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***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*

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27 **Abstract**

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

40
41 **Keywords:** anuran, amphibian, frog, parasite, life cycle, filarial nematode, microfilaria

42 43 **1. Introduction**

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

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

57 Onchocercids of anurans either belong to the subfamilies Icosiellinae Anderson
58 (1958) or Waltonellinae Bain and Prod'Hon (1974). Icosiellinae is monogeneric comprising
59 nine species of *Icosiella* Seurat (1917). Species are recognised based on the presence of
60 two pairs of cephalic submedian spines, a subterminal anus, and few caudal papillae on a
61 relatively short tail. Waltonellinae was erected to accommodate anuran "*Foleyella*"-like
62 filariae and currently comprises five genera, namely *Foleyellides* Caballero (1935),
63 *Ochoterenella* Caballero (1944), *Madochotera* Bain and Brunhes (1968),
64 *Paramadochotera* Esslinger (1986b) and *Paraochoterenella* Purnomo and Bangs (1999)
65 (Table 1).

66 Members of this subfamily are characterised by the absence of submedian cephalic
67 spines; the presence of a pair of lateral, parastomal projections; the anus not being in a
68 sub-terminal position; and several pairs of papillae on a somewhat long tail (Souza et al.,
69 2012; Bain et al., 2013).

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

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

98 The most diverse genus within Waltonellinae is *Ochoterenella*, currently with 16
99 species reported parasitising bufonid, hylid and leptodactylid hosts from the Neotropical
100 and Indomalayan realms (Bain et al., 2013). Caballero (1944) erected the genus and
101 described the type species *Ochoterenella digiticauda* Caballero, 1944 from the body cavity
102 of the Cane toad *Rhinella marina* (Linnaeus, 1758) in Mexico. According to Esslinger
103 (1986b) this species has been reported in several studies from various anurans throughout
104 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

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

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

140 In a recent study on the phylogenetic relationships of Onchocercidae, several
141 species of filarial nematodes parasitising amphibians (*Ochoterenella* spp. and *Icosiella*
142 *neglecta* (Diesing 1851) and reptiles (*Foleyella candezei* (Fraipont, 1882),
143 *Madathamugadia hiepei*, Hering-Hagenbeck, Boomker, Petit, Killick-Kendrick and Bain,
144 2000, *Oswaldofilaria petersi* Bain and Sulahian, 1974 and *Oswaldofilaria chabaudi*
145 Pereira, Souza and Bain, 2010) were included (Lefoulon et al., 2015). This study, based
146 on both nuclear (18S rRNA, 28S rRNA, MyoHC, rbp1, hsp70) and mitochondrial (COI, 12S
147 rDNA) markers, showed Icosiellinae, Waltonellinae, and Oswaldofilariinae (onchocercids
148 from crocodilian and squamate hosts), forming a well-supported clade, sister to all the
149 other taxa evaluated within Onchocercidae. Furthermore, *Foleyella candezei*
150 (Dirofilariinae) found infecting lizard hosts, was shown as distantly related to this group
151 (Oswaldofilariinae, Icosiellinae and Waltonellinae) and clustering with other members of
152 Dirofilariinae (Lefoulon et al., 2015).

153 Excluding Madagascar, the only species described in Africa from Waltonellinae is
154 *Foleyellides duboisi* (Gedoelst, 1916) found in the Democratic Republic of Congo. This
155 species was described by Gedoelst (1916), based on material collected from unidentified
156 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 Readell 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 microfilariiae 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 microfilariiae 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 sphenoccephala* 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

183 35% were infected. The sausage-shaped first-stage appeared from the third day, while first
184 infective larvae appeared from approximately 14 dpi. Furthermore, these authors did not
185 consider *C. pipiens molestus* as the natural intermediate host for *F. duboisi*, suggesting
186 that a more abundant mosquito species with a preference for feeding on frog hosts would
187 be a more likely natural vector (Witenberg and Gerichter, 1944). Later, Crans (1969)
188 elucidated the life cycle of a filarial nematode, later designated as *F. flexicauda* (Schacher
189 and Crans, 1973), from the American Bullfrog *R. catesbeiana* and the amphibian-feeding
190 mosquito *Culex territans* Walker, 1856 in New Jersey. Crans (1969) reported that in the
191 vertebrate host adult filarial nematodes were found encysted in the intestinal mesentery of
192 the body cavity, with microfilariae released in the peripheral blood. In the invertebrate host
193 infective third-stage larvae were detected within ten dpi. Developmental stages appeared
194 throughout the mosquito's haemocoel, in the abdominal fat bodies, the coxal cavities of
195 the legs, and in the head capsule and proboscis (Crans, 1969).

