Institute of Parasitology, Biology Centre CAS
Folia Parasitologica 2017, 64: 035
doi: 10.14411/fp.2017.035

Folia Parasitologica
http://folia.paru.cas.cz

Research Article OPEN ACCESS

Description and phylogenetic position of a new species of *Rhabdias* Stiles et Hassall, 1905 (Nematoda: Rhabdiasidae) from the banded rubber frog, *Phrynomantis bifasciatus* (Smith) (Amphibia: Microhylidae), in South Africa

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Abstract: The lung-dwelling nematode *Rhabdias engelbrechti* sp. n. was found in five of eight examined banded rubber frogs in Limpopo Province, South Africa. The species is differentiated from species of *Rhabdias* Stiles et Hassall, 1905 occurring in the Afrotropical Realm based on the presence of a globular cuticular inflation at the anterior end, the buccal capsule walls being distinctly divided into anterior and posterior parts, the buccal capsule size (6–9 µm × 16–18 µm), and the body length (3.8–6.1 mm). *Rhabdias engelbrechti* is the tenth species of the genus found in Afrotropical anurans. Our molecular phylogenetic analysis based on the complete sequences of the ITS region and partial sequences of large subunit (28S) gene of the nuclear ribosomal RNA demonstrates that the new species is more closely related to the Eurasian species *Rhabdias bufonis* (Schrank, 1788) than to two other species from sub-Saharan Africa represented in the tree. In addition, partial sequences of the mitochondrial protein coding *cox1* and ribosomal 12S genes of the new species have shown significant differences from all previously published sequences of these genes from African species of *Rhabdias.*

Keywords: *Rhabdias engelbrechti* sp. n., amphibians, Limpopo Province, morphology, molecular phylogeny, rDNA, *cox1*

Rhabdias Stiles et Hassall, 1905 is the largest genus of the Rhabdiasidae Railliet, 1915 comprising about 80 nominal species (Kuzmin and Tkach 2017) of lung-dwelling parasites of amphibians and reptiles. Species of the genus are known from all zoogeographical realms, except Antarctica. Currently, 23 species of the genus have been reported from the Afrotropical realm, ten of them as parasites in amphibians. Of these, *Rhabdias bdellophis* Baylis, 1929 is the only parasite of caecilians (Gymnophiona) (Baylis 1929), while the remaining nine species parasitise hosts of the order Anura.

Three species, *R. blommersiae* Kuzmin, Junker, du Preez et Bain, 2013, *R. madagascariensis* Chabaud, Brygoo et Petter, 1961 and *R. vencesi* Junker, Lhermitte-Vallarino et Bain, 2010, are distributed on Madagascar. The six remaining species, *R. africanus* Kuzmin, 2001, *R. collaris* Baker, 1987, *R. ohlerae* Junker, Lhermitte-Vallarino et Bain, 2010, *R. picardiae* Junker, Lhermitte-Vallarino et Bain, 2010, *R. sylvestris* (Baker, 1982) and *R. tanyai* Junker, Lhermitte-Vallarino et Bain, 2010 occur on mainland sub-Saharan Africa, parasitising hosts of the families Arthroleptidae, Bufonidae and Microhylidae (Chabaud et al. 1961, Baker 1982, 1987, Kuzmin 2001, Junker et al. 2010, Kuzmin et al. 2013, Tkach et al. 2014a).

In the present paper we describe a new species of *Rhabdias* collected from the banded rubber frog, *Phrynomantis bifasciatus* (Smith) (Microhylidae), in Limpopo Province, South Africa. Morphological and molecular differentiation (based on *cytochrome oxidase 1* (*cox1*) and 12S mitochondrial genes) of the new species from previously described African species of *Rhabdias* parasitic in anurans is provided along with an analysis of its phylogenetic position based on sequences of the complete ITS region and partial 28S gene of nuclear ribosomal DNA.

MATERIALS AND METHODS

Eight banded rubber frogs were found dead in January 2013 on the farm De Loskop (23°30'S; 29°18'E) in the vicinity of Polokwane, Limpopo Province, South Africa (permit number 001-CPM403-00012 from the Limpopo Department of Economic Development, Environment & Tourism). Collected frogs were transferred on ice to the Laboratory of Parasitology, University of

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Zoobank number for article: urn:lsid:zoobank.org:pub:2D3F9905-F70E-4B46-9479-3E1066A3F7DA

Limpopo and examined for parasites. The frogs had a weight of 4.7–7.9 g (average 6.4 g) and a snout to vent length of 4.0–4.7 cm (average 4.5 cm). All frogs had cestodes in their intestine, seven harboured nematodes in the large intestine and five of them also had rhabdiasid nematodes in their lungs. Prior to examination under a light microscope, the nematodes were cleared in lactophenol (mixture of equal parts of distilled water, phenol, glycerol and lactic acid). Cleared specimens were studied on temporary slides under a Zeiss Axio Imager M1 microscope equipped with differential interference contrast optics and digital imaging system. Apical sections were made manually and examined *en face*. Drawings were made from series of photomicrographs. Measurements were taken with the use of the digital imaging system. Measurements of 20 specimens from three frogs are given in Table 1. All measurements are in micrometres unless otherwise indicated.

