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Amphibiophilus mooiensis n. sp. (Nematoda: Amphibiophilidae), a parasite of *Amietia delalandii* (Duméril & Bibron) (Amphibia: Pyxicephalidae) from South Africa

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Abstract Four species of the genus *Amphibiophilus* Skrjabin, 1916 from pyxycephalid frogs in southern and central Africa are currently recognised as valid. Several specimens of *Amphibiophilus* were found in material from the common river frog, *Amietia delalandii* (Duméril & Bibron) (Amphibia: Pyxicephalidae), collected in Potchefstroom (North-West Province, South Africa). These specimens clearly differ from all previously known species by the shape of the distal end of the spicule, the shape of the gubernaculum and the structure of the synlophe. They are, thus, considered as a new species, *Amphibiophilus mooiensis*. As all other species in the family Amphibiophilidae Durette-Desset & Chabaud, 1981, *A. mooiensis* n. sp. possesses a number of archaic characters, such as a buccal capsule with a well-developed dorsal oesophageal tooth, six inner labial papillae, six outer labial papillae and four cephalic papillae. Molecular data (*cox*1 and ITS-28S rDNA sequences) are provided and host and geographical specificity are discussed.

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Introduction

The genus *Amphibiophilus* Skrjabin, 1916 comprises a small group of primitive intestinal nematodes parasitising anuran amphibians. Hitherto, only a few species of the genus have been reported from Africa, Australia and Asia.

Amphibiophilus acanthocirratus Skrjabin, 1916 was described from frogs of the Pyxicephalidae (reported as Ranidae) in Africa (Skrjabin, 1916). The species has been mentioned only once since its original description. Hsu (1933) reported what are probably young specimens from *Fejervarya limnocharis* (Gravenchorst) (syn. *Rana limnocharis* Gravenchorst) in Amoy, China. *Amphibiophilus natalensis* Travassos, 1937 (syn. *Oswaldocruzia natalensis* Walton, 1935) was described from *Amietia delalandii*

(Duméril & Bibron) (syn. *Rana delalandii* Duméril & Bibron) in KwaZulu-Natal, South Africa. Later, the species was redescribed by Baker (1981) from the type-host and *Strongylopus fasciatus* (Smith) (syn. *Rana fasciata*) in Natal. Recently, one specimen of *A. natalensis* was found in Limpopo Province (South Africa) in material from *Amietia delalandii* [reported as *Amietia angolensis* (Bocage)] (Halajian et al., 2013). *Amphibiophilus versterae* Baker, 1981 was described as coparasite of *A. natalensis* from *Amietia delalandii* (reported as *Rana angolensis* Bosage, but see below) and *S. fasciatus* from Natal (Baker, 1981). *Amphibiophilus chabaudi* Puylaert, 1967 was described from Kenya, also from *Amietia delalandii* (reported as *Rana angolensis*, but see below) (Puylaert, 1967). The two latter species are only known from their original descriptions.

Amphibiophilus egerniae Johnston & Mawson, 1947 was described from an Australian skink. Since, the illustrations of the caudal bursa showed that the arrangement of bursal rays does not correspond to the genus *Amphibiophilus*, Baker (1981) suggested that this species may belong to the family Nicollinidae (Skrjabin & Schultz, 1937) Durette-Desset & Chabaud, 1981, which are parasites of Australian marsupials. Thereafter, Jones (1987) studied type-material of *A. egerniae* and noted that the morphology of the spicules, synlophe and female genital structure is similar to that of the genus *Wanaristrongylus* Jones, 1987. However, only one male with destroyed caudal bursa was available in the type-material. Thus, Jones (1987) preferred not to assign the species to *Wanaristrongylus*, although he suggested that it definitely should not be considered as *Amphibiophilus*.

Amphibiophilus sp. and *A. ranae* Wang, 1978 were reported from *F. limnocharis* in China (Hsu & Hoeppli, 1934). However, based on the morphology of the caudal bursa and synlophe structure, Hasegawa (1989) assigned both to *Batrachonema synaptospicula* Yuen, 1965.

