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New data on the morphology and phylogenetic connections of *Postlepidapedon opisthobifurcatum* (Trematoda, Lepocreadioidea: Lepidapedidae), a parasite of Antarctic and sub-Antarctic fishes

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Summary

The trematode *Postlepidapedon opisthobifurcatum* (Zdzitowiecki, 1990) is a common intestinal parasite of the gadiform fishes of the Southern Ocean. In this work, we supplement the description of the species with the anatomy of the terminal part of the reproductive system and with molecular data. The male terminal genitalia are characterised by the presence of the external seminal vesicle and cirrus-sac. The external seminal vesicle is surrounded by aciniform groups of outer prostatic cells. Groups of outer prostatic cells and proximal parts of their ducts are associated with a thin-walled membrane that is connected to the proximal edge of the cirrus-sac. The cirrus-sac is claviform, with a long proximal part accommodating the tubular, thin-walled internal seminal vesicle and ducts of outer prostatic cells. The female terminal genitalia are represented by a thick-walled metraterm, which is surrounded by aciniform groups of glandular cells. Phylogenetic analysis based on 28S rDNA partial sequences data placed *P. opisthobifurcatum* into the monophyletic group Lepidapedidae, including the species *Myzoxenus insolens* (Crowcroft, 1945), *Intusatrium robustum* Durio et Manter, 1968, and *Postlepidapedon uberis* Bray, Cribb et Barker, 1997. However, we were unable to detect direct phylogenetic connections between *P. opisthobifurcatum* and *P. uberis*.
Keywords: Trematoda; *Postlepidapedon opisthobifurcatum*; Lepidapedidae

Introduction

The trematode *Postlepidapedon opisthobifurcatum* (Zdzitowiecki, 1990) Zdzitowiecki, 1993 is one of the common intestinal parasites of Antarctic and sub-Antarctic fishes of the families Muraenolepididae and Macrouridae (Zdzitowiecki, 1990; Zdzitowiecki & Cielecka, 1997; Walter et al., 2002; Sokolov & Gordeev, 2013; 2015; Gordeev & Sokolov, 2017). This species was originally included in the genus *Neolepidapedon* Manter, 1954 (see Zdzitowiecki, 1990). Zdzitowiecki (1993) subsequently erected a new genus, *Postlepidapedon*, based on the morphology of the cirrus-sac (presence of elongated, thin-walled internal seminal vesicle and narrow, long ejaculatory duct), the position of the intestinal bifurcation, and a number of other characters to accommodate *Neolepidapedon opisthobifurcatus* Zdzitowiecki, 1990. Other than the type species (*P. opisthobifurcatum*), this genus currently includes five other congeners, described from perciform fishes from the waters off Australia, New Caledonia, and the Philippines (Bray *et al.*, 1997; Bray & Cribb, 2001; Machida, 2004). Bray & Cribb (2012) placed the genus *Postlepidapedon* into the family Lepidapedidae.

The aim of the present study is to describe in more detail the morphology of the terminal part of reproductive system of adult *P. opisthobifurcatum* and to define the phylogenetic position of this species based on molecular data.

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Materials and Methods

Specimen collection and morphological study

The digeneans were collected during parasitological examination of specimens of *Muraenolepis marmorata* Günther, 1880 (Gadiformes, Muraenolepididae) caught on 18 March 2013 in the central part of the Ross Sea (75°48S 172°48W). All hosts (length 49 – 55 cm, weight 0.9 – 1.4 kg) were caught by the fishing vessel *Yantar-35* while it was fishing for the toothfish *Dissostichus* spp. at depths ranging from 962 m to 1228 m using bottom longline fishing gear (autoline) "Mustad" (Petrov et al., 2014) inside the Convention for the Conservation of Marine Living Resources (CCAMLR) area. The worms collected for morphological study were fixed in 70 % ethanol under a cover glass without additional pressure and stained with acetocarmine. Trematode species were identified using the publications of Zdzitowiecki (1990; 1993) and Zdzitowiecki & Cielecka (1997). The drawing and dimensions of *P. opisthobifurcatum* from our collection are given in Sokolov & Gordeev (2013).

