This document is the accepted manuscript version of the following article:
Blasco-Costa, I., Seppälä, K., Feijen, F., Zajac, N., Klappert, K., & Jokela, J. (2020). A new species of Atriophallophorus Deblock & Rosé, 1964 (Trematoda: Microphallidae) described from in vitro-grown adults and metacercariae from Potamopyrgus antipodarum (Gray, 1843) (Mollusca: Tateidae). Journal of Helminthology, 94, e108 (15 pp.). https://doi.org/10.1017/S0022149X19000993

A new species of *Atriophallophorus* Deblock & Rosé, 1964 (Trematoda: Microphallidae) described from *in vitro*-grown adults and metacercariae from *Potamopyrgus antipodarum* (Gray, 1843) (Mollusca: Tateidae)

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Short title: A new species of *Atriophallophorus* from New Zealand

Abstract

The adult and metacercaria life stages of a new species of the microphallid genus *Atriophallophorus* Deblock & Rosé, 1964 are described from specimens collected at Lake Alexandrina (South Island, New Zealand). In addition to molecular analyses of ribosomal and mitochondrial genes, metacercariae of *Atriophallophorus winterbourni* n. sp. from the snail host *Potamopyrgus antipodarum* (Gray) were grown *in vitro* to characterise internal and external morphology of adults using light and scanning electron microscopy and histological techniques. *A. winterbourni* n. sp. is readily distinguishable from *A. coxiellae* Smith, 1973 by having a different structure of the prostatic chamber, sub-circular and dorsal to genital atrium, rather than cylindrical, fibrous, elongate and placed between the seminal vesicle and the genital atrium. The new species is most similar to *A. minutus* (Price, 1934) with regards to the prostatic chamber and the morphometric data, but possesses elongate-oval testes and subtriangular ovary rather than oval and transversely-oval in *A. minutus*. Phylogenetic analyses including sequence data for *A. winterbourni* n. sp. suggested a congeneric relationship of the new species to a hitherto undescribed metacercariae reported from Australia, both forming a strongly supported clade closely related to *Microphallus* and *Levinseniella*. In addition, we provide an amended diagnosis of *Atriophallophorus* to accommodate the new species and confirm the sinistral interruption of the outer rim of the ventral sucker caused by the protrusion of the dextral parietal atrial scale at the base of the phallus.

Keywords: *Microphallus*, *in vitro* culture, phylogeny, rRNA genes, mitochondrial genes, life-cycle, freshwater, mud snail, waterfowl, New Zealand.

Introduction

A microphallid species reported as *Microphallus* sp. from New Zealand has been largely used as a model species in parasitology and evolutionary studies for more than three decades (e.g., Lively, 1987; Dybdahl & Lively, 1998; Lively & Dybdahl, 2000; Jokela *et al.*, 2009; Gibson *et al.*, 2016a). Broad knowledge has accumulated on the role of this parasite in coevolutionary dynamics with the host populations. Particularly, in this case the coevolutionary dynamics are suggested to favour sexual reproduction by the host (i.e. "parasite hypothesis" or "Red Queen hypothesis") for maintenance of sex (Jaenike, 1978; Hamilton, 1980; Lively, 2016). However, basic knowledge such as the identity of the species, its genetic diversity and phylogenetic affinities are still lacking, with the consequences that it may be difficult to judge the source of heterogeneity in infection experiments and field surveys.

Microphallids are a diverse and cosmopolitan group of very small worms, typically found in the intestine of birds. Second intermediate hosts tend to be crustaceans but several species present abbreviated life cycles where the metacercariae encyst within the mollusc first intermediate host. Their metacercariae are usually identical to the adult stage in the definitive host. In the definitive host, microphallids tend to mature in a few days and live only for several weeks (Galaktionov & Dobrovolskij, 2003). This feature of microphallids makes them amenable to *in vitro* culture as a method to obtain the mature adults and be able to characterise and describe new species. Such an alternative is nowadays desirable, considering the limitations of obtaining permits to collect potential vertebrate definitive hosts, which are in many cases protected by national or international regulations (Blasco-Costa & Poulin, 2017).

The aim of this study is to morphologically characterise and describe the adult and metacercaria life stages of this microphallid, which represents a new species of the genus *Atriophallophorus* Deblock & Rosé, 1964 instead of *Microphallus* Ward, 1901 as had been previously reported. Metacercariae of microphallid specimens from the snail host *Potamopyrgus antipodarum* (Gray) were grown in vitro and the adults were analysed with light microscopy, scanning electron microscopy and histological techniques to characterise their internal and external morphology. In addition, this study provides an amended diagnosis of *Atriophallophorus*, molecular data for two nuclear and two mitochondrial markers of the new species and an evaluation of its relationship with other microphallids.

Material and methods

Specimens

Potamopyrgus antipodarum snails were collected using a kick net and snorkelling from two sites in Lake Alexandrina (South Island, New Zealand), site "Swamp" (-43.962102, 170.441728) and site "JMS" (-43.937199, 170.459495). Live metacercariae were dissected from four infected snails from each locality and allowed to excyst in Tyrode's salt solution (Sigma) with pancreatin (3 mg/ml; Sigma) and penicillin-streptomycin-neomycin solution (8 % v/v; Sigma) at 39 ºC. Excysted juvenile worms were washed twice with Tyrodes's salt solution supplemented with penicillin-streptomycin-neomycin solution (8 % v/v). Then, juveniles from the same locality were pooled and transferred to culture medium of RPMI 1640 (Gibco) supplemented with horse serum (20 % v/v, Gibco), penicillin-streptomycin-neomycin solution (8 % v/v), HEPES buffer (25 mM; Gibco) and Amphotericin B solution (0.25 mg/ml; Sigma). Juveniles were incubated at 39 ºC and cultured for up to 3 days (72 hours). Fresh culture media was changed daily. Metacercariae right after hatching and *in vitro*-grown adults after 24h, 48h and 72h of culturing were fixed in hot saline and preserved in 75% ethanol for later morphological examination. A subsample of specimens was preserved in hot formalin for examination using scanning electron microscopy (SEM) and histological analysis. Additionally, five metacercariae from each site (from a pool of four snails each) were preserved in 100% ethanol for molecular analyses.

Morphological data

Metacercariae and adult specimens grown *in vitro* were stained using iron acetocarmine, dehydrated through a graded ethanol series, cleared in dimethyl phtalate, and examined as permanent mounts in Canada balsam. Figures were made using a drawing tube mounted on a Zeiss light compound microscope at ×1,600 magnification. Measurements of the specimens were taken from drawings at ×640 magnification. Five specimens were dehydrated in a graded ethanol series, critical point-dried and sputter-coated with gold for SEM examination using a Zeiss DSM 940A (Zeiss AG, Oberkochen, Germany) at an accelerating voltage of 5 kV. Three specimens were used for histology after dehydration through a graded ethanol series followed by propylene oxide and immersed in Epon resin. Histological sections of 1µm were made with an ultramicrotome Reichert-Jung Ultracut E, stained with toluidine blue 0.75% and examined using the light microscope mentioned above. All measurements in the text are in micrometers unless otherwise stated and are given as the range followed by the mean±standard deviation. Permanent mounts of the type material and SEM preparations are

deposited in the Platyhelminthes collection of the Natural History Museum of Geneva, the Institute of Parasitology of the Academy of Sciences of the Czech Republic and the Otago Museum, Dunedin, New Zealand.