In light of the available literature, it becomes clear that all species of *Amphibiophilus* currently considered valid have been described from frogs in southern (South Africa) and central (Kenya, Cameroon) Africa (Halajian et al., 2013; Daniel et al., 2015). All other supposed findings of species of this genus from reptiles or outside the African continent were considered as misidentifications.

Studying nematodes collected in 2016 from *Amietia delalandii* in Potchefstroom (North-West Province, South Africa), we found several specimens of *Amphibiophilus*. These specimens clearly differed from all previously known species based on the synlophe structure, the arrangement of caudal bursal rays and the shape of the spicules and gubernaculum. An illustrated description based on 34 specimens of *Amphibiophilus mooiensis* n. sp., supplied with molecular data, is presented herein.

Materials and methods

Amphibians were collected manually in December of 2016 along the River Mooi in Potchefstroom (North-West Province, South Africa). Frogs were euthanised with an MS222 solution and dissected. Nematodes were removed, washed in saline and fixed in hot 70% ethanol. They were subsequently stored in 70% ethanol. Prior to microscopic examination, nematodes were cleared in lactophenol. The morphology of the nematodes was studied and photomicrographs were taken using a Nikon AZ100 dissecting microscope and a Nikon E800 compound microscope. For scanning electron microscopy (SEM) examination, nematodes were dehydrated in a graded ethanol series, critical-point dried using CO_2 as a transitional fluid, mounted on stubs, coated with gold and examined using a Phenom Pro SEM microscope.

In total, 34 nematode specimens were studied, of which 32 were measured. The synlophe was studied following the method of Durette-Desset (1985). The nomenclature of the caudal bursa follows Durette-Desset & Chabaud (1981). Apical and transverse sections were prepared manually. All measurements in the text and the table are given in micrometres, unless otherwise indicated.

For the molecular data generation, a middle fragment of a male paratype was used. DNA was extracted using the KAPA Express Extraction Kit DKAPKK7103. *Cox*1 amplicons were obtained using the primer pair LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′). The thermocycling profile used was as follows: 2 min denaturation at 94°C, 35 cycles of 94°C for 30 s, 46°C for 30 s, 72°C for 60 s for amplification and 72°C for 10 min for extension. The ITS region (ITS1-5.8S-

ITS2) and the 28S gene were amplified using the forward primer ritf (5′-GCG GCT TAA TTT GAC TCA ACA CGG-3′) and the reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′). The thermocycling profile was as follows: 2 min denaturation at 94°C; 40 cycles of 30 s at 94°C, 30 s at 53°C, 2 min at 72°C; and a final 7 min extension at 72°C. Sequences were obtained using BigDye® Terminator v3.1 Cycle Sequencing on an ABI3500XL sequencer. DNA products were sequenced in both directions using the PCR primers; for the nuclear genes the following additional primers were used: internal primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), 300R (5′-CAA CTT TCC CTC ACG GTA CTT G-3′), ITS5 (5′- GGA AGT AAA AGT CGT AAC AAG G-3′) and ECD2 (5′-CTT GGT CCG TGT TTC AAG ACG GG-3′). Contiguous sequences were assembled, edited using Geneious 9.0 and submitted to GenBank (see below).

Family Amphibiophilidae Durette-Desset & Chabaud, 1981

Subfamily Amphibiphilinae Durette-Desset & Chabaud, 1981

Genus *Amphibiophilus* Skrjabin, 1916

***Amphibiophilus mooiensis* n. sp.**

Type-host: *Amietia delalandii* (Duméril & Bibron) (Amphibia: Anura: Pyxicephalidae).
Type-locality: River Mooi (26°41′03.4″S, 27°05′58.3″E), Potchefstroom, North-West Province, South Africa.
Type-material: Holotype (male, NMB P437), allotype (female, NMB P436), paratypes [NMB P438 (4 males, one used for molecular sequencing, and 12 females) and NMB P439 (4 males and 8 females)] deposited in the National Museum Parasite Collection (Bloemfontein, South Africa).
Site in host: Intestine.
Representative DNA sequences: GenBank MF460455 (*cox*1); MF460456 (ITS-28S).
ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Amphibiophilus mooiensis* n. sp. is urn:lsid:zoobank.org:pub:6E1BAA54-A7DD-4094-B219-DCD24FFEE1A3.
Etymology: The species is named after the River Mooi, the type-locality of the species; (mooi = Afrikaans for beautiful).

