210–280. Lemnisci could not be adequately seen or measured. Cement glands 4. Testes tandem, adjacent to each other, anterior testis 370–510 × 290–420, posterior testis 370–470 × 280–410. Saefftigen's pouch 500 × 250. Genital pore subterminal. Probably terminal pore shifts and becomes subdorsal in the process of worm growth and maturation.

Remarks

Pseudoacanthocephalus smalesi n. sp. can be easily distinguished from all other species of the genus by the size of the basal proboscis hooks, which are more than two-fold shorter than the middle hooks, and in the presence of four cement glands in males. Most other species of the genus have six glands and only *P. nguyenthileae* has eight glands.

Seven previously known species of *Pseudoacanthocephalus* have 16 or more longitudinal hook rows and are clearly differentiated from *P. smalesi* n. sp. possessing 12–13 hook rows. The remaining five species, namely *P. pertensis* Edmonds, 1971, *P. bigueti* (Houin, Golvan & Brygoo, 1965) Golvan, 1969, *P. rhampholeatus* Smales, 2005, *P. reesei*, and *P. bufonis*, have proboscis hook distribution and number similar to that in the new species. Of these, only *P. bigueti* and *P. rhampholeatus* have a proboscis clearly widening from apex to base, similar to the situation in *P. smalesi*. Both these species were described from reptiles in Africa and thus are separated from the new species by substantial geographical distance and host specificity. *P. bigueti* was described from snakes in Madagascar (Houin et al., 1965) while *P. rhampholeatus* was described from chameleons in Tanzania. Besides, *P. smalesi* n. sp. differs from both

Table 4 Pairwise rDNA sequence divergence among the four species of *Pseudoacanthocephalus* studied. Number of variable sites in sequences encompassing ITS1, 5.8S, ITS2, and partial 28S regions of nuclear ribosomal DNA are above the diagonal. Sequences were 1,572–1,608 bp long in different species. Percentages of variable sites in relation to the total number of aligned sites (including gaps) are below the diagonal

Species	*P. nickoli* n. sp.	*P. smalesi* n. sp.	*P. bufonis*	*P. nguyenthileae*
P. nickoli n. sp.	–	339	169	174
P. smalesi n. sp.	21.1	–	310	281
P. bufonis	10.6	19.5	–	65
P. nguyenthileae	11.0	17.8	4.1	–

species by a larger proboscis (260–510 × 250–370 in the new species *vs* 250–300 × 250 in *P. bigueti* and 130–270 × 170–260 in *P. rhampholeatus*) and longer neck (220–400 in the new species *vs* 120–140 in *P. bigueti* and 50–130 in *P. rhampholeatus*).

Molecular data

We compared DNA sequences of the two new species from the Philippines with sequences of the closely related species *P. bufonis* from amphibians in southeastern China and *P. nguyenthileae* from amphibians in Vietnam (Table 1). The amplified nuclear ribosomal DNA fragment encompassed the 3′ end of 18S nuclear rDNA gene, internal transcribed spacer region (ITS1+5.8S+ITS2), and 5′ end of the 28S gene. Upon trimming the sequences to the length of the shortest one, the sequence length of the compared fragment varied from 1,572 base pairs (bp) in *P. nickoli* n. sp. to 1,608 bp in *P. smalesi* n. sp. Although we sequenced from two to six specimens of each *Pseudoacanthocephalus* species and some of them were collected from multiple hosts and localities (Table 1), no intraspecific sequence variability was detected among the different individuals representing each species.

Pairwise comparisons showing the number of nucleotide differences between species pairs are presented in Table 4. The length of the alignments of each pair of species varied from 1,582 to 1,606 bases due to introduced gaps. Overall interspecific variability was very high and varied from 65 (4.1 %) variable sites between *P. bufonis* and *P. nguyenthileae* to 339 (21.1 %) between *P. nickoli* n. sp. and *P. smalesi* n. sp. (Table 4). These molecular data strongly support the status of the *Pseudoacanthoceplaus* specimens from frogs and lizards in the Philippines as two new species.

Discussion

To the best of our knowledge our work represents the first report of *Pseudoacanthocephalus* from the Philippines. Both morphological and molecular data convincingly demonstrated that our specimens of *Pseudoacanthocephalus* from frogs and scincid lizards in the Philippines represent two new species. We have obtained for the first time sequence data for *Pseudoacanthocephalus*. Four species included in the molecular comparison showed very high levels of interspecific sequence divergence. At the same time, sequences obtained from multiple individuals of each species show that there is a lack of intraspecific variablilty, even among specimens collected from multiple hosts and multiple localities. Thus, the selected DNA region is a highly suitable target for species differentiation and diagnostics in this genus.

