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*Dollfustrema dura* n. sp. and *Heterobucephalopsis perarduum* n. sp. (Digenea: Bucephalidae) from the giant moray eel, *Gymnothorax javanicus* (Bleeker) (Anguilliformes: Muraenidae), and proposal of the Heterobucephalopsinae n. subfam.

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## ABSTRACT

Two new species of bucephalid trematode (Platyhelminthes: Digenea) are described from the giant moray eel, *Gymnothorax javanicus* (Anguilliformes: Muraenidae), from off Lizard Island, Australia. We used a combined morphological and molecular-based approach targeting the internal transcribed spacer 2 (ITS2) of the ribosomal DNA (rDNA) and the D1–D3 region of the large subunit (28S) of rDNA to circumscribe the species. *Dollfustrema dura* n. sp. is distinguished from seven congeners in having 5–6 rows of enlarged body spines circling the anterior portion of the rhynchus. From the remaining 10 species, *D. dura* n. sp. differs in body length, and in having a caecum that terminates posteriorly to the confluent arc formed by the vitelline follicles, gonads predominantly anterior to the pharynx, testes in tandem, an anterior testis positioned posteriorly to the vitelline follicles, and the pre-vitelline field 23–40% of the body length. *Heterobucephalopsis perarduum* n. sp. differs from *Heterobucephalopsis gymnothoracis*, the type- and only other reported species, in being two to three times smaller. *Heterobucephalopsis*, currently considered a *genus inquirendum*, is confirmed as valid and is rediagnosed. Bayesian inference analysis of 28S rDNA sequences representing 28 species from nine genera and four subfamilies of bucephalid, indicates that i) subfamily classifications previously based on morphological characters are broadly robust, ii) the sequence representing *H. perarduum* n. sp. is resolved as distinct, and basal, to sequences representing the Bucephalinae, the Prosorhynchinae, the Paurorhynchinae, and the Dolichoenterinae, iii) the Dolichoenterinae and the Prosorhynchinae are monophyletic sister clades, basal to the Bucephalinae and the Paurorhynchinae, iv) sequences representing *Grammatorcynicola*, *Prosorhynchus*, and *Dollfustrema* are also monophyletic, v) the Bucephalinae is paraphyletic relative to the Paurorhynchinae, and vi) the bucephaline genera *Prosorhynchoides*, *Rhipidocotyle*, and *Bucephalus* are each polyphyletic. The morphological and molecular differences observed among the four previously recognised subfamilies in this study lead us to propose Heterobucephalopsinae n. subfam. to accommodate the genus *Heterobucephalopsis*.

## Keywords

Platyhelminthes

Trematoda

Internal transcribed spacer 2 (ITS2) of the ribosomal DNA (rDNA)

Large subunit of rDNA (28S)

## 1. Introduction

The Bucephalidae Poche, 1907 (Platyhelminthes: Digenea) is a large cosmopolitan family currently comprising 380 nominal species, 25 genera, and five subfamilies [1-3]. Flatworms within this group are characterised by an indirect life cycle with sporocysts and cercariae developing in lamellibranch bivalves (molluscan intermediate host), metacercariae encysting within the tissues of fishes (second intermediate host), and sexual adult stages inhabiting the digestive tract, and rarely other sites, in piscivorous teleosts (definitive host) [1]. From Great Barrier Reef (GBR) fishes, there are 23 recorded species, representing five genera and three subfamilies, most of which have been reported since 2003 [4-11]. These parasites are reported from 19 GBR fish species from six families (the Apogonidae, Blenniidae, Carangidae, Labridae, Serranidae, and Scombridae) with most (12 species) infecting species of the Serranidae (the rockcods, coral cods, coral trouts, and groupers or groupers) [4, 10, 11]. In contrast, many other large piscivorous families that are known hosts to bucephalids globally, such as the Belontiidae (needlefishes) and the Sphyraenidae (barracudas), are recorded hosts to few or no species on the GBR, which probably reflects the inadequacy of their study.

The Muraenidae (Pisces: Anguilliformes) is an understudied GBR host group. The concealed habitats and frequently aggressive nature (when disturbed) of moray eels has most likely resulted in their parasite fauna being disproportionately poorly studied [12]. The group is associated primarily with coral formations and/or rocky outcrops in tropical and temperate seas [13]. Currently, the family is reported to harbour 17 species of bucephalid from seven genera globally [14]. Despite this species richness and generic diversity, bucephalids are almost entirely unknown from this host group in Australian waters and are limited to *Dollfustrema gibsoni* Nolan & Cribb, 2010 and *Muraenicola botti* Nolan & Cribb, 2010 from *Gymnothorax woodwardi* McCulloch, from off Western Australia [12]. Here, we capitalise on a continuing study of trematode diversity in Australian fishes and focus our attention on a species of muraenid from off Lizard Island, Queensland, Australia. As a result, we report two new species from the intestine of the giant moray eel, *Gymnothorax javanicus* (Bleeker) (Anguilliformes: Muraenidae: Muraeninae). We rediagnose *Heterobucephalopsis* Gu & Shen, 1983 (currently considered a *genus inquirendum*; see [1]) to which one new species is assigned and propose the Heterobucephalopsinae n. subfam., to accommodate it, based on morphological and molecular data. Finally, we examine relationships among species/genera/subfamilies within the Bucephalidae, and undertake the most in-depth phylogenetic analysis of the family to date, as an introduction to future taxonomic, geographic, and evolutionary studies of bucephalids.

## 2. Materials and Methods

### 2.1. Sample collection

Three *G. javanicus* were collected from off Lizard Island (14.6689°S, 145.4594°E) on the northern Great Barrier Reef, Queensland, Australia, by spearfishing, and were euthanised *via* neural pithing. Immediately upon death the viscera were excised and processed as described by Nolan et al. [15].

## 2.2. Morphological examination of parasites

Fixed worms were washed, stained, and mounted as described by Nolan et al. [15]. Drawings were completed using a drawing tube attached to a compound optical microscope. All measurements, in micrometres, were made using a digital camera and the software SPOT Advanced (version 4.6) (<http://www.spotimaging.com/software/spot-advanced/index.php>). Measurements are provided as length × width × depth, unless otherwise stated. Measurement of morphological characters from the anterior or posterior end of worms reflects the distance from the extremities of each feature. Type-specimens and holotype specimens have been deposited in the Queensland Museum, Australia (QM), and the Smithsonian National Museum of Natural History (USNM) and the Harold W. Manter Museum (HWML), USA.

