Title: Hiding in the swamp: new capillariid nematode parasitizing New Zealand brown mudfish

Authors: Fátima Jorge[1], Richard S. A. White[2] and Rachel A. Paterson[1,3]

Addresses: [1]Department of Zoology, University of Otago, PO Box 56, Dunedin 9054, New Zealand; [2]School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand; [3]School of Biosciences, University of Cardiff, Cardiff, CF10 3AX, United Kingdom

Running headline: Capillariid nematode parasitizing New Zealand mudfish

Corresponding author: Fátima Jorge

Department of Zoology, University of Otago, 340 Great King Street, PO Box 56, Dunedin 9054, New Zealand

e-mail: fatima.esperanca@gmail.com

Abstract

The extent of New Zealand's freshwater fish-parasite diversity has yet to be fully revealed, with host-parasite relationships still to be described from nearly half the known fish community. Whilst advancements in the number of fish species examined and parasite taxa described are being made; some parasite groups, such as nematodes, remain poorly understood. In the present study we combined morphological and molecular analyses to characterize a capillariid nematode found infecting the swim bladder of the brown mudfish *Neochanna apoda*, an endemic New Zealand fish from peat-swamp-forests. Morphologically, the studied nematodes are distinct from other Capillariinae taxa by the features of the male posterior end, namely the shape of the bursa lobes, and shape of spicule distal end. Male specimens were classified in three different types according to differences in shape of the bursa lobes at the posterior end, but only one was successfully molecularly characterized. Molecular analysis indicated that the studied capillariid is distinct fromm other genera. However, inferences about the phylogenetic position of the capillariid reported here will remain uncertain, due to the limited number of Capillariinae taxa molecularly characterized. The discovery of this new capillariid, which atypically infects the swim bladder of its host who itself inhabits a very unique ecosystem, overlays the very interesting evolutionary history of this parasite, which for now will remain unresolved.

Introduction

Understanding parasite diversity and distribution patterns, and what drives them, has long resonated with parasitologists and ecologists alike (Morand, 2015). Despite this, our understanding of host-parasite associations remains limited for many vertebrates, and where described, often at low taxonomic resolution (Poulin & Leung, 2010; Poulin & Presswell, 2016). In New Zealand's freshwater fish community for example, host-parasite relationships have been studied in approximately half of the 40 recognised host species (Hine, 2000; NIWA, 2017), with parasite taxa often identified to genus at best. Whilst such low resolution may be partly attributed to the global loss of taxonomic classification expertise (Poulin & Leung, 2010), taxonomic classification prior to the widespread use of molecular techniques, or the fish species themselves only being identified relatively recently (McDowall & Waters, 2003); for many freshwater fish species, their parasite communities have yet to be examined. Advancements in the number of host species examined continue to be made (e.g. Kelly *et al.*, 2009; Poulin *et al.*, 2012), with molecular approaches often aiding the verification of cryptic parasite taxa (e.g. Blasco-Costa *et al.*, 2017). Although considerable progress has been achieved in regard to trematodes (e.g. Blasco-Costa *et al.*, 2016, 2017), other parasite groups, such as nematodes, remain poorly described (2/14 taxa described to species, Moravec & Taraschewski, 1988; Hine, 2000; Luque *et al.*, 2010).

One such under-studied fish species is the brown mudfish *Neochanna apoda*, an endemic New Zealand fish occupying small (~1.6 m^3) pools in peat-swamp-forests, formed predominantly by sporadic tree-fall events (Eldon, 1968; White *et al.*, 2015a,b). Whilst nationally threatened, this fish species thrives (e.g. 250 fish/m^3, White *et al.*, 2016) in these isolated pools, which are characterised by frequent and unpredictable droughts, high acidity (~3.5 pH) and low oxygen (White *et al.*, 2015a, 2016); conditions lethal to most fish species (Alabaster & Lloyd, 1982). Mudfish move

between pools (up to 112 m, White *et al.*, 2015b) during high rainfall events which flood the forest floor and create connections among isolated pools. Typically, these pools contain isolated mudfish populations, however sympatric populations with banded kokopu *Galaxias fasciatus* may occur in pools within flood plain habitats, where environmental conditions are less stressful to other fish species (White *et al.*, 2015a). The increasing environmental severity gradient from flood plain to forest pool habitats, which limits the distribution of banded kokopu in the peat-swamp-forest landscape, also has the potential to influence the parasite meta-community structure.