In our comparison, the three *Pseudoacanthocephalus* species from amphibians, namely *P. nickoli* n. sp., *P. bufonis*, and *P. nguyenthileae*, were closest genetically with the proportion of variable sites in pairwise comparisons ranging from 4.1 to 11 % (Table 4). When the three species from amphibians were compared to *P. smalesi* n. sp. parasitic in lizards, the percentage of variable sites was much higher and reached from 17.8 to 21.1 % (Table 4). These figures are based on the combined complete ITS and partial 28S region sequences. The proportion of variable sites in the ITS region alone is even greater. ITS sequences have been previously used to separate populations, species, and complexes of cryptic species in other acanthocephalan genera such as *Pomphorhynchus*, *Leptorhynchoides*, and *Neoechinorhynchus*, which showed a genetic divergence in the ITS region ranging from 1 to 20 % (Perrot-Minnot, 2004; Steinauer et al., 2007; Martínez-Aquino et al., 2009).

For our molecular study we used specimens of *P. bufonis* from China that were reported by Bush et al. (2009). These authors found *P. bufonis* in eight species of anuran amphibians from two natural reserves in China. Our sequences of *P. bufonis* collected from four amphibian species confirmed their conspecificity and provided additional evidence of the relatively low host specificity of *P. bufonis*. Still, it is likely that members of the genus are specific with respect to the higher level taxonomic affiliation of their hosts, i.e., either amphibians or reptiles. We examined a large number of amphibians and lizards from the Luzon Island, but did not find *P. smalesi* n. sp. infecting amphibians or *P. nickoli* n. sp. infecting reptiles. The very high level of sequence divergence between *P. smalesi* n. sp. and the three species parasitic in amphibians also suggests that these acanthocephalans represent different, divergent lineages within the genus. The fact that *P. nickoli* n. sp. has demonstrated much greater sequence similarity to the two species from amphibians collected in the mainland China and Vietnam, than to sympatric and symbiotopic

P. smalesi n. sp., indicates that these species probably diverged with early radiations or their hosts and not as a result of recent host switches upon isolation in the Philippines. The interspecific sequence variability between *P. smalesi* n. sp. and the three species parasitic in amphibians is two to four times greater than the variability among the three species from amphibians (Table 4). Therefore, it cannot be excluded that *Pseudoacanthocephalus* from lizards and amphibians may belong to different genera. More sequences of *Pseudoacanthocephalus* species including additional species from reptiles are necessary in order to adequately test this hypothesis.

In addition to the questions of intrageneric taxonomy, the phylogenetic affinities of *Pseudoacanthocephalus* also require additional analysis. Previous molecular phylogenetic studies (García-Varela & Nadler 2005, 2006) have demonstrated the paraphyletic nature of the family Echinorhynchidae as currently recognized. Currently, the relationships of *Pseudoacanthocephalus* with different lineages within the family are unclear. This question, however, is beyond the scope of our study.

The validity of the genus *Pseudoacanthocephalus* is somewhat disputable. The genus was established by Petrochenko (1958) for four species that belonged at the time to *Acanthocephalus* Koelreuther, 1771. The new genus was established by Petrochenko on the basis of the oval eggs lacking polar prolongations of the middle membrane and the holoechinate type of acanthor. Based on the egg morphology and the type of acanthor of these species, Petrochenko (1958) suggested that they have terrestrial life-cycles while *Acanthocephalus* spp. have aquatic life-cycles. However, Grabda-Kazubska (1964) and Golvan (1969) argued that the type of acanthor is less taxonomically important than assumed by Petrochenko. Kennedy (1982) also doubted the importance of the differentiating characters used by Petrochenko (1958) and considered all species possessing these features within the genus *Acanthocephalus*. Nevertheless, the majority of other researchers supported the separation of *Pseudoacanthocephalus* into a separate genus (e.g. Yamaguti, 1963; Golvan, 1969, 1994; Amin, 1985; Khokhlova,1986). Moreover, Petrochenko's suggestion regarding the life-cycles of *Pseudoacanthocephalus* spp. was later confirmed by reports of *Pseudoacanthocephalus* larvae from terrestrial isopods. Muhamadiev et al. (1982) found larvae of *Pseudoacanthocephalus* in *Trachelipus* sp. in Kyrgyzstan; and Ikramov (2002) discovered larvae of *P. bufonis* from *Porcellio fedshenkoi*, *P. latus*, *Porcellio* sp., and *Armadillidium vulgare* in Uzbekistan. The species reported by Ikramov (2002) was most likely conspecific to the species identified as *P. bufonis* by Petrochenko (1958) than to the true *P. bufonis*. Therefore, we agree with the opinion of the authors recognizing *Pseudoacanthocephalus* as a valid genus.

Petrochenko (1958) chose *P. bufonis*, which was originally described from toads in Thailand, as the type-species of *Pseudacanthocephalus*. Petrochenko (1958) did not have access to the type-material of *P. bufonis* (Shipley, 1903). Therefore, he redescribed the species based on his specimens from Central Asia. Some morphological characteristics of these specimens differed substantially from those in the first description by Shipley (1903). For instance, the specimens described by Petrochenko (1958) had 16–20 longitudinal rows of 6–8 hooks in each row while the original description mentioned 14–16 longitudinal rows of 3–4 hooks in each row. This prompted subsequent redescriptions by Kennedy (1982) and Bush et al. (2009). Because of the substantial differences between the Petrochenko's (1958) material and the first description of *P. bufonis*, Kennedy (1982) proposed a new name *P. breviprostatus* for the form described by Petrochenko (1958). However, this systematic change was not supported by the majority of subsequent authors (Amin, 1985; Khokhlova, 1986; Bush et al., 2009). Due to the above contradictions, we differentiated our specimens from *P. bufonis* based on the original description and the redescription Bush et al. (2009).

