Neofoleyellides boerewors n. gen. n. sp. (Nematoda: Onchocercidae) parasitising common toads and mosquito vectors: morphology, life history, experimental transmission and host-vector interaction in situ

Peer-reviewed author version

Netherlands, Edward C.; Svitin, Roman; Cook, Courtney A.; Smit, Nico J.; Brendonck, Luc; VANHOVE, Maarten & Du Preez, Louis H. (2020) Neofoleyellides boerewors n. gen. n. sp. (Nematoda: Onchocercidae) parasitising common toads and mosquito vectors: morphology, life history, experimental transmission and host-vector interaction in situ. In: INTERNATIONAL JOURNAL FOR PARASITOLOGY, 50 (3) , p. 177 -194.

DOI: 10.1016/j.ijpara.2019.11.009
Handle: http://hdl.handle.net/1942/31245

Neofoleyellides boereworsi n. gen. n. sp. (Nematoda: Onchocercidae) parasitising common toads and mosquito vectors: morphology, life history, experimental transmission and host-vector interaction *in situ*

Edward C. Netherlands[a,b]*, Roman Svitin[a,c,h], Courtney A. Cook[a], Nico J. Smit[a], Luc Brendonck[a,b], Maarten P.M. Vanhove[d,e,f,g], and Louis H. Du Preez[a,h]

[a] Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa

[b] Laboratory of Aquatic Ecology, Evolution and Conservation, University of Leuven, Charles Debériotstraat 32, B-3000 Leuven, Belgium

[c] Department of Invertebrate Fauna and Systematics, I.I. Schmalhausen Institute of Zoology NAS of Ukraine, 15 B. Khmelnytskogo str., 01030, Kyiv, Ukraine.

[d] Hasselt University, Centre for Environmental Sciences, Research Group Zoology: Biodiversity & Toxicology, Agoralaan Gebouw D, B-3590 Diepenbeek, Belgium

[e] Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, CZ-611 37 Brno, Czech Republic

[f] Zoology Unit, Finnish Museum of Natural History, P.O.Box 17, FI-00014 University of Helsinki, Helsinki, Finland

[g] Laboratory of Biodiversity and Evolutionary Genomics, University of Leuven, Charles Debériotstraat 32, B-3000 Leuven, Belgium

[h] South African Institute for Aquatic Biodiversity, Somerset Street, Grahamstown 6140, South Africa

*For correspondence. E-mail: ec.netherlands@gmail.com; Telephone number: (+27)18 299 2515; Fax: (+27)18 299 2503.

Abstract

Anuran filarial nematodes are restricted to two comparatively small subfamilies (Icosiellinae and Waltonellinae) of the filariae that currently comprise six genera and 41 recognised species. However, the life histories of only five anuran filarial nematodes, proposed as an ancestral group based on molecular phylogenetic studies, have been elucidated. Furthermore, data on the natural vectors (*in situ*) and parasite transmission is limited. In the current study we elucidate the life history of *Neofoleyellides boereworsi* n. gen. n. sp parasitising the guttural toad, *Sclerophrys gutturalis* and the mosquito vectors *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *Uranotaenia* (*Pfc.*) *montana*. Additionally, we report on the unique host-seeking behaviour of the mosquito vectors that locate their toad hosts using their calls. The complex host-vector relationship and specialised host-seeking behaviour by these mosquitoes indicate biases towards host species and male toad infections.

Keywords: anuran, amphibian, frog, parasite, life cycle, filarial nematode, microfilaria

1. Introduction

Filarial nematodes from the family Onchocercidae Leiper, 1911 (Spirurida), the major group of the suborder Filarioidea, are long thread-like, coelom or tissue dwelling round worms, known for causing medical and veterinary diseases (Bain, 2002). This group is reported infecting a broad range of hosts including amphibians, reptiles, birds and mammals (Lefoulon et al., 2015). These nematodes have a complex life cycle, involving a definitive vertebrate host and invertebrate vector. Within the vertebrate host, adult worms release specialised eggs or microfilaria that migrate to or in the host's lymph or bloodstream (Bain, 2002). Subsequently a haematophagous arthropod vector ingests the microfilaria initiating development that continues from the early first-stage larvae up to the

infective third-stage larvae, all in the same vector individual. Once the infective larvae have been deposited on or inoculated into a new definitive host, via the vector's blood meal, development continues in the vertebrate host to the fourth-stage juvenile and final adult stage (Bain, 2002; Bain et al., 2013).

Onchocercids of anurans either belong to the subfamilies Icosiellinae Anderson (1958) or Waltonellinae Bain and Prod'Hon (1974). Icosiellinae is monogeneric comprising nine species of *Icosiella* Seurat (1917). Species are recognised based on the presence of two pairs of cephalic submedian spines, a subterminal anus, and few caudal papillae on a relatively short tail. Waltonellinae was erected to accommodate anuran "*Foleyella*"-like filariae and currently comprises five genera, namely *Foleyellides* Caballero (1935), *Ochoterenella* Caballero (1944), *Madochotera* Bain and Brunhes (1968), *Paramadochotera* Esslinger (1986b) and *Paraochoterenella* Purnomo and Bangs (1999) (Table 1).

Members of this subfamily are characterised by the absence of submedian cephalic spines; the presence of a pair of lateral, parastomal projections; the anus not being in a sub-terminal position; and several pairs of papillae on a somewhat long tail (Souza et al., 2012; Bain et al., 2013).

Foleyellides currently includes 11 species of cosmopolitan onchocercids reported in ranid, bufonid and dicroglossid hosts from North and Central America, Asia, and Africa (Romero-Mayen and Leon-Regagnon, 2016) (see Table 1). According to García-Prieto et al. (2014), since the establishment of the genus its taxonomic status has been contentious. The type species, *F. striatus*, was described from the body cavity of the ranid frogs *Rana montezumae* Baird, 1854 and *R. sphenocephalus* (Cope, 1886) in Mexico. This species, initially designated as *Chandlerella striata* Ochoterena and Caballero, 1932, was subsequently transferred to *Foleyellides* by Caballero (1935). He distinguished it from representatives of *Foleyella* Seurat (1917) based on differences in cephalic structures and

presence of lateral alae. Later, Witenberg and Gerichter (1944) considered the differences proposed by Caballero (1935) irrelevant, proposing *Foleyellides* as a synonym of with *Foleyella*, which was supported by López-Neyra (1956), Yamaguti (1961), and Anderson and Bain (1976). Regardless, Sonin (1968), considered *Foleyellides* and *Foleyella* as separate genera despite their morphological similarities, thus resurrecting *Foleyellides*. Schacher and Crans (1973) also considered *Foleyellides* as valid. However, based on the morphological differences and host specificity, these authors also divided *Foleyella* into two subgenera, namely *Foleyella* and *Waltonia* Schacher and Crans (1973), parasitising reptiles and amphibians respectively. Nonetheless, the name *Waltonia* was considered *nomen preoccupatum* and replaced with the name *Waltonella* Schacher (1975). Bain and Prod'Hon (1974) elevated *Waltonella* to full genus status, with species of *Foleyellides* included in *Waltonella*. However, according to Esslinger (1986a), following the International Code of Zoological Nomenclature (Article 40, Section a), the genus name *Foleyellides* Caballero (1935) takes precedence over *Waltonella*. Thus, Esslinger (1986a) reinstated *Foleyellides* considering *Waltonella* as its junior synonym, and redescribed *F. striatus* re-establishing it as the type species of the genus by original designation. *Foleyellides* is characterised by the presence of cuticularized parastomal structures, lateral and caudal alae in both sexes, a distinct buccal capsule, as well as the lack of annular bands of longitudinally oriented bosses in the mid-body region (Esslinger, 1986a).

The most diverse genus within Waltonellinae is *Ochoterenella,* currently with 16 species reported parasitising bufonid, hylid and leptodactylid hosts from the Neotropical and Indomalayan realms (Bain et al., 2013). Caballero (1944) erected the genus and described the type species *Ochoterenella digiticauda* Caballero, 1944 from the body cavity of the Cane toad *Rhinella marina* (Linnaeus, 1758) in Mexico. According to Esslinger (1986b) this species has been reported in several studies from various anurans throughout the Neotropical region (Brenes and Hollis, 1959; Travassos and de Freitas, 1960;

105 Marinkelle, 1970; Masi Pallares and Maciel, 1974; Vicente and Santos, 1976; Dyer and
106 Altig, 1977; Vicente and Jardim, 1980). Later, Esslinger (1986b) redescribed the type
107 species *O. digiticauda,* subsequently redefining the genus, and transferring eight species
108 previously included in *'Waltonella'* (*W. convoluta*, *W. scalaris*, *W. vellardi*, *W. guyanensis*,
109 *W. royi*, *W. dufourae*, *W. oumari*, *W. albareti*) to *Ochoterenella* (see Table 1). Esslinger
110 (1986b) also transferred *Ochoterenella guibei* (Bain and Prod'Hon, 1974) to
111 *Paramadochotera*, and considered *Ochoterenella papuensis* Johnston, 1967 described
112 from a frog in Papua New Guinea as *incertae sedis*. Based on the revision of the subfamily
113 and redefinition of the genus (Esslinger, 1986a, b), a further seven species have been
114 described and designated to *Ochoterenella* (Esslinger, 1987, 1988a, b, 1989; Souza et al.,
115 2012). *Ochoterenella* is characterised by the presence of cuticularized parastomal
116 structures, the lack of lateral and caudal alae, the presence of a distinct buccal capsule,
117 and the presence of bands of longitudinally oriented bosses in the mid-body region
118 (Esslinger, 1986a).

119 *Madochotera* is a small group of filariae exclusively described from Malagasy
120 rhacophorids. Currently the genus comprises three species namely *M. alata* Bain and
121 Brunhes, 1968, *M. landauae* Prod'hon and Bain, 1974 and *M. pichoni* Bain and Brunhes,
122 1968. It is characterised by the presence of cuticularized parastomal structures, the
123 presence of lateral alae, the lack of a distinct buccal capsule, the cuticle with sometimes
124 transversely oriented ridges or bosses, and a vulva that is distinctly posterior to the
125 oesophagus (Esslinger, 1986a).

126 *Paramadochotera* is monotypic and as mentioned above, was erected to
127 accommodate *P. guibei* described from a Banded Madagascar frog *Gephyromantis*
128 *redimitus* (Boulenger, 1889) in Madagascar. *Paramadochotera* is characterised by the
129 presence of cuticularized parastomal structures, the lack of lateral and caudal alae, the
130 presence of a distinct cuticularized buccal capsule, the cuticle of the female with

transversely oriented ridges or bosses present on the dorsal and ventral surfaces, and a body that is abruptly attenuated at the extremities (Esslinger, 1986a).

Paraochoterenella is also monotypic and was erected to accommodate *Paraochoterenella javanensis*, described from the mesentery of the Crab-eating frog *Fejervarya cancrivora* (Gravenhorst, 1829) in West Java, Indonesia. *Paraochoterenella* is characterised by the presence of cuticularized parastomal structures, the lack of lateral and caudal alae, the presence of a distinct cuticularized buccal capsule, and the presence of scattered (non-oriented) minute bosses on the cuticle of the midbody region (Purnomo and Bangs, 1999).

In a recent study on the phylogenetic relationships of Onchocercidae, several species of filarial nematodes parasitising amphibians (*Ochoterenella* spp. and *Icosiella neglecta* (Diesing 1851) and reptiles (*Foleyella candezei* (Fraipont, 1882), *Madathamugadia hiepei*, Hering-Hagenbeck, Boomker, Petit, Killick-Kendrick and Bain, 2000, *Oswaldofilaria petersi* Bain and Sulahian, 1974 and *Oswaldofilaria chabaudi* Pereira, Souza and Bain, 2010) were included (Lefoulon et al., 2015). This study, based on both nuclear (18S rRNA, 28S rRNA, MyoHC, rbp1, hsp70) and mitochondrial (COI, 12S rDNA) markers, showed Icosiellinae, Waltonellinae, and Oswaldofilariinae (onchocercids from crocodilian and squamate hosts), forming a well-supported clade, sister to all the other taxa evaluated within Onchocercidae. Furthermore, *Foleyella candezei* (Dirofilariinae) found infecting lizard hosts, was shown as distantly related to this group (Oswaldofilariinae, Icosiellinae and Waltonellinae) and clustering with other members of Dirofilariinae (Lefoulon et al., 2015).

Excluding Madagascar, the only species described in Africa from Waltonellinae is *Foleyellides duboisi* (Gedoelst, 1916) found in the Democratic Republic of Congo. This species was described by Gedoelst (1916), based on material collected from unidentified frogs and then recorded again from the Marsh frog *Pelophylax ridibundus* (Pallas, 1771)

157 from Palestine (Witenberg and Gerichter, 1944). However, these specimens were reported
158 to contain differences in the number of apical papillae and other morphometric characters.
159 Three further blood parasite biodiversity surveys of different anuran families have also
160 been completed in Africa. The first by Readel and Goldberg (2010) in Uganda, who
161 reported on an unidentified microfilaria parasitising *Leptopelis christyi* (Boulenger, 1912)
162 and *L. kivuensis* Ahl, 1929. The second by Aisien et al. (2015) in Nigeria, who reported on
163 microfilariae parasitising *Aubria subsigillata* (Duméril, 1856), *Hoplobatrachus occipitalis*
164 (Günther, 1858), *Sclerophrys regularis* (Reuss, 1833), *S. maculatus*/*pusilla*, and *S.*
165 *galamensis* (Duméril and Bibron, 1841). Lastly, in South Africa, Netherlands et al. (2015)
166 observed microfilariae parasitising *Ptychadena anchietae* (Bocage, 1868) and *Sch.*
167 *carens.*

168 Within Waltonellinae only the life cycles of species of *Foleyellides* have been
169 studied. Causey (Causey, 1939a; Causey, 1939b) was the first to report on the
170 experimental transmission and development of *Foleyellides ranae* (Walton, 1929) from
171 *Rana clamitans* Latreille, 1801 and *Foleyellides dolichoptera* (Wehr and Causey, 1939)
172 from *Rana sphenocephala* Cope, 1886 in the mosquitoes *Aedes aegypti* (Linnaeus in
173 Hasselquist, 1762) and *Culex pipiens* Linnaeus, 1758. Subsequently, Causey (1939c)
174 reported on the development of *Foleyellides brachyoptera* in *Rana catesbeiana* (Shaw,
175 1802) in *A. aegypti*, *Culex quinquefasciatus* Say, 1823, and *C. pipiens*. Although these
176 mosquito species do not naturally feed on amphibians and high mortality of mosquitoes
177 was observed post feeding, development of infective larvae (third-stage) appeared from 13
178 days post-infection (dpi) (Causey, 1939a; Causey, 1939b; Causey, 1939c). The life cycle
179 of *F. duboisi* infecting *Pelophylax* kl. *esculentus* from Palestine has also been studied
180 (Witenberg and Gerichter, 1944). These authors describe the adult, microfilaria and
181 developmental stages in experimentally infected *Culex pipiens molestus*. In that study,
182 twenty per cent of blood fed mosquitoes died within 48 hours, and of those that survived

35% were infected. The sausage-shaped first-stage appeared from the third day, while first infective larvae appeared from approximately 14 dpi. Furthermore, these authors did not consider *C. pipiens molestus* as the natural intermediate host for *F. duboisi*, suggesting that a more abundant mosquito species with a preference for feeding on frog hosts would be a more likely natural vector (Witenberg and Gerichter, 1944). Later, Crans (1969) elucidated the life cycle of a filarial nematode, later designated as *F. flexicauda* (Schacher and Crans, 1973), from the American Bullfrog *R. catesbeiana* and the amphibian-feeding mosquito *Culex territans* Walker, 1856 in New Jersey. Crans (1969) reported that in the vertebrate host adult filarial nematodes were found encysted in the intestinal mesentery of the body cavity, with microfilariae released in the peripheral blood. In the invertebrate host infective third-stage larvae were detected within ten dpi. Developmental stages appeared throughout the mosquito's haemocoel, in the abdominal fat bodies, the coxal cavities of the legs, and in the head capsule and proboscis (Crans, 1969).

Based on previous research of herpetofaunal blood parasites in KwaZulu-Natal (KZN) (Netherlands et al., 2014; Cook et al., 2015, 2016; Cook et al., 2018; Netherlands et al., 2018) and the occurrence of microfilariae in anurans (Netherlands et al., 2015), northern KZN is well suited as a model ecosystem for research on blood parasite diversity and their associated life cycles. Members of Bufonidae are common in this area and have been reported to be parasitised with several blood parasite taxa, including microfilariae (Netherlands et al., 2014; Netherlands et al., 2015). Thus the objectives of this study were first to (1) establish which species of Bufonidae in northern KZN (South Africa) host microfilariae, (2) determine the prevalence, distribution, taxonomic placement, and species identification of any microfilaria species found, and (3) attempt to identify any possible vectors. If the above objectives were achieved, the final objective (4) was to attempt the elucidation of the life history of an anuran filarial nematode.