The description of the terminal part of the reproductive system is based on the study of isolated organs (from 20 specimens) extracted from the bodies using needles. The worms used in the phylogenetic analysis were fixed in 95 % ethanol. Voucher specimens of the studied species were deposited in the Museum of Helminthological Collections, Centre for Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia (IPEE RAS).

DNA extraction, amplification, sequencing, and phylogenetic analysis

Genomic DNA was extracted from single specimens of adult worms following the protocol used by Tkach *et al.* (1999). Nuclear 28S rDNA partial fragment, including D1-D3 domains, was amplified using a polymerase chain reaction by the following primers: DIG12 (5'- AAG CAT ATC ACT AAG CGG-3') and 1500R (5'- GCT ATC CTG AGG GAA ACT TCG-3') (Tkach *et al.*, 2003). The initial polymerase chain reaction was carried out in a total volume of 25 µl containing 0.25 mM of each primer pair, 5 µl DNA in water, 1× Phusion polymerase buffer, 2.5 mM dNTP, and 1 unit of Phusion High-Fidelity DNA Polymerase (New England Biolabs, UK). The amplification of a 1330-bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems) with a 1-min denaturation hold at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C; followed by a 2-min extension hold at 72 °C. Negative and positive controls, using both primers, were used. The PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit, as recommended by the manufacturer, with the internal sequencing primers 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') , ECD2 (5'–CTT GGT CCG TGT TTC AAG ACG GG–3'), 900F (5'-CCG TCT TGA AAC ACG GAC CAA G-3'), and 1200R (Tkach *et al.*, 2003). The sequences obtained have been submitted to NCBI GenBank (Table 1).

Phylogenetic relationships were performed using our data and nucleotide sequences of 28S rDNA of lepidapedids from the NCBI GenBank database (Table 1). Due to the limited data on trematodes of the family Lepidapedidae deposited in the GenBank, we also used an original sequence of *Muraenolepitrema magnatestis* Gaevskaya et Rodjuk, 1988 collected from the same host and in the same region as *P. opisthobifurcatum* (see above, and Sokolov & Gordeev, 2015). Representatives of the families Enenteridae and Gyliauchenidae (Table 1) were selected as the outgroup based on their phylogenetic position relative to the family Lepidapedidae (see Bray & Cribb, 2012). Most of the 28S rDNA sequences of lepidapedids deposited into NCBI GenBank database are about 900 bp long. In this regard, two variants of the tree are given in this paper. The first is built based on 900 bp, and includes the maximum number of species of the Lepidapedidae deposited in GenBank. The second one is built with 1230 bp alignment length and includes a limited number of species. Initially, the sequences were aligned with the aid of ClustalX using default parameters (Jeanmougin *et al.*, 1998), and then they were refined by estimating the number of variable sites and sequence differences using MEGA 6.0 (Tamura *et al.*, 2013). Phylogenetic analysis of the nucleotide sequences was performed using Bayesian inference implemented in MrBayes v.3.2.6 on CIPRES portal (Miller *et al.*, 2010). The analysis was conducted using the GTR+I+G model, where ngen was set to 5×10^6, with two runs each containing four simultaneous Markov ChainMonte Carlo (MCMC) chains and every 10000^{th} tree saved. An evolutionary model for the Bayesian inference analysis was selected using MEGA v.7.0.21 (Kumar *et al.*, 2016). Samples of substitution model parameters and tree and branch lengths were summarised using the parameters "sump burnin = 0.25" and "sumt burnin = 0.25". The significance of the phylogenetic relationships was estimated by posterior probabilities (Huelsenbeck *et al.*, 2001).

Ethical Approval and/or Informed Consent

The conducted research is not related to either human or animals use. Informed consent has been obtained from all individuals included in this study.

Results

Morphology of the terminal part of the reproductive system

The male terminal genitalia are represented by the external seminal vesicle, cirrus-sac, and complex of outer prostatic cells (Fig. 1). The vas deferens is absent; vasa efferentia is joined directly to the external seminal vesicle. The external seminal vesicle is large, 1.5 – 2 times longer than the cirrus-sac, convoluted, and surrounded by aciniform groups of outer prostatic cells radiating into the parenchyma. Groups of outer prostatic cells and proximal parts of their ducts are covered with a thin-walled open-ended membrane. The membrane is divided into two sheets – dorsal and ventral.