Golvan (1994) recognized nine species of *Pseudoacanthocephalus*. Amin et al. (2008) included 11 species in their key to the identification of *Pseudoacanthocephalus* spp. Bush et al. (2009) described another new species *P. reesei* from amphibians in China. In the same year, Arredondo & Gil de Pertierra (2009) have re-examined the type-material of *Acanthocephalus lutzi* (Hamann, 1891), *Acanthocephalus saopaulensis* Smales, 2007, and *Acanthocephalus caspanensis* Fernández & Ibarra Vidal, 1992 and concluded that these species should belong to the genus *Pseudoacanthocephalus*. At the same time, they have demonstrated that *P. lutzi* and *P. saopaulensis* are conspecific and, therefore, *P. lutzi* should be considered a synonym of *P. saopaulensis*. Therefore, with addition of the two

species described in the present work, the genus *Pseudoacanthocephalus* currently comprises 16 species. The key to their identification is provided below.

Key to the species of *Pseudoacanthocephalus*

1a. Proboscis with 20 or more longitudinal hook rows 2
1b. Proboscis with less than 20 longitudinal hook rows 3
2a. Proboscis oval, with 10–12 hooks in each longitudinal hook row. Known from India .... *P. rauschi* Gupta & Fatma, 1986
2b. Proboscis cylindrical, with 6–7 hooks in each longitudinal hook row. Known from Caucasus ..... *P. caucasicus* (Petrochenko, 1953) Petrochenko, 1958
3a. Proboscis with 8–12 longitudinal hook rows. Known from Thailand .... *P. xenopeltidis* (Shipley, 1903) Golvan, 1969
3b. Proboscis with 12–19 longitudinal hook rows 4
4a. Each longitudinal row with 13–15 hooks. Known from China ..... *P. elongatus* (Van Cleave, 1937) Petrochenko, 1958
4b. Each longitudinal row with 3–7 hooks 5
5a. Male with 8 cement glands. Known from Vietnam ..... *P. nguyenthileae* Amin, Van Ha & Heckman, 2008
5b. Male with 4–6 cement glands 6
6a. Basal hooks more than twice shorter than the median hooks. Known from the Philippines .... *P. smalesi* sp. n.
6b. Blades of basal hooks similar in length to the blades of middle hooks 7
7a. Lemnisci shorter or equal in length with proboscis receptacle. Female genital pore terminal. Known from Madagascar .... *P. betsileo* Golvan, Houin & Brygoo, 1969
7b. Lemnisci longer than proboscis receptacle. Female genital pore subterminal 8
8a. Proboscis 132–300. Parasites of reptiles 9
8b Proboscis 350–840. Parasites of amphibians 10
9a. Blades of longest hooks 53–79. Known from Tanzania ..... *P. rhampholeontos* Smales, 2005
9b. Blades of longest hooks reach 100. Known from Madagascar ..... *P. bigueti* (Houin, Golvan & Brygoo, 1965) Golvan, 1969
10a. Trunk widest in anterior third, then narrowing in the middle and somewhat widening again towards posterior end. Known from the Philippines .... *P. nickoli* sp. n.
10b. Body spindle-shaped without narrower region in the middle 11
11a. Proboscis with 12–15 longitudinal rows of 4–5 hooks in each row 12
11b. Proboscis with 16–19 longitudinal rows of 5–7 hooks in each row 13
12a. Males 2.6–3.2 mm long, females 5.1–6.9 mm long. Eggs 45–55 × 20–25. Known from Australia ..... *P. pertensis* Edmonds, 1971
12b. Males 6.0–10.3 mm long, females 11.8–14.8 mm long. Eggs 94–96 × 19–24. Known from China ..... *P. reesei* Bush, Duszynski & Nickol, 2009
13a Eggs 82–93 × 29–42. Known from Argentina and Brazil ..... *P. lutzi* (Hamman, 1891) Arredondo & Gil de Pertierra, 2009
13b. Eggs 55–72 × 19–24 14
14a. Proboscis 408–542. Known from Tanzania, Central and Southeast Asia, China ..... *P. bufonis* (Shipley, 1903) Petrochenko, 1958
14b. Proboscis 700–840 15
15a. Proboscis with 16 longitudinal hook rows. Body length does not exceed 10 mm. Known from Central Asia ..... *P. bufonincola* (Kostylew, 1941) Petrochenko, 1958
15b. Proboscis with 18–19 longitudinal hook rows. Body longer than 10 mm. Known from South America ..... *P. caspanensis* (Fernandez & Vidal, 1992) Arredondo & Gil de Pertierra, 2009

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