To date, our understanding of parasite infections of *Neochanna* fishes (five New Zealand and one Australian species) has been largely limited to external observations (O'Brien, 2005) and incidental reports of parasites encountered during stomach or gonad examinations (Eldon, 1978). Of the five parasite taxa known from brown mudfish, two nematode taxa, *Hedruris spinigera* Baylis, 1931 (family Hedruridae) and an unidentified rhabditoid, have been detected in the stomach (Eldon, 1978). In the present pilot study, several nematode specimens were recovered from the swim bladder of brown mudfish, Westland, New Zealand. These specimens were morphologically distinct from previously described nematode taxa from this host species, and from the only nematode species known to infect the swim bladder of New Zealand freshwater fish, *Anguillicola novaezelandiae* Moravec & Taraschewski (1988). Instead, the new specimens were found to more closely resemble capillariid nematodes, of the subfamily Capillariinae Railliet, 1915 (Nematoda: Capillariidae, following Moravec 2001; Nematoda: Trichuridae, following Gibbons, 2010). The subfamily Capillariinae includes over 300 described species parasitizing a large number of hosts, including fishes, mammals and birds (Moravec, 2001). While the classification of Capillariinae is considered one of the most difficult among nematodes (Moravec, 1982; Anderson, 2000), Moravec (1982) proposed what is now the most accepted systematic of the group. According to this classification, one important morphological feature showing interspecific differences in these

nematodes is the structure of the posterior end of the male. Currently, there are 27 genera assigned to this subfamily according to morphological characteristics (Gibbons, 2010), and only a few have been genetically characterized.

Here, using a combination of morphological and molecular approaches, we characterized the new nematodes parasitizing the swim bladder of brown mudfish. We also investigated the distribution of this parasite in relation to pool habitat types (i.e. floodplain or forest) utilised by this host.

Material and methods

Collection and nematode isolation

Forty-six brown mudfish were collected during November 2013 from the Saltwater Forest, Westland, New Zealand (43°07'S, 170°26'E) from four 'floodplain' pools (occasionally flooded from adjacent streams; 18 fish) and three 'forest' pools (isolated from permanent water sources; 28 fish). Mudfish were captured using unbaited gee minnow traps set overnight, with up to 10 individual fish per pool retained for parasitological examination. Fish were euthanized in an overdose of 2-phenoyethanol (approximately 1 g L^{-1}), then either frozen (-20°C) or preserved in 10% buffered formalin until all internal organs were examined for parasites in the laboratory. Nematodes obtained from the swim bladder were placed in 95% ethanol. Fixed specimens were mounted on temporary slides with a glycerol:water (1:1) solution. Morphological study consisted of microscopic examination of the different specimens at different magnifications using a light microscope (Olympus CX41, Olympus Australia Pty Ltd, Notting Hill Victoria, Australia). All specimens were photographed using a digital Olympus DP25 camera as means of morphological vouchering information, and measured in ImageJ (Schneider *et al.*, 2012). Studied specimens are deposited at the parasite collection of the Evolutionary and Ecological Parasitology group at University of Otago (New Zealand). All measurements are in µm unless otherwise stated, and are given as range with average in parentheses.