## 2.3. Isolation of genomic DNA, PCR, and phylogenetic analysis

Genomic DNA (gDNA) was isolated from single specimens employing a standard proteinase K and phenol-chloroform extraction procedure [16]. For the specific differentiation of bucephalid species, and for future life cycle matching studies, the complete internal transcribed spacer (ITS) 2 of the ribosomal DNA (rDNA) was amplified. To explore the phylogenetic relationships among bucephalids from different genera and subfamilies we targeted a portion of the large subunit (LSU) of rDNA. PCR amplification of ITS2 from bucephalids of Australian fishes was achieved using the primers 3s (forward: 5'-GGTACCGGTGGATCACGTGGCTAGTG-3') and ITS2.2 (reverse: 5'-CCTGGTTAGTTTCTTTTCCTCCGC-3'). The D1–D3 regions of LSU was amplified utilising the primers LSU5 (forward: 5'-TAGGTCGACCCGCTGAAYTTAAGCA-3') and 1500R (reverse: 5'-GCTATCCTGAGGGAACTTCG-3'). PCR was carried out in a 20 µl volume as described by Cutmore et al. [17]. Sequences generated in the United States of America and used in the present study were amplified using the primers ITSF (forward: 5'-CGCCCGTCGCTACTACCGATTG-3') and 1500R, followed by nested amplification using the internal forward primers digl2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'), and 900F (5'-CCGTCTTGAAACACGGACCAAG-3') and the internal reverse primers digl2R (5'-CCGCTTAGTGATATGCTT-3'), 300R (5'-CACTTTCCCTCACGGTACTTG-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3'). All PCR reactions were performed following the procedure described by Tkach et al. [18]. All resultant PCR amplicons were purified and sequenced as described by Nolan et al. [15].

Prior to phylogenetic analysis, the sequences determined herein representing the new species (corresponding to the GenBank accession nos. XXXXX [ITS2] and XXXXX [LSU]) were aligned with 25 and 10 reference sequences (respectively) representing selected bucephalid species/genera/subfamilies, presently available in GenBank (<http://www.ncbi.nlm.nih.gov/>). In addition, we incorporated a further 23 sequences from 20 species in seven genera, generated in the present study (see Table 1). Sequences were aligned using the software MUSCLE version 3.7 [19, 20] with ClustalW sequence weighting and UPGMA

clustering for iterations 1 and 2. The resultant alignment was adjusted manually using the software BioEdit [21]. Total nucleotide distance matrices, corresponding to ITS2 rDNA sequence data, were calculated using the pairwise deletion of gaps/missing data option in the software package MEGA v.5 [22].

Phylogenetic analysis of sequence data from the D1–D3 region of LSU, representing 28 bucephalid species, was conducted by Bayesian inference (BI) utilising Monte Carlo Markov Chain (MCMC) analysis in MrBayes 3.2.5 [23, 24]. The likelihood parameters set for BI analysis were based on the Akaike Information Criteria (AIC) test in jModelTest2 [25]. For these data, we employed the general time-reversible model of evolution with gamma distribution and a proportion of invariable sites (GTR +  $\Gamma$  + I). Posterior probabilities (pp) were calculated *via* 10,000,000 generations, utilising four simultaneous tree-building chains, with every 100<sup>th</sup> tree being saved. At this point, the standard deviation of split frequencies was <0.01, and the potential scale reduction factor (PSRF) approached one. A 50% majority-rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI. The outgroup taxa used in this analysis were *Olssonium turneri* Bray & Gibson, 1980 (Fellodistomidae) (GenBank accession no. AY222283; [26]) and *Pleorchis uku* Yamaguti, 1970 (Acanthocolpidae) (DQ248216; [27]).

### 3. Results

#### 3.1. Morphology

Class Trematoda Rudolphi, 1808

Subclass Digenea Carus, 1863

Order Plagiorchiida La Rue, 1957

Suborder Bucephalata La Rue, 1926

Superfamily Bucephaloidea Poche, 1907

Family Bucephalidae Poche, 1907

Subfamily Prosorhynchinae Nicoll, 1914

Genus *Dollfustrema* Eckmann, 1934

#### 3.2. *Dollfustrema dura* n. sp.

Description (Figs. 1–4): (based on 15 whole mature worms). With features of genus and subfamily. Body linear, 1885–2399 (2195)  $\times$  267–520 (410)  $\times$  325–332 (328), 4.4–7.1 times longer than wide (see Figs. 1, 2). Spines covering entire body surface. Rhynchus truncate, with 5–6 rows of enlarged body spines circling the anterior portion (see Fig. 3), 195–310 (243)  $\times$  241–342 (297)  $\times$  228–272 (250) (see Fig. 1); gland-cells not seen. Mouth opening on ventral surface, medio-sinistral, in posterior half of body, 1320–1690 (1519) from anterior extremity. Pharynx ovoid, 134–216 (149)  $\times$  128–179 (151)  $\times$  118–128 (123),

60–69% of body length from anterior extremity. Oesophagus extending anteriorly, surrounded by gland-cells, 26–45 (37) long. Caecum thick-walled, linear, extending to 520–752 (645) from anterior extremity, 563–816 (666)  $\times$  80–186 (124)  $\times$  118–144 (131), surrounded by gland cells proximally, extending obliquely, left to right (see Fig. 2).

Testes two, ovoid, in tandem; anterior testis anterodextral to pharynx and oesophagus, ventral to caecum, 728–1066 (896) from anterior extremity, 157–224 (188)  $\times$  90–144 (116)  $\times$  128–141 (134); posterior testis anterodextral to mouth, posterior margin overlaps anterior margin of pharynx, lateral to oesophagus, dorsal to pharynx, oesophagus, oesophageal gland cells, and caecum, 559–780 (668) from posterior end, 138–202 (175)  $\times$  112–179 (140)  $\times$  96–138 (117) (see Fig. 1). Cirrus-sac linear, sinistral, 1313–1667 (1525) from anterior extremity, 352–448 (396) or 16–21% of body total length  $\times$  109–163 (134)  $\times$  112–128 (120); 2.8–3.6 times longer than wide. Seminal vesicle ovoid, occupying anterior half of cirrus-sac, dextral, 256–336 (298)  $\times$  54–99 (77)  $\times$  51–80 (66); distal portion extending dorsally to connect with male duct; male duct (proximal) extending antero-obliquely dorsal to seminal vesicle (walls of duct difficult to observe as obscured by seminal vesicle), then posterosinistrally along wall of cirrus-sac, at posterior margin of seminal vesicle extends obliquely across cirrus-sac to dextral margin, mid and distal portions lined by layer of anuclear structures; ejaculatory duct opens into genital atrium at base of genital lobe (see Fig. 4). Genital atrium wide anteriorly, narrows posteriorly, surrounded by gland-cells, 96–160 (118)  $\times$  102–144 (121)  $\times$  112–122 (117). Genital pore opens on ventral surface, sinistral, subterminal.