Genomic DNA of one individual of the new species as well as a specimen of another species from sub-Saharan Africa, *R. africanus* (collected from *Sclerophrys gutturalis* [Power, 1927] and kindly provided by Odile Bain and Kerstin Junker) was extracted according to Tkach and Pawlowski (1999). Fragments of the nuclear DNA region spanning the 3' end of the 18S nuclear rRNA gene, ITS region (ITS1+5.8S+ITS2) and 5' end of the 28S gene (including variable domains D1–D3) were amplified by PCR on an Eppendorf Master Gradient thermal cycler (Hauppauge, NY, USA), using forward primer ritf (5'-GCG GCT TAA TTT GAC TCA ACA CGG-3'), and reverse primer 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3'). PCR reactions were performed using New England Biolabs® (Ipswich, MA, USA) OneTaq® Quick-Load® Master Mix according to manufacturer's instructions. Annealing temperature in these PCR reactions was 53 °C. PCR product was purified using Qiagen Qiaquick™ (Valencia, CA, USA) columns and sequenced directly on an ABI Prism 3100™ (Carlsbad, CA, USA) automated capillary sequencer using ABI BigDye™ chemistry according to manufacturer's protocols. DNA product was sequenced in both directions using the two PCR primers and, additionally, internal primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), 300R (5'-CAA CTT TCC CTC ACG GTA CTT G-3'), 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') and ECD2 (5'-CTT GGT CCG TGT TTC AAG ACG GG-3'). Considering that most of the Afrotropical species of *Rhabdias* are represented in GenBank by partial sequences of mitochondrial *cox1* and ribosomal 12S genes (Junker et al. 2010), we have sequenced these genes from both the new species and *R. africanus*. *Cox1* amplicons were obtained using primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') published by Casiraghi et al. (2004). Partial 12S gene amplicons were obtained using primers 12SF (5'-GTTCCAGAATAATCGGCT A-3') and 12SR (5'-ATTGACGGATG(AG)TTTGTACC-3') published by Casiraghi et al. (2004). An annealing temperature of 45 °C was used in both cases. PCR primers were used as sequencing primers for both mitochondrial genes.

Contiguous sequences were assembled and edited using Sequencher™ ver. 4.1.1 (GeneCodes Corp., Ann Arbor, MI, USA) and deposited in GenBank under accession numbers MG428406, MG428408, MG428410 (*R. engelbrechti* sp. n.) and MG428407, MG428409, MG428411 (*R. africanus*). The phylogenetic analysis included newly obtained nuclear ribosomal sequences of *R. engelbrechti* and *R. africanus* and previously published (Tkach et al. 2006, 2014a,b) sequences of 16 additional species of *Rhabdias* from different continents and host groups. *Pneumonema tiliquae* Johnston, 1916 was chosen as outgroup based on the results of the phylogenetic analysis of the Rhabdiasidae by Tkach et al. (2014b). Names of the taxa and GenBank numbers are presented in Tables 2 and 3.

The new sequences and sequences obtained from GenBank were initially aligned using MEGA6 software (Tamura et al. 2013). The alignments were then manually refined in MEGA6 and saved in FASTA format. NEXUS file for Bayesian analysis was prepared in text editor. The phylogenetic analysis was carried out using Bayesian inference as implemented in the MrBayes program, version 2.01 (Huelsenbeck et al. 2001) with the following nucleotide substitution parameters: lset nst=6, rates=invgamma, ncat=4, shape=estimate, inferrates=yes and basefreq=empirical, that correspond to a general time reversible (GTR) model including estimates of the proportion of invariant sites (I) and gamma (G) distributed among-site rate variation.

The substitution model was selected using Jmodeltest ver. 0.1.1 (Posada 2008) software. Posterior probabilities were approximated over 3,000,000 generations, log-likelihood scores plotted and only the final 50% of trees were used to produce the consensus trees by setting the 'burnin' parameter at 750,000 generations. The resulting phylogenetic trees were visualised using FigTree ver. 1.4 software (Rambaut 2012).

Newly obtained mitochondrial sequences from the new species *R. engelbrechti* and *R. africanus* were compared with compatible sequences of species of *Rhabdias* published by Junker et al. (2010). In the publication by Junker at al. (2010) the GenBank accession numbers for sequences of *R. tanyai* were provided incorrectly and duplicated those of *R. vencesi*. In the present work we use corrected GenBank numbers for sequences of *R. tanyai*. Pairwise nucleotide comparison data and genetic identity matrices for *cox1* and 12S sequences were generated using MEGA6 software.