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

208

209 **2. Material and Methods**

210 *2.1 Toad collection and study area*

211 In a recent survey on the biodiversity of anuran blood parasites, Netherlands et al. (2015)
212 found the Red toad, *Schismaderma carens* parasitised with microfilariae. Thus, for the
213 current study members of Bufonidae were selected as the vertebrate hosts to be screened
214 for microfilariae. A total of 128 individuals, representing four bufonid species, were
215 collected at night via active sampling from several localities throughout northern KZN,
216 South Africa (S1 Table). Species comprised 45 Eastern Olive toads (*Sclerophrys*
217 *garmani*), 73 guttural toads (*Sclerophrys gutturalis*), seven Flat-backed toads (*Sclerophrys*
218 *pusilla*), and three Red toads (*Sch. carens*), identified using field guides (Du Preez and
219 Carruthers, 2009, 2017). Collected specimens were placed in individually marked
220 containers with sufficient moisture and ventilation, and transported back to a field
221 workstation. Toads were collected at these sites during their breeding season and the
222 southern hemisphere warmer months of April, September, November, and December
223 2014, September, November and December 2016, February and November 2017, and
224 January and March 2018. All specimens collected prior to November 2017, were released
225 after taking blood samples. Based on the intensity of microfilariae selected individuals,
226 uninfected (n=1) and infected (n=4), collected during and after the November 2017
227 sampling effort were transported back to the North-West University African Amphibian
228 Conservation Research Group laboratory for further processing and examination. This
229 study received the relevant ethical approval from the North-West University's AnimCare
230 ethics committee (ethics number: NWU-00372-16-A5) and euthanasia of frogs was
231 performed according to approved SOP using tricaine methanesulfonate (MS222) solution
232 (ethics number: NWU-00492-16-S5). Ezemvelo KZN Wildlife provided research permits
233 OP 526/2014, OP 839/2014, OP 4374/2015, OP 4092/2016, and OP 4085/2017 for
234 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 (CO₂) 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)

287 Each of the filarial nematode stages obtained were used for scanning electron microscopy
288 (SEM). Specimens were dehydrated using a gradual ethanol series: nematodes were first
289 added to a small Petri dish with 70% EtOH, continuously and slowly 100% EtOH was
290 added to the Petri dish, and simultaneously the same amount of mixed EtOH removed
291 until concentration reached 100%. After dehydration, nematodes were dried using
292 hexamethyldisilazane as transition fluid (before clear solution worms were dehydrated in a
293 series of hexamethyldisilazane mixed 1:1 with 70°, 80°, 90°, 96° and 100° EtOH) or by
294 means of a critical point drier (Bio-Rad, Bio-Rad Microscience Division, United Kingdom)
295 using liquid CO₂ as transition fluid. In order to prevent shrinking of adult nematodes and
296 third-stage larvae, specimens were dissected into two or more pieces before dehydration.
297 Following the methods of Conradie et al. (2017), fresh blood samples obtained from a
298 highly infected guttural toad (S1 Table, AE180124C1) were also prepared for SEM. Thin
299 blood smears were made on glass coverslips and Whatman® qualitative filter paper
300 (Grade 1), and a drop of Todd's fixative (Todd, 1986) placed on the smears prior to drying.
301 After a minute, the smears were submerged in fresh Todd's fixative for approximately 2 h.
302 The sample was then gently washed with ultrapure water three times each for 15 min. Post
303 fixation was performed with 2% osmium tetroxide (OsO₄) for 90 min. This was followed by
304 rinsing three times in ultrapure water for 10 min, with subsequent dehydration in a gradual
305 ethanol series. The sample was then critical point dried as described above for the filarial
306 nematode stages.

307 Dried specimens and smears were mounted onto 12 mm aluminium stubs with
308 double-sided carbon tape and sputter-coated for 90 seconds with a gold palladium alloy in
309 argon gas at a pressure of 2 atm (SPI-Module™ Sputter Coater, SPI Supplies, West
310 Chester, PA, USA). Specimen stubs were stored in a desiccator for at least 30 min before
311 being examined by SEM at an accelerated voltage of 10 kV (Phenom PRO Desktop SEM,
312 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 COIntF (5'-TGA TTG GTG GTT TTG GTA A-3') and COIntR (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 Cyclor 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