Ovary ovoid, dextral, inter-testicular, abutting posterior testis, dorsal to anterior testis, anterior to pharynx and oesophagus, dorsal to caecum, 995–1170 (1113) from anterior extremity, 715–962 (832) from posterior extremity, 128–176 (147)  $\times$  102–154 (119)  $\times$  86–112 (99). Oviduct, Laurer's canal, oviducal seminal receptacle, vitelline ducts, oötype, and Mehlis' gland not seen. Uterus winds throughout body, extends into anterior third of body (but not past gland-cells of rhynchus), 241–554 (311) from anterior extremity, posteriorly extends to level of genital pore, 46–304 (140) from posterior extremity, dorsal to pharynx, oesophageal gland-cells, and anterior testis, ventral to caecum, posterior testis, and ovary, dorsal and ventral to cirrus-sac; distal portion not obviously differentiated as metraterm, opens into genital atrium (see Fig. 1). Uterine seminal receptacle not seen. Eggs numerous, 26–32 (30) long  $\times$  16–21 (19) wide. Vitelline follicles in confluent arc encompassing intestinal caecum (which does not extend anteriorly beyond level of follicles), 501–906 (646) or 23–40% of body total length from anterior extremity, ventral and dorsal to caecum and ovary, dorsal to pharynx, oesophageal gland cells, anterior testis, and uterus, mostly anterior to gonads; dextral field extends posteriorly to level of ovary, 722–1001 (838) from posterior extremity; sinistral field extends posteriorly to pharynx. Vitelline reservoir not seen.

Excretory vesicle extends anteriorly, dorsal to genital atrium; anterior limit not seen. Excretory pore terminal.

### *3.3. Taxonomic summary*

*Type-host:* *Gymnothorax javanicus* (Bleeker), the giant moray eel (Anguilliformes: Muraenidae: Muraeninae).

*Type-locality:* Off Lizard Island (14.6689°S, 145.4594°E), northern Great Barrier Reef, Queensland, Australia.

*Site:* Intestine.

*Prevalence:* Three of three (100%) *G. javanicus* infected.

*Type-material:* Holotype (QM G234961) and 14 paratypes (QM G234962–QM G234975).

*Molecular sequence data:* ITS2 (complete) and LSU (partial), two identical replicates for each locus.

*GenBank accession numbers:* KT213578 and KT213572 (respectively).

*Etymology:* The epithet *dura* is derived from the Latin *durus*, meaning hard, harsh; tough, strong, enduring, which refers to the physical nature of the host. It stands as a noun in apposition.

### 3.4. *Heterobucephalopsis* Gu & Shen, 1983 emended

Class Trematoda Rudolphi, 1808

Subclass Digenea Carus, 1863

Order Plagiorchiida La Rue, 1957

Suborder Bucephalata La Rue, 1926

Superfamily Bucephaloidea Poche, 1907

Family Bucephalidae Poche, 1907

**Diagnosis:** Modified after Gu & Shen (1983) and Overstreet & Curran (2002). Body elongate, elliptic, spinose. Rhynchus a simple sucker, without central muscular palp, appendages, or spines. Pharynx in anterior half of body, medial. Oesophagus directed posteriorly. Caecum elongate, straight, tubular, directed posteriorly from pharynx. Testes 2, ovoid to spherical, oblique, in posterior half of body; anterior testis lateral to posterior portion of caecum, sinistral; posterior testis posterior to caecum, dextral. Cirrus-sac medial, abutting or adjacent to posterior testis. Seminal vesicle in anterior half of cirrus-sac: proximal portion ovoid, distal portion convoluted, directed posteriorly from proximal portion before reflexing anterosinistrally, then posterodextrally dorsally. Seminal duct convoluted. Pars prostatica short and straight, lined by layer of anuclear structures. Ovary spherical or ovoid, between testes, sinistral, adjacent or entirely posterior to intestinal caecum and anterior testis. Oviduct extending posteriorly from ovary. Laurer's canal convoluted, opening sinistro-submarginally. Oötype posterior to ovary. Mehlis' gland posterior to ovary. Uterus mostly pre-ovarian, extends anteriorly from oötype, filling midbody, extending anteriorly to pharynx and posteriorly past posterior testis. Eggs spherical to ovoid. Vitelline follicles in two lateral rows in middle of body, adjacent to caecum and genital organs. Vitelline ducts pass laterally to genital organs, join dorsal to ovary. Excretory system V-shaped with short excretory vesicle. Excretory pore sub-terminal. Parasites of intestine of marine fishes. Type-species *Heterobucephalopsis gymnothoracis* Gu & Shen, 1983.



### 3.5. *Heterobucephalopsis perarduum* n. sp.

Description (Figs. 5–9): (based on 15 whole mature worms). With features of genus. Body linear, 2392–2893 (2686)  $\times$  410–546 (475)  $\times$  436–579 (485), 4.8–6.7 times longer than wide. Spines small, covering entire surface. Rhynchus spherical to ovoid, 371–455 (397)  $\times$  325–403 (367)  $\times$  325–377 (349) (see Figs. 5, 6). Gland-cells extend posteriorly from rhynchus into parenchyma. Mouth opening on ventral surface, medial, in anterior half of body, 1073–1300 (1186) from anterior extremity. Pharynx spherical or ovoid, 150–195 (170)  $\times$  163–189 (178)  $\times$  143–176 (159); 42–48% of total body length from anterior extremity. Oesophagus surrounded by gland-cells, 163–176 (169) long. Caecum elliptical, medial, 1170–1443 (1300) from anterior extremity, 403–637 (497)  $\times$  46–234 (140)  $\times$  150–215 (182) (see Figs. 5, 7).