RESULTS

Rhabdias engelbrechti sp. n. Figs. 1, 2

ZooBank number for species:
urn:lsid:zoobank.org:act:9636FB19-DFC7-4872-88C4-EF910925AC78

Description (based on the holotype and 4 paratypes, all gravid hermaphrodites; measurements of the holotype are followed by the ranges for the type series in parentheses. Measurements of all studied specimens are given in Table 1). Body length 5.06 mm (4.70–5.49 mm). Body 362 (306–362) wide at mid-body, gradually tapering anteriorly and posteriorly (Fig. 2A). Body width 156 (127–156) at junction of oesophagus and intestine, 145 (119–145) at anus. Anterior end rounded, posterior end tapered. Body cuticle more prominently inflated in anterior and posterior thirds of body, thin at mid-region. Spherical cuticular inflation (cephalic vesicle) present on anterior extremity, more or less distinctly separated from cuticle of remainder of body (Figs. 1A,C, 2B). Slight dilatation of body wall inside cephalic vesicle present at level of anterior end of

Table 1. Measurements of *Rhabdias engelbrechti* sp. n. from *Phrynomantis bifasciatus* (Smith), all measurements are in micrometres unless otherwise indicated.

Characters	Mean	Min.	Max.	SD	CV
Body length (mm)	4.9	3.8	6.1	0.5	10.1
Body width at oesophago-intestinal junction	146	111	158	11.6	8.0
Body width at vulva	316	218	362	28.8	9.1
Body width at anus	119	73	145	14.6	12.3
Buccal capsule width	17	16	18	0.8	4.6
Length of buccal capsule anterior part	5.3	4	7	0.7	12.5
Length of buccal capsule posterior part	2.5	2	3	0.5	20.5
Total length of buccal capsule	7.8	6	9	0.9	11.1
Vestibulum length	4.2	3	5	0.7	17.0
Oesophagus length	361	293	393	25.8	7.1
Oesophagus width at anterior end	34	32	37	1.4	4.2
Oesophagus width at muscular part	38	33	42	2.8	7.2
Oesophagus width at nerve ring	35	27	40	2.7	7.6
Oesophagus, bulb width	61	53	64	2.5	4.2
Distance from anterior end to nerve ring	158	138	171	9.2	5.8
Distance from anterior end to vulva (mm)	2.6	2.1	3.1	0.28	10.7
Tail length	253	166	317	34.5	13.6
Ratios					
Oesophagus length/total body length (%)	7.4	6.0	8.3	0.6	7.9
Distance to vulva/total body length (%)	53.2	48.0	58.4	2.4	4.5
Tail length/total body length (%)	5.2	3.7	6.2	0.6	12.1
Distance from anterior end to nerve ring, in % of oesophagus length	43.8	39.9	53.2	3.7	8.4
Buccal capsule length/width	0.5	0.4	0.5	0.05	10.5

n = 20; SD – standard deviation; CV – coefficient of variation.

oesophagus (Fig. 1A,B). Lateral cuticular pores extending along body.

Oral opening rounded (Fig. 1B). Internal labial papillae minute. Submedian papillae located close to edge of oral opening, lateral papillae at some distance from oral opening. Lips small, in shape of low apical elevations around and posterior to papillae (Fig. 1B). Amphids open as pores just posterior to lateral lips (Fig. 1B).

Vestibulum narrowing at its mid-length (Fig. 1C), 5 (4–5) long, circular in apical view (Fig. 1D). Buccal capsule 17 (17–18) wide, 8 (8–9) long, consisting of thick-walled barrel-shaped anterior part 5 (5–6) long and ring-shaped posterior part 3 long (Fig. 1C). Buccal capsule total length to width ratio 0.47 (0.44–0.50). Both parts circular in apical view, with smooth interior surface of walls (Fig. 1E,F). Apex of oesophagus surrounding posterior part of buccal capsule (Fig. 1C,F).

Oesophagus gradually widening from anterior end posteriorly, from 35 (25–36) at anterior end to 37 (37–40), then forming short dilatation 41 (41–42) wide just anterior to nerve ring (Fig. 1A). Nerve ring located at 159 (159–163) from anterior end of body, surrounding constriction of oesophagus 37 (37–40) wide. Posterior to nerve ring, oesophagus gradually widening, bulbous at its posterior end, 63 (61–64) in diameter. Oesophagus length 393 (377–393), or 7.8% (6.9–8.0%) of body length.

Excretory glands and excretory pore not observed in studied specimens. Intestine thick-walled, narrow at oesophago-intestinal junction, then sharply widening posteriorly (Figs. 1A, 2B). In some specimens, anterior end of intestine wide, enveloping posterior end of oesophageal bulb. Rectum narrow, its walls slightly thicker at proximal end (Fig. 1G).

Genital system amphidelphic, typical of the genus. Vulva with slightly salient lips located at 2.43 mm (2.43–2.96 mm) from anterior end; distance from anterior end to vulva occupying 48% (48–54%) of body length. Vagina short, lined with thin cuticle (Fig. 2C). Uteri joined, similar, thin-walled, filled with numerous eggs; most eggs containing fully developed embryos (Fig. 2A). Egg size 43–59 × 95–108 (n = 13). Both limbs of genital system U-bent at level of seminal receptacles, at 1,128 (908–1,270) from anterior end and at 698 (519–719) from posterior end. Proximal parts of both syngonia significantly overlapping level of vulva. Short testis zone observed closer to level of vulva in posterior syngonium and posterior to level of vulva in anterior one.

Tail gradually tapering from anus posteriorly, more abruptly tapering in posterior third (Fig. 1G). Tail end free from cuticular inflation, narrow, digitiform, with rounded tip. Tail length 297 (256–317), or 5.7% (5.1–5.9%) of body length. Phasmids poorly visible, located at 97 (67–97) from tail tip.