Molecular data

Four metacercariae isolates from Swamp and five from JMS originating from a pool of four infected *Potamopyrgus antipodarum* from each site were characterised molecularly. Due to the small size of these specimens, it was impossible to keep hologenophores of the sequenced specimens. Nonetheless, the specimens used for morphological description herein represent paragenophores and likely genetic clones of the sequenced material. Genomic DNA was extracted from ethanol-fixed metacercariae isolates in 200 µL of a 5% suspension of Chelex® in deionised water and containing 0.1 mg/ml proteinase K followed by incubation at 56 °C for 5 h, boiling at 90 °C for 8 min, and centrifugation at 14,000 g for 10 min. Partial fragments were amplified of the large ribosomal subunit (28S) [1,800 bp; primers U178F: 5′-GCA CCC GCT GAA YTT AAG-3′ and L1642R: 5′-CCA GCG CCA TCC ATT TTC A-3′ (Lockyer *et al.*, 2003)] and internal transcribed spacer 2 (ITS2) [500 bp; primers 3S: 5′-GTA CCG GTG GAT CAC GTG GCT AGT G-3′ and ITS2·2: 5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′ (Morgan & Blair, 1995; Cribb *et al.*, 1998)]. Additionally, two fragments of mitochondrial genes (mt) were amplified: cytochrome oxidase subunit I (Cox I) [1,050 bp; primers JB3: 5′-TTTTTTGGGCATCCTGAGGTTTAT -3′ and microph_rev: 5′- AAT CAT GAT GCA AAA GG-3′ (Bowles *et al.*, 1993; newly designed)] and nicotinamide adenine dinucleotide dehydrogenase subunit 5 (NADH5) [744 bp; primers F2_micND5: 5′-CTTCAACCTTGGTTGCTGCC-3′ and R2_micND5: 5′-TCCCAACGAAACCTAAAACTGC-3′ (newly designed)].

Polymerase chain reaction (PCR) amplifications were performed in 20 µl reactions containing 2 µl of extraction supernatant (~ 10–20 ng of template DNA), 2× MyFi™ Mix (Bioline France, France; containing DNA Polymerase, dNTPs, MgCl2 and enhancers at optimal concentrations) and 0.4 µM of each primer combination. Thermocycling conditions used for amplification of the rDNA regions followed Galaktionov *et al.* (2012). The following thermocycling profile was used for amplification of the mt Cox I and NADH5 fragments: denaturation (95 °C for 3 min); 38 cycles of amplification (94 °C for 50 s, 52 °C for 30 s and 72°C for 1 min); and 4 min extension step at 72 °C. PCR amplicons were purified prior to sequencing using exonuclease I and shrimp alkaline phosphatase enzymes (Werle *et al.*, 1994). Amplicons were cycle-sequenced from both strands using PCR primers and an internal

primer for the 28S fragment [L1200R: 5′-GCA TAG TTC ACC ATC TTT CGG-3′ (Littlewood *et al.*, 2000)] at the commercial facility Macrogen (Amsterdam, The Netherlands). Contiguous sequences were assembled and edited using Geneious® (v. 8.1 Biomatters Ltd., Auckland, New Zealand) and submitted to GenBank (see accession numbers in table 1).

Molecular analyses

Newly generated sequences for the 28S rDNA and the ITS2 fragments were aligned in two independent datasets together with the published sequences of other microphallids from GenBank (see accession numbers in table 1 and figs 3 and 4). The sequences were aligned using default parameters of MAFFT implemented in Geneious®, and the extremes of the alignment were trimmed to match the shortest sequences. The 28S dataset (1280 bp long) included 14 representative sequences of *Microphallus* spp., 12 of *Maritrema* spp., one each of *Levinseniella, Longiductotrema* and an unidentified microphallid of Kudlai *et al.* (2015) and a sequence labelled as *Microphallus fusiformis* (which should be disregarded as a species of *Microphallus*, see Kudlai *et al.* (2015)) retrieved from GenBank (table 1). Additionally, five sequences of species belonging to sister families of the Microphallidae, i.e. Lecithodendriidae, Pleurogenidae and Prosthogonimidae in the Microphalloidea; and three sequences of species in the Plagiorchioidea were retrieved from GenBank and included as outgroups. The ITS2 dataset (401 bp long) included ten representative sequences of *Microphallus* spp.; nine sequences of *Maritrema*; one each of *Levinseniella*, *Probolocoryphe* and *Longiductotrema*; and one of an unidentified microphallid of Kudlai *et al.* (2015). The phylogenetic analyses were run on the two datasets individually under the maximum likelihood (ML) and Bayesian inference (BI) criteria, employing the nucleotide substitution model GTR+Γ. ML analyses were conducted using the program RAxML v. 8.2 (Stamatakis, 2014). All model parameters, bootstrap nodal support values (1000 repetitions) and an extended majority rule consensus topology were estimated using RAxML. BI trees were constructed using MrBayes v. 3.2 (Ronquist *et al.*, 2012), running two independent MCMC runs of four chains with standard settings for 10^7 generations and sampling tree topologies every 10^3 generation. Burn-in periods were set automatically to 25% generations ensuring the remaining trees were obtained after values for standard deviation of split frequencies were < 0.01. A majority rule consensus topology and nodal support estimated as posterior probability values (Huelsenbeck *et al.*, 2001) were calculated from the remaining trees. All MrBayes and RAxML analyses were performed on the computational resource CIPRES (Miller *et al.*,

2010). Genetic divergences amongst taxa were calculated as uncorrected p-distances for each gene region using MEGA v. X (Kumar *et al.*, 2018).

Results

Microphallidae Ward, 1901

Atriophallophorus Deblock & Rosé, 1964

Atriophallophorus winterbourni n. sp.

Taxonomic summary

Synonyms. Metacercaria A of Winterbourn (1974); *Microphallus* sp. of Lively (1987); Lively (1989); Lively & McKenzie (1991); Jokela & Lively (1995); Dybdahl & Lively (1996); Levri & Lively (1996); Lively & Jokela (1996); Dybdahl & Lively (1998); Levri (1999); Krist *et al.* (2000); Levri & Fisher (2000); Jokela *et al.* (2003); Dybdahl & Krist (2004); Krist *et al.* (2004); Lively *et al.* (2004); Osnas & Lively (2004); Levri *et al.* (2005); Fromme & Dybdahl (2006); Osnas & Lively (2006); Koskella & Lively (2007); Lagrue *et al.* (2007); Dybdahl *et al.* (2008); Lagrue & Poulin (2008); Lively *et al.* (2008); Jokela *et al.* (2009); King *et al.* (2009); Koskella & Lively (2009); King *et al.* (2011a); King *et al.* (2011b); Koskella *et al.* (2011); Osnas & Lively (2011); Vergara *et al.* (2013); Paczesniak *et al.* (2014); Vergara *et al.* (2014); Gibson *et al.* (2016a); Gibson *et al.* (2016b); McKone *et al.* (2016); Bankers *et al.* (2017); Bankers & Neiman (2017); Vergara *et al.* (2017); Gibson *et al.* (2018); Paczesniak *et al.* (2019); *Microphallus* sp. "lively" of Hofmann *et al.* (2016); *Microphallus* sp. "livelyi" of Hechinger (2012); Soper *et al.* (2014); Bankers & Neiman (2017); *Microphallus livelyi* (nomen nudum) of Bankers & Neiman (2017).

Type host. *Potamopyrgus antipodarum* (Gray) (Mollusca: Tateidae; first and second intermediate host).

Definitive host. Waterfowl (see Osnas & Lively, 2011).

Type-locality. Lake Alexandrina, South Island, New Zealand (site "Swamp": -43.962102, 170.441728).

Other localities. Lake Alexandrina, South Island, New Zealand (site "JMS": -43.937199, 170.459495).

Site in host. Intermediate host: metacercaria encysted in gonads.