Description (Figs. 1, 2)

General. Body thin, elongated, rounded at anterior extremity, with maximum width near mid-length. Apical structures (Fig. 1B, Fig. 2C, D): oral opening rounded, 4 conspicuous cephalic papillae, 6 comparatively large inner labial papillae, 6 minute outer labial papillae, 2 pore-like amphids. Buccal capsule present, with sclerotised walls and triangular dorsal oesophageal tooth. Body cuticle thin, without conspicuous transverse striations, forming cephalic vesicle at anterior extremity, longitudinal ridges and cervical alae. Cervical alae beginning slightly posterior to cephalic vesicle and transforming into simple crests somewhat posterior to oesophago-intestinal junction. Maximum width of cervical alae at level of distal third of oesophagus. Struts present, clearly visible on transverse sections, reaching extremity of each ala. Oesophagus club-shaped, cylindrical in anterior half, widening posteriorly, with oval posterior bulb. Nerve-ring encircling oesophagus slightly posterior to level of its mid-length. Excretory glands well developed, dissimilar in size, opening with excretory pore at level of oesophago-intestinal junction. Small deirids situated slightly posterior to oesophago-intestinal junction, sometimes asymmetrically (Fig. 1A). Synlophe symmetrical, beginning posterior to cephalic vesicle in both sexes, ending at level of caudal bursa in males and slightly posterior to anus in females. Cervical alae narrow, triangular with rounded top, formed with one increased crest on each side (Figs. 1C, 2E). Number of crests varying from 36 to 44 (including cervical alae) at level of oesophagus in both sexes. About 64 equal crests at mid-body (Fig. 1D).

Male [Measurements are given as ranges for 8 paratypes, followed by the mean values in parentheses, and for the holotype in square brackets]. Body 5.1–7.3 (6.0) [7.2] mm long, 66–111 (83) [115] wide near mid-length (Fig. 2A). Cervical alae (measured in the holotype and one paratype) 7 [6] wide, appearing at 83 [100] and transforming into simple crest at 659 [662] from anterior extremity. Cephalic vesicle