Type host: Banded rubber frog, *Phrynomantis bifasciatus* (Smith) (Amphibia: Anura: Microhylidae).

Site in host: Lungs.

Type locality: De Loskop, Limpopo Province, South Africa (23°30'S; 29°18'E).

Prevalence and intensity of infection: 5 out of 8 (63%) hosts examined with the intensity of 9.6 (2–18).

Type material: 5 specimens (holotype and 4 paratypes). Type specimens are stored in the helminthological collection of the Institute of Parasitology, Biology Centre, CAS, České Budějovice, Czech Republic; IPCAS N-1107.

Etymology: The species is named after Derek Engelbrecht (University of Limpopo) as a tribute to his help in specimen collection.

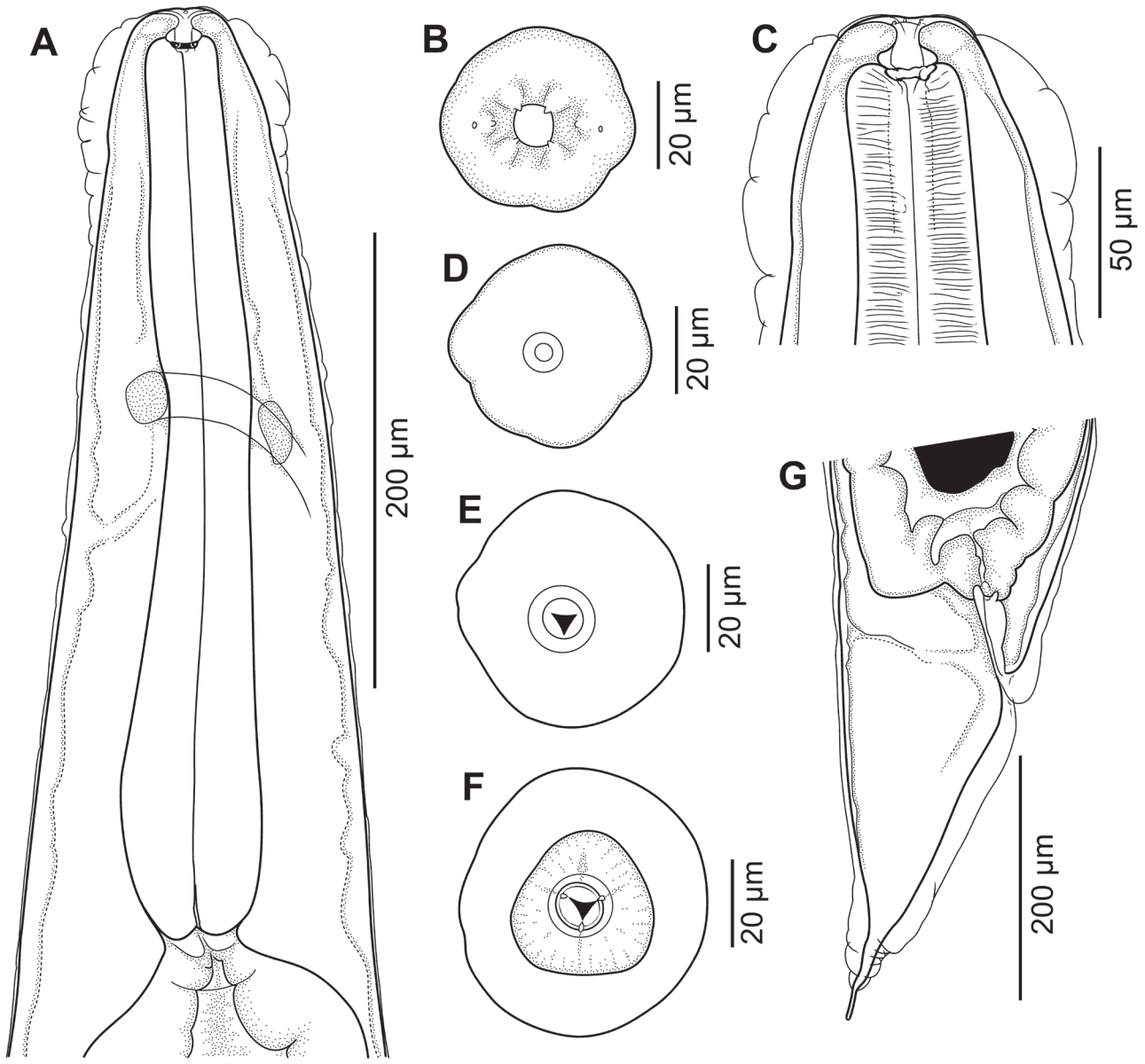

Fig. 1. *Rhabdias engelbrechti* sp. n. from *Phrynomantis bifasciatus* (Smith). **A** – anterior part of body, lateral view; **B** – anterior end, *en face* view; **C** – anterior end, lateral view; **D** – optical transverse section at level of vestibulum; **E** – optical transverse section at level of anterior part of buccal capsule; **F** – optical transverse section at level of posterior part of buccal capsule and apex of oesophagus; **G** – posterior part of body, lateral view. A, C, G – holotype; B, D–F – specimen from additional material.