2. Material and Methods

2.1 Toad collection and study area

In a recent survey on the biodiversity of anuran blood parasites, Netherlands et al. (2015) found the Red toad, *Schismaderma carens* parasitised with microfilariae. Thus, for the current study members of Bufonidae were selected as the vertebrate hosts to be screened for microfilariae. A total of 128 individuals, representing four bufonid species, were collected at night via active sampling from several localities throughout northern KZN, South Africa (S1 Table). Species comprised 45 Eastern Olive toads (*Sclerophrys garmani*), 73 guttural toads (*Sclerophrys gutturalis*), seven Flat-backed toads (*Sclerophrys pusilla*), and three Red toads (*Sch. carens*), identified using field guides (Du Preez and Carruthers, 2009, 2017). Collected specimens were placed in individually marked containers with sufficient moisture and ventilation, and transported back to a field workstation. Toads were collected at these sites during their breeding season and the southern hemisphere warmer months of April, September, November, and December 2014, September, November and December 2016, February and November 2017, and January and March 2018. All specimens collected prior to November 2017, were released after taking blood samples. Based on the intensity of microfilariae selected individuals, uninfected (n=1) and infected (n=4), collected during and after the November 2017 sampling effort were transported back to the North-West University African Amphibian Conservation Research Group laboratory for further processing and examination. This study received the relevant ethical approval from the North-West University's AnimCare ethics committee (ethics number: NWU-00372-16-A5) and euthanasia of frogs was performed according to approved SOP using tricaine methanesulfonate (MS222) solution (ethics number: NWU-00492-16-S5). Ezemvelo KZN Wildlife provided research permits OP 526/2014, OP 839/2014, OP 4374/2015, OP 4092/2016, and OP 4085/2017 for collection and sampling of anurans for this study. Furthermore, authors responsible for

235 sample collection and processing of specimens have undergone specialised training in
236 ethical handling of aquatic ectotherms (NWU Ectothermic Vertebrates Handling and
237 Ethics).
238
239 *2.2 Processing of blood samples and light microscopy screening*
240 To determine prevalence of infection, blood (>0.2 ml) was first taken from each toad via
241 cardiac or femoral venipuncture and thin blood smears prepared, air-dried, fixed and
242 stained using Giemsa-stain following routine practice (Netherlands et al., 2015). When
243 possible, blood smears were screened for microfilariae in the field using a Nikon Eclipse
244 E100 compound microscope. The remaining blood was preserved in 70% ethanol for
245 molecular work (ratio 1:15). Stained blood smears were screened using a Nikon ECLIPSE
246 *Ni* Compound microscope at 1000× and images captured and measured using the imaging
247 software NIS Elements Ver. 4. Parasitaemia was estimated according to the number of
248 microfilariae observed in ten optical fields at 400× magnification. In the current study
249 parasitaemia was regarded as low if one microfilaria per ten fields of view was observed,
250 medium if between one and five microfilariae per field of view was observed, and high if
251 more than five per field of view were observed.
252
253 *2.3 Life history and experimental transmission*
254 Following the screening of blood smears previously collected, the opportunity presented
255 itself to return to one of the collection localities, Sodwana Bay, in November 2017 and
256 again in January and March 2018 (S1 Table), to increase the sample size of toads from
257 this area. The site visited, SB-1 (S27.488591°; E32.664259°), is a permanent and well
258 vegetated wetland with a slow flowing stream. Toads from this area were found positive
259 with microfilariae and during sampling, mosquitoes were observed feeding on calling *S.*
260 *gutturalis*. To investigate if the mosquitoes observed readily feeding on *S. gutturalis* were

261 the responsible vectors, mosquitoes and toads were collected and processed. Table 2
262 provides a summary of the process followed to elucidate the life history of the filarial
263 nematode found parasitising toads from Sodwana Bay.

264 Mosquitoes were euthanized with carbon dioxide (CO_2) and dissected under a
265 stereomicroscope using modified entomology pins. Sausage-shape first-stage larvae were
266 prepared for compound microscopy by smearing the dissected contents on a glass slide
267 and removing the larvae from the mosquito's intestines and fat bodies. Subsequently the
268 glass slides were fixed and stained, as described above for light blood smear preparation,
269 to screen for microfilaria and any larvae that may have been missed. Late sausage-shape
270 first as well as second and third-stage larvae were removed with one hair of a fine tip
271 paintbrush, washed in saline and fixed in hot 70% alcohol.

272 The remaining infected *S. gutturalis* (n=2) were euthanized, dissected, and adult
273 filarial nematodes removed as mentioned above. Prior to microscopical examination, adult
274 nematodes were placed in distilled water for about 20 min and subsequently cleared in
275 lactophenol for 30 min. Apical and transverse sections were prepared manually using a
276 thin razor and examined on temporary mounts. Morphology of the nematodes was studied
277 and photomicrographs were taken using the Nikon E800 and Nikon ECLIPSE *Ni*
278 compound microscopes.

279 All measurements in the text are given in micrometres (µm) unless indicated
280 otherwise. Morphometric data are presented as a range followed by values of holotype or
281 paratype in square brackets and mean values in parentheses. For specimens with a
282 sample size of 30 and above, metrical characters of the coefficient of variation (CV) were
283 calculated as standard deviation (SD) divided by the mean value and presented as a
284 percentage.

285

286 *2.4 Scanning electron microscopy (SEM)*

Each of the filarial nematode stages obtained were used for scanning electron microscopy (SEM). Specimens were dehydrated using a gradual ethanol series: nematodes were first added to a small Petri dish with 70% EtOH, continuously and slowly 100% EtOH was added to the Petri dish, and simultaneously the same amount of mixed EtOH removed until concentration reached 100%. After dehydration, nematodes were dried using hexamethyldisilazane as transition fluid (before clear solution worms were dehydrated in a series of hexamethyldisilazane mixed 1:1 with 70°, 80°, 90°, 96° and 100° EtOH) or by means of a critical point drier (Bio-Rad, Bio-Rad Microscience Division, United Kingdom) using liquid CO_2 as transition fluid. In order to prevent shrinking of adult nematodes and third-stage larvae, specimens were dissected into two or more pieces before dehydration. Following the methods of Conradie et al. (2017), fresh blood samples obtained from a highly infected guttural toad (S1 Table, AE180124C1) were also prepared for SEM. Thin blood smears were made on glass coverslips and Whatman® qualitative filter paper (Grade 1), and a drop of Todd's fixative (Todd, 1986) placed on the smears prior to drying. After a minute, the smears were submerged in fresh Todd's fixative for approximately 2 h. The sample was then gently washed with ultrapure water three times each for 15 min. Post fixation was performed with 2% osmium tetroxide (OsO4) for 90 min. This was followed by rinsing three times in ultrapure water for 10 min, with subsequent dehydration in a gradual ethanol series. The sample was then critical point dried as described above for the filarial nematode stages.

Dried specimens and smears were mounted onto 12 mm aluminium stubs with double-sided carbon tape and sputter-coated for 90 seconds with a gold palladium alloy in argon gas at a pressure of 2 atm (SPI-ModuleTM Sputter Coater, SPI Supplies, West Chester, PA, USA). Specimen stubs were stored in a desiccator for at least 30 min before being examined by SEM at an accelerated voltage of 10 kV (Phenom PRO Desktop SEM, Phenom-World B., Eindhoven, Netherlands).

313

314 *2.5 DNA extraction, PCR amplification and phylogenetic analyses*

315 Ethanol-preserved specimens of the different stages (n=5), sausage-shape first-stage
316 larvae to both adult male and female filarial nematodes, and blood samples from all
317 parasitised toads (n=8) were used for molecular work. Genomic DNA was extracted from
318 the samples following the standard protocol for human or animal tissue and cultured cells
319 as detailed in the NucleoSpin®Tissue Genomic DNA Tissue Kit (Macherey-Nagel, Duren,
320 Germany). Once extracted, DNA was used for polymerase chain reaction (PCR)
321 amplification. The PCR reactions targeted a fragment of approximately 750 nt of the 18S
322 rRNA gene and 650 nt of the cytochrome *c* oxidase subunit I (COI) gene. Sequences were
323 amplified using primer sets obtained from a previous study based on filariae of
324 Onchocercidae (Lefoulon et al., 2015). The 18S rDNA sequence fragment was amplified
325 using F18ScF1 (5′-ACC GCC CTA GTT CTG ACC GTA AA-3′) and F18ScR1 (5′-GGT
326 TCA AGC CAC TGC GAT TAA AGC-3′), and the COI sequence fragment was amplified
327 using COIintF (5′-TGA TTG GTG GTT TTG GTA A-3′) and COIintR (5′-ATA AGT
328 ACG AGT ATC AAT ATC-3′). Conditions for PCR of both primer sets were as follows: 40
329 cycles, entailing a 95°C denaturation for 30 s, annealing at 58°C (18S) and 52°C (COI) for
330 30 s with an end extension at 72°C for 90 s; followed by a final extension of 72°C for 10
331 min. PCR reactions were performed using 12.5 µL OneTaq® 2× Master Mix with Standard,
332 1.25 µL (10 µM) of each of the primer sets mentioned above, and at least 25 ng DNA. The
333 final reaction volume of 25 µL was made up with PCR-grade nuclease-free water (Thermo
334 Scientific). Reactions were undertaken in an Applied Biosystems SimpliAmp Thermal
335 Cycler PCR machine (Thermo Fisher Scientific, Waltham, MA USA). Resulting amplicons
336 were visualized under ultraviolet light on a 1% agarose gel stained with EZ-Vision®
337 Bluelight DNA dye using an E-BOX CX5 imaging system (Vilber Lourmat Deutschland,
338 Eberhardzell, Germany). PCR products from each sample were sent to a commercial

339 sequencing company (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) for
340 purification and sequencing in both directions. Resultant sequences were assembled, and
341 chromatogram-based contigs were generated and trimmed using Geneious R11
342 (http://www.geneious.com, (Kearse et al., 2012)). Sequence and species identity was
343 verified against previously published sequences using the Basic Local Alignment Search
344 Tool (BLAST) (Altschul et al., 1990). Sequences obtained in the current study were
345 deposited in the NCBI GenBank database under accession numbers XXX–XXX.
346 For the partitioned phylogenetic analysis, representative sequences from the different
347 subfamilies of Onchocercidae, were downloaded from GenBank and aligned to the
348 sequences generated in the current study (Table 3). *Filaria latala* [GenBank: 18S:
349 KP760135 and COI: KP760186] was chosen as the outgroup, following Lefoulon et al.
350 (2015). A partition homogeneity test (Farris et al., 1994) (1000 replicates - heuristic
351 search) calculated using PAUP version 4.0a152 (Swofford, 2002) was applied to check
352 whether the 18S rRNA and COI gene trees were sufficiently similar in rates of divergence
353 and branching order and that the datasets could be combined. The partition homogeneity
354 test ($P < 0.97$) supported the combination of the 18S rRNA and COI genes. Concatenated
355 18S rRNA and COI gene sequences were aligned using the Clustal W 2.1 alignment tool
356 (Larkin et al., 2007) under the default settings and implemented in Geneious R11. The
357 GBlocks server was used to remove any alignment gaps and ambiguities selecting the
358 parameters to allow for smaller final blocks with gap positions (Castresana, 2000; Talavera
359 and Castresana, 2007). The final alignment consisted of 54 sequences with a 659 nt 18S
360 rDNA and 577 nt COI, with a total of 1236 nt, 82% of the original 1445 positions. A
361 partitioned Bayesian inference (BI) analysis was performed using MrBayes 3.2.2
362 (Huelsenbeck and Ronquist, 2001) implemented from within Geneious R11. Prior to the
363 analyses, a model test was performed to determine the most suitable nucleotide
364 substitution model according to the Bayesian information criterion using jModelTest 2.1.7

365 (Guindon and Gascuel, 2003; Darriba et al., 2012). The model with the best BIC score for
366 the 18S rDNA sequence alignment was the Kimura 2-parameter model (Kimura, 1980)
367 with an estimated proportion of invariable sites (p-inv = 0.7840) and a discrete gamma
368 distribution (gamma shape = 0.7560) (K80 + I + Γ). For the COI sequence alignment the
369 General Time Reversible model (Tavaré, 1986) with an estimated proportion of invariable
370 sites (p-inv = 0.3160) and a discrete gamma distribution (gamma shape = 0.3430) (GTR +
371 I + Γ) was selected as the model with the best BIC score. For the BI analysis, the
372 alignment was partitioned according to the 18S rRNA (1–659 nt) and COI (660–1236 nt)
373 genes; the Markov Chain Monte Carlo (MCMC) algorithm was run for 10 million
374 generations, sampling every 100 generations, and using the default parameters. The first
375 25% of the trees were discarded as 'burn-in' with no 'burn-in' samples being retained.
376 Results were visualized in Tracer (Rambaut et al., 2018) (implemented from within
377 Geneious R11), to assess convergence and the 'burn-in' period.
378
379

380 ## 3. Results

381 The blood of 128 toads representing four species, *Sclerophrys garmani* (n=45),
382 *Sclerophrys gutturalis* (n=73), *Sclerophrys pusilla* (n=7), and *Schismaderma carens* (n=3)
383 were collected and screened for microfilariae (S1 Table). Eight toads from two species, *S.*
384 *gutturalis* and *S. garmani*, were found positive with microfilariae, all collected from
385 Sodwana, KZN, South Africa. For *S. gutturalis* 13.5% of the males (7/52) and 6.7% of *S.*
386 *garmani* females (1/15) were infected with microfilariae, as compared to 0% of the *S.*
387 *gutturalis* females (0/21) and 0% of the *S. garmani* males (0/30). Based on the morphology
388 of the observed blood stages alone, microfilariae could not be identified to genus or
389 species level. Hence, for further morphological classification (see species description
390 below) both male and female adult specimens were collected from the infected individuals

of *S. gutturalis* (n=4). The number of adults collected ranged from 10 to 52 specimens across the different host individuals dissected.

3.1 Genus description

Phylum: Nematoda Rudolphi, 1808

Class: Chromadorea Inglis, 1983

Order: Rhabditida Chitwood, 1933

Suborder: Spirurida Railliet and Henry, 1915

Superfamily: Filarioidea Weinland, 1858

Family: Onchocercidae Leiper, 1911

Subfamily: Waltonellinae Bain and Prod'Hon, 1974

Genus: *Neofoleyellides* Netherlands, Svitin, Smit and Du Preez n. gen.

Diagnosis: Large elongated nematodes, females generally bigger than males. Oral opening small, oval with two parastomal structures arranged laterally. Buccal capsule small, conspicuous, wider than long. Narrow lateral and caudal alae present in both sexes. Transverse or longitudinal striations absent. Oesophagus visibly divided into muscular and glandular sections. Nerve ring at level of muscular oesophagus posterior quarter. Excretory pore minute, poorly visible, situated at level of oesophageal-intestinal junction. Posterior end of male with narrow caudal alae, four pairs of caudal papillae (one above and three below cloaca). Spicules simply-shaped with sharpened tips, unequal (left longer than right one). Tail tapering with rounded tip. Females viviparous, vulva situated at posterior level of oesophagus. Caudal alae narrow, tail tapering with rounded tip. Parasites found in body cavity and peripheral blood of anurans.

Remarks: According to the keys provided by Esslinger (1986a), Purnomo and Bangs (1999), and Bain et al. (2013), *Neofoleyellides* n. gen. belongs to the family Onchocercidae and subfamily Waltonellinae, based on the presence of distinct parastomal structures, lack of submedian cephalic spines, position of an anus which is not subterminal, possession of a vulva posterior to the nerve ring in females, possession of several pairs of papillae on the somewhat elongated tail in males and the nematode parasitising an amphibian definitive host. Within Waltonellinae, the morphological characters of *Neofoleyellides* n. gen. conform closest with *Foleyellides*, such as the presence of a distinct buccal capsule and parastomal structures, the presence of lateral and caudal alae in both sexes, and the absence of cuticular bends and bosses on the body cuticle. Nonetheless *Neofoleyellides* n. gen. can be easily distinguished from *Foleyellides* and all other genera from Waltonellinae by the absence of dorsal outer cephalic papillae and the absence of small inner cephalic papillae. *Neofoleyellides* n. gen. is characterised by only two enlarged submedian cephalic papillae on the ventral sides of the oral opening, and the absence of other papillae surrounding the oral opening. Since differences in cephalic morphology are commonly used for generic differentiation in many groups of parasitic nematodes and based on the other morphological differences such as asymmetry of cephalic papillae and molecular characterisation (see below), we consider our specimens as belonging to a new onchocercid genus.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:XXX. The LSID for the new genus name *Neofoleyellides* Netherlands, Svitin, Smit and Du Preez n. gen. is urn:lsid:zoobank.org:act:XXX.