Type-material. Holotype (MHNG-PLAT-129859), 22 paratypes (MHNG-PLAT-129860 – MHNG-PLAT-129865) and 7 metacercariae vouchers (MHNG-PLAT-129866 – MHNG-PLAT-129867) deposited in the Platyhelminthes collection of the NHMG, 2 paratypes

(IPCAS D-803) deposited in the helminthological collection of the Institute of Parasitology, České Budějovice, Czech Republic (IPCAS) and 4 paratypes (OMNZ IV110293 – OMNZ IV110295) and 2 voucher metacercariae (OMNZ IV110296) deposited at the Otago Museum, Dunedin, New Zealand.

Representative DNA sequences. 28S rDNA, 4 sequences of specimens from Swamp and 5 of specimens from JMS (GenBank MN342153–MN342154); ITS2 rDNA, 8 identical sequences (4 of specimens from Swamp and 4 from JMS; GenBank MN342155); mt CoxI, 8 identical sequences (3 of specimens from Swamp and 5 from JMS; GenBank MN342156); and mt NADH5, 4 sequences of specimens from Swamp and 5 of specimens from JMS; GenBank MN342157– MN342158).

ZooBank registration. To comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature, details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *A. winterbourni* n. sp. is urn:lsid:zoobank.org:pub:E0E7C4F3-430E-42BD-9FEC-2D11A159DD12.

Etymology. The species is named after Professor Michael Winterbourn in recognition for his contribution to the field of freshwater ecology, in particular our knowledge of freshwater invertebrates of New Zealand, including the first mention of this species.

Description of adult (figs 1a-c and 2a-h; table 2; Supplementary table S1)

[Based on whole mounts of 29 gravid specimens grown *in vitro* culture for 24–72h and SEM preparations. Measurements provided as range and mean±standard deviation for the type-series, variation associated with specimens from each age class (duration of culture) are provided separately in Supplementary table 1].

Body minute, triangular, often curved concave ventrally (body width to length ratio 1:1.3–1.9 (1:1.6±0.4)) with maximum width at posterior level of testes, 145–200 (167±12) × 85–115 (100±8). Tegument bears spines, glands and sensory papillae. Spines palmate, smooth, 5–9 prongs; present in lateral margins and alongside midline, separated by two narrow ventro-lateral regions devoid of spines, sparser towards posterior extremity on dorsal side; anterior forebody spines width 1.5–1.8, inter-spine space 0.6–0.8; mid forebody spines width 1.9–2.2, inter-spines space 0.6–1; anterior dorsal spines width 2.2–2.3, inter-spines space 1.0–1.5; mid dorsal spines width 1.5–1.8, inter-spines space 2.1–4.1; lateral posterior dorsal spines width 1.6–1.8, inter-spines space 1.2–1.5. Forebody 81–125 (97±9) long, representing 52–63 % (58±2 %) of body length. Glands and sensory papillae at anterior extremity, surrounding oral sucker (>14) and lateral margins of body.

Oral sucker subterminal, spherical, 21–29 (25±2) × 21–29 (25±2). Ventral sucker at two-thirds of body length, subspherical, complete, 24–30 (27±1) × 22–33 (28±2); outer rim crescent, interrupted sinistrally by genital pore, bearing spines and 9 glands; oral sucker to ventral sucker length ratio 1: 0.9–1.2 (1:1.1±0.2), width ratio 1: 0.9–1.3 (1:1.1±0.1). Pre-pharynx absent. Pharynx small, oval 11–17 (14±2) × 11–15 (13±1). Pharynx length to oral sucker length ratio 1:1.5–2.4 (1:1.7±0.2). Oesophagus 29–46 (36±5) long. Intestinal bifurcation pre-equatorial, immediately anterior to seminal vesicle. Caeca as long as oesophagus, widely divergent, extend to anterior margin of testes.

Testes two, postovarian, symmetrical, lateral, somewhat diagonal, smooth, slightly elongate-oval, right testis 18–29 (25±4) × 17–28 (20±3); left testis 18–33 (25±5) × 14–24 (19±3). Seminal vesicle arcuate, transversely-oval, intercaecal in mid-body, overlapping anterior margin of ventral sucker dorsally, 12–24 (17±3) × 24–39 (32±3). Seminal vesicle length to ventral sucker length ratio 1:1.1–2.2 (1:1.7±0.3). Seminal proximal duct long entering prostatic chamber (i.e., phallophorus). Prostatic chamber subspherical, with loose fibres, sinistral to ventral sucker, dorsal to genital atrium, reaching proximal part of seminal vesicle. Prostatic glands not observed. Ejaculatory duct sinuous, enters prostatic chamber, opens to small papillae dorso-sinistral in genital atrium, together with prostatic ducts running through to periphery of male duct. Phallus of 'microphalloid'-type, glabrous, turgid, evaginable, with ejaculatory duct in axis, with large dextral triangular distal scale at the base. Gential atrium surrounding phallus, 16–24 (20±3), genital pore large, sinistral to ventral sucker.

Ovary dextral to ventral sucker, pre-testicular, ventral to caeca, adjacent to or slightly overlapping ventral sucker laterally, subtriangular with large cells, 13–26 (21±3) × 17–34 (25±4). Oötype intertesticular, slightly dextral posterior to ovary and ventral sucker. Mehlis' gland surrounding oötype posteriorly. Laurer's canal not observed. Uterus confined posterior to mid-level of ventral sucker, overlapping testes ventrally. Metraterm thin-walled, with widened opening into sinistral wall of genital atrium. Vitellarium in two compact clusters of follicles, disaggregated in older specimens (after 24h cultured), para-, post-testicular or overlapping testes, converging into seminal receptacle next to oötype. Eggs few, large. Excretory vesicle obscured by vitellarium masses and eggs. Flame-cell formula not observed.

Description of metacercaria (fig 1d; table 2; Supplementary table S1)

Overall form highly developed and consistent with adult anatomy except for the absence of eggs.

[Measurements based on 18 encysted specimens; measurements provided as range followed by mean±standard deviation]

Metacercaria folded within small spherical, translucent cyst, 114−130 × 106−120 (123 × 113 ± 4). Cyst wall consisting of three or more hyaline layers, 5−6 (5±0.5) thick. Metacercaria encysted in the gonads of *P. antipodarum*.

Genetic affinities

Two distinct genotypes for the 28S rRNA gene were obtained from the sequences of the nine specimens analysed. The two genotypes differed in two transitions at the nucleotide positions 281, a cytosine in the sequence of specimens originated from Swamp and a thymine in specimens from JMS and 1358, adenine and guanine respectively. However, all specimens shared the same sequence for the ITS2 region and the mt CoxI. Independent phylogenetic analyses of the 28S rDNA and ITS2 regions using BI and ML methods showed congruent results (Fig. 3−4), with the Microphallidae as monophyletic and *M. winterbourni* embedded within. *M. winterbourni* appeared as sister taxon to the sequence of an undescribed microphallid from Australia by Kudlai *et al.* (2015). In the 28S phylogenetic tree (Fig. 3), the sequence of *M. fusiformis* appeared closely related to *A. winterbourni* and the undescribed microphallid with strong nodal support, although considerably divergent. In all trees, *Atriophallophorus* appears closely related to *Microphallus* and *Levinseniella,* but the sister relationship among these three genera could not be established due to the low support of an internal node within the clade (Fig. 3−4).

