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Rhipidocotyle husi n. sp. and three known species of Bucephalidae Poche, 1907 from the East Asian Region: morphological and molecular data

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Research Article

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Abstract

Morphological data and the first molecular data are provided for four species of the trematode family Bucephalidae Poche, 1907 from marine and freshwater teleost fish species of East Asia. A new species, *Rhipidocotyle husi* n. sp., was isolated from *Huso dauricus* from the Amur River, Russia. Adult worms of this species were distinguished from their congeners *Rhipidocotyle illense* and *Rhipidocotyle kovalai* by morphological analysis. Three other known species were identified: *Bucephalus skrjabini* and *Prosorhynchus* cf. *squamatus* were detected in *Siniperca chuatsi* from the Amur River and in *Myoxocephalus* spp. from the Okhotsk Sea, Russia, respectively, while *Prosorynchoides karvei* was extracted from *Strongylura strongylura* from Halong Bay, Vietnam. The 28S ribosomal DNA (rDNA)-based phylogenetic analysis showed that the new species formed a shared polytomy clade with *Rhipidocotyle fennica*. Phylogenetic analysis of all available molecular data showed that four genera, namely *Rhipidocotyle*, *Bucephalus*, *Prosorynchoides* and *Prosorhynchus*, are para- or polyphyletic. Molecular-based phylogenetic analysis of morphologically validated bucephalid species indicated that three genera – *Rhipidocotyle*, *Bucephalus* and *Prosorynchoides* – were monophyletic. The genus *Prosorhynchus* maintained paraphyly, and *P.* cf. *squamatus* was more closely related to *Dollfustrema* spp. than to other *Prosorhynchus* spp. These findings do not exclude the possibility that representatives of *Dollfustrema* and *P.* cf. *squamatus* belong to the same genus.

Introduction

The Bucephalidae Poche, 1907 (Platyhelminthes: Digenea) is a cosmopolitan family comprising many parasitic species of marine and freshwater fish. Most representatives of the family have been identified to the species level based on the morphology of mature worms. Molecular data are only available for relatively few species and do not yet provide a basis for the objective validation of worm species and analysis of the phylogenetic interrelationships of the family (Corner *et al.*, 2020). In our study, morphological and molecular data were obtained for four representatives of the family Bucephalidae, including three from the subfamily Bucephalinae Poche, 1907 and one species of the subfamily Prosorhynchinae Nicoll, 1914 from freshwater and marine fish species of East Asia.

Materials and methods

Collection of trematodes

Trematodes were obtained from the following fish species: *Huso dauricus* (Georgi, 1775) *Siniperca chuatsi* (Basilewsky, 1855) of the Amur River (53°7′N, 140°40′E), *Myoxocephalus tuberculatus* Soldatov et Pavlenko, 1922, *Myoxocephalus ochotensis* Schmidt, 1929 and *Myoxocephalus polyacanthocephalus* (Pallas, 1814) of the Okhotsk Sea (54°14′N, 142°11′E). The above-mentioned fish species were collected during a scientific control catch conducted by the Khabarovsk branch of the Russian Federal Research Institute of Fisheries and Oceanography. Adult worms were also obtained from *Strongylura strongylura* (van Hasselt, 1823) fish in the coastal waters of Cat Ba Island, Vietnam (20°84′N, 106°59′E). Worms were rinsed in distilled water for a very short time, killed in hot distilled water, and preserved in 70% ethanol. Worms for molecular analysis were placed in 96% ethanol after fixation. Whole mounts for adult descriptions were made by staining the specimens with alum carmine, dehydrating the worms in a graded ethanol series and clearing in clove oil, followed by mounting in Canada balsam under a coverslip on a slide. All measurements are given in micrometres.

This material is held in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia; e-mail: petrova@biosoil.ru). It was deposited on 20 November 2020.

DNA extraction, amplification and sequencing

Adult specimens of bucephalid trematodes preserved in 96% ethanol were used for molecular analysis (Table 1). Total DNA was extracted from individual flukes using a 'hot shot' technique (Truett, 2006).

28S ribosomal DNA (rDNA) was amplified with the primers DIGl2 (5′-AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach *et al.*, 2003). The ribosomal ITS1-5.8S-ITS2 fragment was amplified with the primers ITSF (5′-CGC CCG TCG CTA CTA CCG ATT G-3′) (Andres *et al.*, 2014) and S4R (5′-TAT GCT TAA ATT CAG CGG GT-3′) (Besprozvannykh *et al.*, 2019). Initial PCR reaction was performed in a total volume of 25 μL containing 0.25 mM of each primer pair, 25 ng of total DNA in water, 12.5 μL GoTaq Green Master mix (Promega) Amplification of a 1200-bp fragment of 28S rRNA gene was performed in a GeneAmp 9700, Applied Biosystems, with a 5-min denaturation at 96°C, 35 cycles of 1 min at 96°C, 20 s at 55°C and 2 min 30 s at 72°C, and a 7-min extension at 72°C. Negative and positive controls using both primers were used. PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as recommended by the manufacturer, with the internal sequencing primers described by Tkach *et al.* (2003) for 28S rDNA and by Luton *et al.* (1992) for ITS2 rDNA. PCR product sequences were analysed using an ABI 3130 genetic analyser at the Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS. Sequences were submitted to the GenBank database (NCBI).

Alignments and phylogenetic analysis

Ribosomal DNA sequences were assembled with SeqScape v.2.6 software, provided by Applied Biosystems. Alignments and estimations of the number of variable sites and sequence differences were performed using the MEGA 7.1 software (Kumar *et al.*, 2016). Phylogenetic analysis was performed using the Bayesian algorithm with the MrBayes v. 3.1.2 software (Huelsenbeck *et al.*, 2001). The best nucleotide substitution model, TVM + I + G (Posada, 2003) for 28S rDNA and TrN + I + G (Tamura and Nei, 1993) for ITS2 rDNA, were estimated with jModeltest v. 2.1.5 software (Darriba *et al.*, 2012). Bayesian analysis was performed using 10 000 000 generations with two independent runs. Summary parameters and the phylogenetic tree were calculated with a burn-in of 25% of generations. The significance of the phylogenetic relationships was estimated using posterior probabilities (Huelsenbeck *et al.*, 2001). GenBank sequence data for representatives of Bucephallidae and outgroup taxa used in molecular analysis, including references and accession numbers are given in Table 1 and Supplementary Table 1.

Results

Rhipidocotyle husi *n. sp.*

Type-host: *Huso dauricus* (Georgi, 1775), Acipenseridae Bonaparte, 1831.

Type-locality: Amur River (53°7′N, 140°40′E).

Site: Intestine.

Prevalence: 3 of 4 fish.

Intensity of infection: 3–10 specimens.

Deposition: Holotype No. 160–Tr, paratypes No. 161–164–Tr.

Etymology: Species' name in concordance with generic name of definitive host species.

Adult worm (material examined: 5 specimens) (Fig. 1a; Table 2).