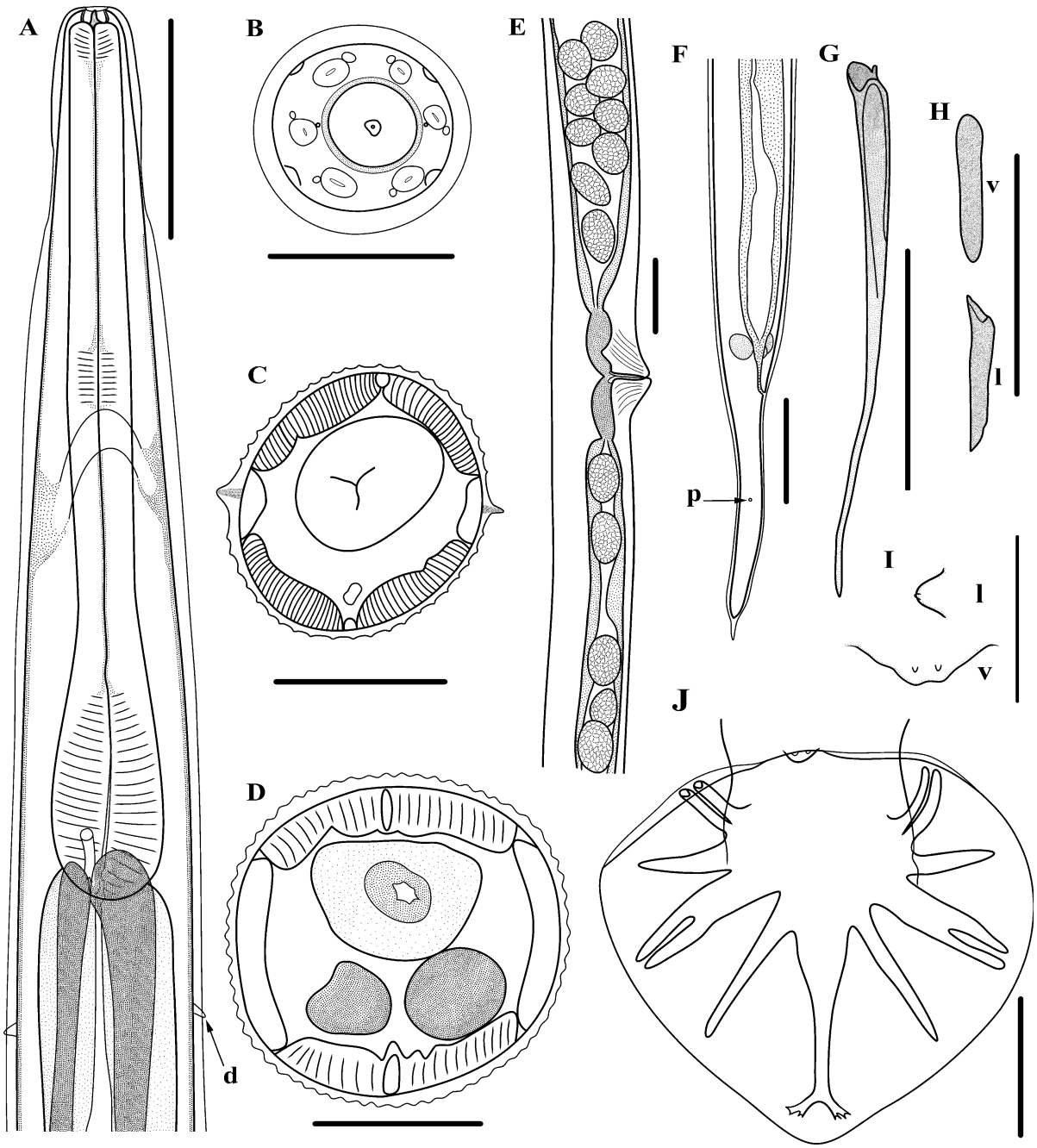

Fig. 1 *Amphibiophilus mooiensis* n. sp. ex *Amietia delalandii*. Paratypes. A, Male, anterior extremity of body, ventral view (d, deirid); B, Female, anterior extremity of body, apical view; C, Male, transverse section at level of oesophageal bulb; D, Female, transverse section at mid-body; E, Female, region of vulva, lateral view; F, Female, posterior extremity, lateral view (p, phasmid); G, Right spicule, lateral view; H, Gubernaculum (v, ventral view; l, lateral view); I, Genital cone (l, lateral view; v, ventral view); J, Caudal bursa, ventral view. *Scale-bars*: A, E–H, J, 100 μm; B, 25 μm; C, D, I, 50 μm

smooth, 67–78 (70) [67] long, 32–38 (34) [41] wide. Buccal capsule 6–8 (7) [7] long, 12–19 (13) [13] wide. Tooth 5–8 (7) [6] long, 3–9 (4) [4] wide at base. Oesophagus 328–446 (371) [402] long; 5.4–6.8 (6.2)% [5.6%] of body length. Oesophagus 17–23 (20) [23], 18–33 (23) [28] and 37–60 (46) [62] wide in