Etymology: The generic name is derived from the close morphological resemblance to representatives of *Foleyellides*, with the new genus possessing morphological characters,

441 such as asymmetry and a reduced number of cephalic papillae generally considered as a
442 progressive character within onchocercids (Anderson and Bain, 1976).
443
444 *3.2 Species description*
445 ***Type species: Neofoleyellides boereworsi*** **Netherlands, Svitin, Smit and Du Preez n.**
446 **sp.**
447 *Type-host*: *S. gutturalis*
448 *Other host*: *S. garmani*
449 *Site in host*: body cavity, subcutaneous. Microfilariae were also observed in peripheral
450 blood obtained from the femoral artery or veins, heart and body cavity.
451 *Vector*: *Uranotaenia* (*Pseudoficalbia*) *mashonaensis, Uranotaenia* (*Pfc*.) *montana*
452 *Site in vector*: stomach, intestine, fat body, thoracic and abdominal cavities, head capsule,
453 proboscis.
454 *Type-locality*: Sodwana, KwaZulu-Natal, South Africa. Coordinates: S27.488591°;
455 E32.664259°.
456 *Type-material*: Holotype (male, NMB PXXX), allotype (female, NMB PXXX), paratypes
457 [NMB PXXX – XXX (XXX males and XXX females)] deposited in the National Museum
458 Parasite Collection (Bloemfontein, South Africa). Microfilaria: Hapantotype and
459 Parahapantotype, 2 × blood smears deposited in the protozoan collection of the National
460 Museum, Bloemfontein, South Africa, under accession number [NMB P XXX and XXX],
461 respectively.

462 *Voucher material*: 1 × male and 1 × female deposited in the collection of the research
463 group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium),
464 under accession number [XXX and XXX]; Microfilaria: 1 × blood smear deposited in the
465 collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University
466 (Diepenbeek, Belgium), under accession number [XXX].

Representative DNA sequences: The 18S rRNA and COI gene sequences were submitted to the GenBank database under the accession numbers XXX.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:XXX. The LSID for the new name *Neofoleyellides boereworsi* n. gen. n. sp. Netherlands, Svitin, Smit and Du Preez is urn:lsid:zoobank.org:act:XXX.

Etymology: The species epithet is derived from the morphological features of this nematode, which resembles Boerewors, a type of traditional sausage and an important part of South African cuisine and culture.

Description: General. Body elongated, cylindrical almost along entire length with rounded anterior and narrowed posterior end. Oral opening small, oval with two parastomal structures arranged laterally (Figs 1B, 2A, 3C, D). Buccal capsule small, conspicuous, wider than long. In both sexes cuticle forming narrow lateral and caudal alae without any other conspicuous transverse and longitudinal striations. Lateral alae beginning at level of anterior end of muscular oesophagus and terminating close to cloaca (Fig 2D). Oesophagus visibly divided into short muscular and longer glandular section. Nerve ring encircling glandular oesophagus at level of its posterior quarter. Minute excretory pore (seen only on SEM images) situated at level of oesophageal-intestinal junction.

Males (n=30). Body 15–28, [20] (22±3.1, 14.3) mm long, 97–138 [105] (120±12.0, 10.0), 151–215 [153] (190±22.1, 11.7) and 135–248 [162] (202±29.3, 14.5) wide at nerve ring, oesophageal-intestinal junction, and mid-body level, respectively (Fig.2B). Buccal capsule, 3–6 [3] (4±0.8, 20.1) long, and 6–12 [8] (9±1.5, 18.0) wide. Glandular portion of oesophagus 190–369 [263] (282±41.8, 14.8) long; 27–57 [32] (38±6.1, 16.0), 25–49 [29] (34±5.4, 16.0) and 32–51 [37] (41±5.9, 14.2) wide at anterior, mid-length and posterior level, respectively. Muscular section of oesophagus 658–1212 [704] (918±145.9, 15.9)

493 long; 44–88 [58] (66±11.8, 18.0), 62–157 [71] (106±23.0, 21.7) and 49–158 [49] (96±27.2,
494 28.4) wide at anterior, mid-length, and posterior level, respectively. Total oesophagus
495 887–1497 [967] (1204±172.1, 14.3) long spanning 4–7 [5] (6±0.6, 11.5) % of body length.
496 Nerve ring situated at 179–270 [207] (222±25.2, 11.3) from the anterior end of body,
497 spanning 14–24 [21] (19±2.7, 14.2) % of total oesophagus length (Figs 1C, 2C).
498 Spicules unequal: left elongated, often extending from cloaca, 50–94 [74] (73±9.1, 12.5)
499 long; right shorter and thicker, 91–158 [152] (137±14.7, 10.7) long (Figs 1H, 2E). Five
500 pairs of sessile papillae located at caudal region: one pair pre-anal, one ad-anal and three
501 post-anal papillae. Size ranges of papillae decrease towards the posterior end. Tail
502 tapering with rounded tip, 50–94 [74] (73±9.1, 12.5) long. Cuticular ornamentation well
503 developed above cloaca. Narrow caudal alae nearly reaching tail's tip (Figs 1I, 2E, F).
504 Females (n=30). Body 16.2–71.5 [6.2] (49.5±15.9, 32.1) mm long, 117–201 [167]
505 (158±23.6, 14.9), 193–390 [368] (291±78.5, 27.0) and 147–441 [402] (330±89.0, 27.0)
506 wide at nerve ring, oesophageal-intestinal junction, and mid-body level, respectively (Fig
507 3A). Buccal capsule 3–7 [5] (4±1.1, 23.6) long, and 5–15 [9] (10±2.1, 22.4) wide (Fig 3C).
508 Glandular section of oesophagus, 215–436 [364] (320±61.1, 19.1) long; 30–56 [30]
509 (40±5.9, 14.7), 25–51 [35] (34±5.2, 15.2) and 25–67 [49] (45±7.7, 17.2) wide at anterior,
510 mid-length and posterior level, respectively. Muscular portion of oesophagus, 490–1798
511 [1798] (1147±312.9, 27.3) long; 37–105 [70] (74±16.2, 21.9), 55–176 [110] (117±29.6,
512 25.3) and 56–184 [143] (104±32.7, 31.4) wide at anterior, mid-length and posterior level,
513 respectively. Total oesophagus 722–2164 [2162] (1467±355.0, 24.2) long spanning 2–5
514 [3] (3±0.6, 20.5) % of body length. Nerve ring at 166–420 [275] (249±56.4, 22.6) from
515 anterior end, spanning 12–31 [13] (17±4.0, 22.8) % of total oesophagus length (Figs 1A,
516 3B).

517 Vulva transversely split, at 687–1882 [1782] (1234±266.1, 21.6) from anterior end,
518 spanning 2–4 [3] (3±0.6, 23.7) % of body length (Figs 1A, 3B, F). Tail tapering, 123–859
519 [331] (274±138.5, 50.6) long. Narrow caudal alae almost reaching tail's tip (Figs 1D, 3E).
520 Microfilaria. Larvae from anuran blood (n=30) small, elongated, coated in relatively
521 thick sheath (Fig 4A, 5C). Anterior and posterior ends rounded, maximum width at level of
522 anterior quarter, 66–92 (77±7.04, 9.15) long, and 4–5 (5±0.22, 4.78) wide. Cuticle smooth
523 along entire body. Unsheathed microfilaria from digestive tract of mosquito (n=30) thin,
524 elongated measuring 82–134 (105±15.14, 14.40) long, and 5–8 (6±0.60, 9.49) maximum
525 width (at anterior quarter) (Fig 4B). Fine transverse striations observed along body cuticle.
526 Anterior end with crown-like structure and small tooth (clearly visible on SEM images) (Fig
527 5A, B).
528 Sausage-shape first-stage larvae (n=37). Body short, almost oval measuring 51–
529 118 (79±18.53, 23.52) long, with maximum width of 12–29 (18±4.03, 22.02) over anterior
530 quarter. Early sausage stage with narrow anterior end, possessing crown-like structure
531 and small tooth (Fig 4C). Late sausage-shape stage with wider and smoother anterior
532 section, deprived of other structures (Figs 1E, 4C, 5E).
533 Second-stage larvae (n=20). Body relatively short, with slightly widened anterior
534 and posterior ends, measuring 315–663 (474) long, and 23–49 (31) wide at mid-body
535 level. Oesophagus narrow at anterior section, uniformly widening towards posterior end,
536 77–286 (157) long, and spanning 16–47 (31) % of body length. Dissected oesophagus
537 visibly divided into glandular (with nerve ring at posterior quarter) and muscular parts. Tail
538 tapering to rounded tip, measuring 21–51 (39) long; and spanning 4–11 (8) % of body
539 length (Figs 1F, 5F, G).
540 Third-stage larvae (n=30). Specimens from body cavity thin and elongated, 752–
541 1090 (927±87.1, 9.4) long, with maximum width of 14–27 (19±2.9, 14.9) at level of anterior
542 third of body. Oesophagus short, measuring 103–196 (142±21.9, 15.4), spanning 11–21

543 (15±2.5, 16.4) % of body length. Nerve ring encircling oesophagus at level of its anterior
544 quarter, measuring 48–107 (75±15.1, 20.0) from anterior end of body, spanning 5–11
545 (8±1.6, 19.5) % and 40–68 (53±5.9, 11.2) % of body and oesophagus length, respectively.
546 Tail short and rounded, measuring 32–55 (45±5.7, 12.7), and spanning 3–6 (5±0.6, 11.5)
547 % of body length. Specimens from mosquito head (n=15) relatively small, measuring 798–
548 978 (893), with a maximum width of 16–23 (19). Oesophagus 124–208 (146) long,
549 comprising 13–22 (16) % of body length. Position of nerve ring varying within posterior half
550 of muscular oesophagus, measuring 44–49 (47) from anterior end of body, spanning 5–6
551 (5) % and 29–37 (33) % of body and oesophagus length, respectively. Tail rounded, 32–
552 51 (44) long, and spanning 3–6 (5) % of body length (Figs 1G, 5H).
553
554 *Remarks*: Several morphological characters are used for differentiation between the
555 various genera and species within Waltonellinae. The most defining characters are based
556 on differences in the apical structures, and the male and female genital system.
557 *Neofoleyellides boereworsi* n. sp. can be easily distinguished from all other described
558 species within Waltonellinae by the absence of dorsal outer cephalic papillae and the
559 absence of small inner cephalic papillae.
560
561 *3.3 Phylogenetic analysis*
562 Amplicons of between 753 and 775 nt (n=15) of the 18S rRNA gene, and between 660
563 and 665 nt (n=15) of the COI gene were obtained. Sequences were derived from the
564 sausage-shape first-stage, second-stage, and third-stage larvae in the mosquito vectors *U.*
565 (*Pfc.*) *mashonaensis* or *U*. (*Pfc*.) *montana*, and from the adult stages in the body cavity of
566 the vertebrate host *S. gutturalis* and from microfilariae in the blood of the hosts *S. garmani*
567 and *S. gutturalis*. All the isolates obtained for *Neofoleyellides boereworsi* n. gen. n. sp.,
568 from the various stages (from microfilaria to adults) and hosts (toads and mosquitoes)

569 were identical for both the 18S rRNA and COI gene sequence fragments. *Neofoleyellides*
570 *boereworsi* n. gen. n. sp. is represented in this analysis by sequences obtained from
571 microfilaria-infected blood samples from *S. gutturalis* and *S. garmani* (as no *S. garmani*
572 were disected for adult stages). For the BI phylogenetic analysis, filariae of Waltonellinae
573 isolated from anuran hosts are the earliest diverging lineages in the ingroup. They are
574 paraphyletic with respect to all other onchocercid taxa used in this analysis, Dirofilariinae,
575 Setariinae, Splendidofilariinae and Onchocercinae, together form a clade with 0.95
576 posterior probability support, in which Dirofilariinae and Onchocercinae are non-
577 monophyletic. *Neofoleyellides boereworsi* n. gen. n. sp., is shown as a sister taxon to
578 Icosiellinae, Oswaldofilariinae and all other onchocercids (Fig 6).
579
580 *3.4 Life history experiment*
581 A total of 146 mosquitoes were collected *in situ* (see Fig 7A-B). Of these, 64 were
582 identified as *U.* (*Pfc.*) *mashonaensis* and 42 as *U*. (*Pfc*.) *montana*. The remaining 40
583 mosquitoes did not take blood meals or died in captivity, and were not included in the
584 experiment. Collected mosquitoes used for experimental transmission consumed blood
585 meals from *S. gutturalis* highly parasitised with microfilariae. Within 24 h post feeding,
586 desheathed microfilaria were observed in the mosquito's intestines, along with undigested
587 erythrocytes. Microfilariae observed in fresh wet blood smears were slightly more active
588 compared to the desheathed microfilaria from mosquitoes (Fig 8A). From three days post-
589 infection (dpi) early sausage-shaped first-stage larvae were observed in the intestine,
590 followed by late sausage-shape first-stage larvae appearing approximately three to four
591 dpi in the fat bodies of its host (Fig 8B). Early sausage-shaped larvae seem to be dormant
592 whereas late sausage-shaped larvae were observed slowly moving the anterior part of the
593 body. Second-stage larvae were found in the fat bodies and body cavity of the abdomen,

594 and in the thorax of the mosquito as from six to 14 dpi (Fig 8C). Second-stage larvae were
595 able to move relatively slowly, although this was faster compared to the first-stage larvae.
596 Third-stage infective larvae were found primarily in the thorax and head capsule of
597 the mosquito host, roughly from 14 to 18 dpi (Fig 8D). Third-stage larvae moved actively,
598 escaping from the dissected cavity, head or proboscis of the mosquito within several
599 seconds. Development from second- to third-stage larvae was progressively prolonged in
600 highly parasitised individuals, which was in contrast to individuals with a low infection level
601 where all second-stage larvae developed into third-stage larvae simultaneously, and
602 accumulated in the head capsule. In a few dissected mosquitoes, third-stage larvae were
603 found positioned in the proboscis of the mosquito vector, seemingly "ready" to enter into
604 the bloodstream of a new host. In the definitive toad host, adult male and female worms
605 were found in the body cavity or subcutaneously, a single individual was parasitised in the
606 eye (see Fig 7C-D), and microfilariae occurred in the peripheral blood (Fig 5A, 8F).
607

608 ## 4. Discussion

609

610 *4.1 Morphology*

611 With the exception of *Madachotera*, *Neofoleyellides* n. gen. and the other genera within
612 Waltonellinae share characters, such as well-developed cuticular parastomal structures
613 and a buccal capsule. Despite the presence of the buccal capsule in most genera of
614 Waltonellinae, past descriptions failed to provide measurements of this structure.
615 Measurements of the buccal capsule of *Neofoleyellides boereworsi* n. gen. n. sp., varied in
616 length and width (in females between 5 and 15) depending on the size of the individual
617 worm (Pearson coefficient of correlation $r = 0.48$ ($p \leq 0.001$) for both sexes). Nonetheless,
618 this sclerotised structure should not be overlooked as it does not alter with fixation and
619 may differ between different species. Cuticular structures e.g. lateral and caudal alae, and

620 bands and bosses over the mid-body region are also used for generic differentiation. As in
621 *Foleyellides*, representatives of *Neofoleyellides* n. gen., possess lateral and caudal alae in
622 both sexes, but lack bands or bosses. Examination of caudal alae and lateral alae on
623 transverse sections, using SEM confirmed their simple shape and small size. However,
624 this was the first study to examine these structures using SEM, thus comparative studies
625 in the future with other species using SEM may reveal additional and new characters for
626 species differentiation. Within Waltonellinae, the male genital system is similar across its
627 different members, possessing an elongated left spicule, a short more cuticularised right
628 spicule, and several large papillae above and below the cloaca. The number and
629 arrangement of the papillae and the ratio of the spicules are used for species
630 differentiation within *Foleyellides* and *Ochoterenella*. However, *Neofoleyellides boereworsi*
631 n. gen. n. sp. contains the lowest number of papillae, one adcloacal and three postcloacal,
632 as in two species of *Foleyellides*, namely *F. confusa* and *F. rhinellae* (García-Prieto et al.,
633 2014). With regard to the female genital structure, only the position of the vulva has been
634 used to distinguish *Madachotera* (with vulva clearly posterior to the oesophagus) from
635 other genera within Waltonellinae. In *Neofoleyellides boereworsi* n. gen. n. sp., the
636 position of the vulva varies from the posterior end of the oesophagus (90% of oesophagus
637 length) to the anterior end of the intestine (103% of oesophagus length). It was also noted
638 that younger (=smaller) females possess a vulva closer to the anterior end, covering about
639 70% of the oesophagus length, while older (=larger) specimens usually possess a vulva at
640 the section posterior to the oesophagus, covering 90–100% of its length. Therefore, in our
641 opinion, the position of the vulva should only be used as a reliable species or generic
642 differentiator if a sufficient sample size of nematodes with different body length is used to
643 determine its location.

