Two new species of *Prosorhynchoides* (Digenea: Bucephalidae) from *Tylosurus crocodilus* (Belonidae) from the Great Barrier Reef and French Polynesia

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Abstract:

We surveyed 14 individuals of *Tylosurus crocodilus* Péron & Lesueur 1821 (Belonidae) collected from the waters around Lizard Island and Heron Island, Great Barrier Reef, Queensland, Australia, and the waters around Moorea, French Polynesia. We describe two new species of bucephaline trematodes from them, *Prosorhynchoides galaktionovi* n. sp. and *P. kohnae* n. sp. They are morphologically distinct from existing *Prosorhynchoides* spp., with molecular data from 28S and ITS-2 ribosomal DNA, as well as *cox*1 mitochondrial DNA, further supporting our morphological findings. Neither species has been observed in other belonid fishes. The new species fall into the clade of species of *Prosorhynchoides* from belonids previously identified in Australian waters. These findings strengthen the observation that groups of bucephaline species have radiated, at least in part, in tight association with host taxa. There are now five species of *Prosorhynchoides* known from two belonid species in Australian waters. We, therefore, predict further richness in the nine other belonid species present.

Keywords: Bucephalidae, *Prosorhynchoides*, 28S rDNA, ITS-2 rDNA, *cox*1 mtDNA, Great Barrier Reef

1. Introduction

Hammond, et al. [1] showed that a single species of the Belonidae, *Tylosurus gavialoides* (Castelnau), collected from Moreton Bay in southern Queensland, has three morphologically and genetically distinct species of bucephalid trematodes from the genus *Prosorhynchoides* [1]. It is presently the only belonid species from which bucephalids have been described in Australian waters.

The Bucephalidae are trematodes found within the gastrointestinal tract of piscivorous fishes. They have been reported from marine, freshwater and brackish environments. Bucephalids differ from

typical trematodes by their anterior sucker being adapted for attachment, known as a rhynchus, rather than being associated with the digestive system, with their ventral sucker being associated, containing a muscular pharynx, and by their distinctive, posteriorly opening terminal genitalia [2].

Here, we explore the bucephalid fauna of *Tylosurus crocodilus* Péron & Lesueur. This species is found in tropical to subtropical waters of the Atlantic and Pacific oceans. In Australia, they occur along the northern coast of Australia, from south-west Western Australia and extending north and east around to northern New South Wales [3]. Four species of bucephalids have previously been described from this fish, three species of *Prosorhynchoides* (*P. fijiensis* (Manter, 1963), *P. southwelli* (Nagaty, 1937) and *P. tylosuris* (Ozaki & Ozaki, 1952)) and *Skrjabiniella uniporus* (Ozaki, 1924) [4-7].

For this study, we collected individuals of *T. crocodilus* from GBR, from the waters around Lizard Island and Heron Island; one individual was collected from the waters around Moorea in French Polynesia. We report two new species of bucephalids, described using a combined morphological and multiple molecular marker approach, and compare them with previously described species of *Prosorhynchoides* from belonids found in Australian waters and elsewhere.

2. Materials and methods

2.1. Sample collection

Specimens of *Tylosurus crocodilus* were collected from waters around three locations; Lizard Island (GBR, 14° 40’ 0”S, 145° 28’ 0”E), Heron Island (GBR, 23° 27’ 0”S, 151° 55’ 0”E) and Moorea (French Polynesia, 17° 32' 25" S 149° 50' 0"W). Hosts were collected *via* line, spear fishing, and seine net.

For each specimen, the digestive tract was isolated, opened and observed in vertebrate saline, and then put through a gut wash, as described by Cribb and Bray [8]. Trematodes were fixed in near-boiling saline, preserved in 70% ethanol, and put into -20°C storage.

2.2. Morphological analysis

Specimens for staining and mounting were washed twice in tap water for 30 min, then stained in Mayer’s Haematoxylin for 30 min, destained with a 1% HCl solution, and neutralised with a 1% ammonia solution. They were then dehydrated using a graded series of ethanol solutions (50%, 70%, 90%, 96% and 100%). Each step was approximately 20–30 min, and the 100% step was repeated. Once dehydrated, they were cleared using methyl salicylate, first in a 50% solution, then in 100% for approximately 30 min each, and then mounted onto slides using Canada Balsam.

Trematodes were observed using a Leica DM 2500 light microscope, a Leica DFC310 FX camera (Leica Microsystems Ltd., Switzerland) and the program Leica Application Suite (LAS) (Leica Microsystems Ltd., Switzerland, ver. 4. 3. 0). Measurements of morphological features were made using the LAS software. All measurements are in micrometres. Holotypes and paratypes have been submitted to the Queensland Museum (QM), Brisbane, Australia.

2.3. Molecular analysis

Molecular and phylogenetic analyses are based on worms from *T. crocodilus* caught from the waters around Lizard Island.

DNA extractions were carried out on individual trematodes using an Isolate II Genomic DNA kit, according to the manufacturer's protocol (Bioline (Aust) Pty Ltd, Alexandria, NSW). Polymerase Chain Reaction (PCR) amplifications were then performed on extracted samples. For this study, two ribosomal DNA (rDNA) markers, 28S and ITS-2, and one mitochondrial DNA (mtDNA) marker, mitochondrial cytochrome *c* oxidase 1 (*cox*1), were amplified. The 28S (1241–1267 nucleotides), ITS-2 (555–562 nucleotides) and the *cox*1 (488–509 nucleotides) sequences were amplified using the oligonucleotide primers; LSU5 and 1500R [9, 10], GA1 and ITS2.2 [11, 12], and Dig_cox1Fa and Dig_cox1R [13], respectively. The 28S and *cox*1 markers were amplified using the Ampli*Taq* Gold® DNA Polymerase kit [Applied Biosystems Inc., Foster City, California, USA], with ITS-2 amplified using My*Taq*™ DNA Polymerase kit [Bioline (Aust) PTY LTD]. For 28S and cox1 markers, 50 µl PCR reactions were set up following Applied Biosystems Inc.'s instructions, with 25 µl of polymerase with buffer, 1 µl of GC enhancer, 1 µl of each primer (10 µM) and 17 µl of autoclaved, Millipore water used, with 5 µl of sample per tube. The PCR cycle for the 28S amplification used the setup as follows; 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min, and a final step of 72°C for 10 min. The PCR cycle for the *cox*1 amplification used the setup as follows; 95°C for 10 min, followed by 35 cycles of; 95°C for 30 s, 45°C for 30 s and 72°C for 1 min, and a final step of 72°C for 10 min. For the ITS-2 marker, fifty µl PCR reactions were set up following Bioline's® instructions, with 10 µl of buffer, 0.25 µl of polymerase, 1 µl of each primer (10 µM) and 32.75 µl of autoclaved, Millipore water used, with 5 µl of sample per tube. The PCR cycle for ITS-2 amplification used the setup as follows; 95°C for 1 min, followed by 30 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 15 s, and a final step of 72°C for 5 min. PCR amplifications were performed on a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, California, U.S.A).