DNA extraction and sequencing

Extractions of new capillariids genomic DNA were performed from six specimens (two females and four males representing the three male morphs, see below). DNA was isolated using the PureLink® Genomic DNA Kit (Invitrogen, Invitrogen New Zealand Ltd, Auckland, New Zealand) according to the manufacturer's protocol. We amplified two nuclear fragments: a 873 bp of the 18S ribosomal DNA (18S) and 455 bp of the ribosomal DNA first internal transcribed spacer (ITS1). The 18S was amplified using the primers Nem 18S F and Nem 18S R from Floyd *et al.* (2005), while the ITS1 was amplified with the primers rDNA2 (Vrain *et al.*, 1992) and rDNA1.58 s (Cherry *et al.*, 1997). Polymerase chain reactions (PCR) were performed in a total volume of 20 µl, comprising 4 µl of MyTaq TM Red reaction buffer (Bioline (Aust) Pty Ltd, Alexandria, New South Wales, Australia), primers at 0.5 µM each, MyTaq TM Red DNA Polymerase (Bioline) at 0.025 units/µl and approximately 10 ng of DNA template. The PCR reactions consisted of 35 iterations of the following cycle: 30 s at 95ºC, 30 s at 54–58ºC (for 18S and ITS1, respectively) and 1 min at 72ºC, beginning with an additional denaturation step of 3 min at 95ºC, and ending with a final extension at 72ºC for 10 min. PCR amplicons were purified and sequenced by a commercial facility (Macrogen Corporation, http://www.macrogen.com/).

Phylogenetic analysis

Sequence chromatograms of the New Zealand mudfish capillariid were edited and trimmed in Geneious v 8.1.4 (http://www.geneious.com/; Kearse *et al.*, 2012). Nematode 18S sequences comprising *Capillaria*, *Aonchotheca*, *Baruscapillaria*, *Eucoleus*, *Trichuris* and other non-identified Trichocephalida sequences were downloaded to Geneious, providing a total of 22 sequences for analysis (Table 1). Since for the ITS1 fragment, maximum query coverage with other Capillariinae nematode available sequences was of 34%, phylogenetic analysis was only performed for the 18S

fragment. *Adoncholaimus*, *Mylonchulus* and *Mermis* were used as outgroups (Table 1). The 18S dataset was aligned using MAFFT (Katoh *et al.*, 2002), in Geneious employing the E-INS-i algorithms (with default parameters). Final alignment consisted of 910 bp including gaps.

To determine the phylogenetic position of the studied capillariid nematode, a Bayesian inference method was employed in MrBayes v 3.2.2 (Ronquist *et al.*, 2012). A reversible-jump Markov chain Monte Carlo (MCMC) was implemented to integrate over the pool of all 203 possible reversible 4×4 nucleotide models. A model *a priori* was specified, allowing for the estimation of base frequencies, the proportion of invariable sites and rate-variation across sites with a gamma distribution. Ten million MCMC generations were sampled every 1000th step, with the first 25% discarded as burn-in. Two independent runs were carried out, each with one cold and three heated chains (T=0.04) and pooled the samples after burn-in was removed. Mixing and convergence of each run were monitored through the statistics provided in MrBayes [values of standard deviation of partition frequencies (<0.01), potential scale reduction factors (PSRF) (1.00), effective sample sizes (ESS) (>200)] and in Tracer v1.6 (Rambaut *et al.*, 2014). Estimates of evolutionary divergence for the 18S pairwise uncorrected differences (*p*-distance) between and within defined lineages were made in MEGA v 6 (Tamura *et al.*, 2013). Newly reported sequences from this study were submitted to GenBank under the accession numbers: 18S: XXXX (18S) and XXXX (ITS1) (AFTER ACCEPTANCE).