Testes two; anterior testis dorsal to caecum, 1339–1729 (1537) from anterior extremity, 221–325 (255)  $\times$  150–215 (193)  $\times$  218–241 (229); posterior testis 507–865 (671) from posterior extremity, 26–267 (205)  $\times$  150–208 (183)  $\times$  189–247 (218). Cirrus-sac conspicuous, elliptical, abutting posterior testis anteriorly, 436–819 (554) or 16–28% of body total length  $\times$  163–237 (197)  $\times$  208–273 (241); 2.4–3.2 times longer than wide; 1794–2236 (2009) from anterior extremity; cirrus-sac wall 10–32 (17) thick. Seminal vesicle ovoid proximally; in anterior half of cirrus-sac, 154–455 (263)  $\times$  91–215 (141)  $\times$  137–254 (195); 1.3–2.3 times longer than wide. Seminal duct distinct, convoluted. Pars prostatica very short, straight. Ejaculatory duct not evident (see Fig. 8). Genital atrium wide anteriorly and medially, narrows posteriorly, 85–115 (97) long  $\times$  78–91 (85) wide. Genital pore opens on ventral surface, medial, subterminal.

Ovary spherical, 176–254 (202)  $\times$  156–228 (181)  $\times$  195–195 (195), posterior to pharynx, in posterior half of body, 1476–1885 (1691) from anterior extremity, 670–956 (811) from posterior extremity, anterior to cirrus-sac, abutting posterior testis, ventral to anterior testis (see Fig. 5). Oviduct originating at posteromedial margin of ovary, extending posteriorly, straight, joining to sinistral margin of oötype (see Fig. 9). Oötype ovoid, lateral to posterior testis. Laurer's canal extending laterosinistrally from oötype before reflexing posteriorly, then dorsosinistrally toward body margin, posterior to oötype, opening submarginally, ventral to oviduct and oötype, surrounded by gland cells (see Fig. 9). Vitelline ducts in posterior half of body, extend posteriorly lateral to caeca and genitalia, except dorsal to posterior testis where branched; dextral common vitelline duct extending anterosinistrally dorsal to posterior testis to connect with sinistral common vitelline duct dorsal to ovary; united vitelline duct extends posteriorly dorsal to ovary to connect with oötype dorsally. Mehlis' gland predominantly posterior to ovary, ventral to oviduct, oötype, and vitelline duct, dorsal to Laurer's canal. Uterus originating posterior to ovary, convoluted, winds throughout body anteriorly to pharynx into anterior third of body (but not past gland-cells of rhynchus), 553–930 (696) from anterior extremity, posteriorly extends past posterior testis to posterior margin of seminal vesicle, 306–780 (485) from posterior extremity, dorsal to pharynx, oesophageal gland-cells, caecum, testes, ovary, and cirrus-sac; distal portion (posterior to seminal vesicle) distinctly narrowed and not containing eggs, opens into genital atrium posterior to opening of ejaculatory duct (see Fig. 5). Uterine seminal receptacle not seen. Eggs numerous, 13–19 (15) long  $\times$  6–14 (11) wide. Vitelline follicles in lateral fields, anteriorly 637–1300 (1087) or 24–47% of body total length from anterior

extremity, posteriorly 429–858 (626) from posterior extremity, dextral field extending posteriorly to posterior testis; sinistral field extending to Mehlis' gland, ventral to caecum, anterior testis, dorsal to posterior testis, cirrus-sac, and Mehlis' gland, ventral and dorsal to uterus. Vitelline reservoir not seen.

Excretory vesicle extends anteriorly, dorsal to genital atrium; anterior limit not seen. Excretory pore subterminal.

### 3.6. Taxonomic summary

*Type-host*: *Gymnothorax javanicus* (Bleeker), the giant moray eel (Anguilliformes: Muraenidae: Muraeninae).

*Type-locality*: Off Lizard Island (14.6689°S, 145.4594°E), northern Great Barrier Reef, Queensland, Australia.

*Site*: Intestine.

*Prevalence*: Two of three (66%) *G. javanicus* infected.

*Type-material*: Holotype (QM G234976) and 14 paratypes (QM G234977–QM G234990).

*Molecular sequence data*: ITS2 (complete) and LSU (partial), two identical replicates for each locus.

*GenBank accession numbers*: KT213577 and KT213571 (respectively).

*Etymology*: The epithet *perarduum* is derived from the Latin *perarduus*, meaning very difficult, referring to the difficulties that may be encountered when collecting the host. It stands as a noun in apposition.

### 3.7. Molecular data

To augment the morphological circumscription of *D. dura* n. sp. and *H. perarduum* n. sp. we sequenced PCR amplicons representing the complete ITS2 region of rDNA. Two replicate sequences were generated from specimens of each species. These sequences were 342 (sequence represented by GenBank accession number KT213578) and 311 (KT213577) nucleotides long (respectively), displayed no intraspecific variation, and differed from each other by 133 bases. Further comparisons with reference data for bucephalids currently available in GenBank, together with additional sequences generated in the present study (see Table 1) indicated both sequence types were new. The sequence represented by KT213578 for *D. dura* n. sp. was 48 nucleotide different from the most similar available sequence, EF198216 [28], which corresponds to *D. vaneyi* (Tseng, 1930), and KT213577 for *H. perarduum* n. sp. was 75 nucleotides different from the most similar available sequence, JX415295 [11], for *Prosorhynchus conorjonesi* Bott & Cribb, 2009.

The D1–D3 region of the 28S rDNA gene was amplified to explore genetic relationships between *D. dura* n. sp. and *H. perarduum* n. sp., and among bucephalid genera and subfamilies, for which sequence data is available. Two genotypes, represented by the GenBank accession numbers KT213572 (*D. dura* n.