Samples that were successfully amplified were then purified using an Isolate II PCR and Gel kit, according to the manufacturer's protocol [Bioline (Aust) PTY LTD]. Samples were then sequenced at the Australian Genome Research Facility (AGRF), in Melbourne, Australia, in both the forward and reverse directions.

2.4. Phylogenetic analysis, inter- and intraspecific variation

All sequences were compared for inter- and intraspecific variation within each marker. ITS-2 sequences were compared using three sequences of worms for both species, *cox*1 sequences were compared using three sequences of *Prosorhynchoides kohnae* n. sp. and two sequences of *P. galaktionovi* n. sp. We also explored the interspecific variation, using *cox*1 sequences, of these two species and the three other species of *Prosorhynchoides* described from belonids in Australian waters; *P. moretonensis*, *P. waeschenbachae* and *P. cutmorei* Hammond, Cribb and Bott [1].

Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, U.S.A, ver. 5.2.4) was used to produce consensus sequences from the forward and reverse sequences that were generated. For 28S analysis, all available sequences of bucephalines were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). For ITS-2, similar sequences were obtained from GenBank. Sequences from GenBank used for the phylogenetic analyses are listed in Table 2. Sequence sets were aligned using the ClustalX [14] accessory application in Bioedit® [15]. Alignments

were trimmed to a point where most of the sequences had started and finished. Bayesian Inference analyses of sequence alignments were conducted using; MrBayes® ver. 3.2.2 [16] and the parameters specified by Aiken, et al. [17], but with five million generations run instead of two million.

3. Results

3.1. Samples

In total, 14 individuals of *Tylosurus crocodilus* were collected, of which 11 were infected with adult bucephalids. Infections of adult bucephalids were found in all eight *Tylosurus crocodilus* collected from Lizard Island, two of five from Heron Island, and the single individual from Moorea was also infected. Two new species of bucephalid trematodes, with morphological characteristics consistent with the diagnosis of *Prosorhynchoides*, were recognised and are described below. All specimens found in the study can be recognised as one of these two species.

3.2. Morphology

Family Bucephalidae Poche, 1907

Subfamily Bucephalinae Poche, 1907

Genus *Prosorhynchoides* Dollfus, 1929

3.3 *Prosorhynchoides galaktionovi* n. sp.

3.3.1 Material studied

Type-host: *Tylosurus crocodilus* (Péron & Lesueur, 1821), Hound needlefish (Belonidae)

Type-locality: Lizard Island (GBR, 14° 40’ 0”S, 145° 28’ 0”E)

Other localities: Heron Island (GBR, 23° 27’ 0”S, 151° 55’ 0”E), Moorea (French Polynesia, 17° 32' 25" S 149° 50' 0"W)

Site and prevalence: Intestine of 9 out of 14 hosts: Lizard Island: 6 of 8, Heron Island: 2 of 5, Moorea: 1 of 1.

Etymology: Named for Dr Kiril Galaktionov for his substantial contributions to trematodology.

Measurements: See Table 1

Specimen lodgement: QM Holotype: QM G237899 Paratypes: QM G237900-G237907

GenBank accession numbers: 28S MN310395 and MN310396, ITS-2 MN310393, *cox*1 MN308456 and MN308457

ZooBank Life Science Identifier: act:28C4214A-20A2-4F75-BCB5-2CE691B0EDF4

3.3.2 Description (Based on nine whole-mounted gravid specimens)

Body small, elongate, widest between one third to half of total body length from anterior end. Tegument spiny throughout. Rhynchus ovoid, a simple sucker, of variable size relative to total body length, with shortest length being approximately one eighth of total body length and longest being approximately one fifth. Pharynx elliptical, muscular, in posterior half of body, usually posterior to posterior testis (drawn specimen exceptional in this respect). Oesophagus approximately one-fifth to one-eighth of total body length, narrow, running anteriorly from pharynx. Caecum short, sac-like, running anterior from pharynx to parallel or anterior to anterior testis or ovary.

Testes two, ovoid, tandem to oblique, sometimes contiguous, dextral. Anterior testis parallel or posterior to caecum, ventral to caecum and posterior testis, dorsal or ventral to ovary. Posterior testis dorsal to pharynx and cirrus-sac. Cirrus-sac elongate, not thick-walled, extends anteriorly for variable length, between level of posterior testis and level of ovary. Seminal vesicle elliptical, centrally aligned in anterior end of cirrus-sac. Pars prostatica straight to slightly curved, glandular. Genital atrium small, ovoid. Form of terminal genitalia could not be discerned.

Ovary ovoid, dextral, anterior to and contiguous with anterior testis, dorsal or ventral to caecum, anterior testis and vitelline follicles. Vitelline follicles in two lateral clusters, with their lengths approximately equal to or greater than their widths, extending anteriorly from ovary to between halfway between ovary and rhynchus to posterior margin of rhynchus. Laurer's canal and Mehlis' gland not seen. Uterus coiled, extends anteriorly, but does not exceed vitelline follicles, dorsal to pharynx, dorsal and ventral to caecum, posterior testis, cirrus-sac, ovary and vitelline follicles, ventral to anterior testis when it overlaps. Eggs small, tanned, ovoid. Excretory pore terminal; anterior extent of excretory vesicle not detected.

3.4 *Prosorhynchoides kohnae* n. sp.

3.4.1 Material studied

Type-host: *Tylosurus crocodilus* (Péron & Lesueur, 1821), Hound needlefish (Belonidae)

Type-locality: Lizard Island (GBR, 14° 40' 0"S, 145° 28' 0"E)

Other localities: Heron Island (GBR, 23° 27' 0"S, 151° 55' 0"E), Moorea (French Polynesia, 17° 32' 25" S 149° 50' 0"W)

Site and prevalence: Intestine of 9 out of 14 hosts: Lizard Island: 7 of 8, Heron Island: 1 of 5, Moorea: 1 of 1.

Etymology: Named for Dr Anna Kohn for her substantial contributions to trematodology.

Measurements: See Table 1

Specimen lodgement: QM Holotype: QM G237908 Paratypes: QM G237909-G237913

GenBank accession numbers: 28S MN310397, ITS-2 MN310394, *cox*1 MN308458-MN308460

ZooBank Life Science Identifier: act:111322DC-054B-4DF0-8FA1-1782AF31AEA2

3.4.2 Description (Based on six whole-mounted gravid specimens)

Body small, elongate, widest between one third to half of total body length from anterior end. Tegument spiny throughout. Rhynchus ovoid, a simple sucker. Pharynx elliptical, muscular, in posterior half of body, parallel or posterior of posterior testis. Oesophagus approximately one-fourth to one-seventh of total body length, narrow, running anteriorly from pharynx. Caecum short, sac- like, running anterior from pharynx to level of anterior testis or ovary.

Testes two, ovoid, tandem to oblique, sometimes contiguous, dextral. Anterior testis parallel or posterior to caecum, dorsal or ventral to caecum, posterior testis and ovary, dorsal to cirrus-sac when they overlap. Posterior testis dorsal to pharynx, ventral to caecum when they overlap. Cirrus- sac elongate, not thick-walled, extends anteriorly for variable length, between level of anterior testis and level of ovary. Seminal vesicle elliptical, centrally aligned in anterior end of cirrus-sac. Pars prostatica straight to curved, glandular. Genital atrium small, ovoid. Form of terminal genitalia could not be discerned.