644 Scanning electron micrographs illustrated some unique characters of unsheathed
645 microfilaria that are not visible under light microscopy, such as a small tooth, a crown-like

646 structure on the anterior end, and fine transverse striations covering the body. The unique
647 structures possessed by the microfilaria gradually disappeared from the early (younger) to
648 late (older) sausage-shape first-stage larvae. We hypothesize that these characters help
649 the microfilaria to easily penetrate the mosquitoes' stomach and intestinal wall. In
650 comparison, second and third-stage larvae contained relatively simple morphological
651 features. Characteristically for other filarial nematodes, both second and third-stages
652 contained a rounded anterior end without conspicuous apical structures, a prominent
653 rectum, and a rounded tail on the posterior end. The oesophagus and intestine were also
654 clearly visible in both stages, whereas in the second-stage larvae the nerve ring
655 surrounding the oesophagus was only visible once dissected.

656 No genital primordiums were observed in any of the collected larval stages. All
657 examined stages were coated in a relatively thick sheath, with the exception of
658 desheathed microfilariae observed in the mosquito blood meal. These unsheathed
659 microfilaria seem to have lost their sheath shortly after ingestion by their mosquito host.
660 During SEM preparation of microfilariae obtained from the guttural toad's blood, the
661 microfilariae effortlessly desheathed after coming into contact with the filter paper, which is
662 in contrast to the second and third-stage larvae that possess a well-attached sheath. Even
663 dissected second and third-stage larvae still contain a strongly attached sheath. It is
664 possible that the thick sheath covers and conceals certain internal organs and structures,
665 such as the excretory glands, and genital primordium.

666 In general, the microfilariae and other larval stages of anuran onchocercids are
667 poorly studied. Only for *Paraochoterenella* the absence of a sheath and the shape of the
668 tail in microfilaria stages is used to distinguish it from other genera within Waltonellinae
669 (Purnomo and Bangs, 1999). In our opinion, meticulous examination of different larval
670 stages can reveal numerous and additional characters for species and generic
671 differentiation.

672 Analyses of the metric characters of both adult and larval stages showed high
673 variability. The smallest coefficient of variation observed was firstly for the values of the
674 nerve ring for both adult sexes, 22.6 in females and 11.3 in males, and secondly for the
675 male spicules comprising 11.3 and 10.7 for left and right spicules respectively. The high
676 variability observed for all the other characters is possibly due to the large sample size and
677 the large size of the nematodes. The metric characters of the different larval stages also
678 varied greatly, possibly due to their intensive growth rate. Only the body length of the third-
679 stage larvae was rather stable (CV less than 10). Based on the above observations, we
680 suggest that species and generic differentiation of members of Waltonellinae should be
681 supported by both qualitative morphological and molecular data.
682
683 *Potential effects of* Neofoleyellides boereworsi *n. gen. n. sp. on its anuran host*
684 The majority of adult specimens of *Neofoleyellides boereworsi* n. gen. n. sp. were removed
685 from the body cavity of dissected guttural toads, with a few exceptions in highly infected
686 hosts. Specifically, one and three adult filarial nematodes were found subcutaneously in
687 the two most highly infected toads respectively. In the latter guttural toad (S1 Table,
688 AE180124C1), three individuals of *Neofoleyellides boereworsi* n. gen. n. sp. were attached
689 to the lymphatic tissue, and one immature specimen was observed in its host's eye (see
690 Fig 7C-D). Although no attempts were made to study the specific pathological effects
691 *Neofoleyellides boereworsi* n. gen. n. sp. has on its host, some observations may suggest
692 such effects. A heavily (52 specimens) infected *S. gutturalis* contained a visibly enlarged
693 spleen, gall bladder, and liver, the latter also appearing darker than normal. Moreover, the
694 same individual's eye was parasitised with a filarial nematode. Although this nematode
695 died within two weeks after its host was collected, it caused swelling, infection and loss of
696 sight to the infected eye. This individual would potentially have been vulnerable to
697 predators and would likely not have survived long in nature.

698

699 *Phylogenetic position of* Neofoleyellides boereworsi *n. gen. n. sp. within Onchocercidae*

700 In the current phylogenetic analysis members of Waltonellinae do not form a clade. The
701 lack of molecular data of filarial nematodes from cold-blooded vertebrates has prevented a
702 detailed phylogenetic comparison of *Neofoleyellides boereworsi* n. gen. n. sp. with the
703 other genera and species within Waltonellinae. Molecular data are only available for three
704 species of *Ochoterenella* (18S rRNA, 28S rRNA, MyoHC, rbp1, hsp70, COI, and 12S
705 rDNA) and one species of *Foleyellides* (COI). In the current study, a BI partitioned
706 phylogenetic analysis was conducted, based on a concatenated dataset of 18S rRNA and
707 COI gene sequences. Although these data revealed differences between *Neofoleyellides*
708 n. gen., *Foleyellides* and *Ochoterenella*, increased sampling of other species and genera
709 from Waltonellinae is required to obtain a more complete overview of the phylogenetic
710 relationships within this subfamily. It would be especially interesting to compare other
711 genera exclusively found parasitising African anurans, such as *Madochotera* and
712 *Paramadochotera*, to see if these genera cluster with *Neofoleyellides* n. gen. Furthermore,
713 members of Oswaldofilariinae, Icosiellinae and Waltonellinae did not from a monophyletic
714 clade as in Lefoulon et al. (2015), this could be due to better resolution provided by the
715 various genetic markers (18S rRNA, 28S rRNA, MyoHC, rbp1, hsp70 and COI, 12S rDNA)
716 used in the latter study.

717

718 *4.2 Host-vector and parasite relationships*

719 Recently, a number of general studies on parasites of African anurans and particularly the
720 guttural toad have been performed (Halajian et al., 2013; Kruger and Du Preez, 2015).
721 The current study is the first record of an adult filarial nematode from an anuran in South
722 Africa. Although in the current study these nematodes were only found in a single locality
723 (Sodwana), it is highly probable that these parasites have a wider distribution, considering

724 the distribution range of their vertebrate hosts and invertebrate vectors (Ingram and De
725 Meillon, 1927; Du Preez and Carruthers, 2017). It is also likely that the presence of these
726 parasites is highly dependent on their mosquito vectors, specialised in feeding on these
727 anurans. However, further investigations on the distribution, host specificity and ecology of
728 these mosquito species are required in order to test these hypotheses.

729 In all previous life cycle studies of representatives of *Foleyellides*, commonly
730 cultured species of mosquitoes of the genera *Aedes* and *Culex* were used, but these are
731 doubtfully the natural vectors in the life cycle of these filarial nematodes (Causey, 1939a;
732 Causey, 1939b; Witenberg and Gerichter, 1944). In the present study, two species of
733 *Uranotaenia* Lynch Arribálzaga, 1891 mosquitoes were used for life history and
734 transmission experiments. These mosquito species were selected based on *in situ*
735 observations made of them feeding on *S. gutturalis* at high prevalences (see Fig 7A-B).
736 Furthermore, we noted that these mosquito species were particularly attracted to the
737 calling male guttural toads. This observation is similar to other reports of species of
738 *Uranotaenia* attracted to calling anurans, namely, *U. unguiculata* Edwards, 1913 from
739 Europe (Camp et al., 2018), *U. lowii* Theobald, 1901 from Costa Rica (Borkent and Belton,
740 2006), and several species of *Uranotaenia* from Japan (Toma et al., 2014). Our
741 observations were based on two experiments using approximately 64 *U.* (*Pfc.*)
742 *mashonaensis* and 42 *U.* (*Pfc.*) *montana* as potential vectors and two highly parasitised
743 male *S. gutturalis* as the definitive host. Unfortunately, the natural conditions could not be
744 simulated *ex situ* and since attempts to culture these mosquito species failed (data not
745 shown), we had to rely on the mosquitoes collected *in situ* to complete the life history
746 experiments. Due to the limited number of mosquitoes collected, a sufficient sample size
747 of individual mosquitoes could not be examined each dpi. As only an average estimation
748 of the time of development can be given from the experiment in the current study, it is
749 essential to consider that this may not truly reflect the timing of development *in situ*.

750 Nonetheless, the data obtained from our experiment conforms to those from previously
751 published experiments, in that first-stage sausage-shaped larvae were observed from the
752 third dpi, and third-stage larvae from the 15th dpi. Although no larvae were observed in the
753 Malpighian tubes, distinct parts of the intestine, or coxal cavities of the legs, a few third
754 stage larvae were observed in the abdominal cavity of the mosquito host, with the majority
755 occurring in the thorax or head, and even in the proboscis. It is also important to note for
756 future experimental work that the mosquito species were highly sensitive to decreases in
757 the level of humidity (all specimens kept in plastic containers without moist cotton wool as
758 a source of water died within 4 hours) and temperature, which could also have affected the
759 rate of development of the different larval stages.

760 In terms of the host-vector and parasite relationships, our observations showed
761 strong evidence of the feeding preference of female *U. (Pfc.) mashonaensis* and *U. (Pfc.)*
762 *montana* to vocalising male *S. gutturalis*. Playing calls of other anurans (*S. garmani*,
763 *Sclerophrys poweri* (Hewitt, 1935), *Sch. carens*, *Leptopelis natalensis* (Smith, 1849),
764 *Phrynobatrachus nataliensis* (Smith, 1849), and *P. anchietae*) found in this area was not
765 as successful in attracting these mosquito species, with only the call of *S. garmani* working
766 but not as effective as the call of *S. gutturalis*. Once a suitable individual is located, the
767 mosquitoes seem to have the opportunity to take a blood meal with minimal interference
768 from the calling toad. This could prevent large numbers of mosquitoes from being
769 consumed by the toads (we observed toads consuming mosquitoes once calling bouts had
770 ended). However, more data are required before these host-vector relationships can be
771 explained with more certainty. No mosquitoes were observed feeding on female toads,
772 and only male *S. gutturalis* were parasitised by *Neofoleyellides boereworsi* n. gen. n. sp.,
773 with the exception of one female *S. garmani* (S1 Table, AE180124F1). The infection of
774 *Neofoleyellides boereworsi* n. gen. n. sp. in this female *S. garmani* seemed unlikely, since
775 female toads do not vocalise, thus having less chance of attracting the mosquito vectors.

776 The infection could have taken place when the female was mistaken for a calling male
777 during breeding activity, or if other vectors not attracted by the call are also able to transmit
778 *Neofoleyellides boereworsi* n. gen. n. sp. A similar situation of higher percentage of males
779 infected was observed for *Trypanosoma tungarae* Bernal and Pinto 2016, parasitising
780 Túngara frogs and potentially being transmitted by eavesdropping frog-biting midges
781 (Bernal and Pinto, 2016).
782 Furthermore, almost all the *U. (Pfc.) mashonaensis* and *U. (Pfc.) montana*
783 specimens which fed on infected *S. gutturalis* contained developing larvae of
784 *Neofoleyellides boereworsi* n. gen. n. sp., some individuals surviving with intensities of
785 more than 50 developing larvae after a single blood meal. However, the majority (n=8) of
786 mosquitoes that fed on one of the highly parasitised *S. gutturalis* (S1 Table, AE180313A1)
787 *in situ*, died one dpi, with only those individuals surviving that did not have sufficient blood
788 meals. This indicates that there may be a threshold in the intensity of infection that an
789 individual mosquito can survive. The high mortality rate of these individual mosquitoes so
790 early post infection could indeed be due to the effect of the microfilariae, which penetrate
791 the gut wall of their host. In the majority of the mosquitoes, larval development was
792 chronological, with only one scenario where sausage-shaped larvae co-occurred with
793 third-stage larvae in the body cavity at the same time. Also, in highly parasitised
794 individuals, development seemed to be gradually prolonged, in that not all the individual
795 larvae developed at the same rate, as compared to the development in mosquitoes with
796 less intense infections. These findings could indicate how *Neofoleyellides boereworsi* n.
797 gen. n. sp., has specifically adapted to maximise the period of available third-stage
798 infective larvae to be transmitted to a new host.
799
800 *4.3 Perspectives*

801 Although as mentioned above, third-stage larvae were found in the proboscis of
802 some of the mosquitoes dissected between 14 and 18 dpi, transmission attempts to
803 uninfected *S. gutturalis* hosts were unsuccessful due to mosquitoes not taking a second
804 blood meal. This could be due to a number of factors, such as mosquitoes not being kept
805 at optimal conditions. Likewise, since all mosquitoes were collected *in situ*, there was no
806 knowledge on the number of previous blood meals taken, and specimens could even have
807 been at the end of their life cycle. In addition, as all infected females were highly gravid,
808 conditions may not have been optimal for them to release their egg clutches, reducing the
809 need for an additional blood meal. Although the ultimate test for any potential vector is
810 transmission to a new and uninfected host, in this case, this may only be possible *ex situ*
811 using a large sample of laboratory cultured and reared mosquitoes under naturally-
812 simulated conditions. Several additional questions remain to be answered, such as: are
813 there any triggers that influence the intensity of microfilariae in blood, for example
814 increased testosterone in the breeding season, a chemical reaction caused from the bite
815 of a mosquito, or even the time of day? Elevated levels of testosterone have been shown
816 to increase parasite transmission potential, especially in male-biased host-parasite
817 occurrences (Grear et al., 2009; Cozzarolo et al., 2019). The avian malarial parasite
818 *Plasmodium relictum* (Grassi and Feletti, 1891) demonstrates increased parasitaemia in
819 hosts exposed to feeding mosquitoes as compared to hosts not exposed (Cornet et al.,
820 2014). This could be a result of chemical cues given off by the host in reaction to the bite
821 or by the parasites' ability to sense the density of mosquitoes feeding (Cornet et al., 2014).
822 Obviously there are several other hormones that could have the same affect, for example
823 species of *Isospora* Schneider, 1881 (Apicomplexa: Eimeriidae) have been shown to
824 synchronize their oocyst output with the nocturnally peaking hormone melatonin which
825 coordinates the hosts circadian rhythm (Dolnik et al., 2011; Martinez-Bakker and Helm,
826 2015). Other heteroxenous parasites have also been shown to maintain or increase

827 certain stages relative to the occurrence or rhythms of their vectors (Martinez-Bakker and
828 Helm, 2015). Microfilariae of *Wuchereria bancrofti* (Cobbold, 1877) are known to alter in
829 intensity based on the activity of the vectors and the time of day (Reece et al., 2017).
830 These scenarios could be the same for anuran filarial nematodes and their host species.
831 Research on vector biology is important for not only for the specific species in question,
832 but it also provides valuable information for other related species, which could provide
833 insights to lager questions or even control efforts of other vector-borne diseases
834 (Valenzuela and Aksoy, 2018).
835
836 *4.4 Concluding remarks*
837 The present study contributes to the limited knowledge on the biodiversity, distribution,
838 evolution, and ecology of this group of neglected anuran parasites. Due to the lack of data
839 on these parasites, the application of molecular methods, as well as life cycle elucidation
840 through natural intermediate hosts, are necessary to gain better knowledge of their
841 phylogenetic relationships, the ecology of these parasites *in situ*, and to be able to link
842 different life stages from various hosts to a particular species. Furthermore, molecular
843 tools are valuable in identifying species, in large scale screening of hosts and possible
844 vectors. Genetic data could shed more light on the history of the interactions between
845 these onchocercids and their amphibian hosts. To obtain our data for this study only a few
846 selected amphibian individuals were sacrificed to yield a maximum number of data.
847 Furthermore, our study provides a template for a full taxonomical account, which includes
848 morphological and molecular data, as well as an approach to elucidate the life history of
849 anuran filarial nematodes such as *Neofoleyellides boereworsi* n. gen. n. sp.

Acknowledgements

We are grateful to Prof M. Coetzee (Wits Research Institute for Malaria, University of the Witwatersrand, South Africa) for assisting in the identification of mosquito vectors, W. Pretorius, for help in the field, W. Landman for SEM work, P. and C. van Jaarsveld, for accommodation and site identification, and to Prof. T. Huyse, Prof. J. Snoeks, Prof. T. Artois and Prof. F. Volckaert for comments on a previous version of this manuscript. Ezemvelo KZN Wildlife is thanked for research permits OP 526/2014, OP 839/2014, OP 4374/2015, OP 4092/2016, and OP 4085/2017. This is contribution number XXX of the North-West University (NWU) Water Research Group.

Funding

This paper forms part of a VLIR-UOS TEAM funded project (ZEIN21013PR396), co-funded by the National Research Foundation (NRF) of South Africa (NRF project CPRR160429163437, grant 105979, NJ Smit, PI). The financial assistance of the NRF towards CAC is also hereby acknowledged (project SFP13090332476), and ECN who is supported by the DAAD-NRF doctoral scholarship (Grant UID: 108803), and the VLIR-OUS university scholarship (ID 0620854 / Contract 000000076310). At the onset of this research, MPMV was supported by the Belgian Directorate-General for Development Cooperation and Humanitarian Aid (CEBioS program at the Royal Belgian Institute of Natural Sciences). Opinions expressed, and conclusions arrived at, are those of the authors and not necessarily those of the funding bodies. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Aisien, M.S.O., Aigbirior, P.O., Ovwah, E., Edo-Taiwo, O., 2015. Blood parasites of some Anurans from southern Nigeria. Trop. Biomed 32, 598–607.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

AmphibiaWeb, 2018. AmphibiaWeb, 2018 ed, University of California, Berkeley, CA, USA.