Body elongate with a narrowed posterior end. Tegument covered with needle-shaped spines. Rhynchus cup-shaped, simple muscular sucker surmounted by a muscular disc with a ventral notch and 10 papillae. Prepharynx not identified. Pharynx round or transversely oval in the middle of posterior half body. Caecum sac-like, thick-walled, located dextral and anterior to the pharynx. Testes tandem, contiguous, entire, oval or round, dextral to the median line of the body posterior to the pharynx. Cirrus sac elongate, club-shaped, sinistral, proximal end at the level of the testis. Seminal vesicle oval. Pars prostatica long, thick-walled with a large number of prostatic cells. Ejaculatory duct short. Genital pore opening ventro-subterminally into the genital atrium. Genital atrium near posterior end body, oval, surrounded by few glandular cells, contains bipartite genital lobe. Ovary spherical, dextral, pretesticular, contiguous with anterior testis. Mehlis' gland between the ovary and anterior testis. Uterus occupies most of the anterior half of the body, region at the level of sinistral vitelline field and posttesticular region. Eggs numerous, oval. Vitelline fields, two, each of 12–13 follicles, lateral, in the middle third of the body, posteriorly reach to anterior margin of the ovary. Excretory vesicle elongate, I-shaped anteriorly, at short distance posterior to rhynchus; pore terminal.

Molecular data

A ribosomal 28S rDNA fragment 1041 bp in length for 15 specimens of *Rhipidocotyle husi n. sp.* ex *Huso dauricus* was generated. All sequences were identical to each other. Nucleotide sequences were submitted to the NCBI database under the following numbers: (will be available after acceptance).

Remarks

Rhynchus structure, organs morphology and relative arrangement of worms extracted from kaluga (*H. dauricus*), correspond to diagnostic criteria of the genus *Rhipidocotyle*. Among representatives of *Rhipidocotyle*, two species have been reported from acipenserids, namely *R. illense* (Ziegler, 1883) and *R. kovalai* (Ivanov, 1967), in the Danube River, the Amudarya River and the Volga-Caspian basin (Skrjabina, 1974). In East Asia, trematodes of this genus have not been found in acipenserid fish species until now. In comparison to the species mentioned above, the worm *R. husi* n. sp. was most similar to *R. illense* by metric and morphological parameters (Table 2). However, these worms differed from *R. illense* by the maximum values of all metric parameters (Table 2) and by the rhynchus structure and arrangement of vitellaria. The rhynchus disc of *R. husi* n. sp., in contrast to that of *R. Illense*, not lobed. Vitelline fields in the new species reached the ovary level, while in *R. illense*, they are distributed well anterior to the ovary. Among other *Rhipidocotyle* spp., *R. husi* n. sp. shows considerable morphometric similarity to *R. tridecapapillata* Curran and Overstreet, 2009, which parasitizes *Morone chrysops* (Rafinesque, 1820), sMoronidae Jordan & Evermann, 1896, from the Luxapalila River, United States (Curran and Overstreet, 2009). They are similar in body and organ sizes, except egg sizes (Table 2), and also both possess a rhynchus with a simple muscular sucker surmounted by a muscular disc, ventrally notched with papillae on the surface. Despite the morphometric similarities, they were identified from very different waterways with no common biogeographical history and belonged to different species based on host specificity and differences in egg sizes and vitellarium arrangement relative to the ovary. In *R.*

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Table 1. List of Bucephalidae species and outgroup taxa used for 28S rDNA-based phylogenetic analysis

Species	*N*	GenBank Accession number	Reference
Aenigmatrema grandiovum	1	MT809145	Corner *et al.* (2020)
Aenigmatrema inopinatum	1	MT809144	Corner *et al.* (2020)
Aenigmatrema undecimtentaculatum	2	MT809141, MT809143	Corner *et al.* (2020)
Bucephalus cynoscion	2	KT273396 – KT273397	Nolan *et al.* (2015)
Bucephalus gorgon	1	KT273400	Nolan *et al.* (2015)
Bucephalus margaritae	1	KT273395	Nolan *et al.* (2015)
Bucephalus polymorphus	2	AY289247 – AY289248	Stunžėnas *et al.* (2004)
Bucephalus skrjabini	5	n/a	This study
Bucephalus varicus	1	MK648266	Pérez-Ponce de León and Hernández-Mena (2019)
Dollfustrema durum	1	MH754947	Cutmore *et al.* (2018)
Dollfustrema gibsoni	1	MH754948	Cutmore *et al.* (2018)
Dollfustrema hefeiensis	1	KT273386	Nolan *et al.* (2015)
Grammatorcynicola brayi	1	KT213573	Nolan *et al.* (2015)
Grammatorcynicola nolani	1	KT213574	Nolan *et al.* (2015)
Heterobucephalopsis perardua	1	KT213571	Nolan *et al.* (2015)
Heterobucephalopsis yongi	1	MH754949	Cutmore *et al.* (2018)
Parabucephalopsis parasiluri	1	AB640884	Baba *et al.* (2012)
Paurorhynchus hiodontis	1	KT273401	Nolan *et al.* (2015)
Prosorhynchus brayi	1	MH754950	Cutmore *et al.* (2018)
Prosorhynchus longisaccatus	1	KT213575	Nolan *et al.* (2015)
Prosorhynchus luzonicus	1	MH754951	Cutmore *et al.* (2018)
Prosorhynchus maternus	1	MH754952	Cutmore *et al.* (2018)
Prosorhynchus pacificus	1	KT273385	Nolan *et al.* (2015)
Prosorhynchus squamatus	10	n/a	This study
Prosorhynchoides caecorum	2	KT273392 – KT273393	Nolan *et al.* (2015)
Prosorhynchoides cutmorei	1	MG953232	Hammond *et al.* (2018)
Prosorhynchoides galaktionovi	2	MN310395 – MN310396	Hammond *et al.* (2018)
Prosorhynchoides gracilescens	1	AY222224	Olson *et al.* (2003)
Prosorhynchoides karvei	4	n/a	This study
Prosorhynchoides kohnae	1	MN310397	Hammond *et al.* (2018)
Prosorhynchoides longoviferus	1	KT273387	Nolan *et al.* (2015)
Prosorhynchoides megacirrus	1	KT273391	Nolan *et al.* (2015)
Prosorhynchoides moretonensis	1	MG953230	Hammond *et al.* (2018)
Prosorhynchoides ovatus	1	KT273399	Nolan *et al.* (2015)
Prosorhynchoides ozakii	1	AB640885	Baba *et al.* (2012)
Prosorhynchoides paralichthydis	1	KT273398	Nolan *et al.* (2015)
Prosorhynchoides scomberomorus	2	KT273388 – KT273389	Nolan *et al.* (2015)
Prosorhynchoides waeschenbachae	1	MG953231	Hammond *et al.* (2018)
Rhipidocotyle angusticolle	1	KT273383	Nolan *et al.* (2015)
Rhipidocotyle campanula	8	JQ346710 – JQ346714, KF184356 – KF184357	Petkevičiūtė *et al.* (2014)
Rhipidocotyle fennica	7	KM068119; KF184361 – KF184364, JQ346715 – JQ346716	Stunžėnas *et al.* (2014); Petkevičiūtė *et al.* (2014)
Rhipidocotyle galeata	1	AY222225	Olson *et al.* (2003)
Rhipidocotyle husi sp. n.	15	n/a	This study
Rhipidocotyle lepisostei	1	KT273390	Nolan *et al.* (2015)