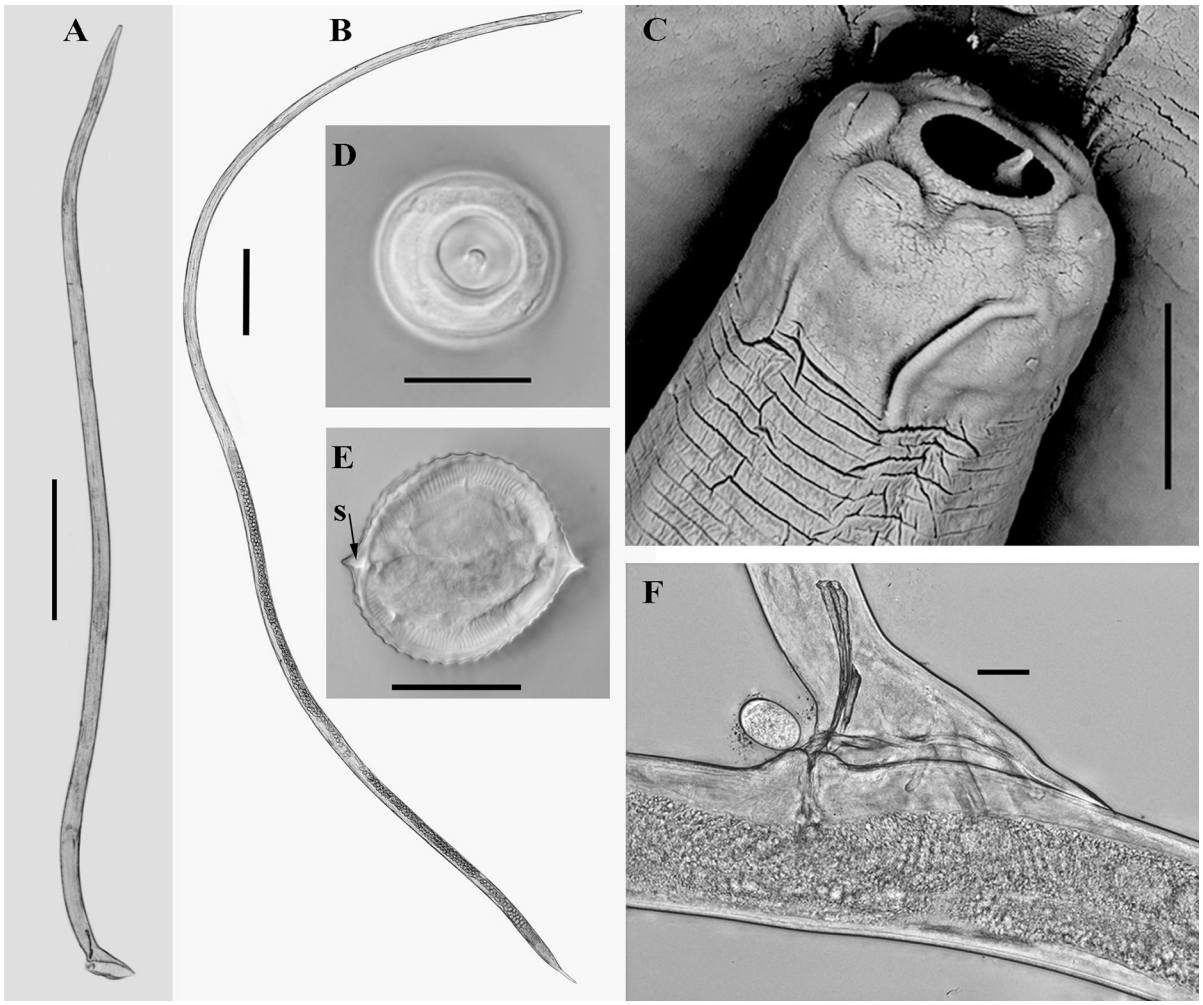

Fig. 2 *Amphibiophilus mooiensis* n. sp. ex *Amietia delalandii*. A, holotype; B, allotype, C–E, paratypes. A, Male, general view; B, Female, general view; C, SEM image of anterior extremity, female, apical view; D, Anterior extremity, female, optical section at a mid-level of buccal capsule; E, Transverse section at oesophago-intestinal junction, male (s, strut); F, Fragment of male and female *in copula*, lateral view. *Scale-bars*: A, B, 1 mm; C, 10 μm; D, 25 μm; E, 50 μm; F, 100 μm