Ovary ovoid, dextral, anterior to and contiguous with anterior testis, dorsal or ventral to caecum and anterior testis, ventral to vitelline follicles. Vitelline follicles in two lateral clusters, with their widths approximately equal to or greater than their lengths, extending anteriorly from ovary to between posterior margin of rhynchus and posterior half of rhynchus. Laurer's canal and Mehlis' gland not seen. Uterus coiled, extends anteriorly, but does not exceed vitelline follicles, dorsal to pharynx, dorsal and ventral to caecum, testes, cirrus-sac and ovary, ventral to vitelline follicles. Eggs small, tanned, ovoid. Excretory pore terminal; anterior extent of excretory vesicle not detected.

3.5 Molecular analysis

3.5.1 Species identification and variation

The intraspecific variation for 28S rDNA, ITS-2 rDNA, and *cox*1 mtDNA sequences for the two species was low. The 28S sequences differed by one base among the three replicates of *P. galaktionovi* n. sp. and identical sequences for the three replicates of *P. kohnae* n. sp. The ITS-2 sequences were identical for both sets of three specimens. The *cox*1 sequences differed by seven bases between the two sequences of *P. galaktionovi* n. sp. and identical sequences for the three sequences of *P. kohnae* n. sp.

The interspecific variation between the two species was eight bases for 28S sequences, 12 bases for ITS-2 sequences and 71–75 bases for *cox*1 sequences. Compared with *cox*1 sequences from Hammond, et al. [1], *P. galaktionovi* n. sp. differed by 91 bases from *P. moretonensis*, 87–92 bases from *P. waeschenbachae*, and 83–85 bases from *P. cutmorei*. *Prosorhynchoides kohnae* n. sp. differed by 87 bases from *P. moretonensis*, 86-88 bases from *P. waeschenbachae*, and 77–79 bases from *P. cutmorei*.

3.5.2 Phylogeny

The 28S rDNA analysis resulted in a sequence alignment of 1342 nucleotides used for the Bayesian analysis and subsequent phylogram (Fig. 3). The two new species described here form a strongly supported clade (node support 100%). They form part of a larger clade of five species of *Prosorhynchoides* from belonid fishes, which is also highly supported (node support 100%). The closest sequence to the two newly described species is *P. cutmorei* Hammond, Cribb and Bott, 2018. The sister taxa to this clade are *P. ovatus* (Linton, 1900), *P. paralichthydis* (Corkum, 1961), and *Bucephalus gorgon* (Linton, 1905). Overall, there is strong support within this tree, with only three clades having less than 89% node support. The topology of the tree is almost identical to that of Hammond, et al. [1], and the same issues surrounding polyphyly are observed.

The ITS-2 rDNA analysis resulted in a sequence alignment of 610 nucleotides used for Bayesian analysis (Fig. 4). The two new species form a strongly supported clade with *P. cutmorei* (node support 99%), but the clade containing *P. galaktionovi* n. sp. and *P. cutmorei* has weak support (node support 64%). Overall, there is also strong support within this tree, with only three clades having less than 99% node support. The sister taxa to the clade containing *Prosorhynchoides* from belonids include two species of *Prosorhynchoides*, *P. ovatus* and *P. paralichthydis*, and *Rhipidocotyle transversalis* Chandler, 1935.

4. Discussion

4.1 Two species from *Tylosurus crocodilus*

The two species reported here are highly similar morphologically. They have small, elongate bodies, widest between one third to half of the total body length from the anterior end. Their pharynges are in the posterior half of the body, variably positioned relative to the posterior testis. Their caeca are anterior to the pharynx and can be parallel to the anterior testis or ovary. Their testes are ovoid, tandem to oblique, and sometimes contiguous. Their cirrus-sacs extend anteriorly as far as parallel to the ovary. Their uteri extend anteriorly but do not exceed the vitelline follicles. These similarities, together with their similar dimensions for several features (Table 1), render the two as almost morphologically cryptic species. However, the form of the vitelline follicles in the two species is a clear distinguishing feature, with the lengths of the clusters being greater than the widths for *P. galaktionovi* n. sp. and the widths of the clusters being greater than the lengths for *P. kohnae* n. sp. For each species, we added the widths of the two vitelline clusters, and compared these to their total widths, resulting in means of 0.52 for *P. galaktionovi* n. sp. and 0.65 for *P. kohnae* n. sp. The vitelline follicles extend anteriorly from the ovary of *P. galaktionovi* n. sp. to between halfway to the rhynchus at its shortest, to the posterior margin of the rhynchus at its longest, while the vitelline follicles of *P. kohnae* n. sp. extend anteriorly from the ovary to between the posterior margin of the rhynchus at its shortest, and the posterior half of the rhynchus at its longest.

Despite the close morphological similarity of these two species, the molecular differences in all three markers make it clear that two species are present. It was thanks to mounted specimens with matching catalogued sequences that we were able to identify the two different species.

4.2 Comparison with previously described species of *Prosorhynchoides*

Prior to the present work, *Prosorhynchoides* was already a large genus comprising 77 accepted species according to WoRMS [18]. The morphological similarity of the two species distinguished here means that great care needs to be taken in considering their status relative to previously described species. Our approach here is to compare them first with species previously reported from *T. crocodilus* and then with species reported from other belonids. We see no evidence that bucephalids from belonids ever infect non-belonid fishes.

4.2.1 Species from *Tylosurus crocodilus*

Three species of *Prosorhynchoides* have been reported from *Tylosurus crocodilus*: *P. fijiensis*, *P. southwelli*, and *P. tylosuris*.

Prosorhynchoides fijiensis was reported from a single specimen found in a host obtained from a fish market in Fiji by Manter [5]. Based on the original and only description, it is immediately distinguishable from the present species by having the pharynx positioned posterior to the posterior testis and unusually close to the posterior extremity. However, it is highly intriguing that we have not encountered this species ourselves, given that its type locality, Fiji, is between the Great Barrier Reef and French Polynesia. Our total sample of *T. crocodilus* (14) is not large so that we may have missed it.

Prosorhynchoides southwelli was initially described by Nagaty from *Strongylura strongylura* (van Hasselt, 1823) from the Red Sea [6]. It was subsequently reported from *T. crocodilus* collected from either the Red Sea or the Gulf of Aden by Parukhin [19]. As originally described, this species exceeds 1 mm in length, and the ovary is always distinctly separated from the anterior testis. These characters appear to distinguish it from the present forms.

Prosorhynchoides tylosuris was described from a host collected from the Pacific waters of Japan by Ozaki & Ozaki [4]. It was considered a synonym of *P. karvei* by Machida & Kuramochi [20]. It appears immediately distinguishable from the present species in reportedly reaching 1400–2250 µm in body length, whereas none of the present specimens exceeds 900 µm. Although the figured specimen of *P. tylosuris* was evidently flattened slightly, it appears to be far longer than the present two species.