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

3. Results

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

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

393

394 3.1 Genus description

395 Phylum: Nematoda Rudolphi, 1808

396 Class: Chromadorea Inglis, 1983

397 Order: Rhabditida Chitwood, 1933

398 Suborder: Spirurida Railliet and Henry, 1915

399 Superfamily: Filarioidea Weinland, 1858

400 Family: Onchocercidae Leiper, 1911

401 Subfamily: Waltonellinae Bain and Prod'Hon, 1974

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

403

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

415

416 *Remarks:* According to the keys provided by Esslinger (1986a), Purnomo and Bangs
417 (1999), and Bain et al. (2013), *Neofoleyellides* n. gen. belongs to the family
418 Onchocercidae and subfamily Waltonellinae, based on the presence of distinct parastomal
419 structures, lack of submedian cephalic spines, position of an anus which is not
420 subterminal, possession of a vulva posterior to the nerve ring in females, possession of
421 several pairs of papillae on the somewhat elongated tail in males and the nematode
422 parasitising an amphibian definitive host. Within Waltonellinae, the morphological
423 characters of *Neofoleyellides* n. gen. conform closest with *Foleyellides*, such as the
424 presence of a distinct buccal capsule and parastomal structures, the presence of lateral
425 and caudal alae in both sexes, and the absence of cuticular bends and bosses on the
426 body cuticle. Nonetheless *Neofoleyellides* n. gen. can be easily distinguished from
427 *Foleyellides* and all other genera from Waltonellinae by the absence of dorsal outer
428 cephalic papillae and the absence of small inner cephalic papillae. *Neofoleyellides* n. gen.
429 is characterised by only two enlarged submedian cephalic papillae on the ventral sides of
430 the oral opening, and the absence of other papillae surrounding the oral opening. Since
431 differences in cephalic morphology are commonly used for generic differentiation in many
432 groups of parasitic nematodes and based on the other morphological differences such as
433 asymmetry of cephalic papillae and molecular characterisation (see below), we consider
434 our specimens as belonging to a new onchocercid genus.

435

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

439 *Etymology:* The generic name is derived from the close morphological resemblance to
440 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].

467

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

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

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

476

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

486 Males (n=30). Body 15–28, [20] (22 ± 3.1 , 14.3) mm long, 97–138 [105] (120 ± 12.0 ,
487 10.0), 151–215 [153] (190 ± 22.1 , 11.7) and 135–248 [162] (202 ± 29.3 , 14.5) wide at nerve
488 ring, oesophageal-intestinal junction, and mid-body level, respectively (Fig.2B). Buccal
489 capsule, 3–6 [3] (4 ± 0.8 , 20.1) long, and 6–12 [8] (9 ± 1.5 , 18.0) wide. Glandular portion of
490 oesophagus 190–369 [263] (282 ± 41.8 , 14.8) long; 27–57 [32] (38 ± 6.1 , 16.0), 25–49 [29]
491 (34 ± 5.4 , 16.0) and 32–51 [37] (41 ± 5.9 , 14.2) wide at anterior, mid-length and posterior
492 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)

were identical for both the 18S rRNA and COI gene sequence fragments. *Neofoleyellides boereworsi* n. gen. n. sp. is represented in this analysis by sequences obtained from microfilaria-infected blood samples from *S. gutturalis* and *S. garmani* (as no *S. garmani* were dissected for adult stages). For the BI phylogenetic analysis, filariae of Waltonellinae isolated from anuran hosts are the earliest diverging lineages in the ingroup. They are paraphyletic with respect to all other onchocercid taxa used in this analysis, Dirofilarinae, Setariinae, Splendidofilarinae and Onchocercinae, together form a clade with 0.95 posterior probability support, in which Dirofilarinae and Onchocercinae are non-monophyletic. *Neofoleyellides boereworsi* n. gen. n. sp., is shown as a sister taxon to Icosiellinae, Oswaldofilarinae and all other onchocercids (Fig 6).

3.4 Life history experiment

A total of 146 mosquitoes were collected *in situ* (see Fig 7A-B). Of these, 64 were identified as *U. (Pfc.) mashonaensis* and 42 as *U. (Pfc.) montana*. The remaining 40 mosquitoes did not take blood meals or died in captivity, and were not included in the experiment. Collected mosquitoes used for experimental transmission consumed blood meals from *S. gutturalis* highly parasitised with microfilariae. Within 24 h post feeding, desheathed microfilaria were observed in the mosquito's intestines, along with undigested erythrocytes. Microfilariae observed in fresh wet blood smears were slightly more active compared to the desheathed microfilaria from mosquitoes (Fig 8A). From three days post-infection (dpi) early sausage-shaped first-stage larvae were observed in the intestine, followed by late sausage-shape first-stage larvae appearing approximately three to four dpi in the fat bodies of its host (Fig 8B). Early sausage-shaped larvae seem to be dormant whereas late sausage-shaped larvae were observed slowly moving the anterior part of the 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

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

Scanning electron micrographs illustrated some unique characters of unsheathed 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 primordia 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 *Paraochoterella* 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.