Remarks. The new species is assigned to the genus *Rhabdias* based on its cuticle being inflated, more so at the anterior and posterior extremities, the presence of a small buccal capsule, the characteristic arrangement of its circumoral structures and parasitism in the lungs of amphibians.

Ten species of the genus were recorded as parasites of amphibians in the Afrotropical Realm (Junker et al. 2010; Tkach et al. 2014a): *R. africanus*, *R. bdellophis*, *R. blommersiae*, *R. collaris*, *R. madagascariensis*, *R. ohlerae*, *R. picardiae*, *R. sylvestris*, *R. tanyai* and *R. vencesi*.

Rhabdias engelbrechti sp. n. is close to *R. africanus*, *R. blommersiae*, *R. madagascariensis*, *R. picardiae* and *R. vencesi* in having a rounded anterior extremity, lacking distinct constrictions (in contrast to *R. tanyai*; see Junker et al. 2010) or dilatations (in contrast to *R. collaris* and *R. ohlerae*; see Baker 1987, Junker et al. 2010), a comparatively small buccal capsule (in contrast to *R. sylvestris*; see Baker 1982, Tkach et al. 2014a) and in its specificity to an anuran host (in contrast to *R. bdellophis* parasitic in Apoda; see Baylis 1929).

Rhabdias engelbrechti differs from *R. africanus* parasitic in South African Bufonidae (Kuzmin 2001) in having a smaller body (3.8–6.1 mm *vs* 12.5–19.8 mm), a smaller buccal capsule (6–9 µm × 16–18 µm *vs* 15–20 µm × 20–23 µm), the mostly post-equatorial position of the vulva, the presence of cuticular inflation at the anterior extremity and the absence of lateral pseudolabia. Molecular differences between the two species were significant at 7.0% in the 12S region (Table 2) and 10.6% in the *cox1* region (Table 3). Additionally, the two species also differed in 47

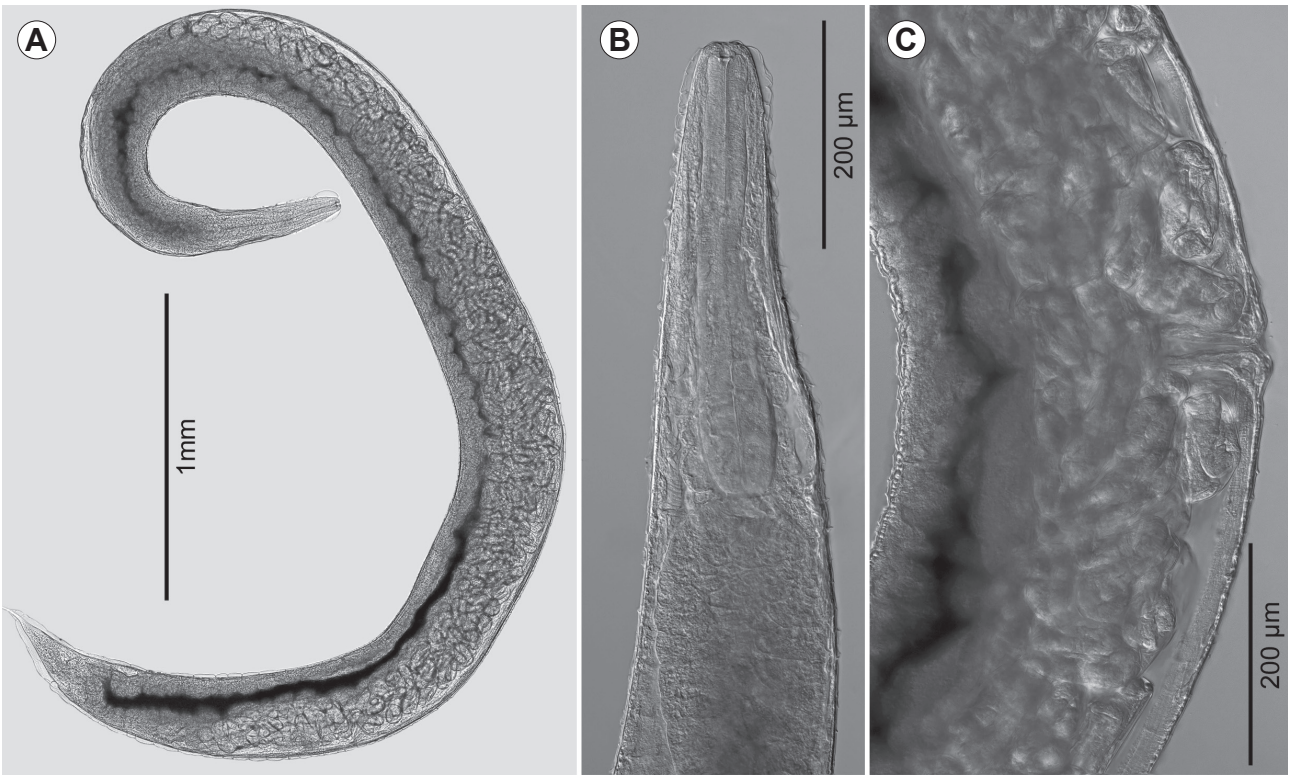

Fig. 2. Paratype of *Rhabdias engelbrechti* sp. n. from *Phrynomantis bifasciatus* (Smith). **A** – general view; **B** – anterior part of body, lateral view; **C** – region of vulva, lateral view.

out of 1,575 (3.0%) nucleotide positions in the ITS+28S region (mostly in the ITS), which is a substantial level of divergence at species level (Tkach et al. 2006).