4.2.2 Species from other Australian belonids

Three species of *Prosorhynchoides* have been described from *Tylosurus gavialoides* from Moreton Bay [1], to the south of the collections sites reported here. All three molecular markers show unambiguously that the three *T. gavialoides* species are distinct from the two from *T. crocodilus*. In terms of morphology, *P. waeschenbachae* is an elongate species easily distinguished from all the other forms. *Prosorhynchoides moretonensis* and *P. cutmorei* both generally resemble the present species in body form. However, *P. moretonensis* has relatively long and straight vitelline fields which immediately distinguish it, and *P. cutmorei* is similarly distinguished by very small and compact vitelline fields, unlike those seen in *P. galaktionovi* n. sp. and *P. kohnae* n. sp.

4.3 Comparison with other previously described species

For morphological comparisons of the species described here with other previously described species, we have restricted the remaining comparisons to the other eight species of *Prosorhynchoides* described from belonids. This is based on the arguments outlined by Hammond, et al. [1], regarding host specificity and reliable reports of *Prosorhynchoides* from belonids.

Of the remaining eight species, some can be quickly excluded from possible identity with the present samples. *Prosorhynchoides ablennus* (Gu & Shen, 1976) and *P. obpyriformis* (Gu & Shen, 1976), while morphologically similar to *P. galaktionovi* n. sp. and *P. kohnae* n. sp., have larger bodies, with lengths exceeding 1000 µm and widths exceeding 500 µm, and are larger in almost all internal structures, with the main exceptions being the rhynchus and eggs. Regarding other morphological differences, *P. ablennus* differs from *P. galaktionovi* n. sp. and *P. kohnae* n. sp. by having a spindle-shaped, rather than elongate, body, the pharynx is in the centre of the body, rather than in the posterior half, and the sinistral vitelline follicles are in a lateral field, rather than clustered. *Prosorhynchoides obpyriformis* differs from both *P. galaktionovi* n. sp. and *P. kohnae* n. sp. by having irregularly shaped testes, rather than ovoid, and the cirrus-sac extends anteriorly to the level of the pharynx, rather than at the level of the ovary.

Prosorhynchoides belonea (Srivastava, 1938) is described as having a body length of at least 1680 µm, vitelline follicles in lateral fields, and large eggs between 34 and 36 µm long. *Prosorhynchoides bennetti* (Hopkins and Sparks, 1958) is described as having a caecum dorsal to the pharynx, a cirrus-sac that does not extend anteriorly beyond the posterior testis, and the ovary parallel to the pharynx. *Prosorhynchoides strongylurae* (Hopkins, 1954) is described as having a body length of at least 2000 µm, and the pharynx variably positioned relative to the ovary, and the caecum dorsal or posterior to the pharynx.

The remaining three species resemble *P. galaktionovi* n. sp. and *P. kohnae* n. sp. This group comprises *P. karvei* (as described by Bhalerao, 1937), *P. lenti* (Nagaty, 1937), and *P. megacetabulus* (Nagaty, 1937). All five species have somewhat elongate bodies, a pharynx variably positioned relative to the posterior testis, the caecum anterior to the pharynx, the cirrus-sac extending anteriorly to at least the anterior extremity of the posterior testis, and vitelline follicles in clusters.

We base our comparisons relative to *Prosorhynchoides karvei* on the original description by Bhalerao [21]. It is our view that the description by Machida and Kuramochi [20], which synonymised species that have morphological features that are quite different to the original description, to be potentially too sweeping given the broad morphologies synonymised. The morphological comparisons of *P. galaktionovi* n. sp. and *P. kohnae* n. sp. to *P. karvei*, *P. lenti* and *P. megacetabulus* are outlined below.

4.3.1 *Prosorhynchoides karvei*

Prosorhynchoides galaktionovi n. sp. differs from *P. karvei* by having a smaller rhynchus (mean dimensions of 119 x 140, compared with 160–246 x 142–227), the cirrus-sac is less than half the length of the body, and does not extend anteriorly beyond the caecum, and it has shorter eggs (longest recorded egg was 18.16 with the range for *P. karvei* at 18–21.5).

Prosorhynchoides kohnae n. sp. differs from *P. karvei* by having a smaller rhynchus (mean dimensions of 135 x 149 compared with 160–246 x 142–227), the cirrus-sac is less than half the length of the body and does not extend anteriorly beyond the caecum, and the vitelline follicles are in clusters with their widths approximately equal or greater than their lengths.

4.3.2 *Prosorhynchoides lenti*

Prosorhynchoides galaktionovi n. sp. differs from *P. lenti* by having vitelline follicles extending anteriorly from the ovary (posterior margin of sinistral group for *P. lenti* is parallel to the ovary), vitelline follicles in clusters (sinistral group for *P. lenti* elongated), and having smaller eggs (mean dimensions of 15.8 x 9.9 µm compared with 29 x 17 µm for *P. lenti*).

Prosorhynchoides kohnae n. sp. differs from *P. lenti* by having a smaller body (longest *P. kohnae* n. sp. is 716 µm, shortest *P. lenti* is 765 µm), vitelline follicles extending anteriorly from the ovary (posterior margin of sinistral group for *P. lenti* is parallel to ovary), vitelline follicles in clusters (sinistral group for *P. lenti* elongated) and having smaller eggs (mean dimensions of 17.3 x 10.1 µm compared with 29 x 17 µm for *P. lenti*). The vitelline follicle clusters in *P. kohnae* n. sp. also have widths that are approximately equal to or greater than their lengths.

4.3.3 *Prosorhynchus megacetabulus*

Prosorhynchoides galaktionovi n. sp. differs from *P. megacetabulus* by having the cirrus-sac extending anteriorly for variable length, with the shortest being at the anterior extremity of the posterior testis and the longest being parallel to the ovary, whilst it does not exceed the posterior testis for *P. megacetabulus*. The vitelline follicles are in clusters for *P. galaktionovi* n. sp. (the sinistral group for *P. megacetabulus* is elongated), the uterus does not exceed the vitelline follicles anteriorly, and it has smaller eggs (mean dimensions of 15.8 x 9.9 µm compared to 21 x 17 µm for *P. megacetabulus*).

Prosorhynchoides kohnae n. sp. differs from *P. megacetabulus* by having a marginally smaller body (longest *P. kohnae* n. sp. is 716 µm, shortest *P. megacetabulus* is 723 µm, with the longest being 910 µm), a cirrus-sac that extends anteriorly for variable length, with shortest being parallel to the anterior testis and longest being parallel to the ovary, whilst it does not exceed the posterior testis for *P. megacetabulus*. Vitelline follicles in clusters (the sinistral group for *P. megacetabulus* is elongated), with their widths approximately equal to or greater than their lengths. The uterus does not exceed the vitelline follicles anteriorly, and it has smaller eggs (mean dimensions of 17.3 x 10.1 µm compared to 21 x 17 µm for *P. megacetabulus*).