Results

Mudfish were found to be infected with nematode and acanthocephalan parasites in the swim bladder and intestinal regions. While acanthocephalans were found in mudfish populations from both ‘forest’ and ‘floodplain’ pools (to be described elsewhere, Paterson *et al.*, *in prep*), nematodes infecting the swim bladder were only found in ‘floodplain’ pools. Twenty-two percent of the mudfish from ‘floodplain’ pools were infected with nematodes, with intensity ranging between one

to three nematodes per fish. In total, nine nematodes (six males and three females) were found, though only five were complete specimens. The nematode specimens of the present material were identified as belonging to the family Capillaridae (following Moravec 2001; Trichuridae, following Gibbons, 2010) subfamily Capillariinae based on the presence of an oesophagus consisting of a long glandular section, i.e. the stichosome (collection of a long chain of glandular cells, the stichocytes), and by the shape of the male posterior end. Morphological variability was observed at the male posterior end, namely in the thickness and extension of the lateral projections of the pseudobursa. Three different male morphotypes were provisionally defined. Male morph 1 (three specimens) was found infecting only one brown mudfish, whereas male morph 2 (one specimen) and morph 3 (two specimens) were found in the same host specimen.

Morphological description

General (based on 5 complete specimens and 4 incomplete specimens): Capillarinae Railliet, 1915. Nematodes with rounded extremities, with anterior end narrower than posterior. Oral aperture terminal. Stichosome composed of one raw of elongate stichocytes with small nuclei, subdivided into transverse annuli (Fig. 1a).

Male: Body 3.83 - 4.58 (4.05, n=4) mm long. Length of the entire oesophagus 1.38-1.61 (1.49, n = 2), representing 37% of the body length. Muscular oesophagus 239.51-278.10 (258.80, n = 2) long, stichosome 1.1-1.37 (1.24, n = 2) mm long, composed of 30 stichocytes (n=1). Well sclerotized spicule, 368.24 - 446.43 (409.43, n=5) µm long, with a tronchanter-like shape at proximal end (Fig. 1h,k), and with narrower and incised-like shape distal end (Fig. 1g). Spicular sheath without spines. Male cloacal aperture subterminal. Excretory pore at the level of the proximal end of spicule (when fully retracted). Caudal lateral alae absent. Membraneous bursa of male supported on either side by one medium round lobe, each lobe provided with a projection ventrally bent (Fig. 1f,g,i,j,l,m). Male morph 1 (n=3; Fig. 1f-h) with a membranous bursa supported by 2 lateral lobes, each with 1

elongate papilla and a dorsal ventrally bent projection extending beyond the papilla, but not reaching the posterior border of bursa (Fig. 1g). Male morph 2 (n=1, Fig. 1i-j) with a small membranous bursa with a bell-like shape (Fig. 1j), with 2 sessile rounded papillae, and 2 dorso-lateral projections (Fig. 1i). Male morph 3 (n=2; Fig. 1l-m), with a membranous bursa, with 2 sessile papillae, and 2 digitiform ventrally bent projections.

Female (gravid): Body 7.80 mm (n=1) long. Length of the entire oesophagus 2.43mm (n=1), representing 31.15% of the body length. Muscular oesophagus 242.75 long, stichosome 2.19 mm long, composed of 37 stichocytes. Vulva situated at the level of the oesophago-intestinal junction, with vulva lips not elevated (Fig. 1b,c). Vulvar appendage not observed. Ovary extending posteriorly to the junction of the intestine and rectum, with anus located subterminal (Fig. 1e). Uterus containing numerous eggs arranged in one row near the vulva and two to three rows more posteriorly (Fig. 1d). Eggs (n= 6) with 58.35 - 62.94 (61.71) in length and 25.94 - 31.23 (27.73) in width. Barrel-shaped eggs appearing with two layers; a hyaline inner layer and outer layer thicker with a fine reticular engrave structure (Fig. 1d).