(*Continued*)

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Table 1. (*Continued.*)

Species	*N*	GenBank Accession number	Reference
Rhipidocotyle transversalis	1	KT273394	Nolan *et al.* (2015)
Rhipidocotyle tridecapapillata	1	KT273384	Nolan *et al.* (2015)
Outgroup			
Heronimus mollis	1	AY116878	Olson *et al.* (2003)
Derogenes varicus	1	AY222189	Olson *et al.* (2003)

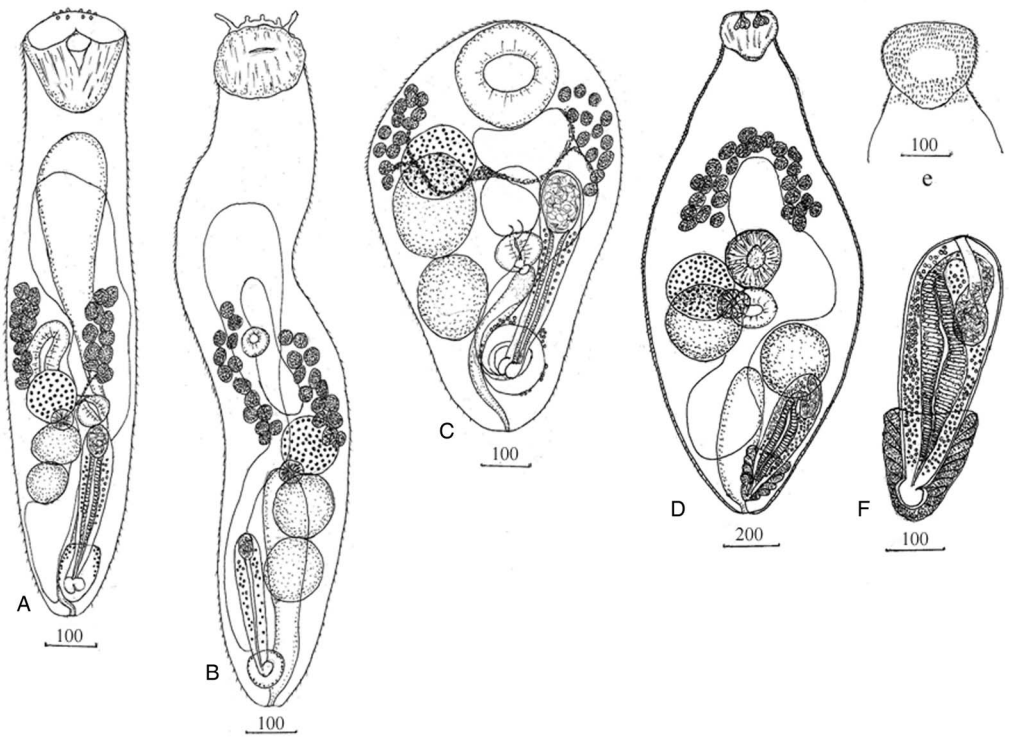

Fig. 1. Adult worms Bucephalidae. (a) *Rhipidocotyle husi* n. sp.; (b) *Bucephalus skrjabini*; (c) *Prosorynchoides karvei*; *Prosorhynchus squamatus*: (d) worm, (e) location of spines on the dorsal surface of Rhynchus, (f) cirrus sac.

tridecapapillata, in contrast to East Asian worms, the vitellarium was arranged at a distance anterior to the ovary. Another species, *R. fennica* Gibson, Taskinen, Valtonen, 1992, is also similar to worms obtained from *H. dauricus*. However, specimens of *R. fennica* have a smaller body and organs (Table 2), do not possess a rhynchus with a muscular disc with papillae on the surface, and infect percid and esocid fish species, which are taxonomically distant from acipenserids (Gibson *et al.*, 1992). Therefore, we infer that the worms obtained from *Huso dauricus* caught in the Amur River represented a new species of the genus *Rhipidocotyle*, named *R. husi* n. sp.

The 28S ribosomal DNA (rDNA)-based phylogenetic tree topology showed that all specimens of *R. husi* n. sp. from our study were within the same highly supported clade as *R. fennica* (Fig. 2). However, these two species demonstrated polytomy rather than a common phylogenetic dichotomy, where *R. fennica* appeared as a well-supported group with a single branch included in the polytomy. Both species were distant from the five other *Rhipidocotyle* spp., including the type species *R. galeata* (Rudolphi, 1819), demonstrating the paraphyly of this genus. *Rhipidocotyle husi* n. sp. differed from *R. fennica* by 1.36 ± 0.35%. Substitution analysis indicated that the 28S rDNA fragment of *R. husi* n. sp. differed from that of *R. fennica* by 14 variable sites, which contained 11 transitions and 3 transversions that, along with morphological differences, support the validity of *R. husi* n. sp.

Bucephalus skrjabini *Akhmerov, 1963*

Host: *Siniperca chuatsi* (Basilewsky, 1855), Percichthyidae Jordan & Eigenmann, 1890.

Locality: Amur river (53°7′N, 140°40′E).

Site: Intestine.

Prevalence: 3 of 5 fish.

Intensity of infection: 1–12 specimens.

Voucher deposition: No. 165–169–Tr.

Adult worm (material examined: 5 specimens) (Fig. 1b; Table 3).

Body elongate with posterior end tapered. Tegument covered with needle-shaped spines. Rhynchus spherical with irregular surface and tentacles contains groups of longitudinal muscles. Prepharynx not identified. Pharynx round or transversely oval immediately pre-equatorial, post-equatorial or equatorial. Caecum sac-like, in the midline, anterior or posterior to the pharynx. Testes tandem, contiguous, entire, oval or round, in the posterior third of the body. Cirrus sac elongated, club-shaped, dextral to testes, extends the level of the posterior end of the anterior

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Table 2. Adult worms *Rhipidocotyle* Diesing, 1858