Table 1. List of taxa, incorporated into molecular analysis.

Species	Family	Reference	GenBank accession number
Bulbocirrus aulostomi Yamaguti, 1965	Lepidapedidae	Bray *et al.* (2009)	FJ788470
Intusatrium robustum Durio et Manter, 1968	Lepidapedidae	Bray *et al.* (2009)	FJ788481
Lepidapedon arlenae Bray et Gibson, 1995	Lepidapedidae	Bray *et al.* (1999)	AJ405262
Lepidapedon beveridgei Campbell et Bray, 1993	Lepidapedidae	Bray *et al.* (1999)	AJ405263
Lepidapedon desclersae Bray et Gibson, 1995	Lepidapedidae	Bray *et al.* (1999)	AJ405264
Lepidapedon discoveryi Bray et Gibson, 1995	Lepidapedidae	Bray *et al.* (1999)	AJ405265
Lepidapedon elongatum (Lebour, 1908)	Lepidapedidae	Bray *et al.* (1999)	AJ405266
Lepidapedon gaevskayae Campbell et Bray, 1993	Lepidapedidae	Bray *et al.* (1999)	AJ405267
Lepidapedon rachion (Cobbold, 1858)	Lepidapedidae	Bray *et al.* (1999)	AJ405261
Lepidapedon sommervillae Bray et Gibson, 1995	Lepidapedidae	Bray *et al.* (1999)	AJ405268
Lepidapedon zubchenkoi Campbell et Bray, 1993	Lepidapedidae	Bray *et al.* (1999)	AJ405269
Muraenolepitrema magnatestis Gaevskaya et Rodjuk, 1988	Lepidapedidae	This study	KY497958
Myzoxenus insolens (Crowcroft, 1945)	Lepidapedidae	Bray *et al.* (2009)	FJ788486
Neolepidapedon smithi Bray et Gibson, 1989	Lepidapedidae	Bray *et al.* (1999)	AJ405270
Postlepidapedon opisthobifurcatum (Zdzitowiecki, 1990)	Lepidapedidae	This study	KY497957
Postlepidapedon uberis Bray, Cribb et Barker, 1997	Lepidapedidae	Bray *et al.* (2009)	FJ788492
Profundivermis intercalarius Bray et Gibson, 1991	Lepidapedidae	Bray *et al.* (1999)	AJ405271
Outgroup			
Koseiria xishaense Gu et Shen, 1983	Enenteridae	Olson *et al.* (2003)	AY222233
Petalocotyle adenometra Hall et Cribb, 2000	Gyliauchenidae	Bray *et al.* (2009)	FJ788504

Distal ends of the membrane's sheets are connected to the proximal edge of the cirrus-sac. The cirrus-sac is 0.237 – 0.331 mm long and 0.05 – 0.06 mm maximal wide, and it is composed of internal seminal vesicle, pars prostatica, ejaculatory duct and eversible cirrus. The cirrus-sac is claviform, with a long proximal part accommodating the proximal part of the internal seminal vesicle and numerous ducts of the outer prostatic cells. The internal seminal vesicle is long, 50 – 70 % the length of the cirrus-sac, tubular, rectilinear or slightly twisted, and thin-walled. The pars prostatica is vesicular. The proximal half of the pars prostatica and distal end of the inner seminal vesicle are surrounded by a field of inner prostatic cells. The cirrus is unarmed and almost cylindrical. The female terminal genitalia are represented by a thick-walled metraterm running over dorsal or dorso-lateral surface of the cirrus-sac, and surrounded by aciniform groups of glandular cells. The length of the metraterm is 0.13 – 0.15 mm, which represents 40 – 60 % of the cirrus-sac length. The male and female canals open into the small genital atrium.

Phylogenetic analysis

Bayesian inference analysis based on sequences containing 900 bp produced topologies in which *P. opisthobifurcatum* formed a strongly supported clade with *Myzoxenus insolens* (Crowcroft, 1945), within a polytomic clade also composed of *Intusatrium robustum* Durio et Manter, 1968 and *Postlepidapedon uberis* Bray, Cribb et Barker, 1997 (Fig. 2a). In turn, the above-mentioned polytomic clade is weakly supported as a sister group to a large clade of lepidapedids, consisting of *M. magnatestis* and two monophyletic clades: *Lepidapedon* spp. and *Neolepidapedon smithi* Bray et Gibson, 1989 + *Profundivermis intercalarius* Bray et Gibson, 1991. The trematode *Bulbocirrus aulostomi* Yamaguti, 1965 is basal taxa to all other Lepidapedidae.