Remarks

Capillariid generic identification is based on specific morphological elements, namely the structure of the posterior end of the male. We followed Moravec (1982) and Gibbons (2010), and restricted the taxonomic discussion to those found in fishes only. Several genera were easily excluded based on the features of stichosome, characters of the male caudal region and female features, i.e. *Capillaria* Zeder, 1800, *Gessyella* Freitas, 1959, *Schulmanela* Ivashkin, 1964, *Freitascapillaria* Moravec, 1982, and *Piscicapillaria* Moravec, 1987. At a general level of the male pseudobursa, the studied specimens share some features with *Paracapillaria* Medonça, 1963, but the latter present large sessile papillae. *Paracapillaroides* Moravec, Salgado-Maldonado and Caspeta-mandujano, 1999 also present some resemblance at the level of pseudobursa, but it presents a spinose spiculer

sheath, which is not observed on the mudfish capillariid specimens. While on basis of shape of the spicule distal end and location in host the new specimens may represent a new species or even a new genus, we refrain from providing a formal description due to low number of examined specimens, together with the uncertainties surrounding the male morphs. In particular, it was uncertain whether the observed morphological variability of the three male morphs also represent genetic variability and if so, if those specimens are or not closely related. In the latter case, we would need to determine to which morph the female morphology described here corresponds to. However, the national conservation status of the host species of the studied nematode restricted the collection of additional specimens in order to clarify such uncertainties.

Phylogenetic analysis

The DNA of a single male (morph 1) was successfully amplified and used for phylogenetic inference of capillariid found in New Zealand mudfish. A phylogenetic inference tree based on the 18S nucleotide sequences is shown in Fig. 2. The molecular analysis supported the morphological identification, placing the 18S sequence obtained in this study together with other sequences identified as belonging to the subfamily Capillariinae, but forming a separate clade. Among the Capillarinae available taxa, the new capillariid seems genetically more closely related to the *Pearsonema* clade (3.4% uncorrected p-distance). This observed genetic divergence is higher than the one observed among other genera, namely between *Pearsonema* and *Aonchotheca* clades (2.6% uncorrected p-distance).

Discussion

On the basis of both morphological and phylogenetic assessments, the nematodes specimens found infecting the swim bladder of the brown mudfish were identified as belonging to the subfamily Capillariinae. At the morphological level, the new nematodes are distinct from other Capillariinae

taxa by the shape of spicule distal end and the shape of the bursa lobes in males. Morphological variation at male posterior end was observed, however we were unable to determine if such variation was an artefact of specimen preservation, or reflects genetic variability within these new nematodes, since only male morph 1 was successfully molecularly characterized. While presenting unique morphological features, we found some similarities between the overall morphological features of the specimens reported here and other capillariid parasites of fishes, i.e. *Paracapillaria* and *Paracapillaroides.* Only a few Capillariinae taxa have been molecularly characterized, so we were unable to properly assess to which level morphological similarities between the mudfish capillariid and other taxa, also match phylogenetic relatedness.

According to Moravec and Justine (2010), eight Capillariinae genera are known to parasitize fish, but none are documented in the swim bladder as observed in the present study. The only recorded trichinelloid nematode found in the swim bladder (Gibbons, 2010), genus *Huffmanela* Moravec, 1987, belongs to a different family (Trichosomoididae Hall, 1916), and is also morphologically distinct from the specimens reported here. Following Moravec's (1982) hypothetical evolution of capillariids based on the structure of the male caudal end and spicule sheath, *Capillaria* was one of the most basal divergences, and *Pearsonema* was more closely related to *Barruscapillaria* than to *Aonchotheca*. In the reported inferred phylogeny, and similar to the one estimated by Tamaru *et al.* (2015), *Aonchotheca* is more closely related to *Pearsonema*. While our results and Tamaru *et al.* (2015) seem to some extend support the validity of Moravec's (1982) taxonomic classification, we highlight the need for thorough analysis of the morphological evolution of this nematode subfamily. We highly recommend that new species descriptions provide a molecular characterization together with the detailed morphological assessment. As several unreported species may await discovery, inferences regarding the evolutionary history of capillariid nematodes will remain challenging if such efforts are not undertaken.