anterior part, at mid-length and level of bulb respectively. Nerve-ring at 159–257 (207) [195] from anterior extremity; 46.0–65.9 (55.7)% [48.5%] of oesophagus length. Excretory pore at 323–390 (353) [387] from anterior extremity; 5.0–6.6 (5.9)% [5.4%] of body length. Deirids small, at 366–463 [355] (419) from anterior extremity, 6.3–8.0 (7.0)% [6.3%] of body length.

Testis beginning close to end of longer excretory gland, extending to posterior extremity, filled with large sperm. Caudal bursa symmetrical (Fig. 1I), heart-shaped, longer than wide. Rays 2 and 3 parallel with common origin, directed anteriorly, almost reaching bursal margin, slightly separated from each other in distal third. Ray 4 originating on base of rays 5 and 6, directed laterally. Rays 5 and 6 parallel with common origin, directed posterolaterally, separated from each other from mid-length onwards. Ray 8 with independent origin, directed exterodorsally. Dorsal ray of bursa bifurcated into 2 rays 10 posterior to base of rays 9. Each ray 9 and 10 slightly bifurcated at tip. Genital cone (measured in the holotype and one paratype) 5 [11] long and 24 [18] wide, with 2 small papillae. Spicules equal (Fig. 1G), 189–248 (209)

[219] long, with tapering, undivided distal extremity. Gubernaculum (measured in the holotype and one paratype) 69 [79] long, evenly rounded rectangular in ventral view and tapering to bevelled tip in lateral view (Fig. 1H).

Female [Measurements are given as ranges for 20 paratypes, followed by the mean values in parentheses, and for the allotype in square brackets]. Body 6.6–17.2 (14.2) [17.0] mm long, 71–188 (111) [185] wide near mid-length (Fig. 2B). Cervical alae (measured in the allotype and one paratype) 9 [8] wide, appearing at 106 [117] and transforming into simple crests at 1,072 [905] from anterior extremity. Cephalic vesicle smooth, 74–102 (84) [92] long, 31–43 (39) [42] wide. Buccal capsule 7–11 (9) [8] long, 7–19 (17) [16] wide. Tooth 4–9 (7) [8] long, 5–8 (6) [7] wide at base. Oesophagus 375–518 (422) [460] long; 2.6–6.2 (3.9)% [2.7%] of body length. Oesophagus 20–28 (25) [28], 23–37 (30) [32] and 39–81 (55) [65] wide in anterior part, at mid-length and level of bulb respectively. Nerve-ring at 181–251 (230) [217] from anterior extremity, 43.4–66.7 (54.8)% [47.2%] of oesophagus length. Excretory pore at 335–481 (418) [484] from anterior extremity, 2.7–5.2 (3.8)% [2.8%] of body length. Deirids small, at 382–635 (503) [564] from anterior extremity of body, 3.2–6.3 (4.6)% [3.3%] of body length. Tail tapering, 198–450 (300) [308] long; tip bearing thin cuticular spike (Fig. 1F). Phasmids situated laterally, at mid-level of tail.

Vulva (measured in the allotype and one paratype) a transverse slit, supported with well-developed muscles (Fig. 1E), 88 [84] deep and 75 [90] wide, situated at 5.0–13.0 (8.7) [12.5] mm form anterior extremity, 72.6–84.3 (76.5)% [72.4%] of body length. Anterior uterus with 127 [211] eggs, posterior uterus with 121 [176] eggs. All eggs observed in uteri, ovejector and vulva at morula stage, 52–72 × 32–47 (n = 31). Division between uterus and infundibulum, infundibulum and sphincter, sphincter and ovejector poorly visible.