	Rhipidocotyle husi sp. n.			*R. illense* (Ziegler, 1883) (Kozicka, 1959) (from Skrjabin, 1962)	*R. kovalai* Ivanov, 1967 (Ivanov, 1967)	*R. tridecapapillata* Curran *et* Overstreet, 2009 (Curran and Overstreet, 2009)	*R. fennica* Gibson *et al.*, 1992 (Gibson *et al.*, 1992) Summarized data
	Holotype	Range	Mean				
Body length (Bl)	1186	832–1894	1168	840–1080	1995–2835	1291–1515	600–1200
Body width (Bw)	231	169–339	220	160–280	525–735	281–295	115–400
Bw/Bl (%)	19.5	17.3–224	18.8	–	–	–	–
Forebody length (Fo)	751	535–1278	737	–	1176–1720	–	–
Fo/Bl (%)	63.3	57.6–67.5	63.1	–	–	–	–
Rhynchus length	196	150–323	198	178–229	420–483	202– 224	144–220
Rhynchus width	192	135–273	189	150–214	525–546	175–213	124–200
Pharynx length	58	50–96	61	51–61	105–189	63–70	46–78
Pharynx width	65	56–96	65	51–61	105–189	65–70	48–78
Ovary length	104	65–166	107	76–102	105–168	99–128	50–115
Ovary width	104	69–135	92	76–102	105–168	74–97	34–96
Anterior testis length	89	73–116	89	96–137	147–231	111–119	42–125
Anterior testis width	96	73–116	93	96–137	147–231	85–97	34–104
Posterior testis length	85	77–131	92	76–137	147–168	108–119	–
Posterior testis width	81	77–108	86	76–137	147–168	94–111	–
Cirrus sac length	331	270–397	315	178–300	420–673	290–340	150–320
Cirrus sac width	62	42–65	53	35–71	105–126	95–106	–
Genital atrium length	116	77–135	105	–	–	85–99	–
Genital atrium width	77	62–135	79	–	–	80–119	–
Post-testicular field length	227	146–335	212	–	–	–	105–285
Eggs length	34–39	34–39	–	–	36–40	26–31	25–42
Eggs width	19–23	19–23	–	–	20	17–18	11–21

testis or anterior end posterior testis. Seminal vesicle oval. Pars prostatica long, thin-walled with a large number of prostatic cells. Genital pore opening ventro-subterminally into genital atrium. Genital atrium near of posterior end body, irregular containing genital lobe. Ovary spherical, pretesticular, contiguous or separated with anterior testis. Mehlis' gland between the ovary and anterior testis. Uterus occupies most of the anterior half of the body, region at the level of dextral vitelline field and partly posttesticular region. Eggs numerous, oval. Vitelline fields, two, each of 13–14 follicles, lateral, in the middle third of the body. The anterior end of the dextral vitelline field is slightly anterior or at the level of the pharynx, sinistral vitelline field is anterior at the level of the pharynx. Posterior end of vitelline fields at level of the ovary. Excretory vesicle elongated, I-shaped extend to ovary, pore terminal.

Molecular data

A ribosomal 28S rDNA fragment 1040 bp in length for 5 specimens of *Bucephalus skrjabini* ex *S. chuatsi* was generated. One of five sequences contained a single T/C transition at nucleotide position #260. Nucleotide sequences were submitted into the NCBI database under the following numbers: (will be available after acceptance).

Remarks

Bucephalus skrjabini was first detected by Akhmerov (1963) in Chinese perch (*S. chuatsi* Basilewsky, 1855) in the Amur River. Mature specimens of *B. skrjabini* were collected from the same fish host in this study. Morphologically, these worms agreed well with the original description. There were differences between these worms in body length and ovary and cirrus sac sizes (Table 3). However, in Akhmerov (1963) publication, one of the specimens presented in the figures does not exceed 1.375 μm (on the basis of the provided scale), a distinct discrepancy in the metric parameters of body length. At the same time, discrepancies in the sizes of the ovary and cirrus sac probably reflect the intraspecific variation. Based on host species, locations, and similarities in morphology, we considered the worms from our study to be *B. skrjabini*.

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Fig. 2. Bayesian phylogenetic tree of Bucephalidae reconstructed on the basis of 28S rDNA sequences. Original sequences are unbolded. Nodal numbers – a posterior probability values (only significant values presented).

Prosorynchoides karvei *Bhalerao, 1937*

Host: *Strongylura strongylura* (van Hasselt, 1823), Belonidae Bonaparte, 1835.

Locality: Halong Bay East; 20°84′N, 106°59′E.

Site: Intestine.

Prevalence: 3 of 5 fish.

Intensity of infection: 1–10 specimens.

Voucher deposition: No. 170–176–Tr.

Adult worm (material examined: 7 specimens) (Fig. 1c; Table 3).

Body small, inversely pear-shaped. Tegument covered with spines. Rhynchus simple muscular sucker, large, spherical. Mouth opening median, postequatorial. Prepharynx short. Pharynx round or transversely oval at level posterior testis. Oesophagus short. Caecum short, sac-like, extends to anteriorly from the pharynx at the level of the anterior testis. Testes tandem, contiguous or partly overlapping, entire, oval or round, dextral to the median line of the body. The anterior testis is mostly located in the anterior half of the body. Posterior testis located in the posterior half body. Cirrus sac elongated extends to the level of the anterior testis. Seminal vesicle oval. Pars prostatica long with a large number of prostatic cells. Ejaculatory duct opening into the genital atrium. Genital atrium near of posterior end body surrounded by few glandular cells, contains bipartite genital lobe. Ovary spherical, pretesticular, or overlapping anterior testis and can be partially covered by rhynchus. Uterus filling most of the anterior body space. Eggs numerous, oval. Vitelline fields, in 2 lateral groups of 10–15 rounded follicles, usually extent from the level middle of rhynchus to level anterior half anterior testis, rare locate at level ovary and anterior of testis. Excretory vesicle elongated, I-shaped extending to pharynx or caecum. Excretory pore terminal.

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Table 3. Adult worms *Bucephalus* Baer, 1827 and *Prosorynchoides* Dollfus, 1929

	Bucephalus skrjabini		*Prosorhynchoides karvei*			*Prosorhynchoides fijiensis*	*Prosorhynchoides galaktionovi*	*Prosorhynchoides kohnae*
	This study	Akhmerov, 1963	This study	Bhalerao, 1937 (from Skrjabin, 1962)	Gupta, 1956	Manter, 1963	Hammond *et al.*, 2020	Hammond *et al.*, 2020
Body length (Bl)	1155–1525	1500–2000	724–1093	690–1520	970–1380	747	526–874	647–716
Body width (Bw)	154–246	200–250	477–662	460–830	460–680	408	200–318	252–312
Bw/Bl (%)	123–17.9	–	53.4–70.2	–	–	–		
Forebody length (Fo)	570–862	–	389–504	460–880	–	–		
Fo/Bl (%)	49.4–57.3	–	40.7–60.1	–	–	–		
Rhynchus length	146–169	130–200	193–235	160–310	150–230	–	89–166	120–155
Rhynchus width	135–177	140–170	213–285	220–370	170–240	201	122–176	140–168
Pharynx length	42–58	50–60	77–108	50–80	60–70	68	44–66	55–61
Pharynx width	50–58	50–60	89–112	50–120	60–90	94	62–81	66–76
Ovary length	100–116	60–80	150–196	110–210	120–160	–	60–162	74–110
Ovary width	85–127	60–70	135–193	120–190	90–160	–	54–122	73–86
Anterior testis length	104–139	110–120	154–262	130–260	140–200	–	73–140	57–113
Anterior testis width	92–119	100–70	123–219	140–260	120–210	–	54–122	85–108
Posterior testis length	85–127	110–120	135–216	–	120–260	–	59–140	81–109
Posterior testis width	92–119	100–70	154–208	–	80–120	–	61–134	81–106
Cirrus sac length	270–308	390	385–631	400–620	350–650	400	199–353	231–287
Cirrus sac width	46–58	60	73–166	130–210	80–100	74	48–79	60–89
Genital atrium length	65–100	–	108–142	–	–	113	57–100	72–124
Genital atrium width	81–85	–	116–169	–	–	85	47–94	64–81
Post-testicular field length	185–289	–	–	–	–	–		
Eggs length	39–46	38–42	15–19	14–22	18	16–20	12.6–18.2	15–21.6
Eggs width	15–19	18–20	12–15	14–19	12	10–13	7.4–12.8	7.6–13