4.4 Morphological conclusions

In our view, a generally robust case can be made for the distinction of the two species described here from all previously described species of *Prosorhynchoides.* However, we freely acknowledge that for several comparisons, the case is not strong. The weakness relates to a combination of factors. The present work shows that distinct species of this genus may be highly similar

morphologically. We have no true understanding of the overall pattern of biogeographical distribution of these species (although the present work suggests that they may indeed be widespread) or the extent to which they are shared between belonid species. Finally, many descriptions lack sufficient detail, and some were based on meagre samples. Thus, the taxonomy of this genus is very-much a best estimate, requiring improvement. We think that a true sense of the status of the taxonomy of this genus now depends on the generation of molecular data. We expect extensive modification of our understanding of the taxonomy of this genus to emerge as these data become available.

4.5 Molecular species recognition

All three genetic markers used here appear effective for the task of species delineation in this group. As expected, the level of base pair distinction was greatest for *cox*1 mtDNA, followed by ITS2 rDNA and smallest for 28S rDNA. Blasco-Costa, et al. [22] recommended that the sequencing of newly described species should use at least two independent loci. Given that most sequences of bucephalids relate to ribosomal DNA, another marker should be considered for species delineation. The mitochondrial *cox*1 marker appears suitable for this role, even though few sequences are available. Bray, et al. [23] used morphological comparisons, host distributions and sequences from ribosomal (28S and ITS-2) and mitochondrial (*cox*1) markers, to identify ten species of *Lepotrema* (Lepocreadiidae), including eight new species. The *cox*1 sequences helped delineate species boundaries, with some combinations of species having no differences in 28S sequences and only one base difference in ITS-2 sequences. Besprozvannykh, et al. [24] used *cox*1 sequences, together with morphological information, to describe a new species of *Metorchis* (Trematoda: Opisthorchiidae). In that study, ITS-2 was not able to distinguish the new species from one that had previously been described and sequenced. Here ITS-2 and *cox*1 both showed unambiguous species-level differences.

It is important to note that the sequences produced in this study were from bucephalids obtained from Lizard Island fishes only. As such, we do not have an understanding of molecular inter- and intraspecific variation based on locality.

4.6 Phylogeny

Phylogenetic analysis of 28S rDNA produced a topology comparable to those produced recently [1, 25], increasing the representation of the clade of species of *Prosorhynchoides* infecting belonids.

The essential problem with this group, as first identified by Nolan, et al. [25] remains; species of the dominant bucephaline genera *Bucephalus* von Baer, 1827, *Prosorhynchoides*, and *Rhipidocotyle* Diesing, 1858, do not form monophyletic clades in phylogenetic trees. In our view, it appears inescapable that these genera are thus not reliable concepts. However, despite the increased molecular representation of the Bucephalinae, it remains premature to attempt to resolve this issue. More sequences from more species are essential. However, we can observe that species of *Prosorhynchoides* from belonid fishes in Australian waters form a well-supported clade. It is therefore at least promising that some bucephalids have radiated in association with definitive host taxa. The extent to which this pattern is widespread remains to be seen. Whether such groups as the *Prosorhynchoides* species of belonids will ultimately require distinct genera remains to be

considered and would require identification of a morphological basis as well as phylogenetic distinction.

Despite the variability of ITS-2, the topology of its tree has many similarities to that of the 28S tree. Both contain a clade for *Prosorhynchoides* from belonids, with species such as *P. ovatus* and *P. paralichthydis* being sister taxa to the clade. There are also clades containing *P. caecorum* and *P. megacirrus*, and *Rhipidocotyle campanula* (Dujardin, 1845) and *Bucephalus polymorphus* von Baer, 1827. The topologies are more similar in this study than those from Hammond, et al. [1], with the species of *Prosorhynchoides* from belonids forming a clade to the exclusion of all other Bucephaline sequences, instead of being basal to the other sequences. It is interesting that, for both ITS-2 trees, *P. paralichthydis*, *P. ovatus* and *R. transversalis* formed a clade with node support of one, despite being from different genera and different host families. The ITS-2 tree generated in this study suggests that this marker has utility for phylogenetic studies in this family.

4.7 Bucephalid diversity

When assessed relative to the findings of three related species of *Prosorhynchoides* in *Tylosurus gavialoides* [1], the identification of two further species here allows some general inferences for the bucephalid fauna of belonids as a whole. First, the present study suggests that these parasite species are capable of being widespread with their hosts, from at least the Great Barrier Reef to French Polynesia (although we acknowledge that they have both been sequenced from only one locality). Second, although sampling of *T. gavialoides* and *T. crocodilus* was at non-overlapping localities, we infer that these species show strong signs of strict (oioxenous) specificity.

Given that both new species of *Prosorhynchoides* were found from all three locations at which we have examined *T. crocodilus*, we predict that these two species are the most abundant bucephalids in this species, at least within Australian waters. *Prosorhynchoides fijiensis* and *P. southwelli* have also been described from *T. crocodilus*, from Fiji and either the Red Sea or the Gulf of Aden, respectively [5, 6]. We are aware of the range over which the host species can be found, but there have been no studies on the range for individuals [3]. If individuals of *T. crocodilus* are able to move vast distances, such as from Fiji to GBR, then it is possible that *P. fijiensis* will also be found in Australian waters, and taking a snapshot collection of a host/locality combination is unlikely to give us an accurate representation of the true parasite assemblage for that host/location. Further research into individual host ranges and the intermediate hosts for the bucephalids that infect them is required.

There have been 44 species of bucephalids described from Australian waters, with 23 species reported from the GBR [1, 25-37]. These have been reported from fish hosts from the families Apogonidae, Belonidae, Bleniidae, Carangidae, Labridae, Muraenidae, Scombridae, and Serranidae. To date, none of the previously reported species has been found in more than one family of hosts. This level of host specificity indicates that species found within a newly surveyed family of hosts are likely to have not been previously described from GBR. However, it is difficult to confirm this without surveying enough hosts from every species of potential hosts, which was estimated to equate to the examination of approximately 60,000 individual fishes [26].

Another group of fishes found in Australian waters that are exceptional hosts for bucephalids is the Serranidae. The species richness of *Tylosurus*, with five species of *Prosorhynchoides* reported from two host species, appears to be comparable to that of *Plectropomus* (Serranidae), which have had ten species of bucephalids (two genera) reported from four host species [38]. Four other species of epinepheline serranids have a further six bucephalid species [39]. At least 40 species of bucephalids reported from serranids globally [38-40]. Further exploration of piscivorous fish, from these groups and others, may uncover further bucephalid species richness.

In combination, the levels of richness (two or three species per host) and host-specificity of species of *Prosorhynchoides* in belonids reported from Australia so far, might suggest that a great deal of richness remains to be detected in the belonids yet to be examined (nine species). According to our records, there are reports of 21 species bucephalids from 14 of the 34 belonid species known globally [3-6, 19, 21, 41-48], although the bucephalid *Neidhartia neidharti* Nagaty, 1937 was reported from a species of *Belone*, but the host was not identified to species level [41]. These reported species of bucephalids include species from four other genera (*Neidhartia* Nagaty, 1937, *Pseuobucephalopsis* Long & Lee, 1964, *Rhipidocotyle*, and *Skrjabiniella* Issaitschikow, 1928). None of these genera has been detected in either *T. crocodilus* or *T. gavialoides*. The pattern of exploitation of belonids is intricate, relating to more genera than just *Prosorhynchoides*, and will require substantial further sampling and (especially) molecular characterisation for it to be satisfactorily understood.