Remarks

The new species belongs to the genus *Amphibiophilus* based on the presence of two circles of labial papillae, a cephalic vesicle, a buccal capsule with a well-developed oesophageal tooth, a symmetrical synlophe, simple spicules, a gubernaculum, and the 2-3 type arrangement of its caudal bursa (Skrjabin, 1916; Durette-Desset et al., 1994).

Amphibiophilus mooiensis n. sp. can be easily distinguished from all other species of the genus by a number of morphological characters. It differs from *A. acanthocirratus*, *A. natalensis*, *A. versterae* and *A. chabaudi* by the shape of the spicules (undivided tips in *A. mooiensis* n. sp. *vs* bifurcated tips in all four species) (see Skrjabin, 1916; Puylaert, 1967; Baker, 1981) and the shape and size of the gubernaculum. *Amphibiophilus mooiensis* n. sp. has an evenly rounded rectangular gubernaculum, 69–79 µm long, whereas *A. natalensis* and *A. versterae* have an elongated gubernaculum bearing a dorsal cap on the proximal extremity, 84–95 and 100 µm long, respectively (see Baker, 1981); the gubernaculum of *A. acanthocirratus* is 90 µm long and spindle-shaped (see Skrjabin, 1916); the gubernaculum of *A. chabaudi* is 95 µm long with a widened distal part (see Puylaert, 1967) in contrast to a shorter, evenly rounded gubernaculum in *A. mooiensis* n. sp. In synlophe structure, *A. mooiensis* n. sp. differs from *A. natalensis* and *A. versterae* in having narrower cervical alae (8-9 µm *vs c.*20 µm in both species) (see Baker, 1981). From *A. acanthocirratus* and *A. chabaudi* the new species is distinguished by the shape of the caudal bursa. Rays 9 and 10 together forming a trident at the tip in *A. acanthocirratus* in contrast to the bifurcated tips of each ray 9 and 10 in *A. mooiensis* n. sp. (see Skrjabin, 1916). The dorsal ray of *A. mooiensis* n. sp. is shorter, not reaching the bursal margin, while in *A. chabaudi* the dorsal ray is relatively longer, reaching the bursal margin (see Puylaert, 1967). From the latter species, *A. mooiensis* n. sp. also differs in the longer tail (350 µm long in a 12.8 mm long female *vs* 250 µm in a 13.1 mm long female of *A. chabaudi*; see Puylaert, 1967).

Morphometric data of the main characters of all known species of *Amphibiophilus* have been summarised in Table 1.

Discussion

Representatives of the genus *Amphibiophilus* possess a number of archaic characters such as a buccal capsule with a well-developed tooth and the presence of six inner and six outer labial papillae as well as four cephalic papillae. It is believed that the genus belongs to

Table 1 Morphometric data for *Amphibiophilus* spp.

Species	*A. mooiensis* n. sp.	*A. acanthocirratus* Skrjabin, 1916	*A. chabaudi* Puylaert, 1967	*A. natalensis* (Walton, 1935)	*A. versterae* Baker, 1981
Host	*Amietia delalandii*	"Frog"	*Amietia delalandii*	*Amietia delalandii*; *Strongylopus fasciatus*	*Amietia delalandii*
Locality	North-West Province, South Africa	Africa	Kenya	KwaZulu Natal, South Africa	KwaZulu Natal, South Africa
Source	Present study	Skrjabin (1916)	Puylaert (1967)	Baker (1981)	Baker (1981)
Male	(n = 9)	–	(n = 1)	(n = 5)	(n = 4)
Body length (mm)	5.1–7.3	7.1–7.4	7.2	6.0–9.4	4.6–5.2
Body width at midlength	66–111	100	120	105	120
Cephalic vesicle length	67–78	–	75	80	73
Cephalic vesicle width	32–38	–	–	40	38
Oesophagus length	328–446	425–450	400	362–469	362–394
Distance from anterior end of oesophagus to nerve-ring	159–257	–	220	213–287	181–222
Distance from anterior end of oesophagus to excretory pore	323–390	–	360	337–459	351–381
Spicule length	189–248	221	210	244–306	206–234
Gubernaculum length	69–79	90	95	84–95	97–115
Female	(n = 21)	–	–	(n = 4)	(n = 4)
Body length (mm)	6.6–17.2	11.7	12.0–18.7	16.9–21.0	8.0–9.2
Body width at midlength	71–188	136	200	150	185
Cephalic vesicle length	74–102	–	80	90	95
Cephalic vesicle width	31–43	–	–	47	55
Oesophagus length	375–518	510	445	480–512	362–500
Distance from anterior end of oesophagus to nerve-ring	181–251	–	270	153–269	216–259
Distance from anterior end of oesophagus to excretory pore	335–481	–	415	419–467	400–481
Distance from anterior end of body to vulva (mm)	5.0–13.0	2.9	9.2–15.2	12.3–15.6	5.9–7.1
Tail length	198–450	220	250	329–468	156–222