Although the life cycle of the studied capillariid remains unknown (i.e. whether it requires a

facultative intermediate invertebrate host), its apparent restricted distribution to ‘floodplain’ pools despite the considerable mobility of its mudfish host (White *et al*., 2015b), suggests this nematode may be less tolerant to the environmental extremes (e.g. drought, high acidity, hypoxia) of the ‘forest’ pools. Whilst mudfish are capable of surviving periods of drought (over thirty days without water; White *et al.*, 2016) by burrowing into the soft peat pool substrate and maintaining a low metabolic rate via cutaneous respiration (Urbina *et al.*, 2014a,b), the environmental extremes of ‘forest’ pools may be lethal to infective stages of this capillariid. Thus adaptations to the harsh environmental conditions in addition to pool isolation may enable brown mudfish to escape some of their enemies, both in the form of parasites and their predators (e.g. banded kokopu).

It remains to be determined whether other mudfish species also harbour this, or related capillariid species given the paucity of parasitological knowledge currently available for the *Neochanna* genus in general. However, the unique habitat niche occupied by brown mudfish in combination with the non-overlapping distribution of mudfish species, suggest that this capillariid is likely to have evolved in isolation together with its host. Furthermore, recent parasitological investigations suggested that other parasite taxa also found in mudfish may be genetically distinct to those infecting other New Zealand freshwater fish species (Paterson & Hernadez-Ortez *pers comm.*). Regarding the capillariid reported herein, future studies should assess host specificity of the new taxa, i.e. whether other fish species (e.g. banded kokopu) in the flood plain habitat are also hosts of this nematode, and assess the diversity of other capillariid present in New Zealand to assess their phylogenetic relatedness. Only then can a proper inference of this parasite’s evolutionary history be made.

In this study we described a new capillariid nematode parasitizing the swim bladder of brown mudfish in New Zealand. Our findings highlight the potential diversity of parasite assemblages which has likely lain hidden in this very unique ecosystem, and establishes a baseline for future

studies. The findings of the study also highlights the necessity of a more collaborative approach between parasitologists and researchers from other fields, in our case fish biologists, which could promote the discovery of host-parasite relationships in New Zealand.

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Statement of interest

None.

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Tables

Table 1. Nematode sequence data included in the phylogenetic analysis with respective family, geographic location, host identity, organ where it is located in the host and GenBank accession number.

GI, gastrointestinal tract

Figure legends

Figure 1. Morphology of the new capillariid from brown mudfish *Neochanna apoda*. (a) stichocytes at middle part of the stichosome; (b) general view of adult female; (c) vulvar region, lateral view; (d) egg; (e) posterior end of female, lateral view; Male morph 1: (f) posterior end of male, lateral view; (g) posterior end of male, ventral view; (h) spicule anterior end; Male morph 2: (i) posterior end of male, lateral view; (j) posterior end of male, ventral view; (k) spicule anterior end; Male morph 3: (l) posterior end of male, lateral view; (m) posterior end of male, ventral view; (n) spicule anterior end. S, spicule; Vu, vulva; An, anus.

Figure 2. Phylogenetic relationships of the new capillariid from brown mudfish *Neochanna apoda* based on Bayesian analysis of 18S rRNA gene sequences. Values represent Bayesian posterior probabilities (values below 0.75 not represented). Sequence newly reported in this study is in bold, and other sequences are presented with the respective GenBank accession number in parentheses. (see Table 1 for additional details).

Figure 1.