Bayesian inference analysis based on sequences containing 1230 bp has revealed that *P. opisthobifurcatum* and *M. insolens* formed a clade that was sister to *P. uberis* with low support (Fig. 2b) and in turn this clade composed of three species was sister to *I. robustum* with high support. The species *M. magnatestis* and *B. aulostomi* form a strongly supported sister clade to the above-mentioned group of lepidapedids. *Postlepidapedon opisthobifurcatum* and *P. uberis* were no more closely related than they were to other lepidapedids.

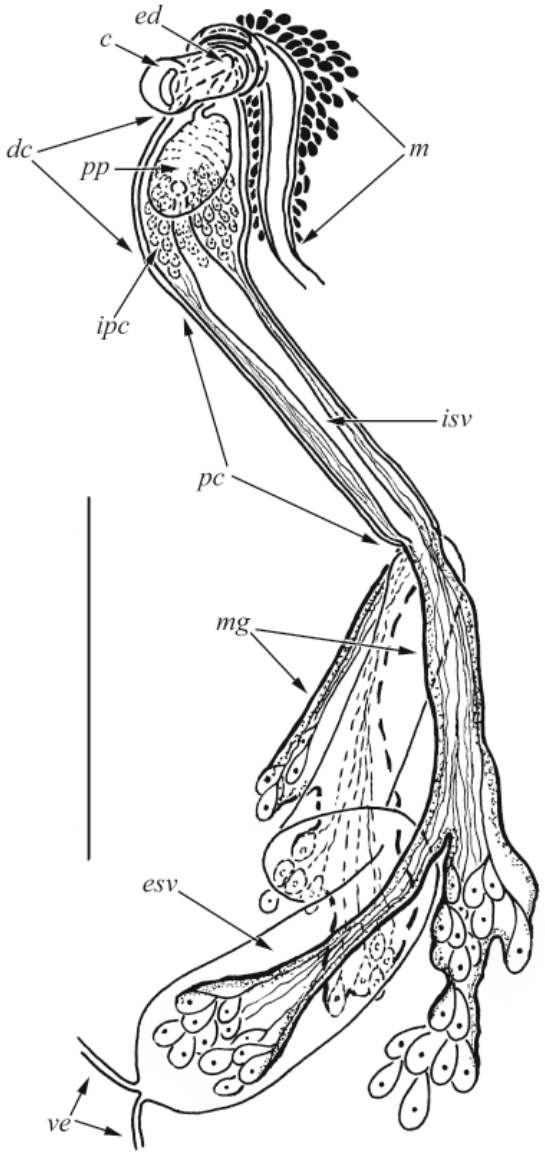

Fig. 1. Male and female terminal genitalia of *Postlepidapedon opisthobifurcatum* with cirrus everted through genital atrium, ventral view, *bar* 0.2 mm; *dc* distal part of cirrus-sac, *pc* proximal part of cirrus-sac, *c* cirrus, *ed* ejaculatory duct, *pp* pars prostatica, *ipc* inner prostatic cells, *isv* internal seminal vesicle, *mg* outer prostatic cells associated with thin-walled membrane, *esv* external seminal vesicle, *ve* vasa efferentia, *m* metraterm surrounded by glandular cells.

Discussion

According to Zdzitowiecki (1990, 1993), the cirrus-sac of *P. opisthobifurcatum* has an elongate-oval or clavate shape and it contains an elongate, but not exceptionally long, thin-walled internal seminal vesicle, a vesicular pars prostatica, a long and narrow ejaculatory duct, and a small cirrus. The external seminal vesicle is long and convoluted, and lies free in the parenchyma. The outer prostatic cells of *P. opisthobifurcatum* were not found by Zdzitowiecki (1990, 1993). However, the proximal edge of the cirrus-sac and the area in which the external seminal vesicle is located are difficult to observe in whole mounts of this parasite, because they are obscured by the ventral sucker, loops of uterus, and vitelline follicles. The study of the isolated terminal part of the reproductive system revealed outer prostatic cells associated with thin-walled open-ended membrane. The membrane, which covers some clusters of outer prostatic cells, is described for other lepidapedids, in particular *Paralepidapedon sebastisci* (Yamaguti, 1938), *M. magnatestis*, and also for some opecoelids (Shimazu & Shimura, 1984; Sokolov & Gordeev, 2015; Shimazu, 2016). Shimazu & Shimura (1984) consider it as the rudiment of the wall of the membranous sac (=proximal portion of cirrus-sac by Shimazu & Shimura's terminology), inherent for many lepidapedids and some opecoelids (see Bray, 2005; Cribb, 2005).