Anderson, R.C., 1958. On the classification of the Filarioidea with special reference to the Filariidae and the Stephanofilariidae. Bull. Soc. Zool. Fr. 83, 144–157.

Anderson, R.C., Bain, O., 1976. Keys to genera of the Order Spirurida. Part 3. Diplotriaenoidea, Aproctoidea and Filarioidea, in: Anderson, R.C., Chabaud, A.G., Willmott, S. (Eds.), CIH Keys to the Nematode Parasites of Vertebrates. No. 3. Commonwealth Agricultural Bureaux, Bucks, England, pp. 59–116.

Bain, O., 2002. Evolutionary Relationships Among Filarial Nematodes, in: Klei, T.R., Rajan, T. (Eds.), World Class Parasites: The Filaria. Springer US, Boston, MA, pp. 21–29.

Bain, O., Brunhes, J., 1968. Un nouveau genre de filaire, parasite de grenouilles malgaches. Bulletin du Museum National d'Histoire Naturelle 40, 797–801.

Bain, O., Kim, D.C., Petit, G., 1979. Diversité spécifique des filaires du genre *Waltonella* coexistant chez *Bufo marinus*. Bull. Mus. Natl. Hist. Nat. 4, 199–212.

Bain, O., Mutafchiev, Y., Junker, K., 2013. Order Spirurida, in: Schmidt-Rhaesa, A. (Ed.), Handbook of Zoology: Gastrotricha, Cycloneuralia and Gnathifera: Nematoda. De Gruyter, Berlin, Germany, pp. 661–732.

Bain, O., Prod'Hon, J., 1974. Homogénéité des Filaires de Batraciens des genres *Waltonella*, *Ochoterenella* et *Madochotera*; création des Waltonellinae n. subfam. Ann. Parasitol. Hum. Comp. 49, 721–739.

Bernal, X.E., Pinto, C.M., 2016. Sexual differences in prevalence of a new species of trypanosome infecting túngara frogs. Int. J. Parasitol. Parasites Wildl. 5, 40–47.

Borkent, A., Belton, P., 2006. Attraction of female *Uranotaenia lowii* (Diptera: Culicidae) to frog calls in Costa Rica. Can. Entomol. 138, 91–94.

Brenes, R.R., Hollis, M.B., 1959. Helmintos de la República de Costa Rica VIII. Nematoda 2. Algunos nemátodes de *Bufo marinus marinus* (L) y algunas consideraciones sobre los géneros *Oxysomatium* y *Aplectana*. Rev. Biol. Trop 7, 35–55.

Caballero, E.C., 1935. Nemátodos parásitos de los batracios de México III. Cuarta contribución al conocimiento de la Parasitología de *Rana montezumae*. An. Inst. Biol. Univ. Nac. Auton. Mex. Ser. Zool. 6, 103–117.

Caballero, E.C., 1944. Estudios helmintologicos de la region oncocercosa de Mexico y de la Republica de Guatemala. Nematoda: Primeira parte. Filarioidea. I. An. Inst. Biol. Univ. Nac. Auton. Mex. Ser. Zool. 15, 87–108.

Camp, J.V., Bakonyi, T., Soltész, Z., Zechmeister, T., Nowotny, N., 2018. *Uranotaenia unguiculata* Edwards, 1913 are attracted to sound, feed on amphibians, and are infected with multiple viruses. Parasit. Vectors. 11, 456.

Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.

Causey, O.R., 1939a. Description of three species of frog microfilariae, with notes on staining methods. Am. J. Hyg. 30, 117-121.

Causey, O.R., 1939b. The development of frog Filaria Larvae, *Foleyella ranae* in *Aëdes* and *Culex* Mosquitoes. Am. J. Hyg. 29, 131–132.

Causey, O.R., 1939c. Development of the larval stages of *Foleyella brachyoptera* in mosquitoes. Am. J. Epidemiol. 30, 69–71.

Conradie, R., Cook, C.A., Preez, L.H., Jordaan, A., Netherlands, E.C., 2017. Ultrastructural comparison of *Hepatozoon ixoxo* and *Hepatozoon theileri* (Adeleorina: Hepatozoidae), parasitising South African anurans. J. Eukaryot. Microbiol. 64, 193–203.

Cook, C.A., Netherlands, E.C., Smit, N.J., 2015. First *Hemolivia* from southern Africa: reassigning chelonian *Haemogregarina parvula* Dias, 1953 (Adeleorina:

928 Haemogregarinidae) to *Hemolivia* (Adeleorina: Karyolysidae). Afr. Zool. 50, 165–
929 173.
930 Cook, C.A., Netherlands, E.C., Smit, N.J., 2016. Redescription, molecular characterisation
931 and taxonomic re-evaluation of a unique African monitor lizard haemogregarine
932 *Karyolysus paradoxa* (Dias, 1954) n. comb. (Karyolysidae). Parasit. Vectors. 9, 347.
933 Cook, C.A., Netherlands, E.C., Van As, J., Smit, N.J., 2018. Two new species of
934 *Hepatozoon* (Apicomplexa: Hepatozoidae) parasitising species of *Philothamnus*
935 (Ophidia: Colubridae) from South Africa. Folia Parasitol. 65, 1–11.
936 Cornet, S., Nicot, A., Rivero, A., Gandon, S., 2014. Evolution of plastic transmission
937 strategies in avian malaria. PLoS Path. 10, e1004308.
938 Cozzarolo, C.-S., Sironi, N., Glaizot, O., Pigeault, R., Christe, P., 2019. Sex-biased
939 parasitism in vector-borne disease: Vector preference? PLoS ONE 14, e0216360.
940 Crans, W.J., 1969. Preliminary observations of frog filariasis in New Jersey. Bull. Wildl.
941 Dis. Assoc. 5, 342–347.
942 Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new
943 heuristics and parallel computing. Nat. Methods 9, 772.
944 Dolnik, O.V., Metzger, B.J., Loonen, M.J., 2011. Keeping the clock set under the midnight
945 sun: diurnal periodicity and synchrony of avian Isospora parasites cycle in the High
946 Arctic. Parasitology 138, 1077–1081.
947 Du Preez, L.H., Carruthers, V., 2009. A complete guide to the frogs of southern Africa.
948 Struik Nature, Cape Town.
949 Du Preez, L.H., Carruthers, V., 2017. Frogs of Southern Africa: A Complete Guide.
950 Penguin Random House.
951 Dyer, W.G., Altig, R., 1977. Helminths of some Ecuadorian anurans. Herpetologica 33,
952 293–296.

Esslinger, J.H., 1986a. Redescription of *Foleyellides striatus* (Ochoterena and Caballero, 1932) (Nematoda, Filarioidea) from a Mexican frog, *Rana montezumae*, with reinstatement of the genus *Foleyellides* Caballero, 1935. Proc. Helminthol. Soc. Wash 53, 218–223.

Esslinger, J.H., 1986b. Redescription of *Ochoterenella digiticauda* Caballero, 1944 (Nematoda: Filarioidea) from the toad, *Bufo marinus*, with a redefinition of the genus *Ochoterenella* Caballero, 1944. Proc. Helminthol. Soc. Wash 53, 210–217.

Esslinger, J.H., 1987. *Ochoterenella caballeroi* sp. n. and *O. nanolarvata* sp. n. (Nematoda: Filarioidea) from the toad *Bufo marinus*. Proc. Helminthol. Soc. Wash 54, 126–132.

Esslinger, J.H., 1988a. *Ochoterenella chiapensis* n. sp. (Nematoda: Filarioidea) from the toad *Bufo marinus* in Mexico and Guatemala. Trans. Am. Microsc. Soc. 107, 203–208.

Esslinger, J.H., 1988b. *Ochoterenella figueroai* sp. n. and *O. lamothei* sp. n. (Nematoda: Filarioidea) from the toad *Bufo marinus*. Proc. Helminthol. Soc. Wash 55, 146–154.

Esslinger, J.H., 1989. *Ochoterenella complicata* n. sp. (Nematoda: Filarioidea) from the toad *Bufo marinus* in Western Colombia. Trans. Am. Microsc. Soc. 108, 197–203.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1994. Testing significance of incongruence. Cladistics 10, 315–319.

García-Prieto, L., Ruiz-Torres, N., Osorio-Sarabia, D., Merlo-Serna, A., 2014. *Foleyellides rhinellae* sp. nov. (Nematoda, Onchocercidae) a new filaria parasitizing *Rhinella marina* (Anura, Bufonidae) in Mexico. Acta Parasitol. 59, 478–484.

Gedoelst, L., 1916. Notes sur la faune parasitaire du Congo Beige. Rev. Zool. Afr 5, 1–90.

Grear, D.A., Perkins, S.E., Hudson, P.J., 2009. Does elevated testosterone result in increased exposure and transmission of parasites? Ecol. Lett. 12, 528–537.

978 Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large
979 phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.
980 Halajian, A., Bursey, C.R., Goldberg, S.R., Luus-Powell, W., 2013. Helminths of six
981 species of anurans from the Republic of South Africa: *Amietophrynus garmani*,
982 *Amietophrynus gutturalis*, *Amietophrynus maculatus*, *Schismaderma carens*
983 (Bufonidae), *Amietia angolensis*, and *Strongylopus grayii* (Pyxicephalidae), with a
984 review of South African anuran helminths. Comp. Parasitol. 80, 80–95.
985 Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic trees.
986 Bioinformatics 17, 754–755.
987 Ingram, A., De Meillon, B., 1927. A Mosquito Survey of Certain Parts of South Africa, with
988 Special Reference to the Carriers of Malaria and their Control. Part I. South African
989 Institute for Medical Research 4, 1-81.
990 Jupp, P.G., 1996. Mosquitoes of Southern Africa: Culicinae and Toxorhynchitinae.
991 Ekogilde Publishers, Hartebeespoort, South Africa.
992 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S.,
993 Cooper, A., Markowitz, S., Duran, C., 2012. Geneious Basic: an integrated and
994 extendable desktop software platform for the organization and analysis of sequence
995 data. Bioinformatics 28, 1647–1649.
996 Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions
997 through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
998 Kruger, N., Du Preez, L.H., 2015. Reproductive strategies of the kangaroo leech,
999 *Marsupiobdella africana* (Glossiphoniidae). Int. J. Parasitol. Parasites Wildl. 4, 142–
1000 147.
1001 Larkin, M.A., Blackshields, G., Brown, N., Chenna, R., McGettigan, P.A., McWilliam, H.,
1002 Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., 2007. Clustal W and Clustal X
1003 version 2.0. Bioinformatics 23, 2947–2948.

1004 Lefoulon, E., Bain, O., Bourret, J., Junker, K., Guerrero, R., Cañizales, I., Kuzmin, Y.,
1005 Satoto, T.B.T., Cardenas-Callirgos, J.M., de Souza Lima, S., Raccurt, C.,
1006 Mutafchiev, Y., Gavotte, L., Martin, C., 2015. Shaking the tree: multi-locus
1007 sequence typing usurps current onchocercid (filarial nematode) phylogeny. PLoS
1008 Negl. Trop. Dis. 9, e0004233.
1009 López-Neyra, R.C., 1956. Revision de la superfamilia Filarioidea (Weinland, 1858). Rev.
1010 Iber. Parasitol 16, 1–225.
1011 Marinkelle, C.J., 1970. Observaciones sobre la periodicidad de las microfilarias de
1012 *Ochoterenella* en *Bufo marinus* de Colombia. Rev. Biol. Trop 16, 145–152.
1013 Martinez-Bakker, M., Helm, B., 2015. The influence of biological rhythms on host–parasite
1014 interactions. Trends Ecol. Evol. 30, 314–326.
1015 Masi Pallares, R., Maciel, S., 1974. Helminthes en batracios del Paraguay (1ra. Parte),
1016 con descripción de una nueva especie *Aplectana pudenda* (Oxyuridae:
1017 Cosmocercinae). Rev. Paraguaya Microbiol. 9, 55–60.
1018 Molin, R., 1858. Versuch einer Monographie der Filarien. Sitzber. Saechs. Akad. Wiss.
1019 Leipz. Math-Natwiss. Kl 28, 365–461.
1020 Netherlands, E.C., Cook, C.A., Du Preez, L.H., Vanhove, M.P.M., Brendonck, L., Smit,
1021 N.J., 2018. Monophyly of the species of *Hepatozoon* (Adeleorina: Hepatozoidae)
1022 parasitizing (African) anurans, with the description of three new species from
1023 hyperoliid frogs in South Africa. Parasitology 145, 1039–1050.
1024 Netherlands, E.C., Cook, C.A., Kruger, D.J.D., Du Preez, L.H., Smit, N.J., 2015.
1025 Biodiversity of frog haemoparasites from sub-tropical northern KwaZulu-Natal,
1026 South Africa. Int. J. Parasitol. Parasites Wildl. 4, 135–141.
1027 Netherlands, E.C., Cook, C.A., Smit, N.J., 2014. *Hepatozoon* species (Adeleorina:
1028 Hepatozoidae) of African bufonids, with morphological description and molecular

1029 diagnosis of *Hepatozoon ixoxo* sp. nov. parasitising three *Amietophrynus* species
1030 (Anura: Bufonidae). Parasit. Vectors. 7, 552.
1031 Petit, G., Yen, P.K.F., 1979. *Waltonella malayensis* n. sp. une. nouvelle filaire de
1032 Batracien, en Malaisie. Bull. Mus. Natl. Hist. Nat. 1, 213–218.
1033 Prod'hon, J., Bain, O., 1974. *Madochotera landauae* n. sp., nouvelle Filaire de Grenouille
1034 malgache. Bull. Mus. Natl. Hist. Nat. 156, 813–817.
1035 Purnomo, Bangs, M.J., 1999. *Paraochoterenetta javanensis* gen. et sp. n. (Filarioidea:
1036 Onchocercidae) from *Rana cancrivora* (Amphibia: Anura) in West Java, Indonesia.
1037 J. Helminthol. Soc. Wash. 66, 187–193.
1038 Rambaut, A., Drummond, A.J., Xie, D., Baele, G., Suchard, M.A., 2018. Posterior
1039 summarisation in Bayesian phylogenetics using Tracer 1.7. Syst. Biol. 67, 901–904.
1040 Readel, A.M., Goldberg, T.L., 2010. Blood parasites of frogs from an equatorial African
1041 montane forest in western Uganda. J. Parasitol. 96, 448–450.
1042 Reece, S.E., Prior, K.F., Mideo, N., 2017. The life and times of parasites: rhythms in
1043 strategies for within-host survival and between-host transmission. J. Biol. Rhythms
1044 32, 516–533.
1045 Romero-Mayen, A.R., Leon-Regagnon, V., 2016. A new species of *Foleyellides*
1046 (Nematoda: Onchocercidae) parasite of *Lithobates* spp.(Amphibia: Ranidae) from
1047 Mexico with a key for the species of the genus. Zootaxa 4170, 581–586.
1048 Schacher, J.F., 1975. *Waltonella*, nom. n. for subgenus *Waltonia* (Nematoda: Filaroidea)
1049 Schacher and Crans, 1973, preoccupied by *Waltonia* Davidson, 1850
1050 (Brachiopoda). J. Parasitol. 61, 58.
1051 Schacher, J.F., Crans, W.J., 1973. *Foleyella flexicauda* sp. n. (Nematoda: Filarioidea) from
1052 *Rana catesbeiana* in New Jersey, with a review of the genus and erection of two
1053 new subgenera. J. Parasitol. 59, 685–691.

1054 Schmidt, G.D., Kuntz, R.E., 1969. Nematode parasites of Oceanica. VI. *Foleyella confusa*
1055 sp. nov., *Icosiella hoogstraali* sp. nov.(Filarioidea), and other species from
1056 Philippine amphibians. Parasitology 59, 885–889.
1057 Seurat, L.G., 1917. Filaires des Reptiles et des Batraciens. Bull. Soc. Hist. Nat. Afr. Nord
1058 8, 236–242.
1059 Sonin, M.D., 1968. Filariata of animals and man and diseases caused by them. Part 2.
1060 Diplotriaenoidea., in: Skrjabin, K.I. (Ed.), Essentials of Nematodology. Academy of
1061 Sciences of the URSS, Moscow, pp. 243–244.
1062 Souza, L.S., Marun, B., Alves, P.V., Bain, O., 2012. *Ochoterenella esslingeri* n.
1063 sp.(Nematoda: Onchocercidae: Waltonellinae) from *Bokermannohyla luctuosa*
1064 (Anura: Hylidae) in Minas Gerais, Brazil, with notes on *Paraochoterenella* Purnomo
1065 & Bangs, 1999. Parasite 19, 341–350.
1066 Swofford, D.L., 2002. PAUP*. Phylogenetic analysis using parsimony (* and other
1067 methods), Ver. 4. Sinauer Associates, Sunderland, Massachusetts.
1068 Talavera, G., Castresana, J., 2007. Improvement of phylogenies after removing divergent
1069 and ambiguously aligned blocks from protein sequence alignments. Syst. Biol. 56,
1070 564–577.
1071 Tavaré, S., 1986. Some probabilistic and statistical problems in the analysis of DNA
1072 sequences. Lect. Math. Life Sci. 17, 57–86.
1073 Todd, W.J., 1986. Effects of specimen preparation on the apparent ultrastructure of
1074 microorganisms. Springer.
1075 Toma, T., Miyagi, I., Tamashiro, M., 2014. Blood meal identification and feeding habits of
1076 *Uranotaenia* species collected in the Ryukyu Archipelago. J. Am. Mosq. Control
1077 Assoc. 30, 215–218.
1078 Travassos, L., 1929. Filaridés des batraciens du Brésil. C. R. Seances Soc. Biol. 100,
1079 967–968.