Molecular data

Ribosomal 1034 bp 28S and 556 bp ITS2 rDNA fragments were generated for 4 and 5 specimens of *Prosorhynchoides karvei* ex *S. strongylura,* respectively. All 28S rDNA sequences were identical to each other and for ITS 2 three variable singleton substitutions were revealed. Nucleotide sequences were submitted into the NCBI database under the following numbers: (will be available after acceptance).

Remarks

The genus *Prosorynchoides* is cosmopolitan and comprises numerous morphologically similar species that are intestinal parasites of a wide range of definitive hosts, including marine and freshwater fish species. The considerable morphological similarity is typical, for example, for *Prosorynchoides ozakii* (Nagaty, 1937); *P. karvei*, with a number of synonymous species; *P. fijiensis* Manter, 1963; *P. galaktionovi* Hammond, Cribb, Nolan, Bott, 2020; and *P. kohnae* Hammond, Cribb, Nolan, Bott, 2020 (Bhalerao, 1937; Manter, 1963; Urabe *et al*., 2007; Maurya *et al*., 2018; Hammond *et al*., 2020). The geographic distribution of these worms ranges from coastal waters of Japan to India, as well as coastal waters of Fiji, Australia and French Polynesia. Species differentiation and synonymization of most known representatives of *Prosorynchoides* based only on morphological data create difficulties in the validation or invalidation of a great number of representatives in the genus *Prosorynchoides*. This challenges the estimation of both interrelationships within *Prosorynchoides* and intergeneric relationships within the subfamily.

The morphological characteristics of worms from Vietnamese *S. strongylura* unambiguously indicated membership of these worms in *Prosorynchoides*; they are morphologically similar to *P. karvei*, *P. fijiensis*, *P. galaktionovi* and *P. kohnae*. Specimens of *P. karvei* have been detected in fish species of the families Schilbeidae Bleeker, 1858 and Belonidae Bonaparte 1832, in particular in Indian *S. strongylura*. Specimens of *P. fijiensis* have been detected in *Strongylura gigantea* (Teraminck and Schlegel, 1846) from the coastal waters of Fiji. In that case, *P. fijiensis* was described on the basis of a single mature specimen (Manter, 1963), and the species was delimitated from *P. karvei* by the presence of a relatively long prepharynx and extension of the excretory bladder. Comparative analysis of morphometric data (Table 3) showed no difference for most of the parameters between worms from Vietnam and specimens of *P. karvei* from the studies of Bhalerao (1937), Gupta (1956) and Maurya *et al.* (2018) and specimens of *P. fijiensis* from Manter (1963). We think that the criteria used by Manter (1963) for differentiation of *P. fijiensis* and *P. karvei*, namely differences in prepharynx length and excretory bladder extension, are not sufficient to delimit these species. In our material, some specimens had a prepharynx and some did not. For *P. karvei*, Maurya *et al.* (2018) denote considerable variation in relative organ reciprocal arrangement and extension of the excretory bladder. Given this variability, and given the shared infection of belonid fish species as definitive hosts in the Indo-Malaysian region and Oceania it cannot be excluded that *P. fijiensis* represents a junior synonym of *P. karvei*. The final conclusion about the membership of these worms to the same or different species can only be made by generating additional molecular data for specimens from India and Fiji. As we mentioned above, the worms from our study are morphologically similar to *P. galaktionovi* and *P. kohnae*, parasites of belonid fish species from Australia and French Polynesia. However, there were differences in most of the metric parameters (Table 3). Moreover, *P. galaktionovi* and *P. kohnae* are more similar to each other than to Vietnamese *Prosorynchoides* species based on body and organ sizes. On the basis of the aforementioned information, we identify the new specimens from Vietnamese *S. strongylura* as *P. karvei*.

Phylogenetic reconstructions showed that *P. karvei* is closely related to *P. kohnae* and *P. galaktionovi* within a highly supported polytomic clade (Fig. 2). The genetic p-distance values between *P. karvei* and two closely related species, *P. kohnae* and *P. galaktionovi*, were 0.34 ± 0.18% and 0.66 ± 0.25%, respectively. Additionally, *P. karvei* differed from *P. kohnae* by four transitions, namely three A/G and one T/C substitution. Because *P. kohnae* was represented by a single 28S rDNA sequence, all four substitutions were classified as singletons until additional data on this species become available. *Prosorhynchoides karvei* and *P. galaktionovi* differed by seven fixed transitions, namely four A/G and three T/C substitutions. Of these, five substitutions were similar in *P. karvei* and *P. kohnae*, and two in *P. galaktionovi* and *P. kohnae*. These results clearly demonstrated that *P. karvei* and *P. galaktionovi* differ from each other at the interspecific level. However, a statement cannot be made regarding *P. karvei* and *P. kohnae*; in other words, there were no significant differences between *P. karvei* and *P. kohnae* to be interpreted as interspecific based on the 28S rDNA sequence data. According to the available data, the only differences between these species were metric parameters of the body and organs (Table 3). *Prosorhynchoides kohnae*, *P. karvei* and *P. galaktionovi* were located within the same monophyletic clade with *P. cutmorei*, *P. waeschenbachae* and *P. moretonensis* (Fig. 2). The genetic p-distance within this clade ranged from 0.34 ± 0.18% to 1.87 ± 0.41%. Another well-supported sister clade consisted of four species of the genus *Bucephalus*, namely *B. cynoscion*, *B. varicus*, *B. margaritae* and *B. gorgon*, and three *Prosorhynchoides* spp., *P. gracilescens*, *P. ovatus* and *P. paralichthydis*. Within this clade, there were two lineages, each including representatives of both genera with internal p-distance values from 1.94 ± 0.41% (*B. varicus*/*P. gracilescens*) to 5.42 ± 0.47% (*B. varicus*/*B. cynoscion*) and from 3.63 ± 0.52% (*B. gorgon*/*P. paralichthydis*) to 4.32 ± 0.52% (*P. ovatus*/*P. paralichthydis*). Differentiation between these lineages ranged from 3.53 ± 0.56% (*P. paralichthydis*/*P. gracilescens*) to 5.52 ± 0.68% (*B. cynoscion*/*B. gorgon*). *Rhipidocotyle transversalis* was a sister to these two clades, with p-distance values from 4.26 ± 0.59% to 5.82 ± 0.69% and from 4.31 ± 0.63% to 5.52 ± 0.68%, respectively. These values corresponded with p-distances both within and between two observed clades. These results indicated that the first clade could be recognized as the monophyletic genus *Prosorhynchoides*, whereas the second clade may have united representatives of one or several genera that belong to neither *Bucephalus* nor *Prosorhynchoides*, as well as worms identified as *R. transversalis* that do not belong to the genus *Rhipidocotyle*.