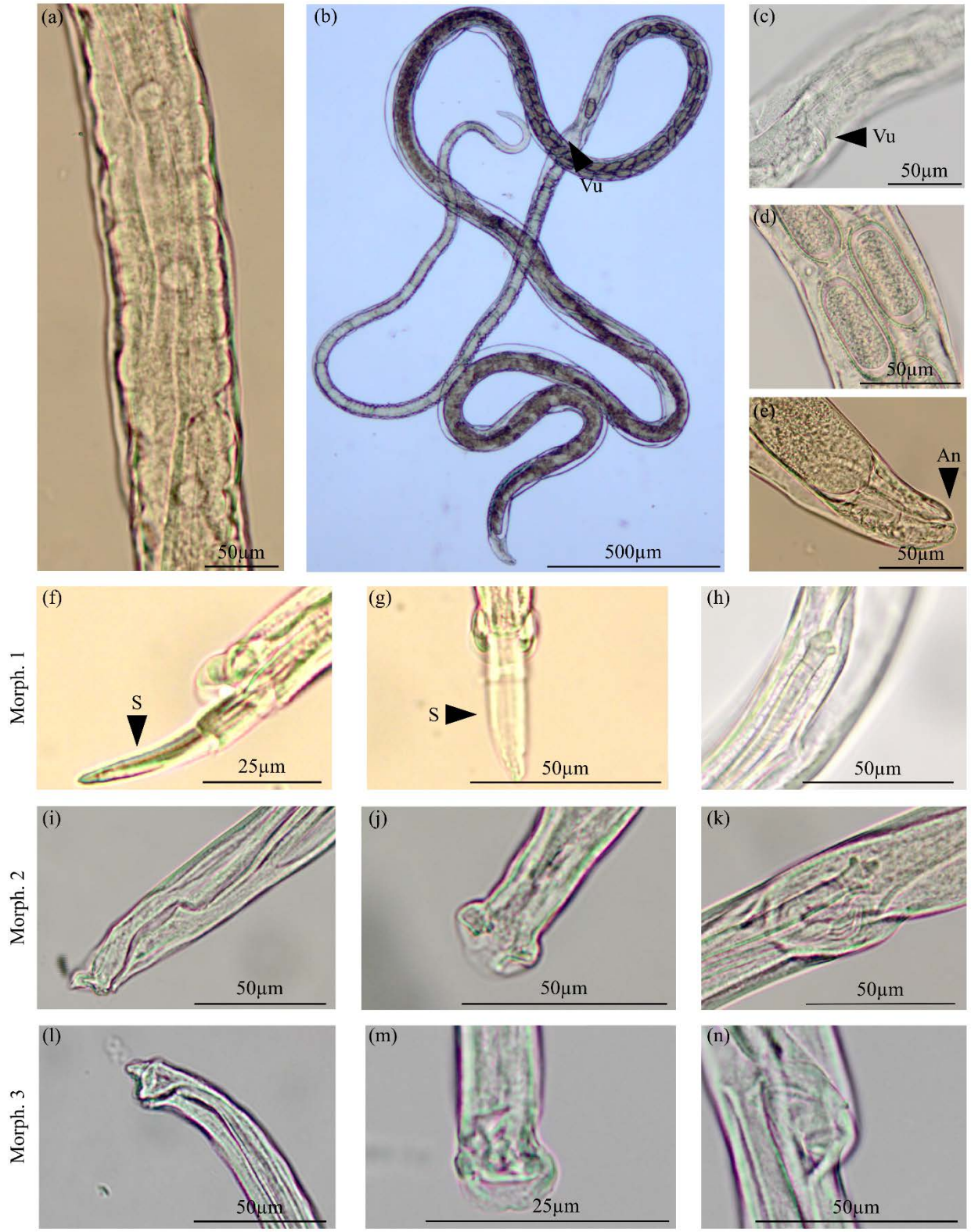
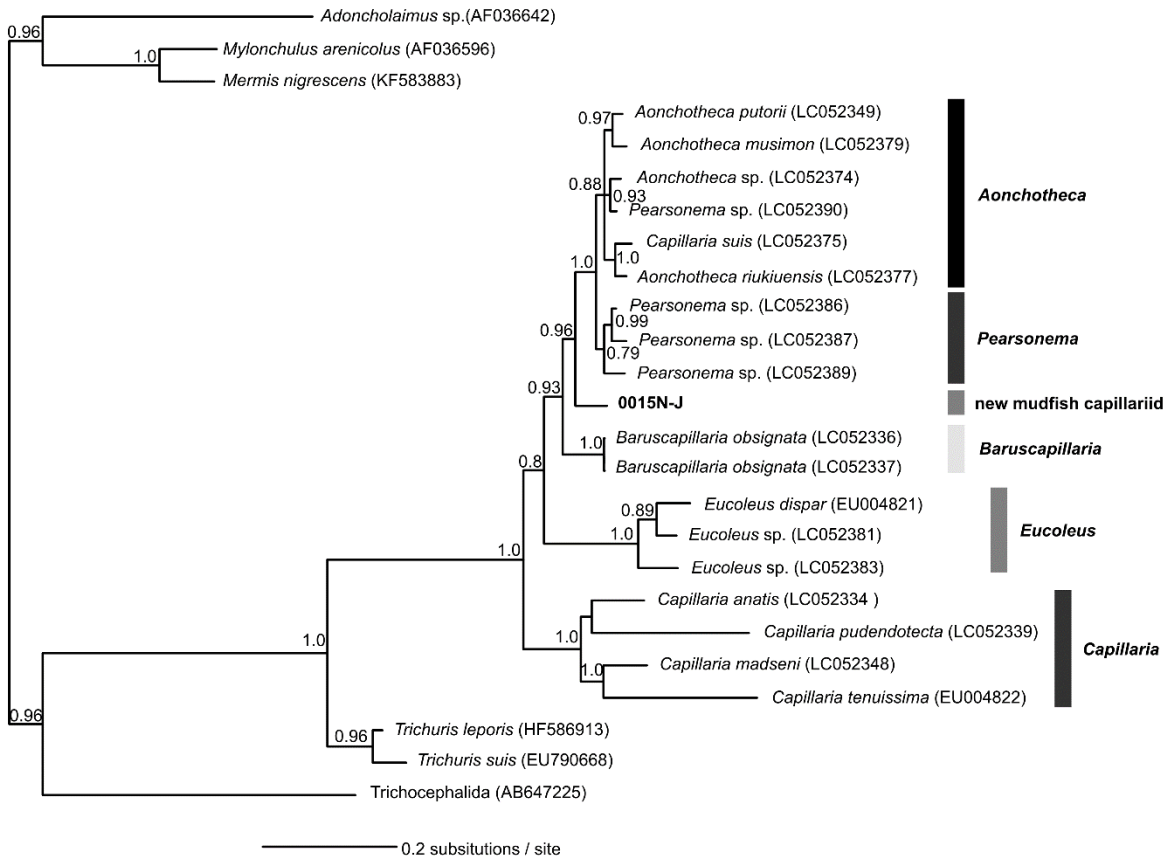