Funding

MDH was provided with an Australian Society for Parasitology Travel grant to complete aspects of this study at the University of Queensland and is supported financially by an RMIT University administered Australian Postgraduate Award. RMIT University provided funding that facilitated the participation of MDH and NJB in this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

All applicable institutional, national and international guidelines for the care and use of animals were followed.

Acknowledgements

The authors thank Dr Rodney Bray, Dr Scott Cutmore, Dr Andrea Waeschenbach, Russell Yong, Daniel Hutson, and Storm Martin for their assistance with field collections and advice. The authors thank Prof Ian Beveridge, Assoc Prof Abdul Jabbar, and Dr Lea Indjein for their assistance with

illustrations and advice. The authors also thank Dr Andrea Waeschenbach for her assistance with mtDNA primer design.

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Tables and figures

Table 1. Measurements of *P. galaktionovi* n. sp. and *P. kohnae* n. sp. Mean measurement with the range in parentheses (all measurements in µm).

	P. galaktionovi n. sp. (n = 9)	*P.kohnae* n. sp. (n = 6)
Length	712 (526–874)	686 (647–716)
Width	256 (200–318)	276 (252–312)
Length/Width	2.8 (2.53–3.12)	2.5 (2.12–2.63)
Rhynchus length	119 (89–166)	135 (120–155)
Rhynchus width	140 (122–176)	149 (140–168)
Rhynchus length/Length	0.17 (0.13–0.2)	0.2 (0.19–0.22)
Pharynx length	58 (44–66)	59 (55–61)
Pharynx width	71 (62–81)	71 (66–76)
Oesophagus length	127 (89–160)	125 (108–138)
Caecum length	109 (80–161)	107 (82–126)
Caecum width	83 (53–114)	85 (64–124)
Anterior testis length	93 (73–140)	97 (57–113)
Anterior testis width	81 (54–122)	94 (85–108)
Posterior testis length	84 (59–140)	91 (81–109)
Posterior testis width	91 (61–134)	94 (81–106)
Cirrus-sac length	262 (199–353)	260 (231–287)
Cirrus-sac width	65 (48–79)	71 (60–89)
Seminal vesicle length	50 (28–105)	52 (27–67)
Seminal vesicle width	37 (25–48)	44 (30–62)
Pars prostatica length	227 (165–274)	214 (201–225)
Pars prostatica width	27 (20–39)	30 (18–40)
Genital atrium length	78 (57–100)	98 (72–124)
Genital atrium width	67 (47–94)	70 (64–81)
Genital lobe length	65 (44–84)	78 (51–103)
Genital lobe width	56 (40–82)	60 (54–65)
Ovary length	90 (60–162)	85 (74–110)
Ovary width	72 (54–122)	82 (73–86)
Dextral vitelline field	104 x 62 (78–172 x 44–85)	87 x 78 (78–103 x 60–92)
Sinistral vitelline field	131 x 71 (104–224 x 51–89)	86 x 100 (76–96 x 80–123)
Uterus	444 (308–591)	400 (356–455)
Uterus/Length	0.62 (0.54–0.68)	0.58 (0.52–0.64)
Eggs	15.8 x 9.9 (12.6–18.2 x 7.4–12.8)	17.3 x 10.1 (15–21.6 x 7.6–13)
Distance to pharynx	405 (334–510)	401 (376–442)
Distance to caecum	257 (215–347)	249 (222–314)
Distance to anterior testis	302 (236–379)	289 (257–300)
Distance to cirrus-sac	337 (222–449)	316 (241–377)
Distance to ovary	234 (186–305)	234 (201–264)
Distance to closest vitelline field	130 (106–155)	124 (101–144)
Distance to uterus	147 (130–166)	161 (124–213)

Table 2. Sequences used for phylogenetic analyses. References in parentheses after accession numbers.

Taxon	28S	ITS-2	*Cox*1
Bucephalus cynoscion	KT273397.1 [25]	-	-
Bucephalus gorgon	KT273400.1 [25]	-	-
Bucephalus margaritae	KT273395.1 [25]	-	-
Bucephalus minimus	-	-	KF880428.1, KF880479.1 [49]
Bucephalus polymorphus	AY289238.1 [50]	AY289239.1 [50]	-
Dollfustrema hefeiensis (Outgroup)	KT273386.1 [25]	KT273386.1 [25]	-
Parabucephalopsis parasiluri	AB640884.1 [51]	-	-
Paurorhynchus hiodontis	KT273401.1 [25]	-	-
Prosorhynchoides apogonis	KT213576.1 [25]	-	-
Prosorhynchoides caecorum	KT273393.1 [25]	KT273392.1 [25]	-
Prosorhynchoides cutmorei	MG953232.1 [1]	MG953235.1 [1]	MG953242.1, MG953243.1, MG953244.1 [1]
Prosorhynchoides gracilescens	AY222224.1 [52]	-	-
Prosorhynchoides longoviferus	KT273387.1 [25]	-	-
Prosorhynchoides megacirrus	KT273391.1 [25]	KT273391.1 [25]	-
Prosorhynchoides moretonensis	MG953230.1 [1]	MG953233.1 [1]	MG953236.1 [1]
Prosorhynchoides ovatus	KT273399.1 [25]	KT273399.1 [25]	-
Prosorhynchoides ozakii	AB640885.1 [51]	-	-
Prosorhynchoides paralichthydis	KT273398.1 [25]	KT273398.1 [25]	-
Prosorhynchoides scomberomorus	KT273389.1 [25]	-	-
Prosorhynchoides waeschenbachae	MG953231.1 [1]	MG953234.1 [1]	MG953237.1, MG953238.1, MG953239.1, MG953240.1, MG953241.1 [1]
Rhipidocotyle angusticolle	KT273383.1 [25]	-	-
Rhipidocotyle campanula	KF184355.1 [53]	KF184360.1 [53]	-
Rhipidocotyle fennica	KF184361.1 [53]	-	-
Rhipidocotyle galeata	AY222225.1 [52]	-	-
Rhipidocotyle lepisostei	KT273390.1 [25]	-	-
Rhipidocotyle sp.	-	-	KM538111.1 [54]
Rhipidocotyle transversalis	KT273394.1 [25]	KT273394.1 [25]	-
Rhipidocotyle tridecapapillata	KT273384.1 [25]	-	-
Telorhynchus arripidis (Outgroup)	-	-	AY504854.1 [55]

Figure 1. *Prosorhynchoides galaktionovi* n. sp., ventral view (terminal genitalia could not be discerned). Scale bar 200 µm.

Figure 2. *Prosorhynchoides kohnae* n. sp., ventral view (terminal genitalia could not be discerned). Scale bar 200 µm.