The position of *P. uberis* in the obtained phylograms (Fig. 2), separated from *P. opisthobifurcatum*, is consistent with differences in the morphology of the male terminal genitalia. *Postlepidapedon uberis* has a subglobular cirrus-sac with a small proximal part, containing a convoluted internal seminal vesicle. The ejaculatory duct is relatively short and thick-walled. The complex of the outer prostatic cells is absent in *P. uberis* (see Bray & Cribb, 2001). In addition, this species differs from *P. opisthobifurcatum* in the position of vitelline follicles. In *P. uberis* vitelline follicles form two lateral fields that are arranged in hindbody. These fields overlap the intestinal branches ventrally, laterally and dorsally (Bray & Cribb, 2001). In *P. opisthobifurcatum*, in addition to the lateral fields arranged in hindbody, there are two longitudinal intercaecal rows of the vitelline follicles that lie on the dorsal side of the body. Lateral fields of vitelline follicles in this species overlap the intestinal branches only ventrally and partly laterally (Zdzitowiecki, 1990).

Four other species of the genus *Postlepidapedon*: *Postlepidapedon philippinense* Machida, 2004, *Postlepidapedon secundum* (Durio et Manter, 1968), *Postlepidapedon spissum* Bray, Cribb et Barker, 1997, and *Postlepidapedon quintum* Bray et Cribb, 2001 also differ from *P. opisthobifurcatum* in the shape of the cirrus-sac (oval or elongate-oval without detached proximal part) (Bray *et al.*, 1997; Bray & Cribb, 2001; Machida, 2004). The species *P. philippinense, P. secundum,* and *P. quintum* do not have a complex of the outer prostatic cells. The distal end of the external seminal vesicle of *P. spissum* is encircled by glandular cells, lying unconfined in the parenchyma. (Bray *et al.*, 1997). Moreover, *P. secundum* has a coiled thin-walled internal seminal vesicle, while *P. spissum* and *P. quintum* have a rectilinear, but thick-walled, internal seminal vesicle. *Postlepidapedon philippinense* has rectilinear thin-walled internal seminal vesicle (Machida, 2004). The placement of lateral fields of the vitellarium relative to the intestinal branches in *P. quintum P. secundum,* and *P. spissum* is the same as in *P. uberis* (Bray *et al.*, 1997; Bray & Cribb, 2001). Accurate information about mutual location of lateral fields and intestinal branches in *P. philippinense* is absent (see Machida, 2004). We think it is likely that