Genetic divergence between *A. winterbourni* and the unidentified microphallid from Australia was 1.2-1.3% for the 28S and 3.2% for the ITS2, which are within the lower limit of the range observed among congeneric species of *Microphallus* (28S: 0.8-9.1%, ITS2: 1.0-9.9%) and *Maritrema* (28S: 0.6-9.1%, ITS2: 0.3-12.3%). The 28S sequences of *A. winterbourni* and the unidentified microphallid diverged 10.5-10.8% from the sequence of *M. fusiformis*, which fell within range of intergeneric distances in the 28S region of microphallids (*Maritrema* - *Microphallus*: 7.5-12.6%, *Microphallus* - *Levinseniella*: 5.5-10.0%, *Maritrema* - *Levinseniella*: 9.1-11.4%, *Longiductotrema* - Levinseniella: 7.7%, *Longiductotrema* - *Maritrema*: 7.8-11.1%, *Longiductotrema* - *Microphallus*: 6.5-10.6%).

Sequence data for the COI marker resulted in identical sequences for all specimens (five from JMS and three from Swamp, one sample from swamp did not amplify and another one produced chromatograms with double peaks), whereas sequences for the NADH5 marker

showed variability at the position 191 with specimens from Swamp having a thymine and specimens from JMS a cytosine.

Discussion

Species differentiation

Following the key to the Microphallidae provided by Deblock (2008), the new species described from *in vitro* grown adults conform to the general morphology of the Microphallidae in having a typically very small body, longer than broad, densely covered with squamous spines, suckers well-separated and sub-equal, short digestive tract with divergent caeca not extending posteriorly beyond ventral sucker. Ovary pretesticular, in the opposite side of the body to the genital pore. Two testes, lateral and symmetrical, with male terminal genitalia intercaecal and anterior to ventral sucker. These specimens fit to the diagnosis of the supersubfamily Microphallidi Ward, 1901 by having the terminal genitalia free in the parenchyma and the Microphallinae Ward, 1901 by presenting a genital atrium that closely envelops the phallus. However, the opening of the genital atrium and the presence of the scale at the base of the phallus of the new species were found to alter the outer rim of the ventral sucker. Whereas this feature is characteristic of the monotypic Endocotylinae, our specimens differ from the genus *Endocotyle* in lacking a connection between the cavity of the sucker and the genital pore, and presenting a scale at the base of a phallus of the 'microphalloid-type'. Our specimens fall within the Microphallini tribe by having a fleshy and muscular phallus and the opening of the metraterm on the sinistral wall of the genital atrium. The new species exhibits features consistent with the genus *Atriophallophorus*: body of triangular shape; male genital pouch absent; proximal ejaculatory ducts very long and entering a large prostatic chamber (described as 'phallophorus' by Deblock & Rosé (1964)), subcircular and similar in size to the ventral sucker and dorsal to the genital atrium, with long prostatic ducts running through the periphery of the male duct; genital atrium containing a phallus of the 'microphalloid-type' and at its base a large dextral parietal atrial scale often protruding from the genital pore; a metraterm that widens at its opening into the sinistral wall of the genital atrium; and few but large eggs.

Currently the genus contains only two species, *A. minutus* (Price, 1934) and *A. coxiellae* Smith, 1973. *A. coxiellae* was described from metacercariae infecting *Coxiella badgerensis* in a freshwater lake in Tasmania (Smith, 1973). However, the metacercaria of *A. winterbourni* n. sp. is readily distinguishable from *A. coxiellae* by having a different structure of the prostatic chamber, sub-circular and dorsal to genital atrium, rather than cylindrical,

343 fibrous, elongate and placed between the seminal vesicle and the genital atrium, as well as
344 smaller pharynx and testes, and somewhat smaller oral sucker and ovary, which barely
345 overlap the lower limit of the range of *A. coxiellae*.
346 The new species is most similar to *A. minutus* with regards to the prostatic chamber
347 and the morphometric data. But *A. minutus* possesses oval testes and transversely-oval ovary,
348 versus elongate-oval and subtriangular, respectively in *A. winterbourni*. In addition, the
349 original description of *A. minutus* based on adult specimens from *Aythya affini*s from the
350 Caribbean (Price, 1934) reported *A. minutus* had shorter oesophagus than the new species,
351 although this feature might vary greatly depending on the position of the specimen.
352 Morphometric data provided by Stunkard (1958) for the redescription of *A. minutus* was
353 based on adults experimentally grown in white mice from metacercariae infecting *Hydrobia*
354 *minuta* and *Amnicola limosa* in the east coast of U.S.A. However, Stunkard's specimens were
355 measured alive whereas our measurements were taken from fixed, stained and mounted
356 worms. Therefore, the morphometric data are not directly comparable. Furthermore, Stunkard
357 noted that fixed specimens were slightly smaller than alive, which suggests that if the
358 measurements of *A. minutus* were taken after mounting they would be slightly smaller than
359 those of *A. winterbourni.* Nonetheless, live specimens of *A. minutus* also show smaller
360 dimensions for several metrical features (body length, ventral sucker width and testes width)
361 extending outside the lower range for *A. winterbourni*, and eggs 3-times more numerous and
362 with size overlapping the lower range of variation of the new species.

363 Metrical data of *A. minutus* described by Deblock & Rosé (1964) from France
364 supports our statement above. Whereas the specimens examined by Deblock & Rosé (1964)
365 were of comparable size to the largest in our sample, their ovary width is smaller than that of
366 *A. winterbourni*, and the ventral sucker width overlapped and extended below the lower range
367 of variation of *A. winterbourni*. *A. minutus* also shows smaller values for the ratios oral sucker
368 size to ventral sucker size and seminal vesicle length to ventral sucker length (estimated from
369 the original illustrations) than *A. winterbourni*.

370 *In vitro* cultivation of the metacercaria and adult allowed us to observed
371 developmental changes through time in the shape of the vitellarium, the seminal vesicle and
372 testes (see also Supplementary table 1). In metacercariae, the testes are well visible, the
373 seminal vesicle is small, indistinct and quite empty and the vitellarium forms two masses of
374 tight follicles. After 24h of growth, the adults are already gravid, testes become indistinct, the

seminal vesicle is full and extended and the follicles constituting the vitellarium masses start to disaggregate slightly. After 48h or more, the testes are faintly visible, the seminal vesicle stays fully extended and the vitellarium follicles are drawn further apart in the posterior region of the body. When descriptions shall be based on specimens collected from the wild birds, this variation in the sample of specimens should be taken into account.

Previous researchers working on *Atriophallophorus* spp. have emphasized the difficulty of observing the features of specimens of such tiny size. Thus, it is highly recommended to describe new species with the support of genetic data and using a holistic biological approach (Blasco-Costa *et al.*, 2016a). Despite *A. winterbourni* showing subtle morphological differences from *A. minutus*, we consider them sufficient to distinguish the new species given the intrinsic difficulties of this group. Molecular results showed convincingly that specimens of *A. winterbourni* belong to a different microphallid genus to those already represented by sequence data. The new species appeared sister to an unidentified microphallid recovered from *Posticobia brazieri* (Smith) (Gastropoda, Tateidae) and *Caridina indistincta* Calman (Decapoda, Atyidae) in Australia by Kudlai *et al.* (2015). These authors suggested that their specimens were likely closely related to (or even conspecific) with the material reported as *Microphallus* sp. 'livelyi' by Hechinger (2012), which is considered a junior synonym of *A. winterbourni* herein. Divergence between our sequences and those of the Australian microphallid of Kudlai *et al.* (2015) suggests that they represent two distinct but congeneric species as the authors anticipated.

The examination of specimens of *A. winterbourni* with SEM has allowed us to confirm a sinistral interruption of the outer rim of the ventral sucker caused by the protrusion of the dextral parietal atrial scale, while light microscopy and histological sections show that internally the ventral sucker is complete. Deblock & Rosé (1964) mentioned the possibility of the scale being united to the rim of the ventral sucker (p. 229). However, this feature went unnoticed and was never confirmed by a later redescription or new species description for the genus, nor was mentioned in the most recent diagnosis provided by Deblock (2008). Since the three species known for the genus so far are characterised by the presence of the large atrial scale, it is likely that in all three cases it has resulted in the same modification of the ventral sucker. Thus, we consider this feature of diagnostic value for the genus and amend herein the generic diagnosis of *Atriophallophorus* as follows.