Additionally, we performed an analysis of ITS2 sequence data of *P. karvei* and closely related species. The obtained results showed a genetic differentiation of 0.6 ± 0.34% to 0.8 ± 0.39% within *P. karvei* (including GenBank data), 0.81 ± 0.39% to 1.44 ± 0.54% between *P. karvei* and *P. galaktionovi*, and 1.43 ± 0.52% to 2.07 ± 0.67% between *P. karvei* and *P. kohnae*. Additionally, 1.01 ± 0.45% to 1.21 ± 0.47% of Differentiation from 1.01 ± 0.45% to 1.21 ± 0.47% was revealed between *P. karvei* and *P. cutmorei*. Phylogenetic analysis based on ITS2 rDNA sequences showed that *P. karvei* and *P. galaktionovi* were within a polytomic clade, which provides no basis for the differentiation of these two species (Fig. 3, Supplementary Fig. 1). *Prosorhynchoides cutmorei* was sister to the *P. karvei* + *P. galaktionovi* clade, and *P. kohnae* was a basal relative to these three species. These data confirmed the species validity of *P. cutmorei* and *P. kohnae*. Despite some metric differences between *P. karvei* and *P. galaktionovi*, the conspecificity of these worms cannot be excluded on the basis of available molecular data and accepting the morphological similarity of these trematodes.

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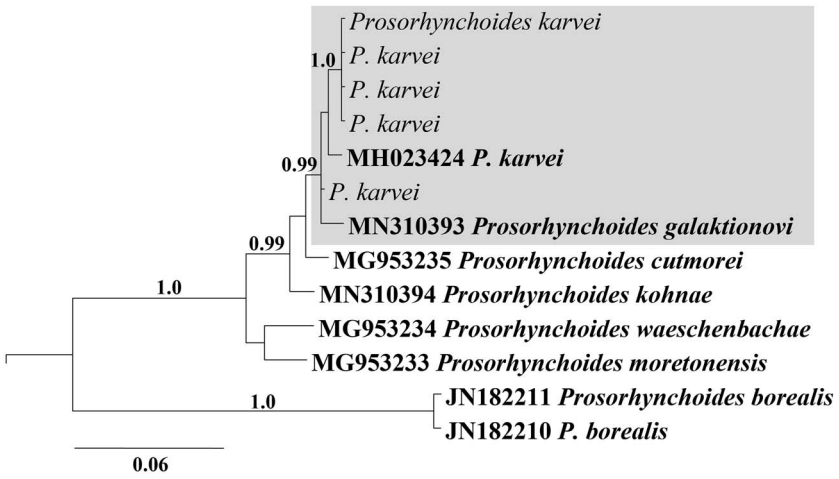

Fig. 3. The fragment of Bayesian phylogenetic tree of *Prosorhynchoides* species reconstructed on the basis of ITS2 rDNA sequences. Original sequences are unbolded. Nodal numbers – a posterior probability values (only significant values presented).

Prosorhynchus *cf.* squamatus *Odhner, 1905*

Host: *Myoxocephalus polyacanthocephalus* (Pallas, 1814), *M. ochotensis* Schmidt, 1929, *M. tuberculatus* Soldatov *et* Pavlenko, 1922, Cottidae Bonaparte, 1831.

Locality: Okhotsk Sea (54°14′N, 142°11′E).

Site: Intestine.

Prevalence: 1 of 1 in three species of fish.

Intensity of infection: 2–12 specimens.

Voucher deposition: No. 177–181–Tr.

Adult worm (material examined: 5 specimens from M. polyacanthocephalus) (Fig. 1d–f; Table 4).

Body fusiform is widest at the level of the middle of the body. Tegument covered with spines. Rhynchus apex, cup-shaped, with longitudinal muscles and six drop-shaped glandular cells, a ventral surface without spines, dorsal surface spines absent from the central region. Prepharynx not identified. Pharynx spherical or subspherical immediately pre-equatorial, post-equatorial or equatorial. Caecum sac-like, thick-walled, located in midline anteriorly to the pharynx. Testes two, spherical, opposite,

Table 4. Adult worms *Prosorhynchus* Odhner, 1905 and *Dollfusitrema* Eckmann, 1934

	Prosorhynchus squamatus			*Prosorhynchus brayi*	*Dollfusitrema gibsoni*
	This study	*P. squamatus*[a]	*Prosorhynchus cubiculum*[b]	Cutmore *et al.*, 2018	Nolan and Cribb, 2010
Body length (Bl)	1694–2110	1000–2378	1750–3400	1090–1410	1152–1203
Body width (Bw)	770–986	689–980	530–1300	371–462	512–515
Bw/Bl (%)	37.0–54.5	–	–	–	58–59
Forebody length (Fo)	739–1155	–	–	577–727	662–704
Fo/Bl (%)	43.6–56.8	–	–	–	
Rhynchus length	177–193	127–200	350–670	193–222	144–147
Rhynchus width	162–208	90–180	280–502	163–192	189–195
Pharynx length	131–142	106–160	140–190	102–115	96–99
Pharynx width	135–158	117–160	140–190	113–123	122–125
Ovary length	200–246	159–262	150–280	77–97	170–179
Ovary width	200–246	159–262	163–300	76–94	176
Anterior testis length	216–277	127–371	230–400	105–142	163–195
Anterior testis width	200–277	159–265	240–430	114–142	128–173
Posterior testis length	216–277	127–371	230–400	103–142	138–166
Posterior testis width	216–277	159–265	240–430	108–151	160–170
Cirrus sac length	477–570	392–640	560–620	254–325	333–349
Cirrus sac width	154–169	148–300	250–300	124–141	102–115
Genital atrium length	169–231	–	–	90–128	128–141
Genital atrium width	154–169	–	–	56–89	90–102
Post-testicular field length	431–447	–	–	–	291–314
Eggs length	31–35	27–41	25–36	29–33	22–32
Eggs width	19–23	18–25	15–27	17–20	14–21

[a]Summarized data from Odhner, 1905, Isaiitschikow, 1928, Layman, 1930, Miller, 1941.
[b]Summarized data from Odhner, 1905, Ozaki, 1928, Yamaguti, 1938, Layman, 1930, Linton, 1940 (from Skrjabin, 1962).