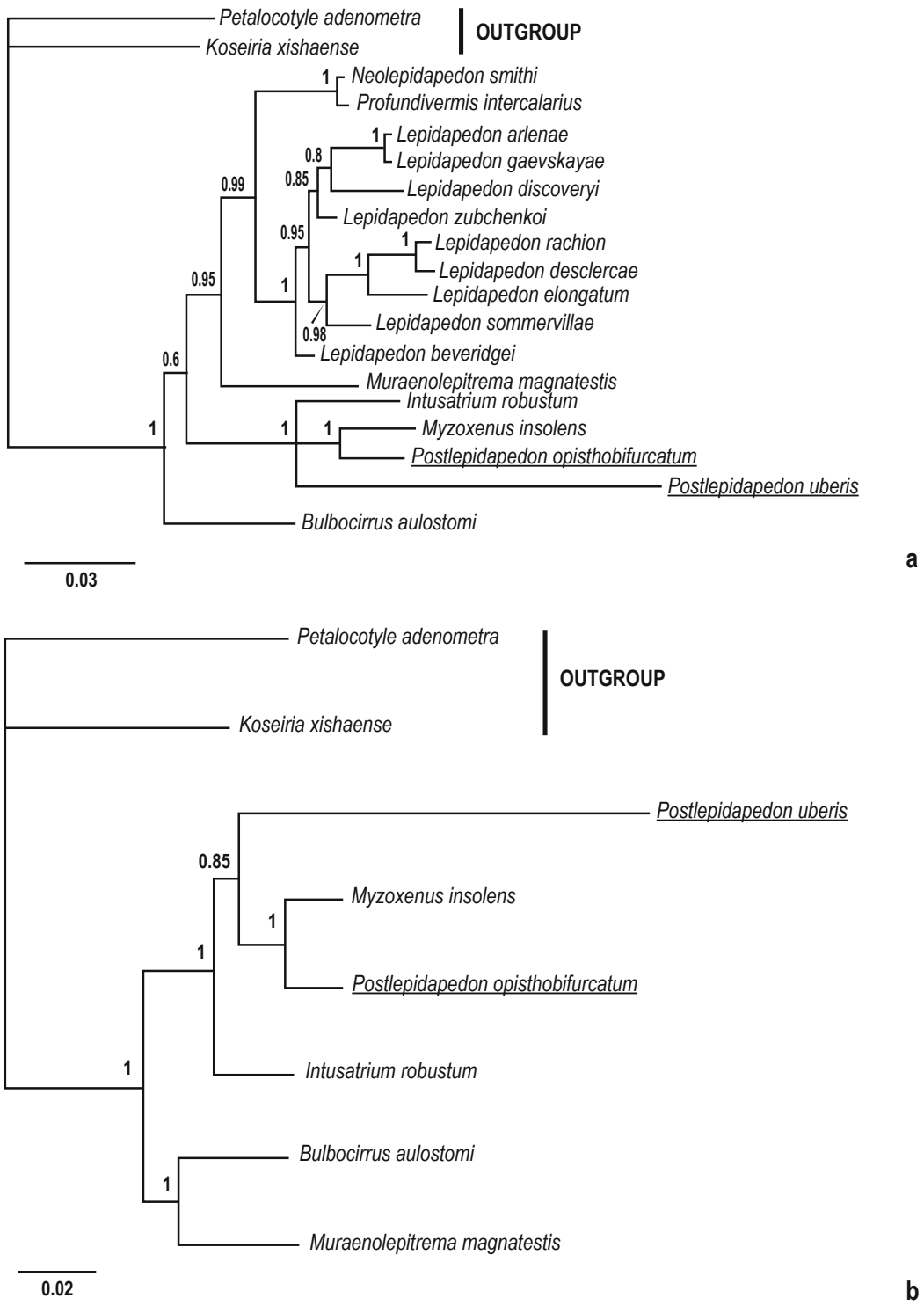

Fig. 2. Phylogenetic position of *Postlepidapedon opisthobifurcatum* within the Lepidapedidae based on 28S rDNA sequences containing 900 bp (a) and 1230 bp (b) analysed by Bayesian inference; nodal numbers indicate posterior probabilities. Scale bar shows substitutions per site.

only *P. opisthobifurcatum* among all the species currently recognised as representatives of genus *Postlepidapedon* will ultimately be proven to belong to this genus.

Phylogenetic analysis supports the position of *M. insolens* as the sister taxon of *P. opisthobifurcatum.* This result is unexpected because the indicated species are similar only by the signs common to many lepidapedids (see Bray & Cribb, 1998, 2012; Bray, 2005). Definitive hosts of *P. opisthobifurcatum* are gadiform fishes, and for *M. insolens* – perciform fishes (Bray & Cribb, 1998). Life cycles of these parasites are not known. In turn, *P. opisthobifurcatum* + *M. insolens* clade form a well-supported monophyletic group with the species *I. robustum* and *P. uberis*. In the phylogenetic model of Lepocreadioidea proposed by Bray *et al*. (2009) using partial 28S rDNA and *nad1* sequences, this species group (without *P. opisthobifurcatum*) was named "clade III." Bray *et al*. (2009) noted the absence of a general morphological synapomorphy in representatives of the clade III (at least for adult specimens); therefore, taxonomic reorganisation or revision of this clade is premature.

Conflict of Interest

Authors state no conflict of interest.

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