Genus *Atriophallophorus* Deblock & Rosé, 1964

Body piriform or triangular, small (150-200 μm). Resembles *Microphallus*. Ventral

sucker postequatorial, outer rim interrupted sinistrally by genital pore. Oesophagus medium or short. Caeca short, divergent, in mid-body. Testes symmetrical in hindbody. Male genital pouch absent. Seminal vesicle intercaecal in mid-body, ovoid; prostatic gland caps distal part of seminal vesicle; proximal ejaculatory duct very long, supported by envelope acting as large prostatic chamber, either: (i) subcircular with diameter of ventral sucker, dorsal to genital atrium (formation described as 'phallophorus' (apparently bearing phallus)) and with long prostatic duct running through to periphery of male duct inside phallus; or (ii) cylindrical, fibrous, elongate between seminal vesicle and genital atrium, adjacent to margin of ventral sucker; bundle of long prostatic ducts enter as far as mid-part of cylindrical prostatic chamber (not inside phallus). Phallus of 'microphalloid'-type, more or less turgid, with ejaculatory duct in axis. Genital atrium present, envelopes phallus, with enormous dextral parietal atrial scale; genital pore sinistral to ventral sucker. Ovary dextral to ventral sucker. Uterus postcaecal, with few coils around testes. Metraterm long, with widened opening into sinistral wall of genital atrium. Eggs not numerous, relatively large. Vitellarium formed of two clusters of follicles, paratesticular and post-testicular in hindbody; vitelline ducts short, arched, post-testicular. Excretory vesicle short, Y-shaped, post-testicular. In intestine of birds (Anseriformes, Charadriiformes); cosmopolitan.

Type-species. *Atriophallophorus minutus* (Price, 1934) (*Synonym A. samarae* Deblock & Rosé, 1964; *synonym lapsus calami Atriophallus samarae* in Figure 1 of Deblock & Rosé, 1964).

Life cycle and putative definitive hosts

Compared to other microphallid genera, the known species diversity of *Atriophalophorus* is quite low. So far, the four members of this genus (including the yet undescribed but molecularly characterised lineage from Australia of Kudlai et al. (2015)) show an abbreviated life cycle with the absence of the cercarial stage. Furthermore, *A. winterbourni* also lacks daughter sporocyst parthenitae bearing germ cells (or has a very reduced life span) so that germ balls and embryonic metacercariae are observed free in the visceral mass of the snail host and appear to develop directly into encysted metacercariae as reported by Krist & Lively (1998).

Based on the experimental exposure of snails to the faeces of different waterfowl species from Lake Alexandrina, Osnas & Lively (2011) concluded that the likely definitive hosts of *A. winterbourni* are mallard ducks (*Anas platyrhynchos* L.), grey ducks (*Anas superciliosa*

Gmelin), their hybrids and the New Zealand scaup (*Aythya novaeseelandiae* Gmelin). Their conclusion agrees with the known distribution of both the putative hosts and *A. winterbourni* throughout the South Island. Furthermore, recent studies of the parasite fauna of mallards have discovered new species to science of microphallids and strigeids in New Zealand (Presswell *et al.*, 2014; Blasco-Costa *et al.*, 2016b). Altogether, these results highlight the still scarce knowledge on the parasites of birds, the most diverse group of vertebrates native to New Zealand, and the need for more biodiversity studies to address this gap.

Acknowledgements

We thank Pilar Ruga Fahy from the University Medical Centre of the *Pôle Facultaire de Microscopie Ultrastructurale* at the University of Geneva for carrying out the histological sections and procedures, Janik Pralong (Natural History Museum of Geneva) for preparation of the permanent mounts and Dr. André Piuz (Natural History Museum of Geneva) for his help with the scanning electron microscopy. We are also grateful to Dr. Rodney Bray and Tomáš Scholz for discussions on the interpretation of the morphology, and one anonymous reviewer and the editor for very useful comments that helped us improve the manuscript.

Financial Support

This work has been supported by the Natural History Museum of Geneva, and indirectly by the Swiss National Science Foundation (Grant No. 31003A_169211/1 to IBC).

Conflict of interest

None.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of Switzerland and New Zealand and our institutional guides on the care and use of wild invertebrate animals.

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Table 1. List of taxa included in the phylogenetic analyses, GenBank accession numbers and references.

Taxa	28S rDNA	ITS2	References
Haematoloechidae			
Haematoloechus longiplexus	AF387801		(Snyder & Tkach, 2001)
Lecithodendriidae			
Paralecithodendrium parvouterus	AY220617		(Tkach *et al.*, 2003)
Pycnoporus heteroporus	AF151918		(Tkach *et al.*, 2000)
Microphallidae			
Atriophallophorus winterbourni n. sp.	MN342153–MN342154	MN342155	**This study**
Levinseniella sp.	MG783585	MG783580	(Galaktionov & Blasco-Costa, 2018)
Longiductotrema tethepae	KX712084	KX712086	(Kudlai *et al.*, 2016)
Maritrema arenaria	AY220629	HM584171	(Galaktionov *et al.*, 2012)
Maritrema brevisacciferum	KT355818	KT355824	(Kudlai *et al.*, 2015)
Maritrema corai	KT880222		(Hernandez-Orts *et al.*, 2016)
Maritrema deblocki	KJ144173		(Presswell *et al.*, 2014)
Maritrema eroliae	JF826247	HQ650132	(Al-Kandari *et al.*, 2011)
Maritrema heardi	AY220632		(Tkach *et al.*, 2003)
Maritrema madrynense		KF575167	(Diaz & Cremonte, 2010)
Maritrema neomi	AF151927		(Tkach *et al.*, 2000)
Maritrema novaezealandense	KJ144178	KJ540203	(Presswell *et al.*, 2014)
Maritrema oocysta	AY220630	HM584170	(Tkach *et al.*, 2003; Galaktionov *et al.*, 2012)
Maritrema poulini	KJ144175		(Presswell *et al.*, 2014)
Maritrema prosthometra	AY220631		(Tkach *et al.*, 2003)
Maritrema subdolum	AF151926	HM584172	(Tkach *et al.*, 2000; Galaktionov *et al.*, 2012)
Maritrema sp. 1		KC012521	(Gilardoni *et al.*, 2011)
Maritrema sp. 2		KC222022	(Gilardoni *et al.*, 2011)
Microphallidae gen. sp.	KT355820	KT355826	(Kudlai *et al.*, 2015)
Microphallidae gen. sp.	AB974360		(Kakui, 2014)
Microphallus (?) fusiformis	AY220633		(Tkach *et al.*, 2003)
Microphallus abortivus	AY220626	HM584173	(Tkach *et al.*, 2003; Galaktionov *et al.*, 2012)
Microphallus basodactylophallus	AY220628		(Tkach *et al.*, 2003)
Microphallus calidris	HM584125	HM584183	(Galaktionov *et al.*, 2012)
Microphallus kurilensis	HM584140	HM584185	(Galaktionov *et al.*, 2012)
Microphallus minutus	KT355822	KT355828	(Kudlai *et al.*, 2015)
Microphallus ochotensis	HM584142	HM584175	(Galaktionov *et al.*, 2012)
Microphallus piriformes	HM584122	HM584181	(Galaktionov *et al.*, 2012)
Microphallus primas	AY220627		(Tkach *et al.*, 2003)
Microphallus pseudopygmaeus	HM584126	HM584198	(Galaktionov *et al.*, 2012)
Microphallus pygmaeus	HM584133	HM584190	(Galaktionov *et al.*, 2012)
Microphallus similis	HM584138	HM584178	(Galaktionov *et al.*, 2012)
Microphallus triangulatus	HM584139	HM584195	(Galaktionov *et al.*, 2012)