Rhabdias engelbrechti differs from *R. blommersiae* parasitic in Malagasy Mantellidae in its somewhat larger body size, when compared to the body length of 3.2–3.6 mm in the latter species (Kuzmin et al. 2013). In *R. engelbrechti* the lateral lips are smaller than the submedian ones, whereas in *R. blommersiae* all lips are of approximately equal size. The buccal capsule in the new species is wider than that in *R. blommersiae* (11–12 µm wide), and the distinct posterior part was not observed at all in the buccal capsule of the latter species. The vulva in *R. blommersiae* is pre-equatorial, whereas in *R. engelbrechti* it is mostly post-equatorial. Excretory glands were not observed in gravid specimens of *R. engelbrechti*, whereas they are apparent in *R. blommersiae* (see Kuzmin et al. 2013).

Rhabdias engelbrechti differs from *R. madagascariensis* parasitic in Malagasy Ranidae (Chabaud et al. 1961) in the absence of distinct excretory glands in gravid specimens, the presence of a globular cuticular inflation at the anterior extremity and a rounded, not truncated, anterior end. Body width near vulva in *R. engelbrechti* is 218–362 µm, whereas in the type specimen of *R. madagascariensis* the body is merely 150 µm wide, though its body length (3.5 mm) is close to that in the smallest studied specimen of the new species (3.8 mm). A distinct rounded dilatation of the oesophagus just anterior to the nerve ring is absent in *R. engelbrechti* but present in *R. madagascariensis*, as seen on fig. 1C in Chabaud et al. (1961).

The new species is slightly smaller than *R. picardiae* from Bufonidae in South Africa, which is 8.0–8.4 mm long (Junker et al. 2010). *Rhabdias picardiae* lacks the distinct anterior spherical cuticular inflation typical of the new species. The buccal capsule in the new species is smaller than in *R. picardiae* (16–18 µm wide *vs* 23–25 µm wide). The oesophagus in *R. engelbrechti* is shorter than in *R. picardiae* not only in absolute dimensions (293–393 µm *vs* 690–790 µm), but also in relation to body length (8.6–9.9% *vs* 6.0–8.3%). The DNA sequence divergence between the two species was lower in the more conserved 12S gene (1.7% of differences; see Table 2), but much more substantial in the more variable *cox1* fragment (8.4%; see Table 3).

Rhabdias engelbrechti differs from *R. vencesi* parasitic in Mantellidae on Madagascar (Junker et al. 2010) in having a smaller body (3.8–6.1 mm *vs* 9.3–13.2 mm), narrower buccal capsule (16–18 µm *vs* 19–22 µm) and smaller eggs (95–108 µm × 43–59 µm *vs* 110–148 µm × 56–72 µm). Besides, *R. vencesi* lacks the anterior cuticular swelling and the division of the buccal capsule into a distinct anterior and posterior part, characteristic of the new species. The oral opening is laterally elongated in *R. vencesi* while it is rounded in the new species. Sequence divergence between the two species was also significant with a 7.5% base pair difference in the 12S gene (Table 2) and 9.9% in *cox1* (Table 3).

The new species differs from *R. tanyai* described from *Astylosternus rheophilus* Amiet (Arthroleptidae) in Cameroon (Junker et al. 2010) not only in the shape of anterior extremity, as mentioned above, but also in the smaller size

Table 2. Number (above diagonal) and percentage (below diagonal) of variable sites based on pairwise comparison within 360 bp long alignment of partial sequences of 12S gene of *Rhabdias engelbrechti* sp. n. from *Phrynomantis bifasciatus* (Smith) and other published sequences of species of *Rhabdias* from Africa.

Species	*R. engelbrechti* sp. n. MG428408*	*R. africanus* MG428409*	*R. tanyai* FN434108*	*R. vencesi* FN434098*	*R. picardiae* FN434093*
R. engelbrechti sp. n.	0	25	19	27	6
R. africanus	7.0	0	30	22	24
R. tanyai	5.3	8.4	0	29	19
R. vencesi	7.5	6.2	8.1	0	25
R. picardiae	1.7	6.7	5.3	7.0	0

* GenBank accession numbers.

Table 3. Number (above diagonal) and percentage (below diagonal) of variable sites based on pairwise comparison within 599 bp long alignment of partial sequences of *cox1* gene of *Rhabdias engelbrechti* sp. n. from *Phrynomantis bifasciatus* (Smith) and other published sequences of species of *Rhabdias* from Africa.