Figure 3. Bayesian inference analysis of bucephaline 28S rDNA. Host family in parentheses, *denotes bivalve host. Functional outgroup is *Dollfustrema hefeiensis*.

Figure 4. Bayesian inference analysis of bucephaline ITS-2 rDNA. Host family in parentheses, *denotes bivalve host. Functional outgroup is *Dollfustrema hefeiensis*.

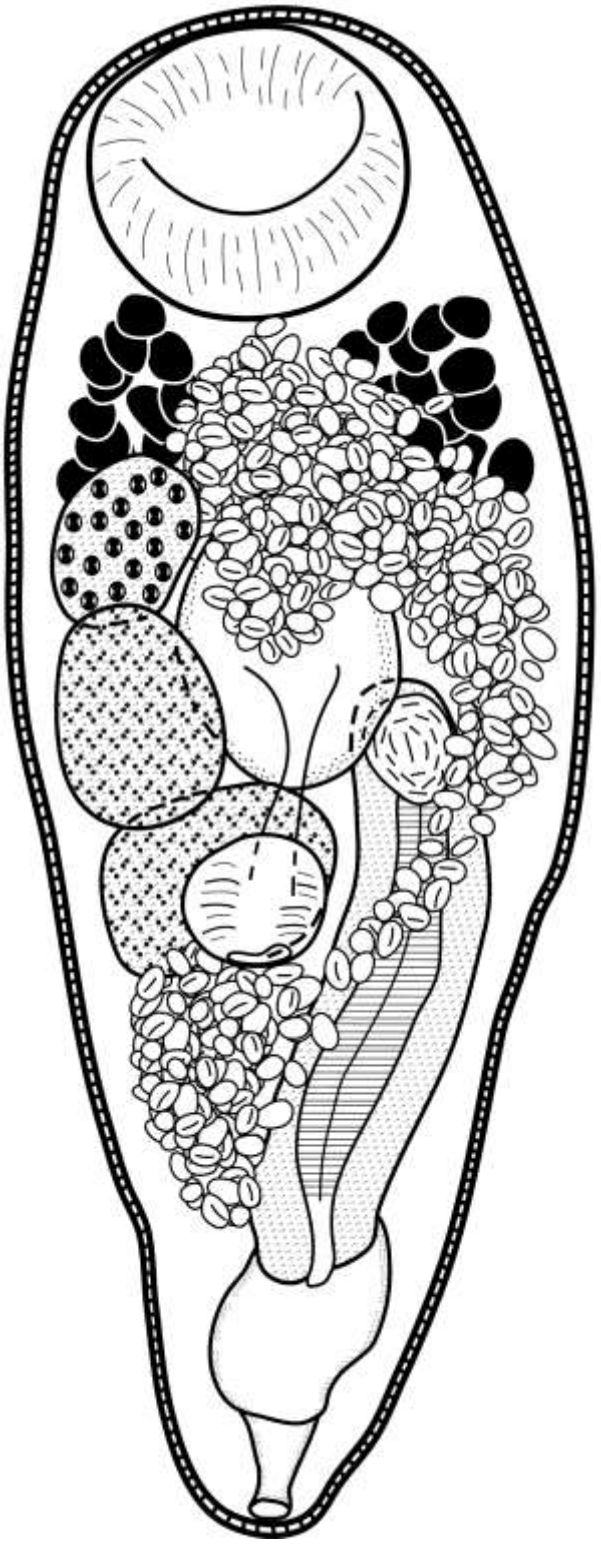

Figure **1**

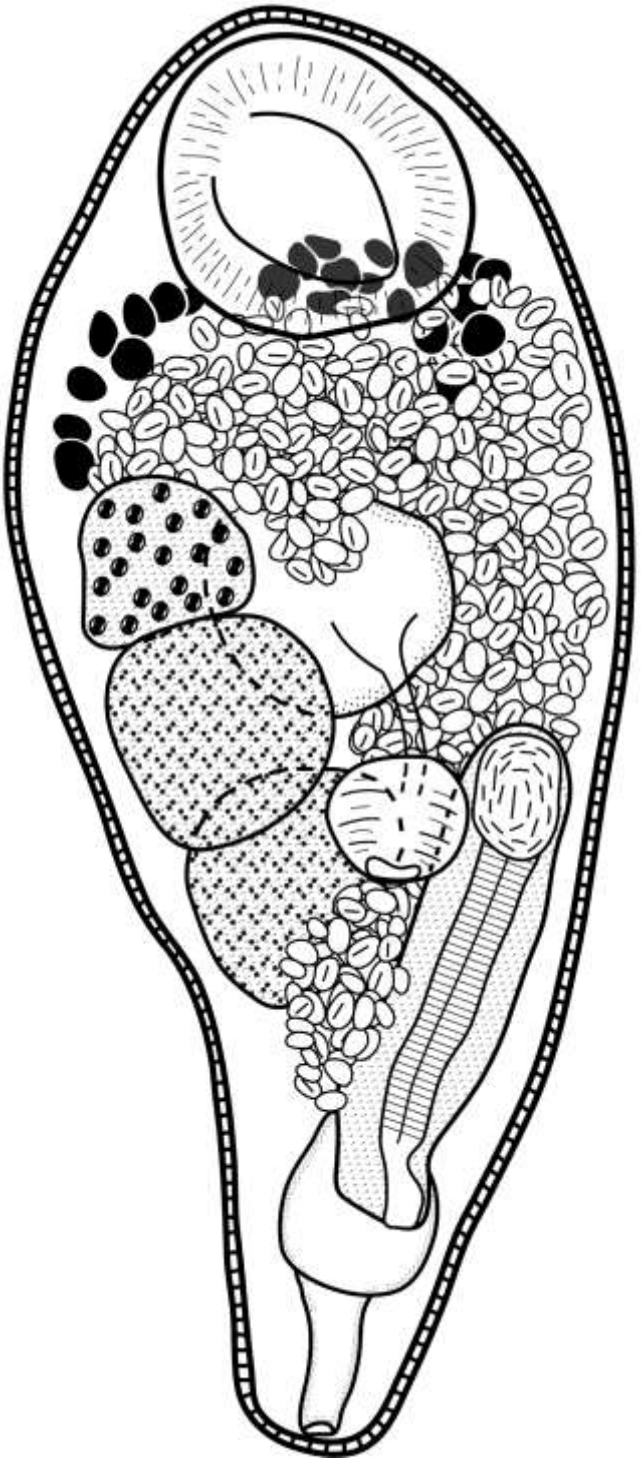

Figure 2

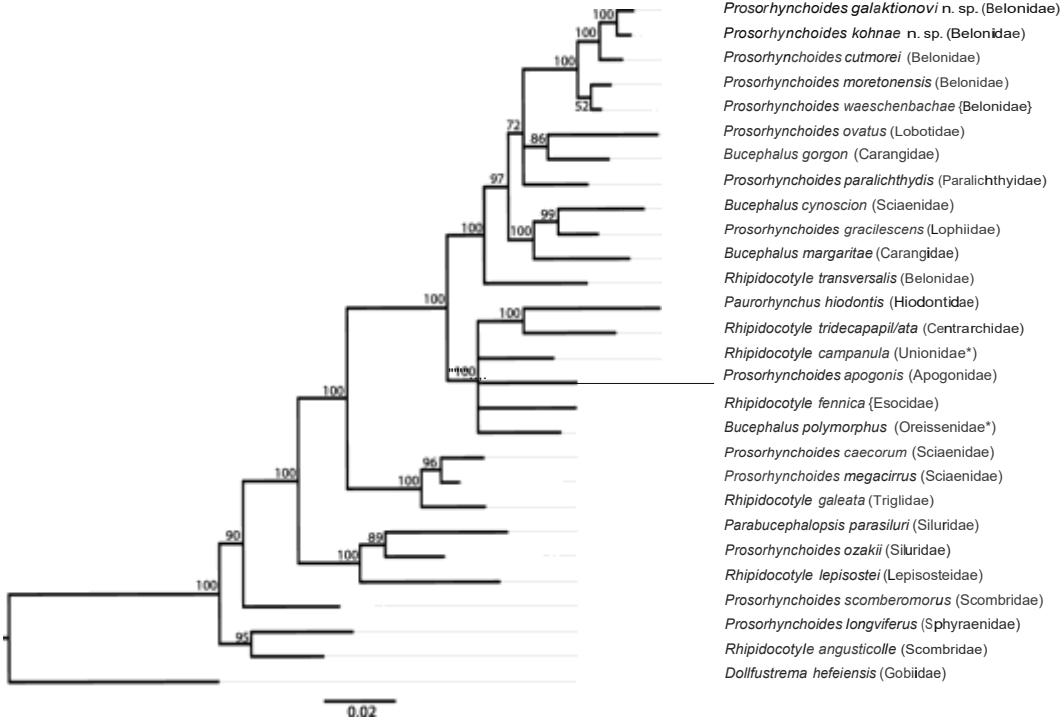
100
100
100
52
72
86
97
99
100
100
100
100
100
100
100
96
100
90
89
100
100
95
0.02
Prosorhynchoides galaktionovi n. sp. (Belonidae)
Prosorhynchoides kohnae n. sp. (Belonidae)
Prosorhynchoides cutmorei (Belonidae)
Prosorhynchoides moretonensis (Belonidae)
Prosorhynchoides waeschenbachae (Belonidae)
Prosorhynchoides ovatus (Lobotidae)
Bucephalus gorgon (Carangidae)
Prosorhynchoides paralichthydis (Paralichthyidae)
Bucephalus cynoscion (Sciaenidae)
Prosorhynchoides gracilescens (Lophiidae)
Bucephalus margaritae (Carangidae)
Rhipidocotyle transversalis (Belonidae)
Paurorhynchus hiodontis (Hiodontidae)
Rhipidocotyle tridecapapil/ata (Centrarchidae)
Rhipidocotyle campanula (Unionidae*)