Travassos, L., de Freitas, J.F.T., 1960. Excursão a Maicurú, Estado do Pará. Atas Soc. Biol. Rio. J. 4, 13–15.

Valenzuela, J.G., Aksoy, S., 2018. Impact of vector biology research on old and emerging neglected tropical diseases. PLoS Negl. Trop. Dis. 12, e0006365.

Vicente, J.J., Jardim, C.R., 1980. Filarideos da coleção helmintologica do Institute Oswaldo Cruz. I. Peixes, anfibios e répteis. Atas Soc. Biol. Rio. J. 21, 47–57.

Vicente, J.J., Santos, E., 1976. Fauna helmintológica de *Leptodactylus ocellatus* (L., 1758) de Volta Redonda, Estado do Rio de Janeiro. Atas Soc. Biol. Rio. J. 18, 27–42.

Walton, A.C., 1929. Studies on some nematodes of North American frogs. I. J. Parasitol. 15, 227–240.

Wehr, E.E., Causey, O.R., 1939. Two new Nematodes (Filarioidea: Dipetalonematidae) from *Rana sphenocephala*. Am. J. Hyg. 30, 65–68.

Witenberg, G., Gerichter, C.H., 1944. The morphology and life history of *Foleyella duboisi* with remarks on allied filariids of amphibia. J. Parasitol. 30, 245–256.

Yamaguti, S., 1961. Systema Helminthum. Interscience Publishers.

Legends to figures

Fig 1. Illustrations of adult and larval stages *Neofoleyellides boereworsi* n. gen. n. sp. A – female, anterior part of body, lateral view; B – female, anterior part of body, apical view; C – male, anterior part of body, lateral view; D – female, posterior part of body, lateral view; E – first-stage sausage-shape larva, lateral view; F – second-stage larva, lateral view; G – third-stage larva, lateral view; H – male, spicules, lateral view; I – male, posterior part of body, ventral view. Scale bars: A, C, D, F-H – 100; B, E – 20; I – 50.

Fig 2. Photomicrographs of *Neofoleyellides boereworsi* n. gen. n. sp. male. A – anterior part of body, apical view (SEM image); B – entire body, lateral view; C – anterior

1106 part of body, lateral view; D – cross-section at mid-body level; E – posterior part of body,
1107 lateral view; F – posterior part of body, lateral view (SEM image). Scale bars: A – 10; B –
1108 1mm; C – 100; D-F – 50.
1109
1110 **Fig 3. Photomicrographs of *Neofoleyellides boereworsi* n. gen. n. sp. female.** A –
1111 entire body, lateral view; B – anterior part of body, lateral view; C – anterior part of body,
1112 head region, lateral view; D – anterior part of body, apical view (SEM image); E – posterior
1113 part of body, lateral view; F – region of vulva, ventral view (SEM image). Scale bars: A –
1114 2mm; B, E – 100; C, D, F – 20.
1115
1116 **Fig 4. Photomicrographs of *Neofoleyellides boereworsi* n. gen. n. sp. stained larvae.**
1117 A – microfilaria from amphibian blood; B – unsheathed microfilaria from mosquito blood
1118 meal; C – sausage-shape first stage. All images captured from the deposited slides (XXX)
1119 Scale bar: 10μm.
1120
1121 **Fig 5. Photomicrographs of *Neofoleyellides boereworsi* n. gen. n. sp. larval stages.**
1122 A – exsheathed microfilaria from amphibian blood, anterior part of body, subdorsal view
1123 (SEM image); B – sheathed microfilaria from amphibian blood, anterior part of body, lateral
1124 view (SEM image); C – sheathed microfilaria from amphibian blood, entire body, subdorsal
1125 view (SEM image); D – early sausage-shape first-stage, entire body, lateral view (SEM
1126 image); E – late sausage-shape first-stage, entire body, lateral view; F – second-stage,
1127 anterior part of body, apical view (SEM image); G – second-stage, entire body, lateral
1128 view; H – third-stage, entire body, lateral view. t – tooth, cs – crown-like structure. Scale
1129 bars: A-D, F – 10; E-H – 100.
1130

1131 **Fig 6. Phylogeny of selected filarial nematodes from Onchocercidae, based on**
1132 **partitioned concatenated datasets of 18S rDNA, and COI sequences using Bayesian**
1133 **Inference.** The phylogram shows the relationship of *Neofoleyellides boereworsi* n. gen. n.
1134 sp., compared to species of anuran and other onchocercids. *Filaria latala* [GenBank: 18S:
1135 KP760135 and COI: KP760186] was chosen as outgroup. Clades with posterior probability
1136 support values lower than 0.80 were removed. The total length of datasets is 1136 nt, and
1137 containing 54 taxa. Pictograms are used to illustrate host taxa. The scale bar represents
1138 0.05 nucleotide substitutions per site.
1139
1140 **Fig 7. Mosquito vectors *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *U.* (*Pfc.*)**
1141 ***montana* feeding on the host *Sclerophrys gutturalis in situ*, as well as the infected**
1142 **eye of one highly parasitised individual.** A – Several *U.* (*Pfc.*) *mashonaensis* and *U.*
1143 (*Pfc.*) *montana* (arrows) taking a blood meal from a male *S. gutturalis in situ*. B – Close up
1144 photograph of engorged mosquito (arrow) taking its blood meal from *S. gutturalis*. C –
1145 *Neofoleyellides boereworsi* n. gen. n. sp. (arrow) infecting the eye of the definitive guttural
1146 toad host, *S. gutturalis.* D – Close up photograph showing *S. gutturalis* infected eye with
1147 *Neofoleyellides boereworsi* n. gen. n. sp. (arrow) compared to uninfected eye.
1148
1149 **Fig 8. Graphical representation of the life history of *Neofoleyellides boereworsi* n.**
1150 **gen. n. sp. in the invertebrate mosquito vectors *Uranotaenia* (*Pseudoficalbia*)**
1151 ***mashonaensis* and *U.* (*Pfc.*) *montana*, and in the vertebrate definitive host**
1152 ***Sclerophrys gutturalis*.** A-D – *Neofoleyellides boereworsi* n. gen. n. sp. development in
1153 the mosquito vector. A – Represents unsheathed microfilaria in the blood meal of the
1154 mosquito, observed up to three days post infection (dpi). B – Sausage-shaped first-stage
1155 larvae, observed from between three and seven dpi. C – Second-stage larvae, observed
1156 from between six and 14 dpi. D – Third-stage infective larvae, observed from between 14

and 18 dpi. E-F – *Neofoleyellides boereworsi* n. gen. n. sp. development in the definitive guttural toad host, *S. gutturalis*. E – Male and female adult stages. F – Sheathed microfilaria in the peripheral blood of *S. gutturalis*. Images not drawn to scale.

Supporting Information

S1 Table. Sampling data of toads collected from several sampling localities in South Africa. Table shows host field number, species, positive, locality, coordinates, and sex.

1167 **Table 1: Summary of valid species of Waltonellinae, with type host, host family and type country.** Type host according to

1168 AmphibiaWeb (2018)

Genus	Species and Authority († = type species)	Type host (syn.)	Host family	Country of type locality	Reference
Foleyellides Caballero, 1935	*Foleyellides mayenae* Romero-Mayén and León-Règagnon, 2016	*Rana psilonota* (syn. *Lithobates psilonota*)	Ranidae	Mexico	(Romero-Mayen and Leon-Regagnon, 2016)
	Foleyellides americana (Walton, 1929)	*Rana pipiens* (syn. *Lithobates pipiens*)	Ranidae	USA	(Walton, 1929)
	Foleyellides brachyoptera (Wehr and Causey, 1939)	*Rana sphenocephala* (syn. *Lithobates sphenocephalus*)	Ranidae	USA	Wehr and Causey (1939)
	Foleyellides confusa (Schmidt and	*Fejervarya vittigera* (syn.	Dicroglossidae	Philippines	Schmidt and Kuntz

Kuntz, 1969)	*Rana limnocharis vittigera*)			(1969)
Foleyellides dolichoptera (Wehr and Causey, 1939)	*R. sphenocephala* (syn. *L. sphenocephalus*)	Ranidae	USA	(Wehr and Causey, 1939)
Foleyellides duboisi (Gedoelst, 1916)	*Pelophylax ridibundus* (syn. *Rana esculenta ridibunda*)	Ranidae	DRC	Gedoelst (1916)
Foleyellides flexicauda (Schacher and Crans, 1973)	*Rana catesbeiana* (syn. *Lithobates catesbeianus*)	Ranidae	USA	Schacher and Crans (1973)
Foleyellides malayensis (Petit and Yen, 1979)	*Pulchrana glandulosa* (syn. *Rana glandulosa*)	Ranidae	Malaysia	Petit and Yen (1979)
Foleyellides ranae (Walton, 1929)	*R. catesbeiana* (syn. *L. catesbeianus*)	Ranidae	USA	Walton (1929)
Foleyellides rhinellae García-Prieto, Ruiz-Torres, Osorio Sarabia and	*Rhinella marina*	Bufonidae	Mexico	García-Prieto et

	Merlo-Serna, 2014				al. (2014)
	Foleyellides striatus (Ochoterena and Caballero, 1932) †	*Rana montezumae* (syn. *Lithobates montezumae*)	Ranidae	Mexico	Esslinger (1986a)
Madochotera Bain and Brunhes, 1968	*Madochotera alata* Bain and Brunhes, 1968 †	*Rhacophorus* sp.	Racophoridae	Madagascar	Bain and Brunhes (1968)
	Madochotera landauae Prod'hon and Bain, 1974	*Rhacophorus* sp.	Racophoridae	Madagascar	Prod'hon and Bain (1974)
	Madochotera pichoni Bain and Brunhes, 1968	*Rhacophorus* sp.	Racophoridae	Madagascar	Bain and Brunhes (1968)
Ochoterenella Caballero,	*Ochoterenella albareti* (Bain, Kim and Petit, 1979)	*R. marina* (syn. *Bufo marinus*)	Bufonidae	French Guyana	Bain et al. (1979)

Ochoterenella caballeroi Esslinger, 1987	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico	Esslinger (1987)
Ochoterenella chiapensis Esslinger, 1988	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico	Esslinger (1988a)
Ochoterenella complicata Esslinger, 1989	*R. marina* (syn. *B. marinus*)	Bufonidae	Columbia	Esslinger (1989)
Ochoterenella convoluta (Molin, 1858)	*Leptodactylus pentadactylus*	Leptodactylidae	Brazil	Molin (1858)
Ochoterenella digiticaudata Caballero, 1944 †	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico	Caballero (1944)
Ochoterenella dufourae (Bain, Kim and Petit, 1979)	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana	Bain et al. (1979)
Ochoterenella esslingeri Souza Lima and Bain, 2012	*Bokermannohyla luctuosa*	Hylidae	Brazil	(Souza et al., 2012)

Ochoterenella figueroai Esslinger, 1988	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana	Esslinger (1988b)
Ochoterenella guyanensis (Bain and Prod'Hon, 1974)	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana	Bain and Prod'Hon (1974)
Ochoterenella lamothei Esslinger, 1988	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico	Esslinger (1988b)
Ochoterenella nanolarvata Esslinger, 1987	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico	Esslinger (1987)
Ochoterenella oumari (Bain, Kim and Petit, 1979)	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana	Bain et al. (1979)
Ochoterenella royi (Bain, Kim and Petit, 1979)	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana	Bain et al. (1979)
Ochoterenella scalaris (Travassos, 1929)	*Leptodactylus macrosternum* (syn. *L. ocellatus*)	Leptodactylidae	Brazil	Travassos (1929)

	Ochoterenella vellardi (Travassos, 1929)	*R. marina* (syn. *B. marinus*)	Bufonidae	Brazil	Travassos (1929)
Paramadochotera (Bain and Prod'Hon, 1974)	*Paramadochotera guibei* (Bain and Prod'Hon, 1974) †	*Gephyromantis redimitus* (syn. *Mantidactylus redimitus*)	Mantellidae	Madagascar	Bain and Prod'Hon (1974)
Paraochoterenella Purnomo and Bangs, 1999	*Paraochoterenella javanensis* Purnomo and Bangs, 1999†	*Fejervarya cancrivora* (syn. *Rana cancrivora*)	Dicroglossidae	Indonesia	Purnomo and Bangs (1999)

1169 * USA = United States of America; DRC = Democratic Republic of the Congo

1170

1171

1172

1173

1174

1175 **Table 2. Step by step summary of field sampling of infected toads and mosquito collection *in situ* and experimental**

1176 **transmission and life cycle elucidation *ex situ*.**

November 2017	Field sampling	• Sampling took place in Sodwana Bay at site SB-1 (S27.488591°; E32.664259°). • Collect *Sclerophrys gutturalis* (n = 8) and blood samples as detailed earlier. • During sampling, mosquitoes observed feeding on calling *S. gutturalis*. • Blood fed mosquitoes (n = 5) were collected using an aspirator and temporarily housed in small glass jars with moist cotton wool.
	Laboratory	• Collected mosquitoes identified using Jupp (1996) – fixed whole in 70% ethanol. • Two *S. gutturalis* parasitised with microfilaria – euthanized using tricaine methanesulfonate (MS222) solution and dissected. • Adult filarial nematodes removed from body cavity, washed in saline, and fixed hot and stored in 70% ethanol.
January & March 2018 field sampling	Host collection	• Two subsequent sampling expeditions to Sodwana Bay followed. • Collected toads *S. gutturalis* (n = 35) and *S. garmani* (n = 3) – transported to a field workstation to be processed and blood screened as detailed earlier. • Positive specimens were kept in individual containers – the rest released at capture site.

Vector collection	• Infected toads (n = 3) were transported back to site SB-1 to be used as enticement for collecting mosquitoes *in situ*.
	• Mosquitoes were collected:
	  ○ Via a modified portable Centre for Disease Control (CDC) mosquito trap - fitted with a speaker (instead of a light) to play the call of *S. gutturalis* as a lure.
	  ○ Using an aspirator – directly from infected *S. gutturalis* enticed to call using playbacks (playing of the call back through a speaker).
	  ○ With a glass tank fixed with a fine mesh funnel and baited with an infected *S. gutturalis* enticed to call.
	• Calls from several anuran species from the area were played with CDC trap without the same success.

Laboratory phase following January & March 2018 field sampling	• Three individuals, two parasitised with microfilariae and one not infected – transported to NWU frog lab and housed in vivarium to monitor and complete the life history observations *ex situ.* • Collected mosquitoes (n = 146) were maintained in plastic jars (350 ml) lined at the base with moist cotton wool and transported to NWU. • Mosquitoes collected that did not take a blood meal *in situ*, were released in a glass tank containing a highly infected guttural toad *ex situ*. • Mosquitoes were enticed to feed on the infected toads in the lab at night using playbacks for approximately 4–8 hours. • All engorged mosquitoes were kept separately, according to when their blood meal was taken, and supplied daily with fresh water and a 10% sucrose solution. • At least one mosquito was dissected every day for the first five dpi, followed by one or two mosquitoes being successively dissected every three days, spanning a period of 20 days. • This experiment was repeated twice (January and March 2018 field sampling) – fatalities were dissected as soon as possible post-mortem.