Species	*R. engelbrechti* sp. n. MG428410*	*R. africanus* MG428411*	*R. tanyai* FN434107*	*R. vencesi* FN434104*	*R. picardiae* FN434095*
R. engelbrechti sp. n.	0	63	66	59	50
R. africanus	10.6	0	74	72	72
R. tanyai	11.1	12.4	0	87	73
R. vencesi	9.9	12.1	14.6	0	56
R. picardiae	8.4	12.1	12.2	9.4	0

* GenBank accession numbers.

of the body (>13.9 mm in *R. tanyai*) and the buccal capsule (13 µm × 23 µm in *R. tanyai*). Sequence divergence between *R. engelbrechti* and *R. tanyai* was significant at 5.3% in the sequenced region of the 12S gene and 11.1% in *cox1* (Tables 2, 3).

Phylogenetic analysis. The alignment of the sequenced regions of the nuclear ribosomal DNA (see Materials and Methods) included newly obtained sequences of *R. engelbrechti* sp. n. and *R. africanus*, as well as previously published sequences of other species of *Rhabdias* and the sequence of *Pneumonema tiliquae* as an outgroup. The alignment was 1,588 bp long and required insertion of only a few gaps due to the overall high sequence similarity among ingroup taxa. Only 22 nucleotide positions had to be excluded from the analysis due to ambiguous homology. In the phylogenetic tree resulting from the Bayesian analysis, the new species formed a 100% supported clade with *R. bufonis* (Fig. 3). In turn, this clade clustered (100% support) with the group of three species of *Rhabdias* parasitic in various amphibians in Asia, namely *R. bermani* Rausch, Rausch et Atrashkevich, 1984, *R. bulbicauda* Sarkar et Manna, 2004 and *R. kongmongthaensis* Kuzmin, Tkach et Vaughan, 2005. Another South African species, *R. sylvestris*, appeared on the tree as the taxon basal to the rest of the ingroup.

DISCUSSION

Rhabdias engelbrechti sp. n. lacks the pronounced morphological characters found in some species of *Rhabdias* parasitising Afrotropical anurans, such as the modification of the body wall in the anterior part observed in *R. collaris*, *R. ohlerae* (dilatation in both) and *R. tanyai* (constriction), or the presence of onchia and an enlarged buccal capsule found in *R. sylvestris* (Baker 1982, 1987, Junker et al. 2010). The buccal capsule morphology in *R. engelbrechti* is similar to that observed in *R. picardiae*, *R. ohlerae* and *R. tanyai* from Afrotropical anurans (Junker et al. 2010). In the latter three species the buccal capsule consists of a longer anterior part and shorter posterior part.

Rhabdias engelbrechti, *R. picardiae* and *R. tanyai* (all possessing divided buccal capsules) seem to be more similar to each other in the relatively conservative, non-coding 12S gene sequences. Differences between the former three species were from 1.7% to 5.3%, whereas each of them differed from *R. africanus* and *R. vencesi* (both lacking buccal capsule division) by 7.0–8.4% (Table 2). Further molecular phylogenetic studies including broader taxonomic diversity and additional genes, may provide data allowing for a better understanding of the origins of certain morphological features in this group of nematodes, including the structure of the buccal capsule.

Comparison of pairwise sequence variability between five species of Afrotropical species of *Rhabdias* demonstrated that *cox1* and 12S genes show different levels of divergence among these nematode species (Tables 2, 3), which is fully expected considered the different levels of variation observed in these genes (Junker et al. 2010). However, the two genes have also indicated somewhat different levels of relatedness between compared taxa. For instance, sequences of 12S genes suggested that *R. tanyai* and *R. africanus* were the most genetically distant species at 8.4% pairwise sequence difference while in the *cox1* gene *R. tanyai* and *R. vencesi* showed the highest level of divergence at 14.6%. Comparison between other pairs of species (Tables 2, 3) shows additional inconsistencies in the results derived from the two genes. This raises a question regarding the phylogenetic signal and potential bias that may be introduced if these genes were used for broader phylogenetic analyses of the Rhabdiasidae. Currently