Prosorhynchoides apogonis (Apogonidae)
Rhipidocotyle fennica (Esocidae)
Bucephalus polymorphus (Oreissenidae*)
Prosorhynchoides caecorum (Sciaenidae)
Prosorhynchoides megacirrus (Sciaenidae)
Rhipidocotyle galeata (Triglidae)
Parabucephalopsis parasiluri (Siluridae)
Prosorhynchoides ozakii (Siluridae)
Rhipidocotyle lepisostei (Lepisosteidae)
Prosorhynchoides scomberomorus (Scombridae)
Prosorhynchoides longviferus (Sphyraenidae)
Rhipidocotyle angusticolle (Scombridae)
Dollfustrema hefeiensis (Gobiidae)

Figure 3

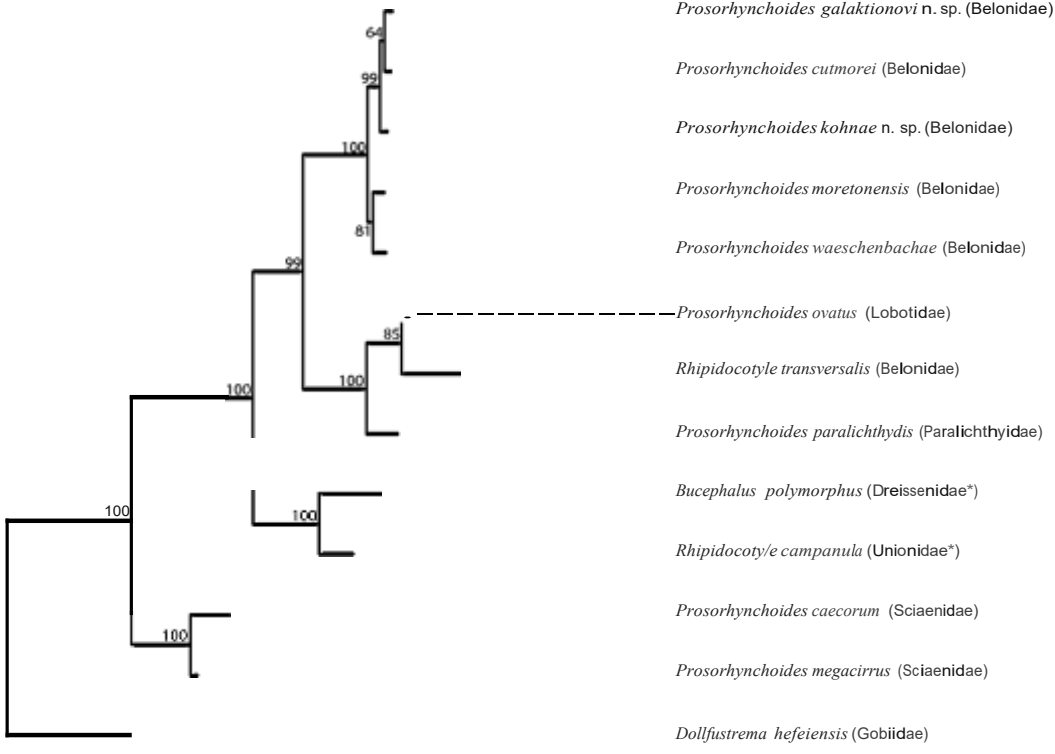
Prosorhynchoides galaktionovi n. sp. (Belonidae)
Prosorhynchoides cutmorei (Belonidae)
Prosorhynchoides kohnae n. sp. (Belonidae)
Prosorhynchoides moretonensis (Belonidae)
Prosorhynchoides waeschenbachae (Belonidae)
Prosorhynchoides ovatus (Lobotidae)
Rhipidocotyle transversalis (Belonidae)
Prosorhynchoides paralichthydis (Paralichthyidae)
Bucephalus polymorphus (Dreissenidae*)
Rhipidocoty/e campanula (Unionidae*)
Prosorhynchoides caecorum (Sciaenidae)
Prosorhynchoides megacirrus (Sciaenidae)
Dollfustrema hefeiensis (Gobiidae)
64
99
100
81
99
85
100
100
100
100
100
0.07

Figure 4