sp.) and KT213571 (*H. perarduum* n. sp.), were sequenced and were 1183 and 1162 nucleotides in length (respectively). These sequences were aligned with 33 reference sequences representing bucephalid species from the Bucephalinae Poche, 1907 (n = 27), the Prosorhynchinae Nicoll, 1914 (n = 3), the Paurorhynchinae Dickerman, 1954 (n = 1), and the Dolichoenterinae Yamaguti, 1958 (n = 2), together with AY222283 [26] (Fellodistomidae) and DQ248216 [27] (Acanthocolpidae) (see Table 1), for outgroup comparisons. All 37 sequences (including outgroups), 25 of which are new, were aligned over 1142 positions (trimmed to match the shortest sequence length). Phylogenetic analysis of this dataset resulted in the bucephalids forming a monophyletic clade, to the exclusion of outgroup taxa. The sequence representing *H. perarduum* n. sp. resolved here as a distinct clade, which was basal to all other bucephalid taxa. The Dolichoenterinae and the Prosorhynchinae resolved as monophyletic sister clades, which were in turn basal to the Bucephalinae/Paurorhynchinae clade. Sequences representing *Grammatorcynicola* Bott & Cribb, 2005 (Dolichoenterinae), and *Prosorhynchus* Odhner, 1905 and *Dollfustrema* (Prosorhynchinae) each resolved as monophyletic, with strong support (pp = 0.76–1.00). The sequence representing *D. dura* n. sp. grouped with a reference sequence, represented by the accession no. KT273386, for *Dollfustrema hefeiensis* Liu, 1999, as expected based on ITS2 sequence comparisons. The Bucephalinae was paraphyletic, based on the inclusion of a single sequence representing *Paurorhynchus hiodontus* Dickermann, 1954 from the Paurorhynchinae; nesting of *P. hiodontus* among bucephalines was well supported (pp = 1.00). Within the Bucephalinae, the genera *Prosorhynchoides* Dollfus, 1929, *Rhipidocotyle* Diesing, 1858, and *Bucephalus* Baer, 1827 were each polyphyletic relative to each other, *Parabucephalopsis* Tang & Tang, 1963 (based on a single sequence representing *Parabucephalopsis parasiluri* Wang, 1985), and *Paurorhynchus*. Components of these genera formed several well-supported clades (pp = 0.80–1.00); definitive host distribution in either marine or freshwater environments appears to parallel these clades. With the exception of sequences representing *Prosorhynchoides ozakii* (Nagaty, 1937) Bott & Cribb, 2005 and *Parabucephalopsis parasiluri* Wang, 1985, which both occur in the silurid *Silurus biwaensis* (Tomoda), and those representing *Prosorhynchoides caecorum* (Hopkins, 1956) Bott & Cribb, 2005 and *Prosorhynchoides megacirrus* (Riggin & Sparks, 1962) Overstreet, Cook & Heard, 2009, from the sciaenid *Sciaenops ocellatus* (Linnaeus), 28S sequences did not group based on the phylogenetic relatedness of the host families (i.e. belonging to the same order) and/or species (i.e. belonging to the same family) (see Fig. 10).

## 4. Discussion

### 4.1. *Dollfustrema*

Of the 17 reported species of *Dollfustrema* [29], *D. dura* n. sp. shows clear affinity to species previously reported from muraenids since it possesses at least four rows of enlarged body spines circling the anterior portion of the rhynchus as outlined by Nolan and Cribb [12]. As a result of this distinction, we only compare the present specimens with species of *Dollfustrema* previously reported from muraenids (n = 10). The other seven species are not considered further, and include *D. fochowense* Tang & Tang, 1963

(senior syn. of *D. sinipercae* Wang, 1985, according to Wang & Wang [30]), *D. sinicum* Gu & Shen, 1976, *D. cociellae* (Gu & Shen, 1976) Wang & Wang, 1998, *D. hippocampi* (Shen, 1982) Wang & Wang, 1998, *D. bagarii* Moravec & Sey, 1989, and *D. vaneyi*. In addition, although Chen et al. [28] use molecular data to differentiate *D. vaneyi* from '*D. hefeiensis* Liu', we have not been able to trace the original description of *D. hefeiensis*. *Dollfustrema dura* n. sp. differs from *D. bipapillosum* Manter & Pritchard, 1961, *D. bengalense* Madhavi, 1974, and *D. stromborhynchum* Manter & Pritchard, 1961 in possessing testes that are in tandem, as opposed to them being positioned obliquely (see [31, 32]). In having vitelline follicles confluent anteriorly, *D. dura* n. sp. differs from *D. macracanthum* Hanson, 1950 and *D. xishaense* Gu & Shen, 1983 in which the vitelline follicles appear as distinctly separate lateral fields (see [33, 34]). *Dollfustrema dura* n. sp. differs from *D. californiae* Montgomery, 1957 in having an anterior testis that does not extend anterior to the vitelline follicles (see [35]) and from *D. gravidum* Manter, 1940 in having a caecum that terminates posteriorly to the confluent arc formed by the vitelline follicles (see [36]). *Dollfustrema gymnothoracis* Nahhas & Cable, 1964 is distinct in possessing gonads that are predominantly posterior to the pharynx (see [37]). *Dollfustrema muraenae* Sogandares-Bernal, 1959 is somewhat similar to the present material; however, the vitelline follicles in *D. muraenae* extend all the way to the rhynchus (see [38]) whereas in *D. dura* n. sp. the pre-vitelline field is 23–40% of the body total length. Finally, in having a body 1885–2399 long, *D. dura* n. sp. is longer than *D. gravidum* (840–1512) [36], *D. muraenae* (1273–1350) [38], *D. bipapillosum* (1253–1561) [31], *D. gymnothoracis* (1030–1660) [37], and *D. gibsoni* (1152–1203) [12], but shorter than *D. stromborhynchum* (2700–2854) [31] and *D. xishaense* (2567–4629) [33].

Of the 10 muraenid-infecting species, *D. dura* n. sp. appears morphologically most similar to *D. gibsoni* from *Gymnothorax woodwardi* from off Western Australia. Both species occur off Australia and are generally similar in the specific arrangement of the genitalia. However, *D. dura* n. sp. differs in possessing i) a body 1885–2399 (2195) long with a length/width ratio of 4.4–7.1, ii) a linear elongate caecum, iii) a pharynx that is posterosinistral to the testes, iv) testes that are in tandem, v) a posterior testis that is anterosinistral to the cirrus-sac, vi) a cirrus-sac that occupies 16–21% of the body total length, and vii) an ovary positioned between the testes. In contrast, *D. gibsoni* possesses i) a body 1152–1203 (1178) long with a length/width ratio of 2.2–2.4, ii) a short, sac-like, caecum, iii) a pharynx positioned between the testes, dextrally, iv) testes that are oblique, v) a posterior testis that is lateral to the anterior portion of the cirrus-sac, vi) a cirrus-sac that occupies 28–30% of the body total length, and vii) an ovary that is posterolateral to the anterior testis [12].

Sequence comparisons among ITS2 data generated in this study (see Table 1), together with data currently available in GenBank from four relatively recent studies (see [11, 28, 39, 40]), are consistent with *D. dura* n. sp. being a distinct species. As stated by Nolan et al. [15], (typically) the major advantage of using ITS2 sequence data for molecular taxonomic investigations of digeneans is that substantial genetic data are already available, which can be employed for comparative analyses on a global scale. This does not apply to species of the Bucephalidae. Prior to this study, genetic data was available for only 21 species in six genera. The major determining factor contributing to the limited availability of sequence data is associated with the fact that most species, including type-species, were described prior to the advent of

molecular technologies and have not been recorded again. Given the numerous difficulties associated with the validity of many species/genera in this large cosmopolitan family (see [1]), the lack of sequence data linked to specimens deposited in museum collections (i.e. hologenophores) represents a major impediment to unravelling bucephalid taxonomy and ascertaining the importance of morphological characters currently employed to define species, genera, and subfamilies.