Analyses of the metric characters of both adult and larval stages showed high variability. The smallest coefficient of variation observed was firstly for the values of the nerve ring for both adult sexes, 22.6 in females and 11.3 in males, and secondly for the male spicules comprising 11.3 and 10.7 for left and right spicules respectively. The high variability observed for all the other characters is possibly due to the large sample size and the large size of the nematodes. The metric characters of the different larval stages also varied greatly, possibly due to their intensive growth rate. Only the body length of the third-stage larvae was rather stable (CV less than 10). Based on the above observations, we suggest that species and generic differentiation of members of Waltonellinae should be supported by both qualitative morphological and molecular data.

Potential effects of Neofoleyellides boereworsi n. gen. n. sp. on its anuran host

The majority of adult specimens of *Neofoleyellides boereworsi* n. gen. n. sp. were removed from the body cavity of dissected guttural toads, with a few exceptions in highly infected hosts. Specifically, one and three adult filarial nematodes were found subcutaneously in the two most highly infected toads respectively. In the latter guttural toad (S1 Table, AE180124C1), three individuals of *Neofoleyellides boereworsi* n. gen. n. sp. were attached to the lymphatic tissue, and one immature specimen was observed in its host's eye (see Fig 7C-D). Although no attempts were made to study the specific pathological effects *Neofoleyellides boereworsi* n. gen. n. sp. has on its host, some observations may suggest such effects. A heavily (52 specimens) infected *S. gutturalis* contained a visibly enlarged spleen, gall bladder, and liver, the latter also appearing darker than normal. Moreover, the same individual's eye was parasitised with a filarial nematode. Although this nematode died within two weeks after its host was collected, it caused swelling, infection and loss of sight to the infected eye. This individual would potentially have been vulnerable to predators and would likely not have survived long in nature.

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 form 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 natalensis* (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 (Gear 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 larger 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.

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872

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1096 **Legends to figures**

1097 **Fig 1. Illustrations of adult and larval stages *Neofoleyellides boereworsi* n. gen. n.**

1098 **sp.** A – female, anterior part of body, lateral view; B – female, anterior part of body, apical
1099 view; C – male, anterior part of body, lateral view; D – female, posterior part of body,
1100 lateral view; E – first-stage sausage-shape larva, lateral view; F – second-stage larva,
1101 lateral view; G – third-stage larva, lateral view; H – male, spicules, lateral view; I – male,
1102 posterior part of body, ventral view. Scale bars: A, C, D, F-H – 100; B, E – 20; I – 50.

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1104 **Fig 2. Photomicrographs of *Neofoleyellides boereworsi* n. gen. n. sp. male. A –**

1105 anterior part of body, apical view (SEM image); B – entire body, lateral view; C – anterior

part of body, lateral view; D – cross-section at mid-body level; E – posterior part of body, lateral view; F – posterior part of body, lateral view (SEM image). Scale bars: A – 10; B – 1mm; C – 100; D-F – 50.

Fig 3. Photomicrographs of *Neofoleyellides boereworsi* n. gen. n. sp. female. A – entire body, lateral view; B – anterior part of body, lateral view; C – anterior part of body, head region, lateral view; D – anterior part of body, apical view (SEM image); E – posterior part of body, lateral view; F – region of vulva, ventral view (SEM image). Scale bars: A – 2mm; B, E – 100; C, D, F – 20.

Fig 4. Photomicrographs of *Neofoleyellides boereworsi* n. gen. n. sp. stained larvae. A – microfilaria from amphibian blood; B – unsheathed microfilaria from mosquito blood meal; C – sausage-shape first stage. All images captured from the deposited slides (XXX) Scale bar: 10µm.

Fig 5. Photomicrographs of *Neofoleyellides boereworsi* n. gen. n. sp. larval stages. A – exsheathed microfilaria from amphibian blood, anterior part of body, subdorsal view (SEM image); B – sheathed microfilaria from amphibian blood, anterior part of body, lateral view (SEM image); C – sheathed microfilaria from amphibian blood, entire body, subdorsal view (SEM image); D – early sausage-shape first-stage, entire body, lateral view (SEM image); E – late sausage-shape first-stage, entire body, lateral view; F – second-stage, anterior part of body, apical view (SEM image); G – second-stage, entire body, lateral view; H – third-stage, entire body, lateral view. t – tooth, cs – crown-like structure. Scale bars: A-D, F – 10; E-H – 100.

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.

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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.

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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

1157 and 18 dpi. E-F – *Neofoleyellides boereworsi* n. gen. n. sp. development in the definitive
1158 guttural toad host, *S. gutturalis*. E – Male and female adult stages. F – Sheathed
1159 microfilaria in the peripheral blood of *S. gutturalis*. Images not drawn to scale.