Microphallus sp.	KJ868216		(O'Dwyer *et al.*, 2014)
Probolocoryphe uca		GQ377842	(Al-Kandari & Al-Bustan, 2010)
Plagiorchiidae			
Plagiorchis vespertilionis	AF151931		(Tkach *et al.*, 2000)
Pleurogenidae			
Parabascus duboisi	AY220618		(Tkach *et al.*, 2003)
Pleurogenes claviger	AF151925		(Tkach *et al.*, 2000)
Prosthogonimidae			
Prosthogonimus ovatus	AF151928		(Tkach *et al.*, 2000)
Telorchiidae			
Telorchis assula	AF151915		(Tkach *et al.*, 2000)

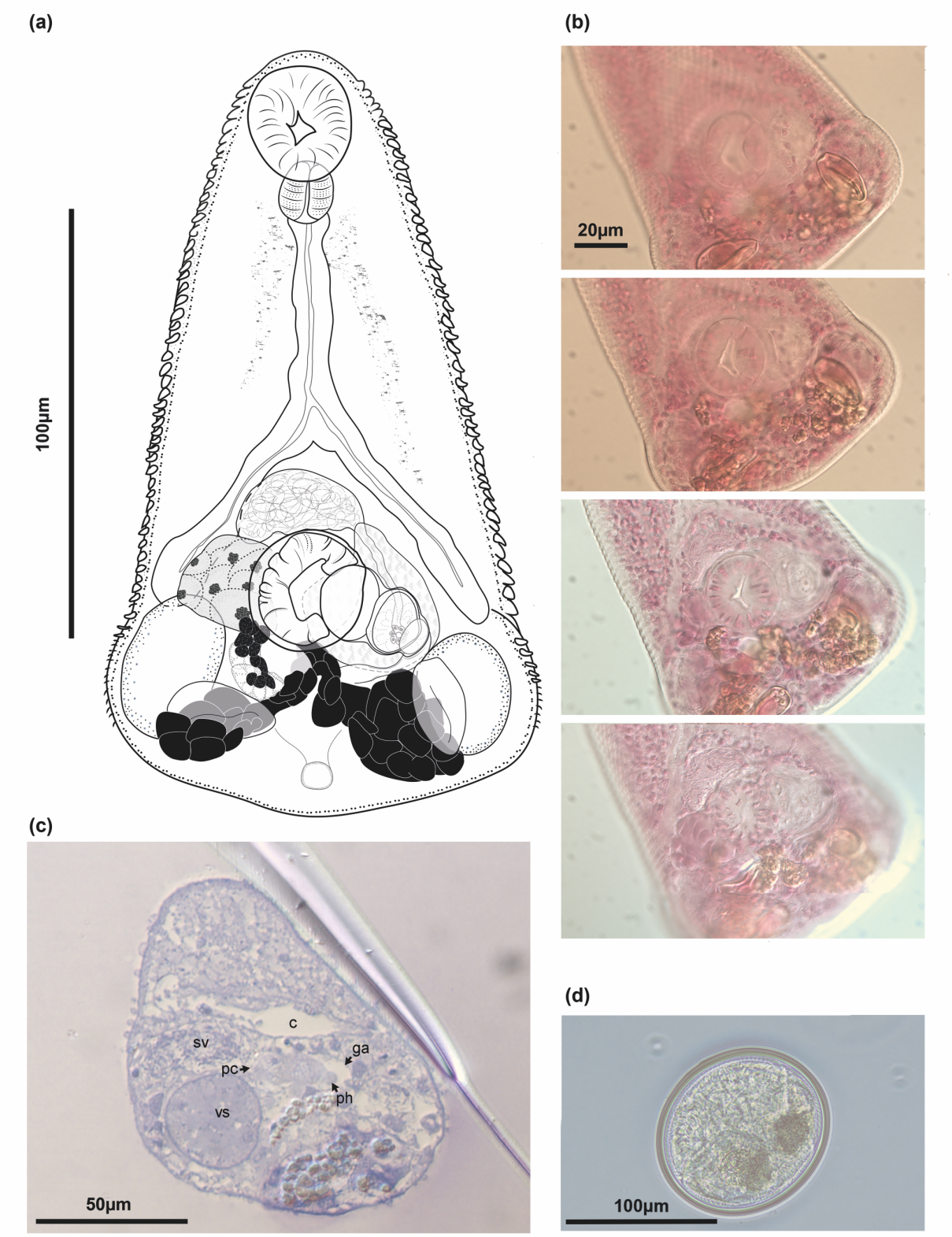
704 Table 2. Comparative metrical data for *Atriophallophorus* spp.

Species	***A. winterbourni* n. sp.**				***A. minutus* (Price, 1934) Deblock & Rosé, 1964**			***A. coxiellae* Smith, 1973**	
Stage	Adult		Metacercaria		Adult	Adult	Metacercaria	Metacercaria	
Hosts	*In vitro* cultured		*Potamopyrgus antipodarum* (Mollusca: Tateidae)		*Anas platyrrhynchos* L. (Aves: Anatidae)	*Aythya affinis* (Eyton) (Aves: Anatidae)	*Ecrobia truncata* (Vanatta) and *Amnicola limosa* (Say) (Mollusca: Hydrobiidae) and mice (experimental)	*Coxiella badgerensis* (Johnston) (Mollusca: Tomichiidae); *Charadrius cucullatus* Vieillot and *C. ruficapillus* Temminck (Aves: Charadriidae)	
Distribution	New Zealand				North America, Caribbean Islands and Europe (France)			Tasmania	
Source	Present study		Present study		Deblock & Rosé, 1964	Price (1934)	Stunkard 1958	Smith, 1973	
	Range (n=29)	Mean ± SD	Range (n=9)	Mean ± SD	Range or Mean		Range	Range	Mean
Body length	145–200 (28)	167±12	165–193 (9)	175±9	170–200	153–180	120–200	143–229	195
Body width	85–115 (28)	100±8	98–118 (9)	106±7	90–100	105–112	90–120	72–125	90
Forebody	81–125 (28)	97±9	97–115 (9)	104±5					
Oral sucker length	21–29 (29)	25±2	23–28 (9)	25±2	29*		27*		
Oral sucker width	21–29 (28)	25±2	21–28 (9)	24±2	22–25	23–25	23–30	26–34	29
Pharynx length	11–17 (26)	14±2	12–14 (9)	14±1	12*		12*	16–44	20
Pharynx width	11–15 (26)	13±1	11–15 (9)	13±1	12–15	10–18	10–18	15–20	17
Oesophagus	29–46 (22)	36±5	40–50 (8)	44±4		18–30	16–40		
Ventral sucker length	24–30 (28)	27±1	28–35 (9)	31±2	23*		26*		
Ventral sucker width	22–33 (28)	28±2	24–31 (9)	28±2	19–25	22–27	20–28	26–31	29
Right testis length	18–29 (13)	25±4	26–34 (8)	30±3					
Right testis width	17–28 (12)	20±3	24–29 (9)	26±2	20–30	28	12–29	36–40	39
Left testis length	18–33 (10)	25±5	26–35 (7)	30±3					
Left testis width	14–24 (10)	19±3	23–29 (9)	26±2	25–30				
Seminal vesicle length	12–24 (27)	17±3	9–15 (2)	13±3	13–19		14–20		
Seminal vesicle width	24–39 (28)	32±3	35–44 (3)	40±6	13–26		26*	29–36	34