1177

1178

1179

1180 **Table 3. Summary of filarial species, their host, accession numbers and locality, used in phylogenetic analyses in this study.**

Subfamilies	Species	Definitive host	Accession numbers (18S; COI)		Locality
Dirofilariinae	*Dofilaria immitis* (Leidy, 1856)	*Canis familiaris*	KP760134	KP760185	Italy
	Foleyella candezei (Fraipont, 1882)	*Agama agama*	KP760136	KP760187	Togo
	Foleyella candezei	*Agama agama*		FR823336	Togo
	Foleyella furcata (Linstow, 1899)	*Furcifer oustaleti*		KM234627	Madagascar
	Foleyella furcata	*Furcifer* sp.		KM234628	Madagascar
	Pelecitus fulicaeatrae (Diesing, 1861)	*Podiceps nigricollis*	KP760161	KP760206	Spain
	Loa loa (Cobbold, 1864)	*Homo sapiens*	KP760143	KP760194	France
Icosiellinae	*Icosiella neglecta* (Diesing, 1851)	*Pelophylax ridibunda*	KP760137	KP760188	Ukraine
	Icosiella neglecta	*Pelophylax* kl. *esculeta*	KP760138	KP760189	France
	Icosiella sp.	*Conraua goliath*		MH182623	Cameroon

Onchocercinea	*Acanthocheilonema odendhali* (Perry, 1967)	*Callorhinus ursinus*	KP760116	KP760168	Alaska
	Acanthocheilonema vitaea (Krepkogorskaya, 1933)	*Meriones unguiculatus*	KP760117	KP760169	FR3 strain
	Breinlia jittapalapongi Veciana, Bain, Morand, Chaisiri, Douanghoupha, Miquel and Ribas, 2015	*Rattus tanezumi*	KP760119	KP760170	Laos
	Brugia malayi (Brug, 1927)	*Meriones unguiculatus*	KP760120	KP760171	FR3 strain
	Brugia pahangi (Buckley and Edeson, 1956)	*Meriones unguiculatus*	KP760121	KP760172	FR3 strain
	Brugia timori Partono, 1977	*Homo sapiens*	KP760122	KP760173	Indonesia
	Cercopithifilaria bainae Almeida and Vicente, 1984	*Canis familiaris*	KP760123	KP760175	experimental
	Cercopithifilaria rugosicauda (Böhm and Supperer, 1953)	*Capreolus capreolus*	KP760124	KC610815	France
	Cruorifilaria tuberocauda Eberhard, Morales	*Hydrochoerus*	KP760125	KP760176	Venezuela

and Orihel, 1976	*hydrochaeris*			
Dipetalonema caudispina (Molin, 1858)	*Ateles paniscus*	KP760126	KP760177	Guyana
Dipetalonema gracile (Rudolphi, 1809)	*Cebus olivaceus*	KP760128	KP760179	Venezuela
Dipetalonema graciliformis (Freitas, 1964)	*Saimiri scuireus*	KP760131	KP760182	Peru
Dipetalonema robini Petit, Bain and Roussilhon, 1985	*Lagothrix poeppigii*	KP760132	KP760183	Peru
Litomosoides brasiliensis Lins de Almeida, 1936	*Carollia perspicillata*	KP760139	KP760190	Peru
Litomosoides hamletti Sandground, 1934	*Glossophaga soricina*	KP760141	KP760192	Peru
Litomosoides solaria Guerrero, Martin, Gardner and Bain, 2002	*Trachops cirrhosus*	KP760142	KP760193	Venezuela
Loxodontofilaria caprini Uni and Bain, 2006	*Naemorhedus crispus*	KP760144	AM749237	Japan
Mansonella perforate Uni, Bain and Takaoka, 2004	*Cervus nippon*	KP760145	AM749265	Japan

Mansonella ozzardi (Manson, 1897)	*Homo sapiens*	KP760147	KP760195	Haiti
Madathamugadia heipei Hering-Hagenbeck, Boomker, Petit, Killick-Kendrick and Bain, 2000	*Pachycactylus turneri*	KP760146	JQ888270	South Africa
Monanema martini Bain, Bartlett and Petit, 1986	*Arvicanthis niloticus*	KP760148	KP760196	Senegal
Onchocerca armilatta Railliet and Henry, 1909	*Bos taurus*	KP760153	KP760200	Cameroon
Onchocerca dewittei japonica Uni, Bain and Takaoka, 2001	*Sus scrofa leucomystax*	KP760154	KP760203	Japan
Onchocerca eberhardi Uni and Bain, 2007	*Cervus nippon*	KP760155	AM749268	Japan
Onchocerca gutturosa Neumann, 1910	*Bos taurus*	KP760156	KP760201	Cameroon
Onchocerca ochengi Bwangamoi, 1969	*Bos taurus*	KP760157	KP760202	Cameroon
Onchocerca skrjabini Ruklyadev, 1964	*Cervus nippon*	KP760158	AM749269	Japan

	Wuchereria bancrofti (Cobbold, 1877)	*Homo sapiens*	AF227234	JN367461	Mali
	Yatesia hydrochoerus (Yates, 1980)	*Hydrochoerus hydrochaeris*	KP760166	KP760210	Venezuela
Oswaldofilariinae	*Oswaldofilaria petersi* Bain and Sulahian 1974	*Crocodilurus amazonicus*	KP760160	KP760205	Peru
	Oswaldofilaria chabaudi Pereira, Souza and Bain, 2010	*Tropidurus torquatus*	KP760159	KP760204	Brazil
Setariinae	*Setaria tundra* Bain, 1974	*Rangifer tarandus*	KP760165	KP760209	Finland
	Setaria labiatopapillosa (Alessandrini, 1848)	*Bos taurus*	KP760164	KP760208	Cameroon
Splendidofilariinae	*Aproctella alessandroi* Bain, Petit, Kosek and Chabaud, 1981	*Saltator similis*	KP760118	FR823335	Brasil
	Madathamugadia hiepei Hering-Hagenbeck, Boomker, Petit, Killick-Kendrick and Bain, 2000	*Pachycactylus turneri*	KP760146	JQ888270	South Africa

	Rumenfilaria andersoni Lankester and Snider, 1982	*Rangifer tarandus*	KP760163	JQ888273	Finlande
Waltonellinae	*Foleyellides* sp.	*Rana pustulosa*		KC130677	Mexico
	Foleyellides sp.	*Rana pustulosa*		KC130679	Mexico
	Ochoterenella sp. 1	*Rhinella granulosa*	KP760151	KP760198	Venezuela
	Ochoterenella sp. 2	*Rhinella marina*	KP760152	KP760199	Venezuela
	Ochoterenella sp. 3	*Phyllomedusa bicolor*	KP760150	KP760197	French Guyana
	Neofoleyellides boereworsi n. gen. n. sp.	*Sclerophrys garmani*	XXX	XXX	South Africa
	Neofoleyellides boereworsi n. gen. n. sp.	*Sclerophrys gutturalis*	XXX	XXX	South Africa
Outgroup	*Filaria latala* Chabaud and Mohammad, 1989	*Panthera leo*	KP760135	KP760186	South Africa