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1161 **Supporting Information**

1162 **S1 Table. Sampling data of toads collected from several sampling localities in South**
1163 **Africa.** Table shows host field number, species, positive, locality, coordinates, and sex.

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

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AmphibiaWeb (2018)

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

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

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

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

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

Ochoterenella vellardi (Travassos, 1929) *R. marina* (syn. *B. marinus*) Bufonidae Brazil Travassos (1929)

| | | | | | |
|-----------------------|---|--|-------------|------------|----------|
| <i>Paramadocho</i> | <i>Paramadochotera guibei</i> (Bain and | <i>Gephyromantis redimitus</i> | Mantellidae | Madagascar | Bain and |
| <i>tera</i> (Bain and | Prod'Hon, 1974) † | (syn. <i>Mantidactylus redimitus</i>) | | | Prod'Hon |
| Prod'Hon, | | | | | (1974) |
| 1974) | | | | | |

| | | | | | |
|---------------------|-------------------------------------|------------------------------------|----------------|-----------|-----------|
| <i>Paraochotere</i> | <i>Paraochoterenella javanensis</i> | <i>Fejervarya cancrivora</i> (syn. | Dicroglossidae | Indonesia | Purnomo |
| <i>nella</i> | Purnomo and Bangs, 1999† | <i>Rana cancrivora</i>) | | | and Bangs |
| Purnomo and | | | | | (1999) |
| Bangs, 1999 | | | | | |

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

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

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transmission and life cycle elucidation *ex situ*.

| | | |
|---|--|---|
| Field sampling | <ul style="list-style-type: none">• Sampling took place in Sodwana Bay at site SB-1 (S27.488591°; E32.664259°).• Collect <i>Sclerophrys gutturalis</i> (n = 8) and blood samples as detailed earlier.• During sampling, mosquitoes observed feeding on calling <i>S. gutturalis</i>.• Blood fed mosquitoes (n = 5) were collected using an aspirator and temporarily housed in small glass jars with moist cotton wool. | |
| | November | |
| | 2017 | |
| | Laboratory | <ul style="list-style-type: none">• Collected mosquitoes identified using Jupp (1996) – fixed whole in 70% ethanol.• Two <i>S. gutturalis</i> 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 | <ul style="list-style-type: none">• Two subsequent sampling expeditions to Sodwana Bay followed.• Collected toads <i>S. gutturalis</i> (n = 35) and <i>S. garmani</i> (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. |

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- 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.

Vector
collection

- 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.
-

-
- 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.
- Laboratory phase following
January & March 2018
field sampling
-

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

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

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

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

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

Rumenfilaria andersoni Lankester and Snider,

Rangifer tarandus KP760163 JQ888273 Finlande

1982

| | | | | |
|----------------------|--|-------------------------------|-------------------|------------------|
| Waltonellinae | <i>Foleyellides</i> sp. | <i>Rana pustulosa</i> | KC130677 | Mexico |
| | <i>Foleyellides</i> sp. | <i>Rana pustulosa</i> | KC130679 | Mexico |
| | <i>Ochoterenella</i> sp. 1 | <i>Rhinella granulosa</i> | KP760151 KP760198 | Venezuela |
| | <i>Ochoterenella</i> sp. 2 | <i>Rhinella marina</i> | KP760152 KP760199 | Venezuela |
| | <i>Ochoterenella</i> sp. 3 | <i>Phyllomedusa bicolor</i> | KP760150 KP760197 | French Guyana |
| | <i>Neofoleyellides boereworsi</i> n. gen. n. sp. | <i>Sclerophrys garmani</i> | XXX XXX | South Africa |
| | <i>Neofoleyellides boereworsi</i> n. gen. n. sp. | <i>Sclerophrys gutturalis</i> | XXX XXX | South Africa |
| Outgroup | <i>Filaria latala</i> Chabaud and Mohammad, 1989 | <i>Panthera leo</i> | KP760135 KP760186 | South Africa |

1181

1182

Figure 1

[Click here to access/download;Figure;Fig.1_Illustrations-01.jpg](#)