Ejaculatory duct length	21–34 (11)	27±3			20–25				
Ejaculatory duct width	1.5–2.3 (13)	2.0±0.4			2.5				
Genital atrium width	16–24 (10)	20±3	22–23 (2)	22±1	20–25		20–35		
Ovary length	13–26 (20)	21±3	23–31 (9)	25±2	19–25	18	15–20		
Ovary width	17–34 (20)	25±4	17–31 (9)	26±5	13–16	22	21–24	30–48	35
Right vitellarium length	15–26 (4)	20±5	20–28 (9)	25±3	20		5–6 follicles		
Right vitellarium width	23–35 (4)	29±5	28–54 (8)	37±9	30				
Left vitellarium length	15–28 (4)	21±5	20–23 (9)	22±1					
Left vitellarium width	23–37 (4)	28±6	20–35 (9)	31±5					
Egg number	1–12 (29)	5±3			20–25		10–30		
Egg length × width	20–26 × 11–15				21–25 × 10–13	22 × 13	20–22 × 12–13		
Oral to ventral sucker length ratio	0.9–1.2	1.1±0.2	1.1–1.4	1.3±0.1	0.8*		1.0*	0.9*	
Oral to ventral sucker width ratio	0.9–1.3	1.1±0.1	0.9–1.3	1.1±0.1	1.1		0.7*	0.9*	
Body width to length ratio	1.3–1.9	1.6±0.4	1.5–1.8	1.7±0.1	1.8*		–	1.6*	
Seminal vesicle to ventral sucker length ratio	1.1–2.2	1.7±0.3	2.1–3.8	2.8±0.9	0.7*		1.0*	1.9*	
Pharynx to oral sucker length ratio	1.5–2.4	1.7±0.2	1.7–2.0	1.8±0.1	2.4*		2.3*	1.3*	
Forebody as percentage of body length	52–63	58±2	57–62	60±1	62*		–	63*	
Metacercaria cyst length			114–130	123±4					
Metacercaria cyst width			106–120	113±4			≤100	88–125	

Metacercaria cyst wall	5–6	5±0.5	8.0–10

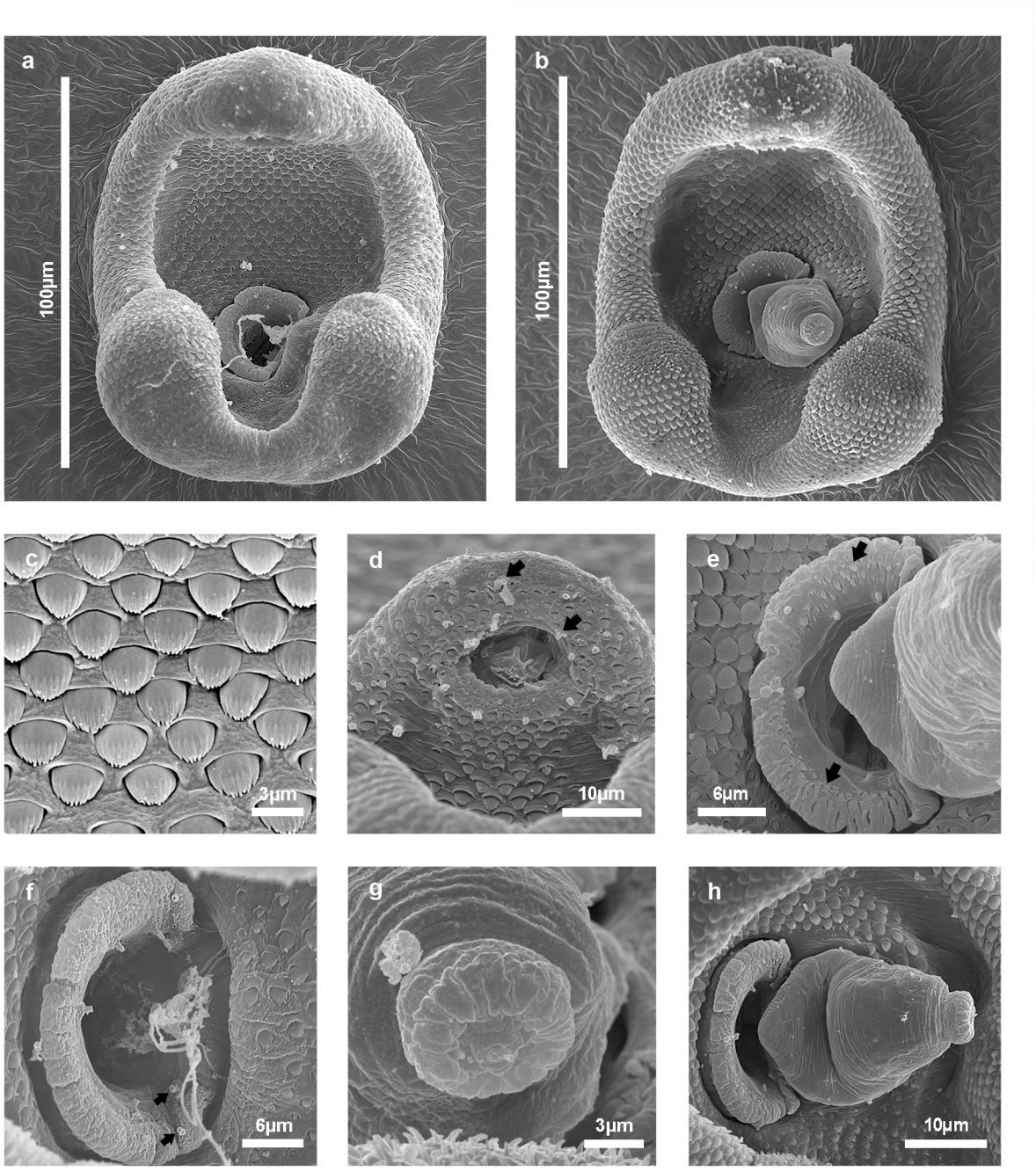
*, estimated from the published illustration; n, number of specimens measured; SD, standard deviation of the mean



706

707 **Figure 1.** *Atriophallophorus winterbourni* n. sp. (a) Illustration of the holotype, 24h *in vitro*
708 grown adult in ventral view. (b) Microphotographs of the terminal genitalia of the holotype
709 using light microscopy. (c) Histological oblique section of a paratype at the level of the
710 ventral sucker: c, caeca, ga, genital atrium, pc, prostatic chamber, ph, phallus, sv, seminal

711 vesicle, and vs, ventral sucker. (d) Microphotograph of an encysted metacercariae ex
712 *Potamopyrgus antipodarum* (Gray).



713

714 **Figure 2.** Scanning electron micrographs of *Atriophallophorus winterbourni* n. sp. (a) Adult.
715 (b) Adult with phallus protruded. (c) Palmate spines on the ventral surface of the body. (d)
716 Detail of the oral sucker, arrows point at a gland opening and a sensory papilla surrounding
717 the oral sucker. (e) Detail of the outer rim of the ventral sucker with spination and the parietal
718 atrial scale at the basis of the phallus. (f) Outer rim of the ventral sucker interrupted sinistrally

by the opening for the genital papilla with arrows pointing at glands. (g) Tip of the phallus evaginated, which appears as a flower-like structure when invaginated. (h) Detail of the configuration of the protruded phallus and the sinistrally interrupted outer rim of the ventral sucker.