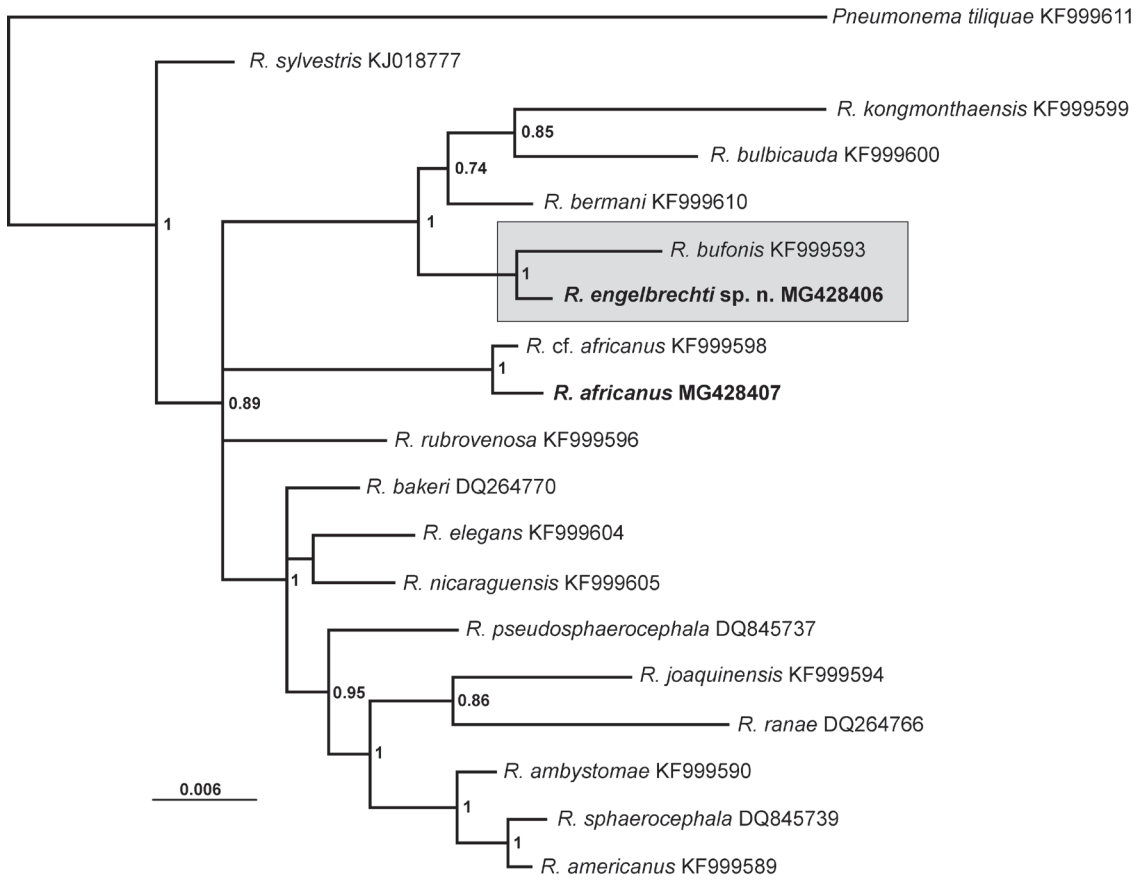

Fig. 3. Phylogenetic relationships among 17 taxa of *Rhabdias*, including *R. engelbrechti* sp. n. from *Phrynomantis bifasciatus* (Smith), resulting from Bayesian analysis (3,000,000 generations) based on sequences of the nuclear ribosomal DNA region sequences spanning the 3' end of 18S rRNA gene, ITS region (ITS1+5.8S+ITS2) and 5' end of the 28S rRNA gene. Posterior probabilities are shown at the internodes. New sequences in bold.

available sequence data do not allow for a more conclusive answer on this matter.

As expected, the phylogenetic tree obtained in our study is overall very similar to the trees recently published by Tkach et al. (2014a,b). Inclusion of the new and some additional species of *Rhabdias* in our tree compared to that published by Tkach et al. (2014a) outlined a strongly supported (100%) group of five taxa which incorporates a 100% supported sub-clade of *R. engelbrechti* and *R. bufonis*. A highly conspicuous feature of this group of 5 taxa is that each of them parasitises a different family of amphibians. Moreover, *R. bermani* is a parasite of a caudatan, the Siberian newt *Salamandrella keyserlingii* Dybowski. From a geographic viewpoint, the taxa in this otherwise small clade are distributed from the Eastern Palearctic and Southeast Asia to the nearly southernmost part of the Afrotropical realm. This suggests that this clade is not only evolutionary quite ancient, but also conducive to easy host switching. The close, well supported relationship between *R. engelbrechti* from South Africa and *R. bufonis* distributed in the Palaearctic is particularly remarkable and somewhat unexpected. It suggests the existence of other, yet undescribed or merely not sequenced for ribosomal genes, Afrotropical species of *Rhabdias* belonging to this clade. The huge geographic gap between the distribution areas of these two undoubtedly closely related species would be difficult to explain otherwise.

Of the four Afrotropical species of *Rhabdias* included in our phylogenetic analysis, only two, *R. africanus* and *R.* cf. *africanus*, turned out to be close relatives (Fig. 3). Based on molecular data, it is clear that the species previously provisionally identified as *R.* cf. *africanus*, in fact represents a new species awaiting formal description.

Denser geographic and taxonomic sampling of *Rhabdias* and inclusion of additional species in future phylogenetic analyses should allow to better resolve phylogenetic interrelationships not only among Afrotropical *Rhabdias*, but also their relationships with members of the genus from different continents.

Acknowledgements. This work is based on the research supported by the South African Research Chairs Initiative (SARChI) of the Department of Science and Technology and National Research Foundation of South Africa (Grant no. 101054). We are grateful to anonymous referees for the thorough revision and very useful suggestions that allowed to improve our manuscript.

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Received 17 September 2017 Accepted 7 November 2017 Published online 13 December 2017

Cite this article as: Kuzmin Y., Halajian A., Tavakol S., Luus-Powell W.J., Tkach V.V. 2017: Description and phylogenetic position of a new species of *Rhabdias* Stiles et Hassall, 1905 (Nematoda: Rhabdiasidae) from the banded rubber frog, *Phrynomantis bifasciatus* (Smith) (Amphibia: Microhylidae), in South Africa. Folia Parasitol. 64: 035.