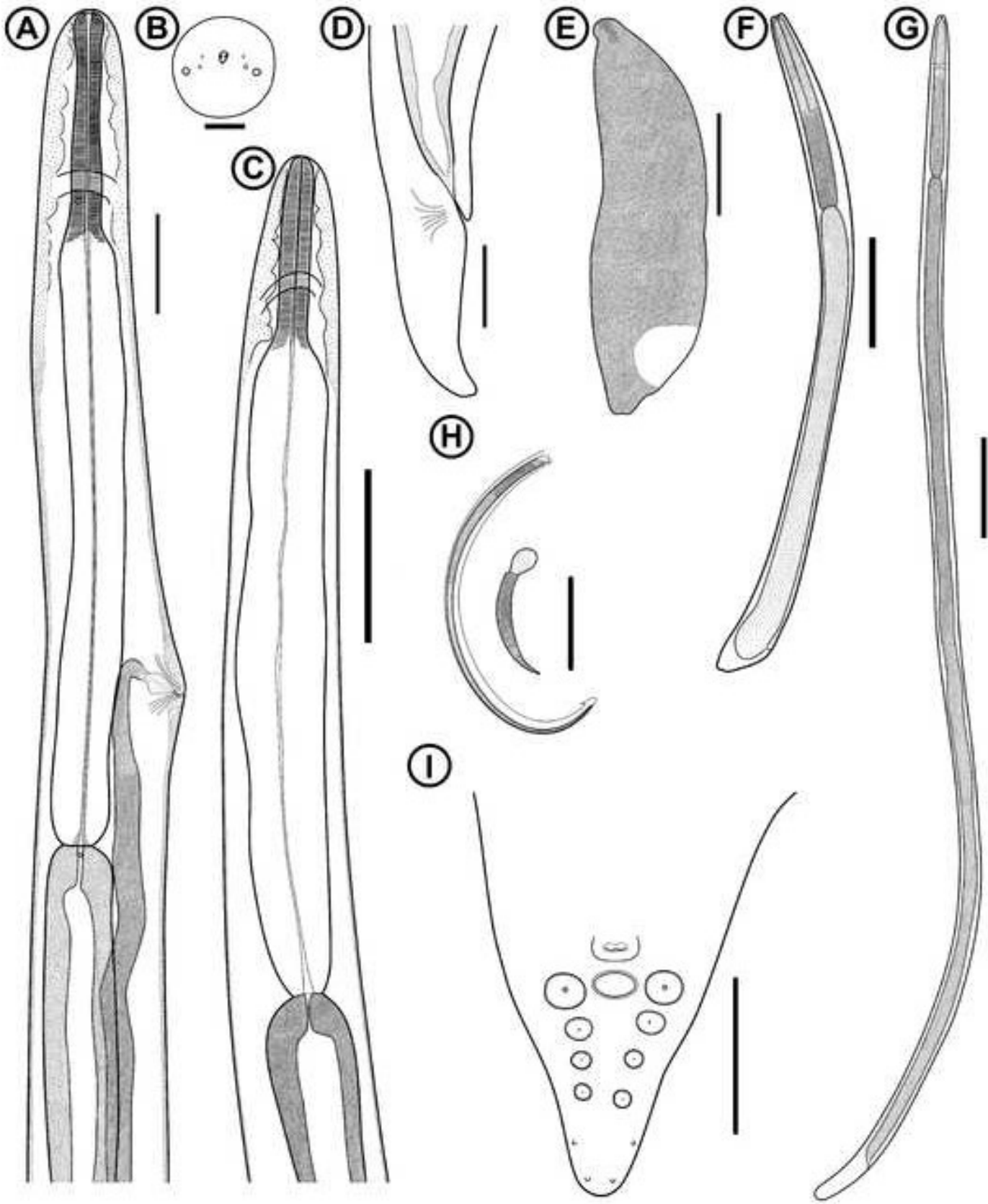


Figure 2

[Click here to access/download;Figure;Fig.2_male-01.jpg](#)