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diagonally, dextral testis at the level of pharynx, sinistral testis posterior to the pharynx. Cirrus sac elongated, club-shaped, sinistrally anteriorly reaches at a level of the left testis. Seminal vesicle elongated, curved. Pars prostatica long, thick-walled, glandular with a large number of prostatic cells. The ejaculatory duct is short, narrow. Genital pore opening ventral – subterminally into of genital atrium. Genital atrium near the posterior end of the body, elongate with genital lobe, surrounded by numerous large glandular cells. Ovary spherical, dextral to the pharynx and overlapping with the right testis. Mehlis' gland at a level to region overlap of the ovary and testis. Uterus extend anterior to caecum, posteriorly reaches of the level genital atrium. Eggs numerous, oval. Vitelline fields, two, each of 13–14 follicles in a confluent arc in the middle of the anterior half of body, posteriorly of vitelline fields reaches of level end caecum. Excretory vesicle elongated, I-shaped reaches level sinistral of testis, pore terminal.

Molecular data

A ribosomal 28S rDNA fragment 1040 bp in length was generated for 5 specimens of *Prosorhynchus squamatus* from three fish species. One specimen of *P. squamatus* ex *Myoxocephalus tuberculatus* differs from trematodes from the other two host species by three variable sites, contained two T/C transitions at sites #536 and #665 and one T/A transversion at site #944. Nucleotide sequences were submitted into the NCBI database under the following numbers: (will be available after acceptance).

Remarks

From our study, worms collected from *Myoxocephalus* Tilesius, 1811 from coastal waters of Kamchatka, were morphologically similar to the cosmopolitan *Prosorhynchus crucibulum* (Rudolphi, 1819) Odhner, 1905 and *P. squamatus* Odhner, 1905, which were first reported in the intestines of marine eels (*Conger* (Linnaeus, 1758)) and gobies (*Cottus* Linnaeus, 1758; = *Myoxocephalus*), respectively. Subsequent studies reported *P. squamatus* from fish in the families of Rajidae Bonaparte, 1831, Cottidae Bonaparte, 1831 and Salmonidae Cuvier, 1816 (Pratt and McCauley, 1961; Cheung, 1993; McDonald and Margolis, 1995; Benz and Bullard, 2004; Kuklin *et al*., 2012). Fish species from these three families, denoted as definitive hosts for *P. crucibulum*, and Hemitripteridae Cuvier, 1829 and Hexagrammidae Gill, 1889 have been identified as definitive hosts of *P. squamatus* (Shvetsova and Pozdnyakov, 1999). Assessments about the validity of these worms are controversial because of morphological similarities. Some authors believed that *P. squamatus* is a junior synonym of *P. crucibulum* (Eckmann, 1932; Nagaty, 1937; Dawes, 1947; Zhukov, 1960), while others considered these worms a separate valid species (Polyansky, 1955; Brinkmann, 1957). Moreover, the type species of the genus *Prosorhynchus* was recognized as either *P. crucibulum* by Skrjabin (1962) or *P. squamatus* by Overstreet and Curran (2002). As mentioned above, worms from our study had no significant morphological or pronounced metric differences (Table 4) relative to *P. crucibulum* and *P. squamatus*. Morphological similarity of the worms can be evidence of its belonging to the same species. Nevertheless, the results of some studies indicate that morphological similarity of mature worms is not always sufficient for regarding those worms as the same species. For this reason, the question of the taxonomic status of *S. squamatus* and *P. crucibulum* could be resolved using molecular data for these worms from type locations from representatives of *Myxocephalus* и *Conger,* respectively. We identify the worms from our material as *P. squamatus* on the basis of its occurrence in Myxocephalus (Cottidae) – the type host for this trematode, whereas for *P. crucibulum* the type host is species of the genus *Conger* (Congridae Kaup, 1856).

It is important to note that, based on morphological characteristics, worms of the discussed species were similar to *Prosorhynchus brayi* Cutmore, Nolan and Cribb, 2018, obtained from *Epinephelus* Bloch, 1973 in Moreton Bay, Queensland, Australia (Cutmore *et al*., 2018). However, *P. brayi* differed from both *P. crucibulum* and *P. squamatus* by most metric parameters (Table 4).

Phylogenetic analysis showed that, for the general phylogenetic tree (Fig. 2), the genus *Prosorhynchus* was paraphyletic. *Prosorhynchus* cf. *squamatus* is closely related to species of the genus *Dollfustrema* Eckmann, 1934 within a highly supported polytomic clade, with p-distance values from 4.06 ± 0.58% to 5.03 ± 0.68%. Five *Prosorhynchus* spp. from the GenBank database formed a distinct, highly supported clade, sister to *Prosorhynchus* cf. *squamatus*/*Dollfustrema* (Fig. 1). Within this clade, the p-distances ranged from 1.55 ± 0.4% (*P. maternus* Bray, Justine, 2006/*P. luzonicus* Velasquez, 1959) to 4.95 ± 0.63% (*P. longisaccatus* Durio and Manter, 1968/*P. brayi*). Genetic differentiation between representatives of these two clades ranged from 5.05 ± 0.66% (*P.* cf. *squamatus*/*P. luzonicus* Velasquez, 1959) to 7.18 ± 0.77% (*D. hefeiensis* Liu, 1999/*P. longisaccatus*). At the same time, *P.* cf. *squamatus* and *D. gibsoni* Nolan, Cribb, 2010 from one clade and *P. brayi* from another clade were characterized by close metric values (Table 2), and morphological similarity based on the body form, relative arrangement of organs, and the structure of vitellaria. By the last character, *Prosorhynchus cf. squamatus* is similar to both *D. durum* and *P. longisaccatus* as well. Alongside this, *Prosorhynchus cf. squamatus* differs from *D. durum* by elongate body and ovary arrangement. Nevertheless, these two species are within the same subclade. *Prosorhynchus longisaccatus* from the sister subclade most similar to *D. durum* by body form and ovary arrangement relative to the anterior testis (from the right side or partially behind anterior testis) (Durio and Manter, 1968; Nolan *et al*., 2015). *Prosorhynchus pacificus* Manter, 1940, *P. maternus*, and *P. luzonicus* from corresponding subclades have an elongate body and vitellaria fields do not unite anteriorly (confluent arc) (Manter, 1940; Bray and Justine, 2006; Cutmore *et al*., 2018). Thus, within this clade there are species that are both similar to each other morphologically and some of them differ from each other in body form, vitellaria structure and ovary arrangement relative to the anterior testis. Unfortunately, we were unable to trace the original description of *D. hefeiensis* as well as *D. vaneyi*, which was distinguished from *D. hefeiensis*, based on molecular data (Chen *et al*., 2007).