(a)
50µm
(b)
Vu
500µm
(c)
Vu
50µm
(d)
50µm
(e)
An
50µm
Morph. 1
(f)
S
25µm
(g)
S
50µm
(h)
50µm
Morph. 2
(i)
50µm
(j)
50µm
(k)
50µm
Morph. 3
(l)
50µm
(m)
25µm
(n)
50µm

Figure 2.


Adoncholaimus sp.(AF036642)
Mylonchulus arenicolus (AF036596)
Mermis nigrescens (KF583883)
Aonchotheca putorii (LC052349)
Aonchotheca musimon (LC052379)
Aonchotheca sp. (LC052374)
Pearsonema sp. (LC052390)
Capillaria suis (LC052375)
Aonchotheca riukiuensis (LC052377)
Pearsonema sp. (LC052386)
Pearsonema sp. (LC052387)
Pearsonema sp. (LC052389)
0015N-J
Baruscapillaria obsignata (LC052336)
Baruscapillaria obsignata (LC052337)
Eucoleus dispar (EU004821)
Eucoleus sp. (LC052381)
Eucoleus sp. (LC052383)
Capillaria anatis (LC052334)
Capillaria pudendotecta (LC052339)
Capillaria madseni (LC052348)
Capillaria tenuissima (EU004822)
Trichuris leporis (HF586913)
Trichuris suis (EU790668)
Trichocephalida (AB647225)
0.2 subsitutions / site
Aonchotheca
Pearsonema
new mudfish capillariid
Baruscapillaria
Eucoleus
Capillaria