#### 4.2. *Heterobucephalopsis*

*Heterobucephalopsis gymnothoracis* Gu & Shen, 1983 (type-species) was described from the small intestine of *Gymnothorax undulatus* (Lacepède) (Muraenidae) from off the Xisha Islands, Guangdong Province, China [33]. Gu and Shen [33] stated that the species was similar to *Bucephalopsis* (Nicoll, 1914) (which is currently considered to have subgeneric status; see [1]) in general morphology, but differed in having the ovary between the testes rather than anterior to them. *Heterobucephalopsis* was also differentiated from *Pseudobucephalopsis* Long & Le, 1964 (currently considered a *genus inquirendum*; see [1]) by the position of the pharynx and the uterus, the shape of the caecum, and the distribution of the vitelline follicles. More recently, Overstreet and Curran [1] observed that *H. gymnothoracis* closely resembled *Prosorhynchoides ovatus* (Linton, 1910) (Bucephalinae), based on similarities of the rhynchus and digestive caecum, but differed in having the ovary between oblique testes, and vitelline follicles positioned in lateral fields and posteriorly in the body. Additionally, it was suggested the genus might belong in the Dolichoenterinae but that, until examination of type-material and of the finer points of the terminal genitalia could be conducted, the genus should be considered a *genus inquirendum*.

Following examination of the present material from *Gymnothorax javanicus* from off Lizard Island, we conclude that the overall morphology of the second putative species reported here fits well with the differentially diagnostic characters previously used to define *Heterobucephalopsis*, which included possession of a rhynchus as a simple sucker, a caecum that is elongate and directed posteriorly from the pharynx and reaching to the level of the anterior testis, testes that are oblique, a seminal vesicle that is convoluted, an ovary that is dextral and between the testes, and vitelline follicles in two lateral bands in the middle of the body [1, 33]. Although previously considered a *genus inquirendum*, we consider it clearly valid given these morphological features. *Heterobucephalopsis* presently contains just one other species, *H. gymnothoracis*. *Heterobucephalopsis perarduum* n. sp. differs from this species in possessing a pharynx that is 42–48% of the body total length from the anterior end, a body 2392–2893 (2686) × 410–546 (475), a caecum 403–637 (497) long, an anterior testis 221–325 (255) × 150–215 (193) and the posterior one 26–267 (205) × 150–208 (183), an ovary 176–254 (202) × 156–228 (181), and a cirrus-sac 436–819 (554) × 163–237 (197) (all measurements indicate length × width). In contrast, *H. gymnothoracis* possesses a mouth opening in the anterior third of the body, and is generally (overall) two to three times larger, having a body 4624–6953 × 1598–2193, a digestive caecum 1598–2448 long, an anterior testis 510–595 × 459–765 and the posterior one 442–646 × 425–595, an ovary 340–459 × 391–476, and a cirrus-sac 714–1139 × 340–357 [33].

Our phylogenetic analysis of the D1–D3 region of the 28S rDNA locus (see Fig. 10) suggests that

*Heterobucephalopsis* forms a clade separate to the Dolichoenterinae and the Bucephalinae (to which it is currently assigned), from which it differs morphologically by the combined possession of a distinct and convoluted seminal duct, a very short (relative to the length of the cirrus-sac) and straight pars prostatica, and a cirrus-sac with a wall less than 40 µm thick. It also differs from the Prosorhynchinae by possessing a straight pars prostatica. It differs from members of the Paurorhynchinae in having a seminal duct and a well-developed and sucker-like rhynchus (see Fig. 6), and in inhabiting the intestine of fishes. In contrast, the Paurorhynchinae lack a seminal duct, possess a rhynchus that is a small and weakly developed pad, and has members that occur within the swim bladder and/or body cavity of their host [41]. From the Macrorchirhynchinae Bilqees, Ibrahim, Khan, Ajazuddin & Talat, 2010, for which we do not have molecular data, the new material differs in the combined possession of a spinose body surface, the rhynchus lacking papilla, the pharynx situated anterior to the testes, and the testes in the posterior half of the body. In contrast, the Macrorchirhynchinae possesses a smooth body, a rhynchus with prominent lateral and dorsal papillae, a pharynx and intestine positioned posterior to the testis, and testes that are located anteriorly, close to the rhynchus [3]. Given the morphological and genetic distinctions among sequences representing the four subfamilies analysed here (see Fig. 10), we conclude that *Heterobucephalopsis* requires separate subfamily status. Therefore, we propose and diagnose a new subfamily as follows:

***Heterobucephalopsinae* n. subfam.**

Body elongate, elliptical, spinose. Rhynchus a simple sucker, without dorsal hood, lobe, muscular palp, appendages, retractile tentacles or spines. Pharynx in anterior half of body. Oesophagus directed posteriorly from pharynx. Caecum elongate, tubular, directed posteriorly from pharynx. Testes 2, oblique, spherical to ovoid, in posterior half of body; anterior testis lateral to portion of caecum, sinistral; posterior testis posterior to caecum, dextral. Cirrus-sac medial, 2.0–3.5 times longer/wide. Seminal vesicle ovoid. Seminal duct present, coiled. Pars prostatica usually straight and very short. Ejaculatory duct straight, extending through posteromedial margin of cirrus-sac, opening into genital atrium. Ovary spherical or ovoid, between testes, sinistral. Oviduct extending posteriorly from ovary. Laurer's canal convoluted, opening sinistrally and submarginal. Uterus mostly pre-ovarian, extends anteriorly from oötype, filling midbody, extending anterior to pharynx and posteriorly past posterior testis. Vitelline follicles in two lateral rows in middle of body, adjacent to caecum and genital organs. Excretory system V-shaped with short excretory vesicle. Excretory pore sub-terminal. Parasites of the intestine of muraenid fishes. Type- and only genus *Heterobucephalopsis* Gu & Shen, 1983.

**4.3. Phylogenetic relationships among the Bucephalidae**

This study is the first to conduct an in-depth phylogenetic analysis of sequence data representing the Bucephalidae. Previous reports using sequencing methods have predominantly focused on ITS rDNA to illustrate its utility for species identification and phylogenetic estimation [28, 40], augment and enhance morphology-based taxonomy [11], and link life-cycle stages [39]. Here, we analysed 37 28S rDNA

sequences, representing 28 species from nine genera and four subfamilies, to explore phylogenetic relationships.