The problem of the taxonomy of the species studied here is not resolved by analysis of morphological characteristics or by the definitive host specificity of these trematodes. *Prosorhynchus* cf. *squamatus* and *Dollfustrema* spp. each forms a clade that infects fish of the Scorpaeniformes Greenwood *et al*., 1966 and Anguilliformes, respectively (Nolan and Cribb, , 2010; Cutmore *et al*., 2018). However, if the worms identified as *P. squamatus* and *P. crucibulum* (type host fish species of Anguilliformes) are recognized as a single species, then fish of Anguilliformes will be considered definitive hosts alongside others for these trematodes, as well as for *Dollfustrema* spp. Within sister clade, only *Prosorhynchus* spp. with definitive hosts from Perciformes are presented. Accepting the above-mentioned morphological characteristics of representatives of the considered clades and similar values of molecular differentiation within and between clades, the hypothetical congeneric nature of all representatives from these clades cannot be excluded. However, final conclusions in this respect require additional data on definitive host specificity and molecular data both nuclear and mitochondrial markers, *Prosorhynchus cf. squamatus* was subdivided into two lineages, including specimens obtained from *Myoxocephalus polyacanthocephalus* (Pallas, 1814) and *Myoxocephalus ochotensis* Schmidt (voucher numbers 1811

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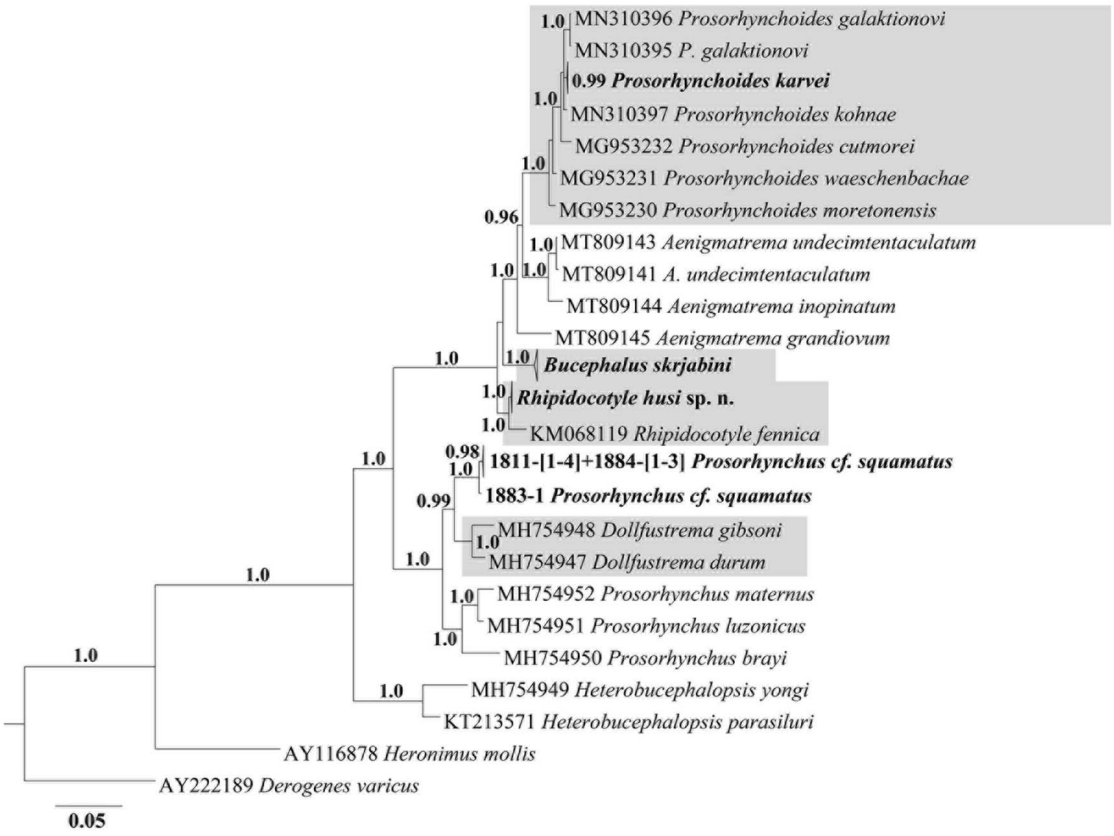

Fig. 4. Bayesian phylogenetic tree of morphologically validated species of Bucephalidae reconstructed on the basis of 28S rDNA sequences. Original sequences are unbolded. Monophyletic genera are framed. Nodal numbers – a posterior probability values (only significant values presented).

and 1884) and single specimens obtained from *M. tuberculatus* (voucher number 1883; Figure 2, Table 1). Variation in the 28S rDNA of these trematodes can presently be characterized as intraspecific. However, additional material from *M. tuberculatus* is needed to explore the depth of this variation.

Discussion

Corner *et al.* (2020) reported that complex data, including the simultaneous generation of morphological and molecular characteristics, are needed to resolve systematic and phylogenetic relationships within Bucephalidae. The identification of worm species using molecular data but without general morphological description does not resolve the issue when species from different genera cluster in one clade. The complexity of the problem can be seen from the phylogenetic structure of Bucephalidae (Fig. 2). In several cases, worms identified as members of different genera clustered together, suggesting that they belong to the same genus. On the basis of general reconstruction using overall molecular data (Fig. 2), the family Bucephalidae is monophyletic, but the genera *Rhipidocotyle*, *Bucephalus*, *Prosorhynchoides* and *Prosorhynchus* are poly- or paraphyletic, an outcome that has also been shown in previous studies (Nolan *et al.*, 2015; Hammond *et al.*, 2018, 2020; Corner *et al.*, 2020). For this reason, we also performed phylogenetic analysis of Bucephalidae using our material and GeneBank molecular data on bucephalid species supported by morphological descriptions (Fig. 4). This restricted analysis includes representatives of seven genera (*Bucephalus* with only one representative). Of these, *Aenigmatrema* and *Prosorhynchus* are not monophyletic. *Rhipidocotyle* represents a monophyletic subclade with a single specimen of *R. fennica* and specimens of *R. husi* n. sp. The genus *Prosorhynchoides* is also monophyletic, *P. karvei* is closely related to *P. galaktionovi* and *P. kohnae*, forming a shared terminal subclade with highly supported polytomy. Another three species from Australia, *P. cutmorei*, *P. waeschenbachae* and *P. moretonensis* (Genbank data), appeared as distinct branches within a monophyletic clade, including the *P. karvei* + *P. galaktionovi* + *P. kohnae* group.

Similar to the full tree, in the reduced phylogenetic tree, the genus *Prosorhynchus* maintained paraphyly, and *Prosorhynchus cf. squamatus* was closely related to *Dollfustrema* species, supporting our suggestion relative to the taxonomic status of *Prosorhynchus* and *Dollfustrema* provided above.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182022000208.

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Conflict of interest. The authors declare there are no conflicts of interest.

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