1181

1182

Figure 1

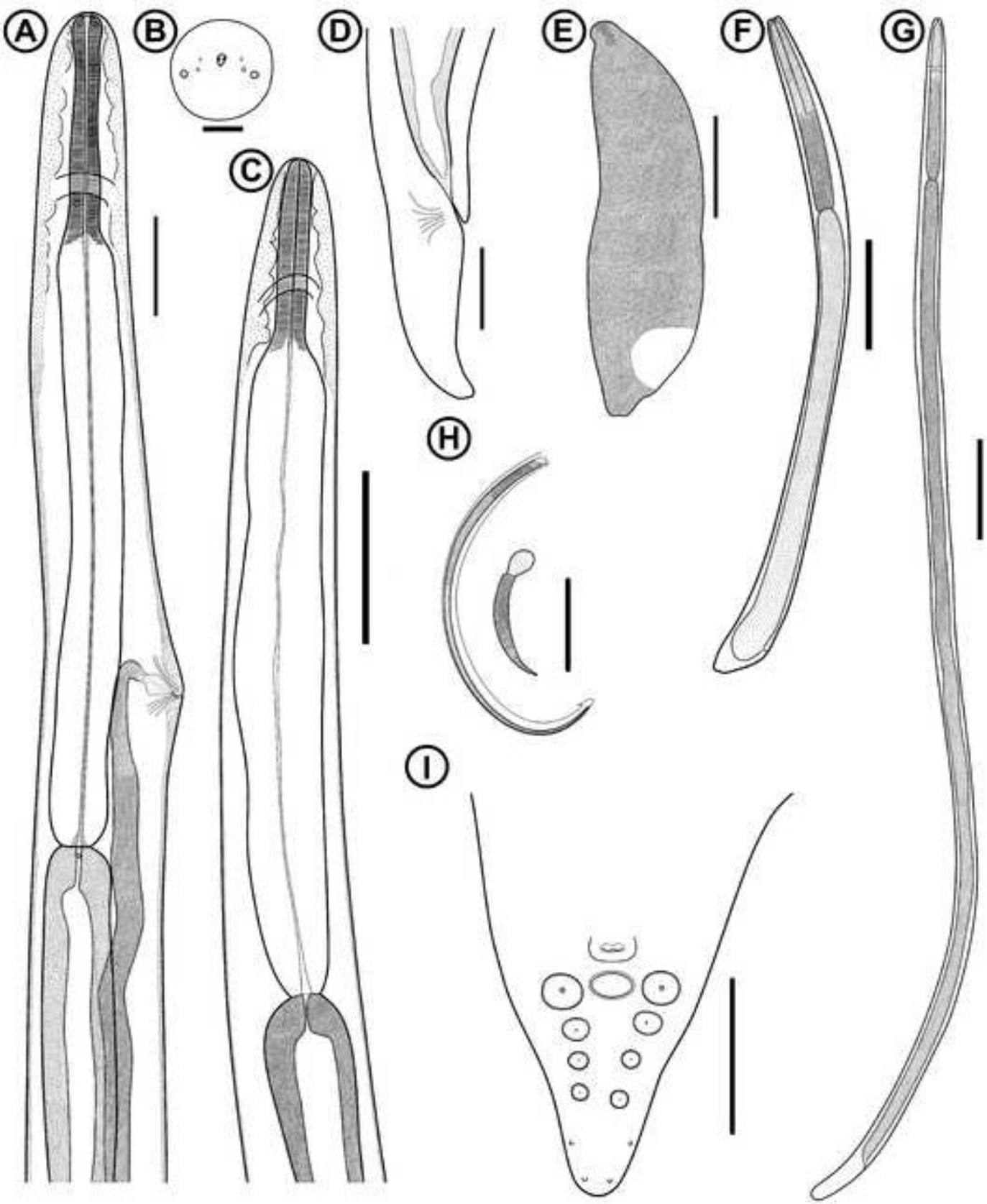

Figure 2

Click here to access/download;Figure;Fig.2_male-01.jpg

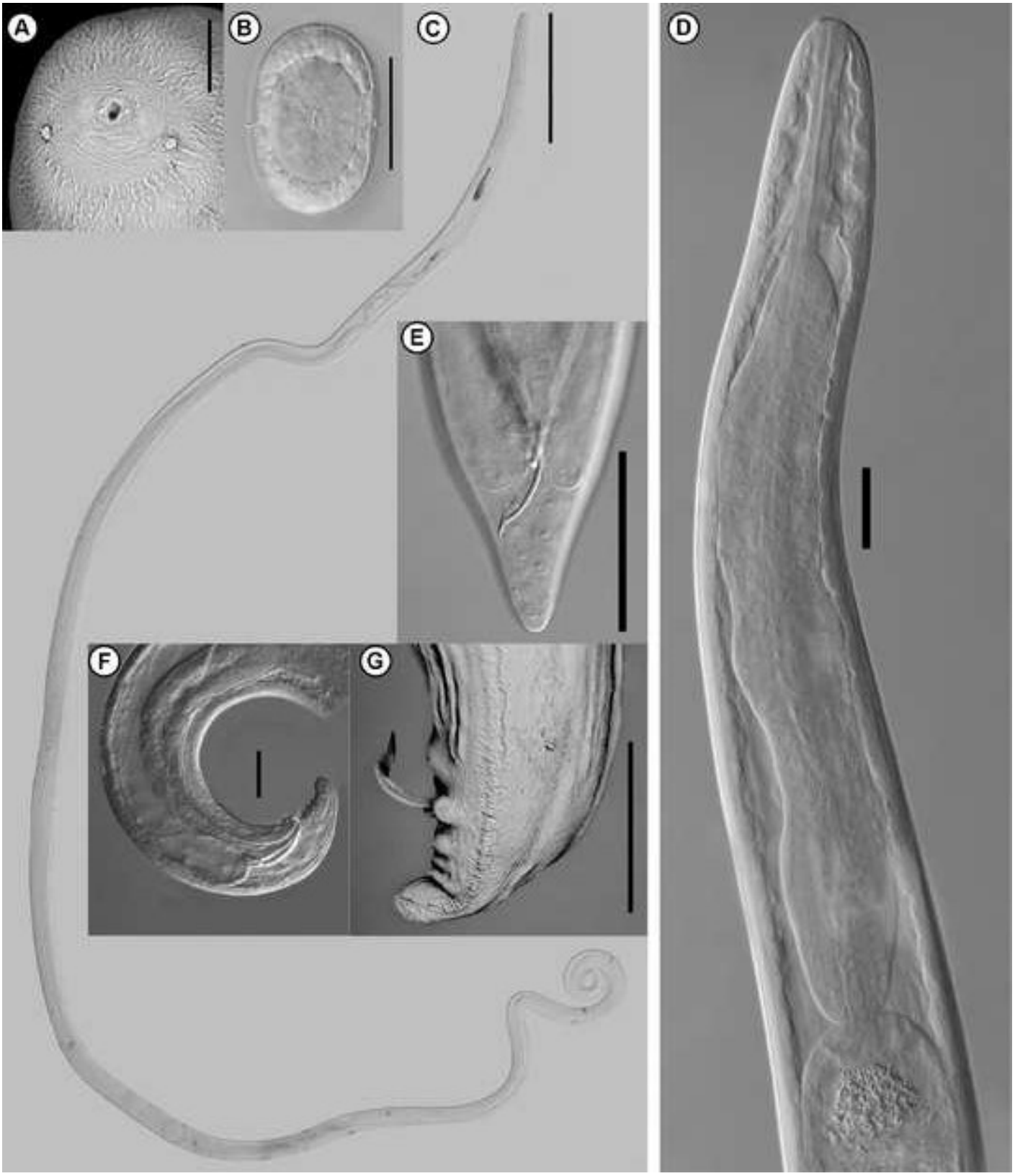

Figure 3

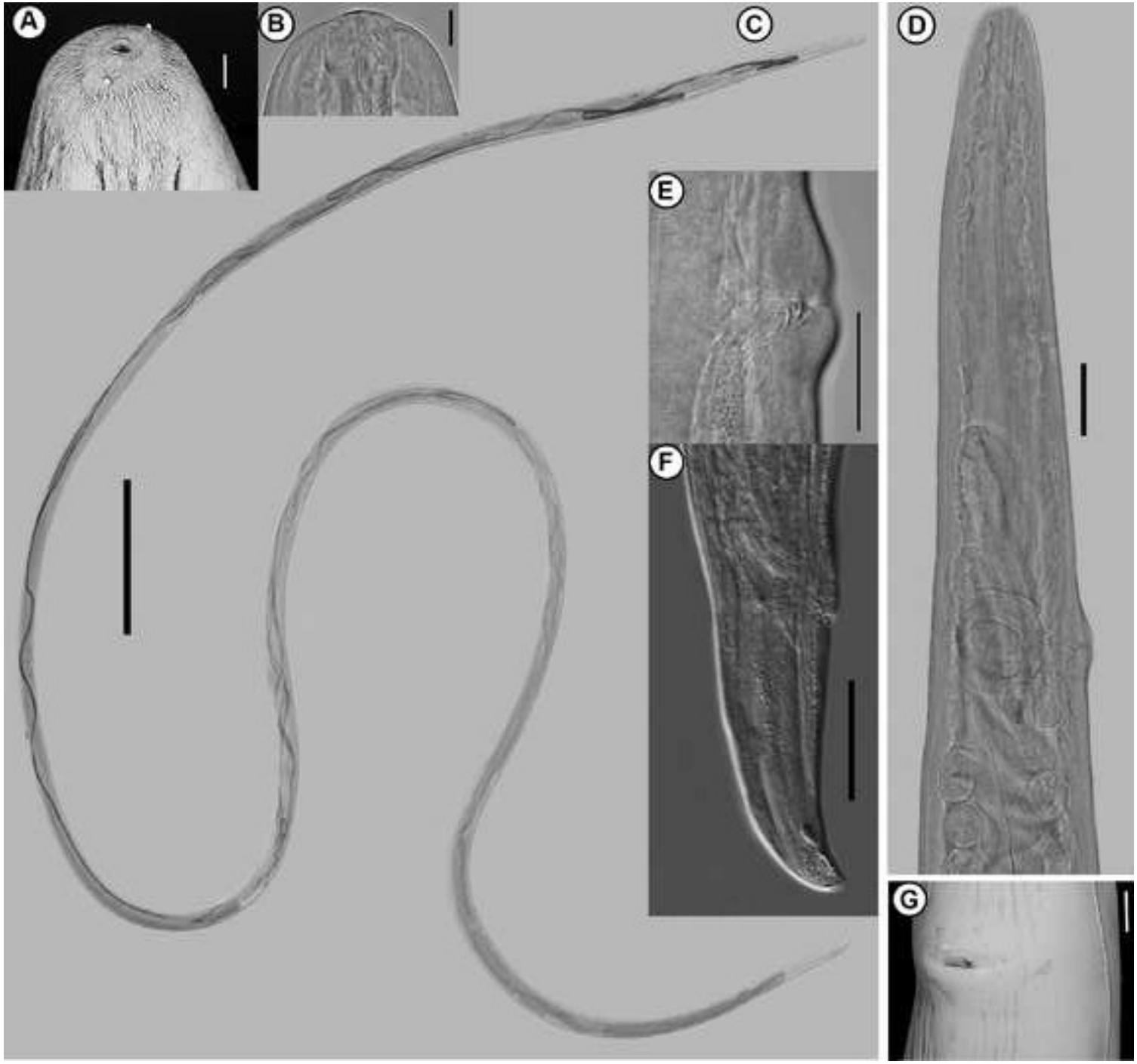

Figure 4

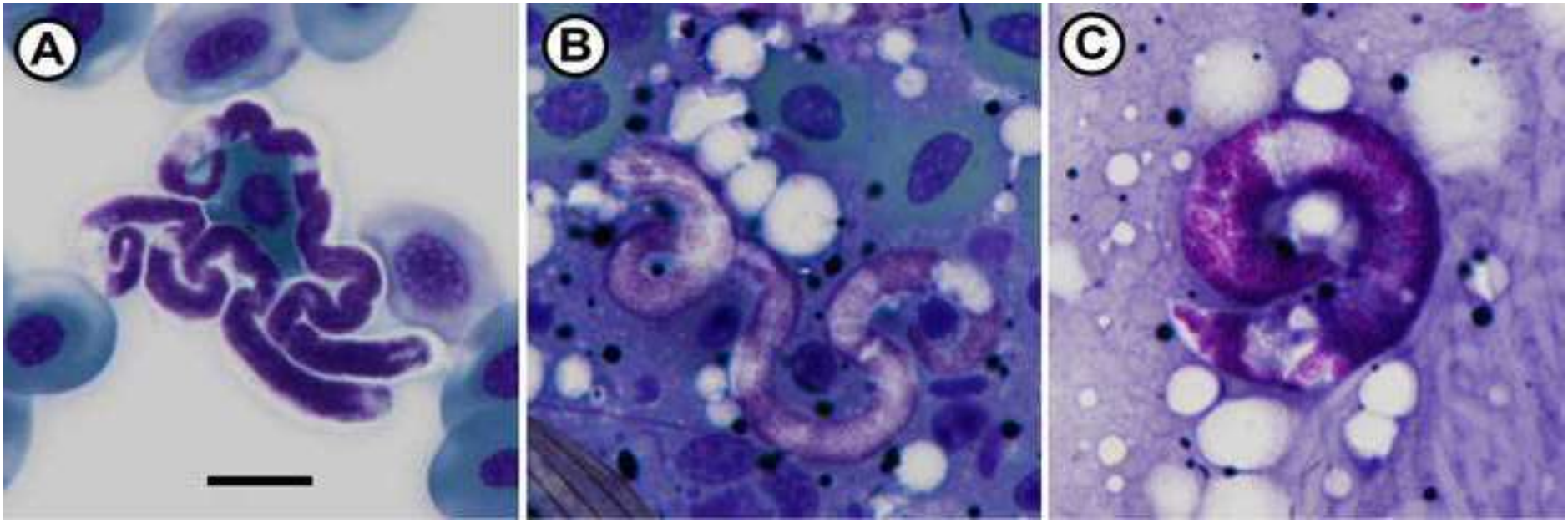

Figure 5

Click here to access/download;Figure;Fig.5_larvae-01.jpg

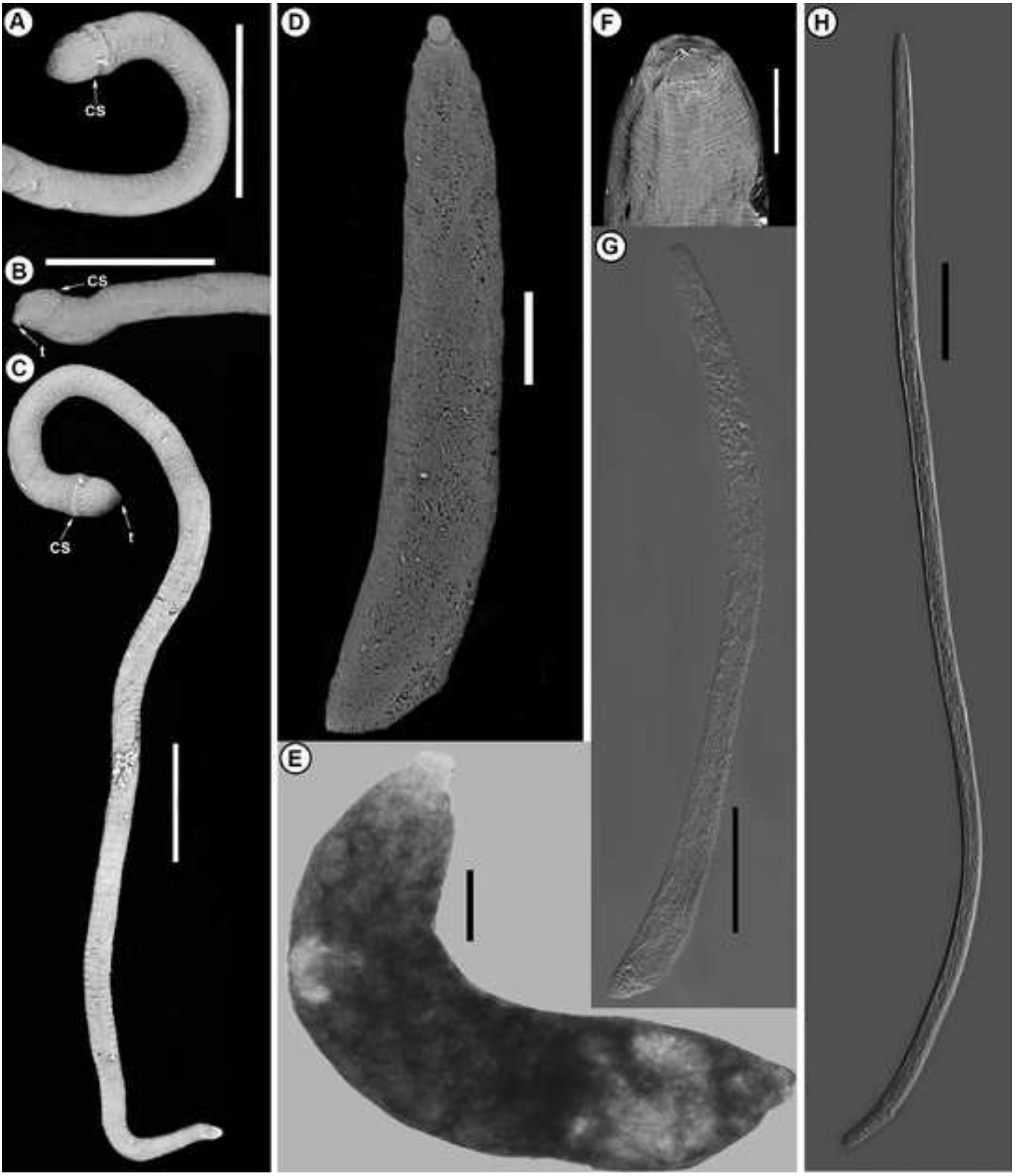

Figure 6

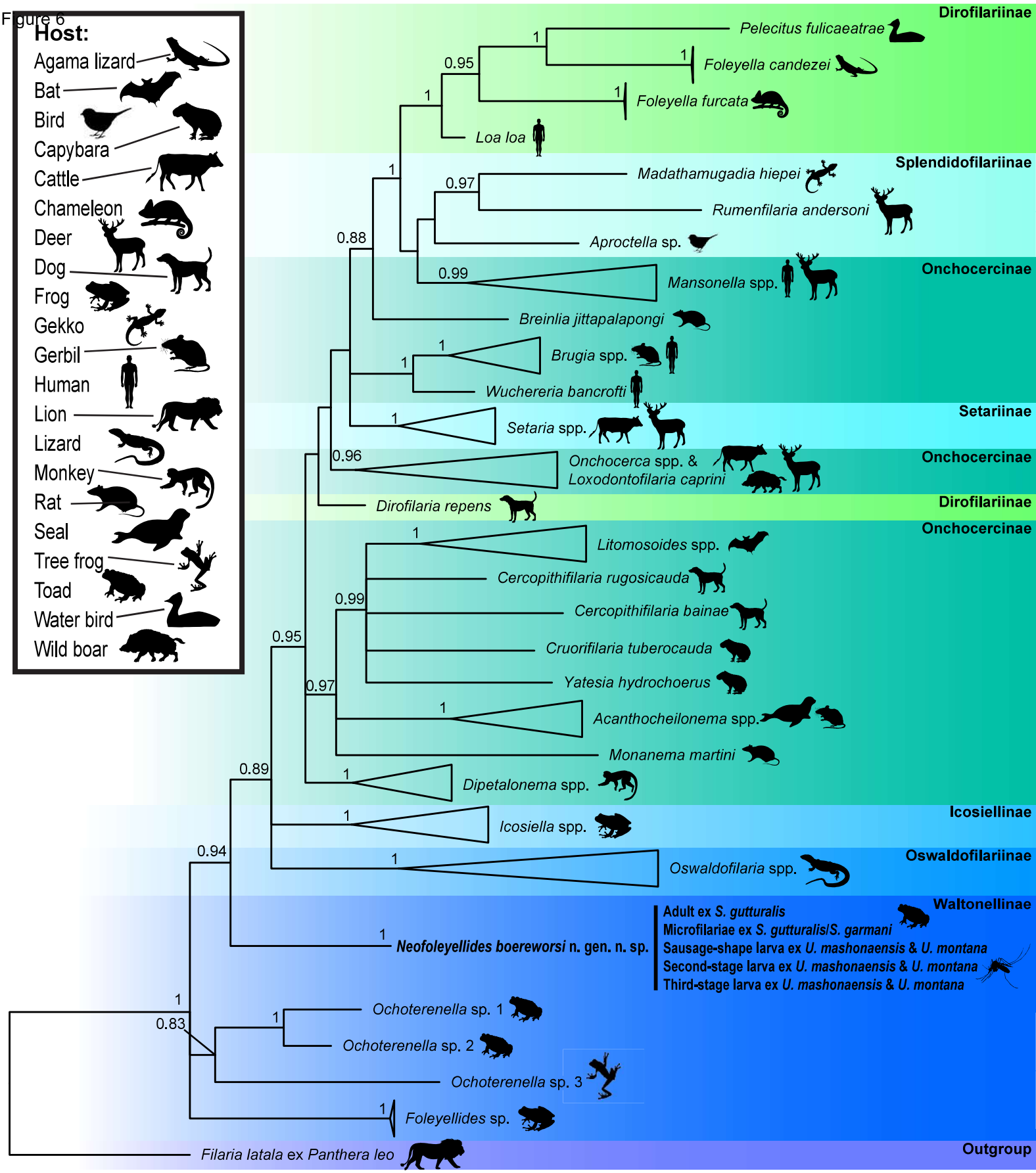
Host:
Agama lizard
Bat
Bird
Capybara
Cattle
Chameleon
Deer
Dog
Frog
Gekko
Gerbil
Human
Lion
Lizard
Monkey
Rat
Seal
Tree frog
Toad
Water bird
Wild boar
Dirofilariinae
Pelecitus fulicaeatrae
Foleyella candezei
Foleyella furcata
Loa loa
Splendidofilariinae
Madathamugadia hiepei
Rumenfilaria andersoni
Aproctella sp.
Onchocercinae
Mansonella spp.
Breinlia jittapalapongi
Brugia spp.
Wuchereria bancrofti
Setariinae
Setaria spp.
Onchocercinae
Onchocerca spp. &
Loxodontofilaria caprini
Dirofilariinae
Dirofilaria repens
Onchocercinae
Litomosoides spp.
Cercopithifilaria rugosicauda
Cercopithifilaria bainae
Cruorifilaria tuberocauda
Yatesia hydrochoerus
Acanthocheilonema spp.
Monanema martini
Dipetalonema spp.
Icosiellinae
Icosiella spp.
Oswaldofilariinae
Oswaldofilaria spp.
Waltonellinae
Neofoleyellides boereworsi n. gen. n. sp.
Adult ex S. gutturalis
Microfilariae ex S. gutturalis/S. garmani
Sausage-shape larva ex U. mashonaensis & U. montana
Second-stage larva ex U. mashonaensis & U. montana
Third-stage larva ex U. mashonaensis & U. montana
Ochoterenella sp. 1
Ochoterenella sp. 2
Ochoterenella sp. 3
Foleyellides sp.
Outgroup
Filaria latala ex Panthera leo
1
0.95
1
1
1
1
0.97
0.88
0.99
1
1
1
0.96
1
0.99
0.95
0.97
1
1
0.89
1
1
0.94
1
1
1
0.83
1
1
0.05

Figure 7

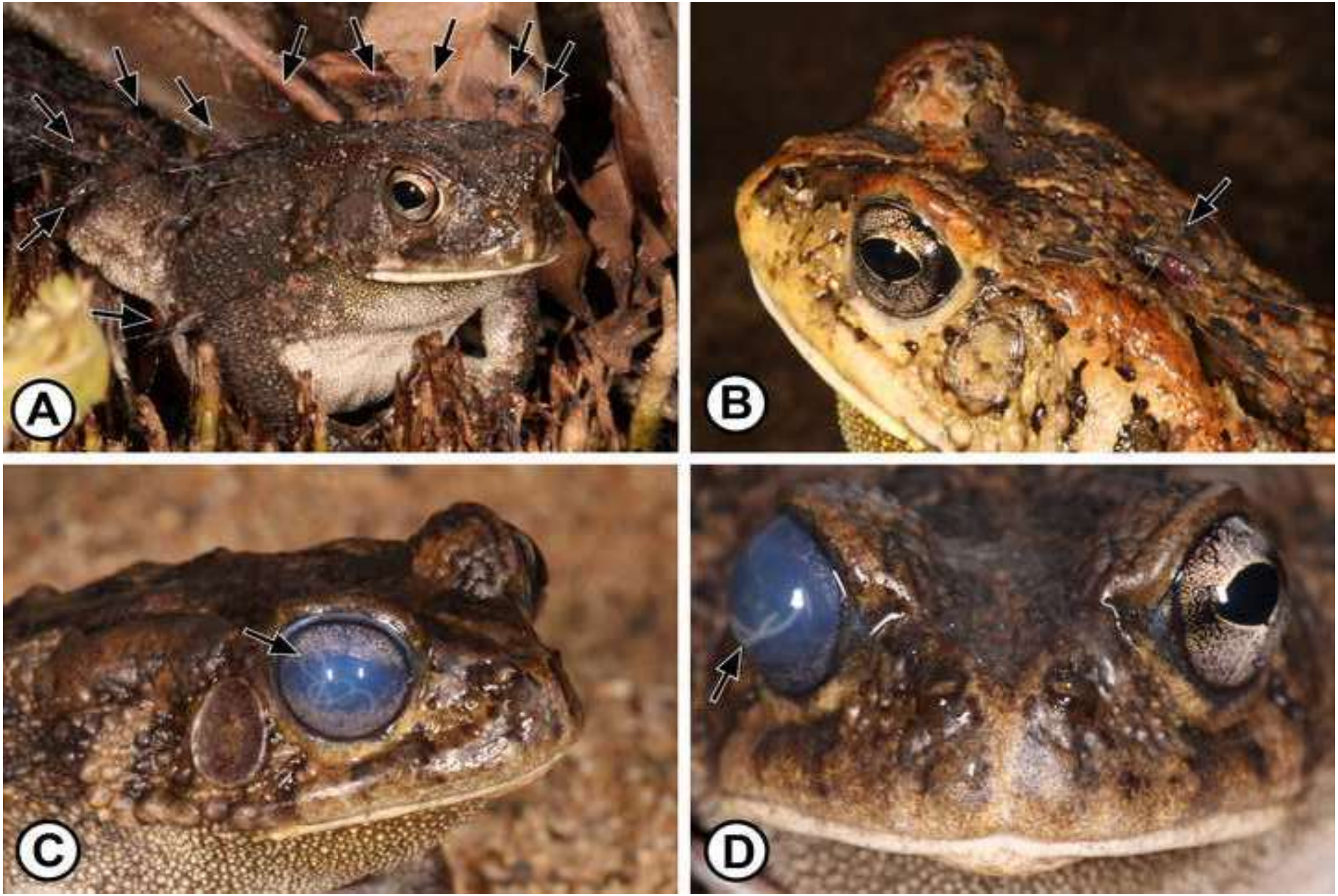

Figure 8

Click here to access/download;Figure;Fig.8_Life_cycle-01.jpg