Our analysis found that subfamily classifications based to date entirely on morphological characters (i.e. the cirrus-sac, seminal vesicle and duct, pars prostatica, and ejaculatory duct) are broadly robust (see Fig. 10). The sequence representing *H. perarduum* n. sp. and those representing species of the Dolichoenterinae and the Prosorhynchinae all resolved as strongly supported monophyletic clades. Each of these was basal to the Bucephalinae/Paurorhynchinae. The embedding of *Paurorhynchus* (albeit based on a single sequence) within the Bucephalinae is interesting, but not surprising. The subfamily is biologically unique, occurring in the swim bladder, body cavity, and/or stomach of the host [41]. We suggest that the distinctive morphology of this group, which includes a small rhynchus, a mouth that opens in the anterior third of the body, lobed testes positioned in the posterior two-fifths of the body, the cirrus-sac only half as long as the posterior testis, a short pars prostatica, an ovary variable in shape and number of lobes, and vitelline follicles in linear series [41], arose in association with an ecological shift. A similar interpretation can be made for *Aphalloides* Dollfus, Chabaud & Golvan, 1957. The species of this genus infect the body cavity of gobies and are morphologically quite unlike other cryptogonimids, but molecular analysis shows that it is nested within the family (see [42]). Regardless of its phylogenetic position, we do not think it necessary to modify the classification of the Paurorhynchinae on the basis of a single sequence. However, we do suggest that, when more sequence data become available, this subfamily assignment should be reconsidered.

Analysis of 28S rDNA sequences representing species of *Grammatorcynicola* (Dolichoenterinae), and *Prosorhynchus* and *Dollfustrema* (Prosorhynchinae) resolved these as monophyletic clades, with strong support (pp = 0.76–1.00). However, within the Bucephalinae, sequences representing species of *Prosorhynchoides*, *Rhipidocotyle*, and *Bucephalus* all formed polyphyletic clades relative to each other, *Parabucephalopsis*, and *Paurorhynchus*. These clades typically had strong support (pp = 0.99–1.00), which suggests that relationships depicted in Fig. 10 are ‘real’, based on currently available data. Additionally, and with the exception of sequences representing *Prosorhynchoides ozakii* and *Parabucephalopsis parasiluri*, which occur in the silurid *Silurus biwaensis*, and those representing *Prosorhynchoides caecorum* and *P. megacirrus* from the sciaenid *Sciaenops ocellatus*, 28S sequences generally did not group based on the parasite’s host family and/or species (see Fig. 10); however, sequences did cluster based on distribution in either marine or freshwater environments. Similar patterns of disintegration have recently been seen in other large trematode genera following molecular phylogenetic analysis, such as the opecoelid genus *Macvicaria* Gibson & Bray, 1982 (see [43]) and the gorgoderid genus *Phyllodistomum* Braun, 1899 (see [17]).

*Prosorhynchoides*, *Rhipidocotyle*, and *Bucephalus* are large genera, with 88, 64, and 79 species, respectively. They are distinguished mainly on the basis of the form of the rhynchus. According to Overstreet and Curran [1], *Bucephalus* possesses a rhynchus with tentacles, *Prosorhynchoides* a simple sucker without a central muscular palp, and *Rhipidocotyle* species with a simple sucker covered by a simple muscular hood or hood with three to five large fleshy lobes. Manter [44] highlighted some of the difficulties associated with recognising features of the rhynchus, including i) failure to recognise the genus *Bucephalus*

due to the inability to note the presence of tentacles when they are completely retracted and all but invisible, and ii) that the ‘hood’ surmounting the sucker of *Rhipidocotyle* assumes a variety of forms, and may bear papillae (i.e. *Rhipidocotyle galeata* (Rudolphi, 1819)), which may be extendable (i.e. *Rhipidocotyle longleyi* Manter, 1934) and probably homologous with the tentacles of *Bucephalus*. To further compound the difficulty with their circumscription, species in these three genera share a mouth that opens near the mid-body, a pre-ovarian pharynx, a sac-like caecum, testes that are oblique, a pre-testicular ovary, and vitelline follicles in two lateral fields (grouped or in rows) [1]. These difficulties are highlighted by the fact that, since their original description, 11 species (see [45]) have been reassigned from one of these three genera to another. Based on the phylogenetic analysis presented here, we conclude that there are clear issues with the circumscription of the bucephaline genera. Establishing the true extent to which patterns reported here exist between/among all recognised species in each genus, and dealing with this difficult and challenging situation systematically, will require far more genotyping and phylogenetic analysis, especially of type-species, followed by iterative reassessment of morphology, host distribution, and ecology.

#### **Conflict of interest**

The authors declare they have no competing interests.

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**Table 1**

Sequence data representing species of the Bucephalidae determined in the present study, together with key 28S reference sequences (see GenBank accession nos.) and epidemiological information.