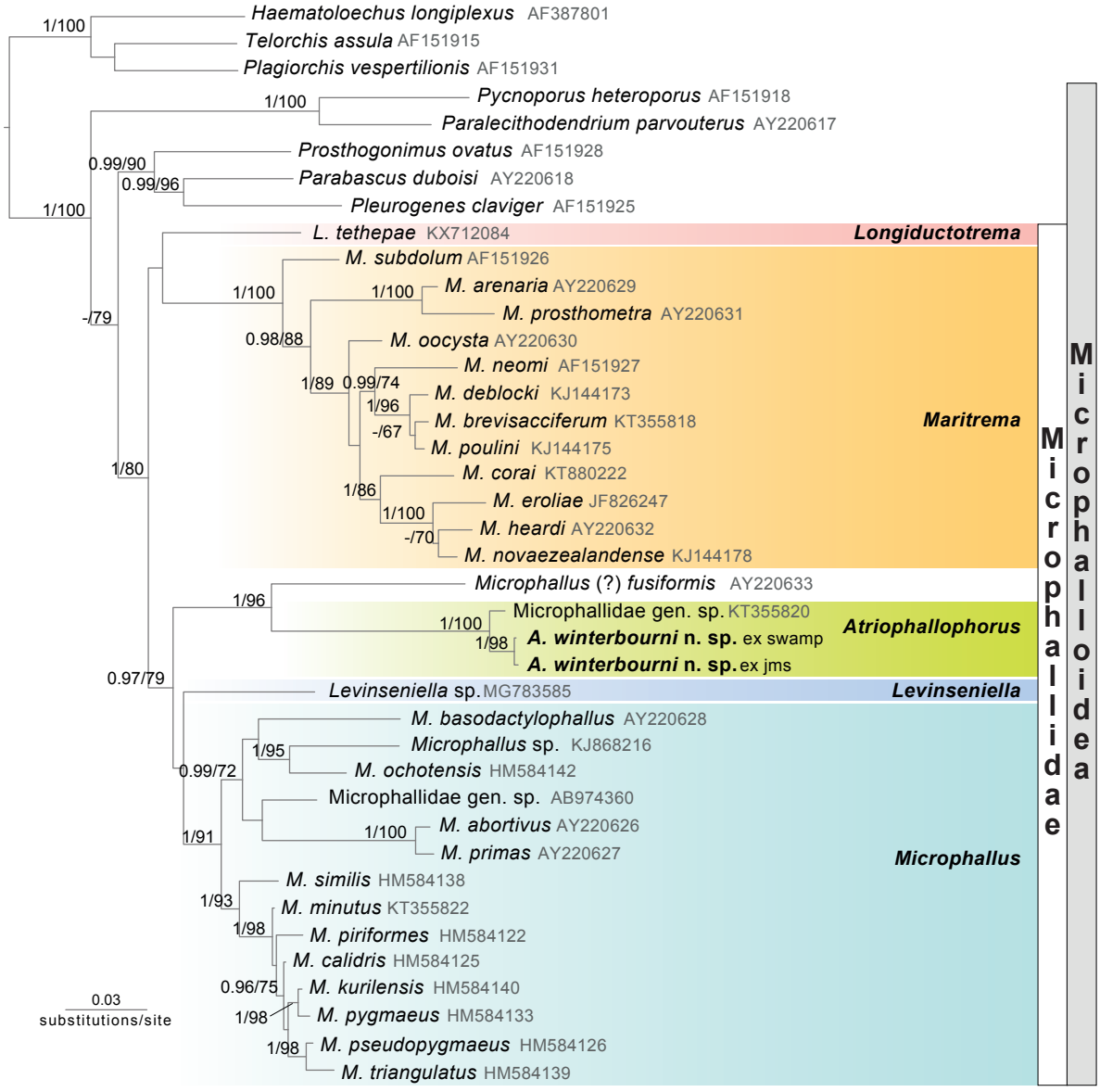
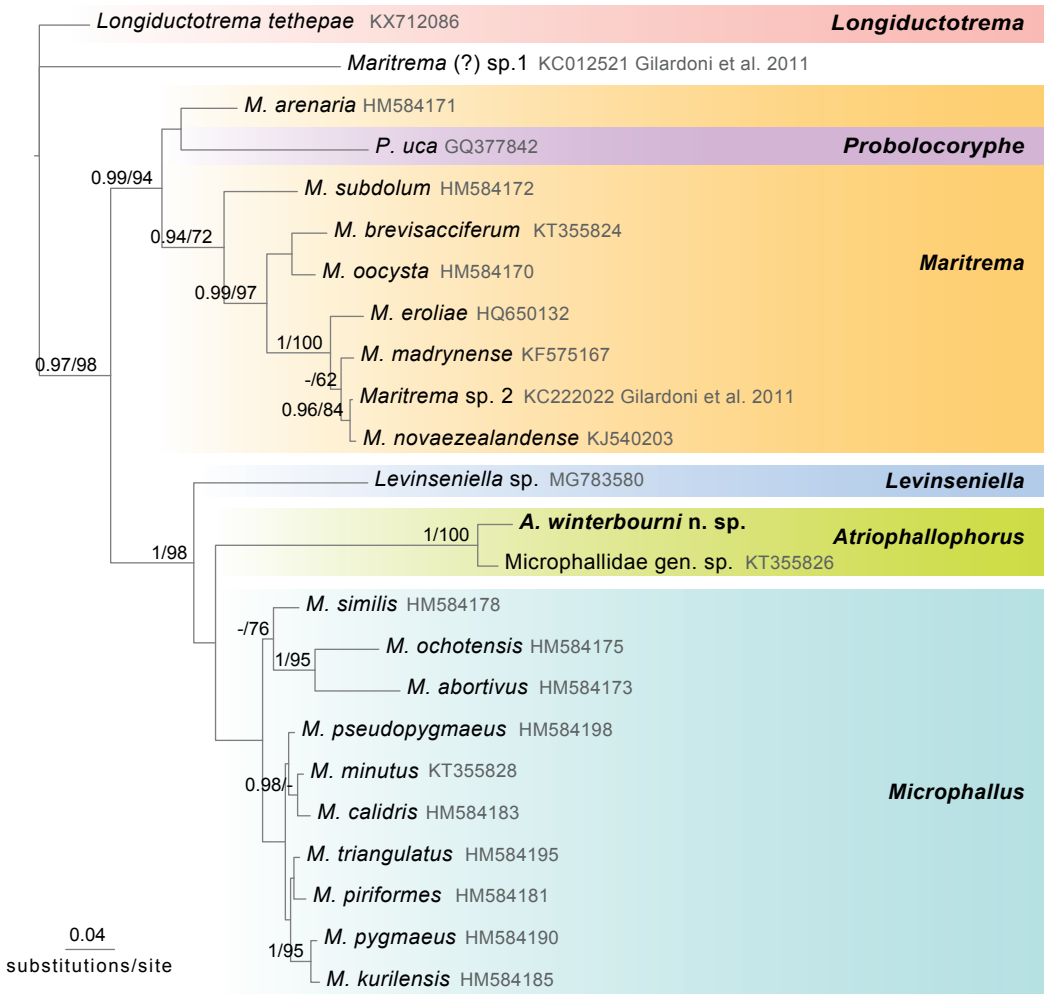


Figure 3. Phylogenetic relationships for representatives of the family Microphallidae, inferred by maximum likelihood analysis of 28S rDNA sequence data. The newly generated sequences are indicated in bold. Values on the branches correspond to posterior probabilities > 0.95 followed by bootstrap support > 60, values below these thresholds were not reported.



729

730 **Figure 4.** Phylogram for representatives of the family Microphallidae, inferred by maximum
731 likelihood analysis of sequence data for the internal transcribed spacer 2 of the rRNA genes.
732 The newly generated sequences are indicated in bold. Values on the branches correspond to
733 posterior probabilities > 0.95 followed by bootstrap support > 60, values below these
734 thresholds were not reported.