a relict group of amphibian parasites and represents the early evolution of bursate nematodes (Baker, 1981; Durette-Desset et al., 1994). Unfortunately, the lack of molecular data did not allow us to study the phylogeny of *Amphibiophilus*. However, using the BLAST searching method, we observed that, based on nematode sequences available in the GenBank database, the species most closely related to *Amphibiophilus* belong to the genera *Patricialina* Inglis, 1968, *Mackerrastrongylus* Mawson, 1960 and *Litoditis* Sudhaus, 2011, which are represented by primitive parasitic and even free-living marine nematodes (Chilton et al., 2006; 2015). In our opinion, this may be considered additional evidence of the primitive character of the family Amphibiophilidae, though it unquestionably should be confirmed through molecular, morphological and

ecological investigations of a wider range of parasite species.

Skrjabin (1916) mentioned that all specimens of *A. acanthocirratus* studied by him were fixed *in copula*, resembling representatives of the genus *Syngamus* Siebold, 1836. However, specimens *in copula* have not been recorded for any other *Amphibiophilus* species. In our study of 34 specimens, we collected two males and two females *in copula* (Fig. 2F). Therefore, we suppose that the duration of copulation in *Amphibiophilus* is rather shorter than in *Syngamus*, though it might be longer than in other bursate nematodes.

All species of *Amphibiophilus* have been described from frogs in southern and central Africa. *Amphibiophilus natalensis*, *A. versterae* and *A. chabaudi* were identified in material from *Amietia angolensis* (syn. *R. angolensis*) (see Baker, 1981; Halajian et al., 2013; Puylaert, 1967). However, this frog is restricted to its type-territory in Angola and, unlike the widespread *Amietia delalandii,* does not occur in the areas where the nematodes were found (Channing et al., 2016). Thus we suppose that the mentioned species of *Amphibiophilus* in fact were identified from the common river frog *Amietia delalandii*. *Amphibiophilus mooiensis* n. sp. appears to be the fourth species described from the same host. All four species, i.e. *A. natalensis*, *A. chabaudi*, *A. versterae* and *A. mooiensis*, are described from distant localities and distinguished mostly by the shape of the spicules and gubernaculum, which are significant interspecific characters. Therefore we suppose that geographical distribution might be strictly determined for *Amphibiophilus* spp. The same appears to be true for host specificity. No specimens of *Amphibiophilus* were found in material from frogs of the families Brevicipidae, Ptychadenidae, Pipidae and from the toads *Sclerophrys gutturalis* (Power) and *S. garmani* (Meek) (Halajian et al., 2013; Kruger et al., 2017; our unpublished data). Therefore, nematodes of the genus *Amphibiophilus* and frogs of the family Pyxicephalidae may represent examples of long-term co-evolutionary history, the study of which is necessary for our understanding of the evolution of parasitic nematodes and their host-parasite relationships.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed. North-West University ethics approval no NWU-00380-16-A5 and frogs were collected under North-West Province READ Permit no HQ 24t10/16-267 NW.

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