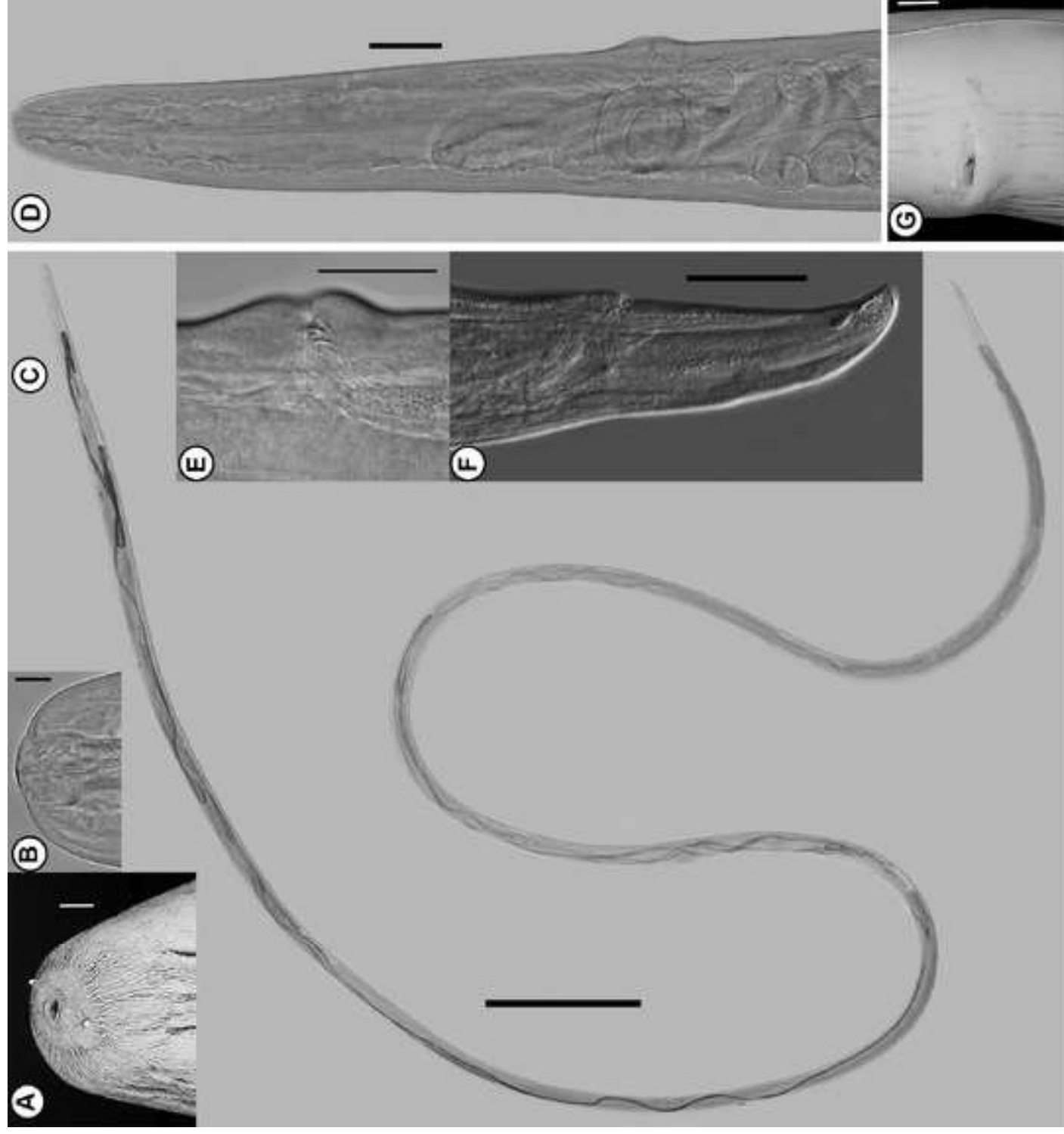


Figure 4

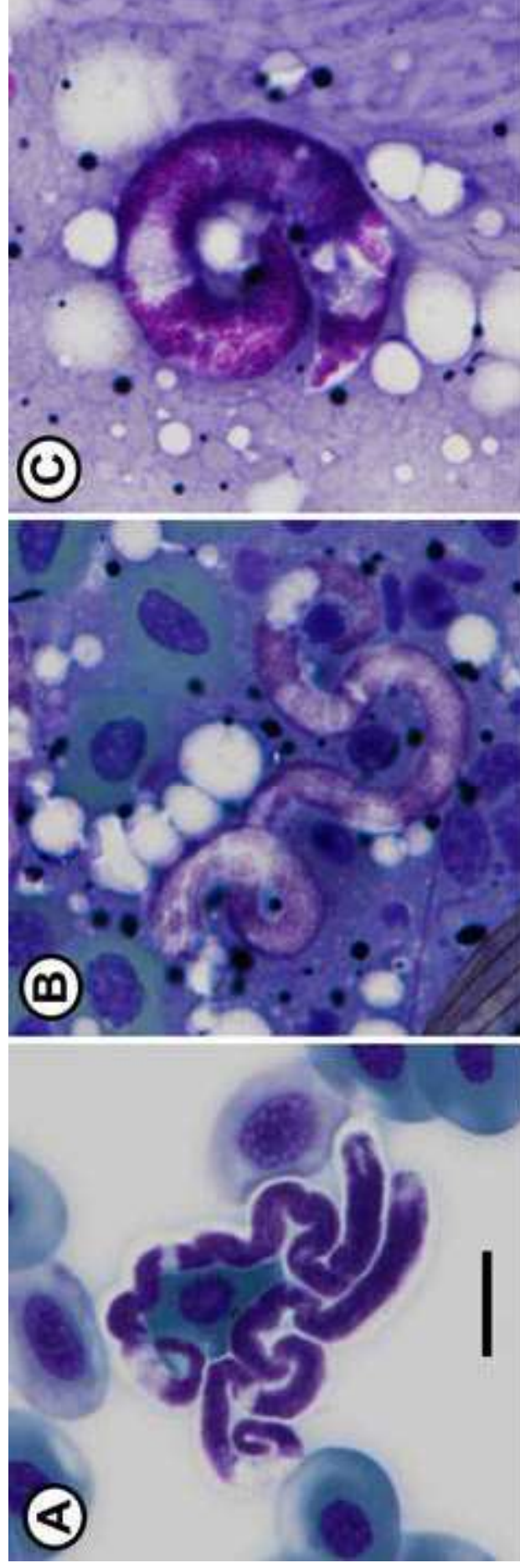


Figure 5

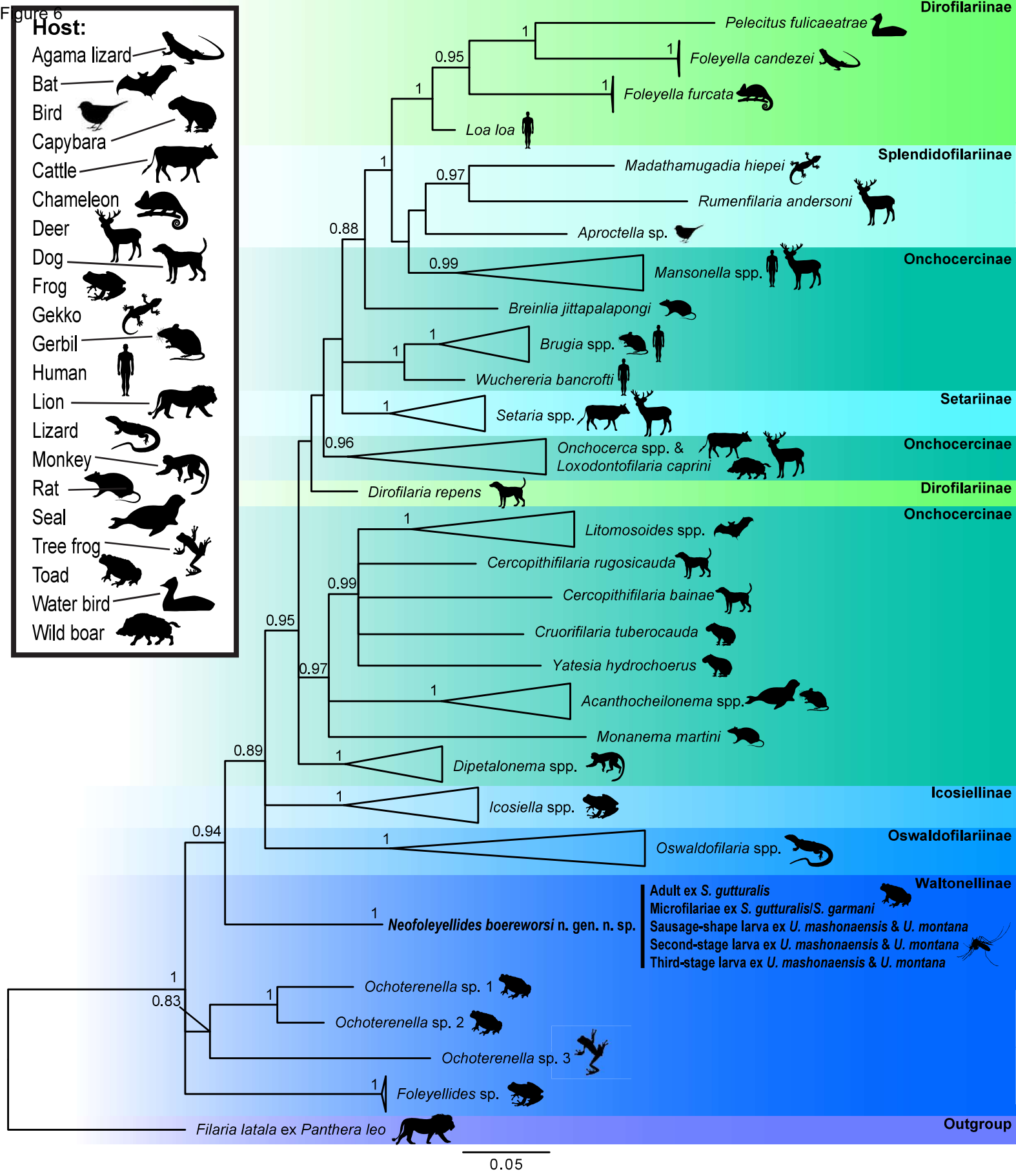
[Click here to access/download;Figure;Fig.5_larvae-01.jpg](#)



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



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*



Figure 7