Subfamily Species	Host	Locality	ITS2 replicates	28S replicates	QM <sup>a</sup>	USNM <sup>b</sup>	GenBank accession nos.	References
<b>Heterobucephalopsinae n. subfam.</b>								
<i>Heterobucephalopsis perarduum</i> n. sp.	<i>Gymnothorax javanicus</i> (Bleeker)	Off Lizard Island, Queensland, Australia	2	2	G234976– G234990	-	KT213577, KT213571	This study
<b>Dolichoenterinae Yamaguti, 1958</b>								
<i>Grammatorcynicola brayi</i> Bott & Cribb, 2005	<i>Grammatorcynus bicarinatus</i> (Quoy & Gaimard)	Swain Reefs off Queensland, Australia	1	1	-	-	KT213579, KT213573	This study
<i>Grammatorcynicola nolani</i> Bott & Cribb, 2005	<i>Grammatorcynus bilineatus</i> (Rüppell)	Off Lizard Island, Queensland, Australia	1	1	-	-	KT213580, KT213574	This study
<b>Prosorhynchinae Nicoll, 1914</b>								
<i>Dollfustrema dura</i> n. sp.	<i>Gymnothorax javanicus</i> (Bleeker)	Off Lizard Island, Queensland, Australia	2	2	G234961– G234975	-	KT213578, KT213572	This study
<i>Dollfustrema hefeinensis</i> Liu, 1999 <sup>c</sup>	<i>Rhinogobius giurinus</i> (Rutter)	Fish market, Shoaguan, Guandong, China	2	2	-	-	KT273386	This study
<i>Prosorhynchus longisaccatus</i> Durio & Manter, 1968	<i>Epinephelus undulatostratus</i> (Peters)	Off Heron Island, Queensland, Australia	1	1	-	-	KT213581, KT213575	This study
<i>Prosorhynchus pacificus</i> Manter, 1940	<i>Mycteroperca microlepi</i> (Goode & Bean)	North central Gulf of Mexico	7	9	-	1283064– 1283067	KT273385	This study
<b>Bucephalinae Poche, 1907</b>								
<i>Bucephalus cynoscion</i> Hopkins, 1956	<i>Bairdiella chrysoura</i> (Lacepède)	Back Bay, Biloxi, Mississippi, USA	4	4	-	1283068– 1283071	KT273397	This study
	<i>Cynoscion arenarius</i> Ginsburg	Off Ocean Springs, Mississippi, USA	1	1	-	1283072	KT273396	This study
<i>Bucephalus gorgon</i> (Linton, 1905) Eckmann, 1932	<i>Seriola dumerili</i> (Risso)	North central Gulf of Mexico	6	6	-	1283073, 1283074	KT273400	This study
<i>Bucephalus margaritae</i> Ozaki & Ishibashi, 1934	<i>Caranx crysos</i> (Mitchill)	North central Gulf of Mexico	9	6	-	1283077– 1283080	KT273395	This study
<i>Bucephalus polymorphus</i> von Baer, 1827	<i>Perca fluviatilis</i> Linnaeus	Curonian Lagoon, Lithuania	-	-	-	-	JQ346717	[40]
	<i>Dreissena polymorpha</i> (Pallas) <sup>d</sup>	Belarus	-	-	-	-	AY289243	[46]
<i>Parabucephalopsis parasiluri</i> Wang, 1985	<i>Silurus biwaensis</i> (Tomoda)	Seta river, Shiga, Japan	-	-	-	-	AB640884	[47]
<i>Prosorhynchoides apogonis</i> Bott & Cribb, 2005	<i>Cheilodipterus macrodon</i> (Lacepède)	Off Heron Island, Queensland, Australia	1	1	-	-	KT213582, KT213576	This study
<i>Prosorhynchoides caecorum</i> (Hopkins, 1956) Bott & Cribb, 2005	<i>Bairdiella chrysoura</i>	Davis Bayou, Mississippi, USA	4	2	-	1283081, 1283082	KT273393	This study

	<i>Sciaenops ocellatus</i>	Off Deer Island, Mississippi, USA	1	1	-	1283083, 1283084	KT273392	This study
<i>Prosorhynchoides longoviferus</i> (Manter, 1940) Overstreet, Cook & Heard, 2009	<i>Sphyræna barracuda</i> (Edwards)	Off Key West, Florida, USA	2	2	-	1283086, 1283087	KT273387	This study
<i>Prosorhynchoides megacirrus</i> (Riggin & Sparks, 1962) Overstreet, Cook & Heard, 2009	<i>Sciaenops ocellatus</i>	Off Deer Island, Mississippi, USA	3	3	-	1283088– 1283091	KT273391	This study
<i>Prosorhynchoides ovatus</i> (Linton, 1900) Dollfus, 1929	<i>Lobotes surinamensis</i> (Bloch)	Off Deer Island, Mississippi, USA	2	2	-	1283092– 1283098	KT273399	This study
<i>Prosorhynchoides ozakii</i> (Nagaty, 1937) Bott & Cribb, 2005	<i>Silurus biwaensis</i>	Uji river, Kyoto, Japan	-	-	-	-	AB640885	[47]
<i>Prosorhynchoides paralichthydis</i> (Corkum, 1961) Overstreet, Cook & Heard, 2009	<i>Paralichthys lethostigma</i> Jordan & Gilbert	Off Deer Island, Mississippi, USA	10	10	-	1283099– 1283102	KT273398	This study
<i>Prosorhynchoides scomberomorus</i> (Corkum, 1968) Overstreet, Cook & Heard, 2009	<i>Scomberomorus cavalla</i> (Cuvier)	North central Gulf of Mexico	1	1	-	1283103– 1283105	KT273389	This study
	<i>Scomberomorus maculatus</i> (Mitchill)	Off Horn Island, Mississippi	1	1	-	1283106	KT273388	This study
<i>Rhipidocotyle angusticollis</i> Chandler, 1941	<i>Euthynnus alletteratus</i> (Rafinesque)	Off Louisiana, USA	2	2	-	1283107– 1283110	KT273383	This study
<i>Rhipidocotyle campanula</i> (Dujardin, 1845)	<i>Anodonta anatina</i> (Linnaeus) <sup>d</sup>	River Smolka, Ukraine	-	-	-	-	KF184356	[40]
	<i>Unio crassus</i> Philipsson <sup>d</sup>	River Kiauna, Lithuania	-	-	-	-	KF184357	[40]
	<i>Anodonta anatina</i> <sup>d</sup>	Kaunas water reservoir on the Nemunas River, Lithuania	-	-	-	-	JQ346713	[40]
<i>Rhipidocotyle fennica</i> Gibson, Taskinen & Valtonen, 1992	<i>Anodonta anatina</i> <sup>d</sup>	Lake Saravesi, Finland	-	-	-	-	JQ346715	[40]
	<i>Esox lucius</i> Linnaeus	Lake Vilkoksnis, Lithuania	-	-	-	-	KM068119	[48]
<i>Rhipidocotyle galeata</i> (Rudolphi, 1819)	<i>Eutrigla gurnardus</i> (Linnaeus)	North Sea, UK	-	-	-	-	AY222225	[26]
<i>Rhipidocotyle lepisostei</i> Hopkins, 1954	<i>Lepisosteus occeus</i> (Linnaeus)	Pascagoula River, Mississippi, USA	4	4	-	1283111– 1283113	KT273390	This study
<i>Rhipidocotyle transversale</i> Chandler, 1935	<i>Strongylura marina</i> (Walbaum)	Off Deer Island, Mississippi, USA	4	5	-	1283114– 1283117	KT273394	This study
<i>Rhipidocotyle tridecapapillata</i> Curran & Overstreet, 2009	<i>Micropterus salmoides</i> (Lacepède)	Luxapalila River, Mississippi, USA	2	4	-	-	KT273384	This study
<b>Paurohynchinae Dickerman, 1954</b>								
<i>Paurohynchus hiodontus</i> Dickermann, 1954	<i>Hiodon alosoides</i> (Rafinesque)	Red River, Minnesota, USA	2	6	-	1283118	KT273401	This study

<sup>a</sup> Accession numbers for specimens lodged with the Queensland Museum, Brisbane, Australia

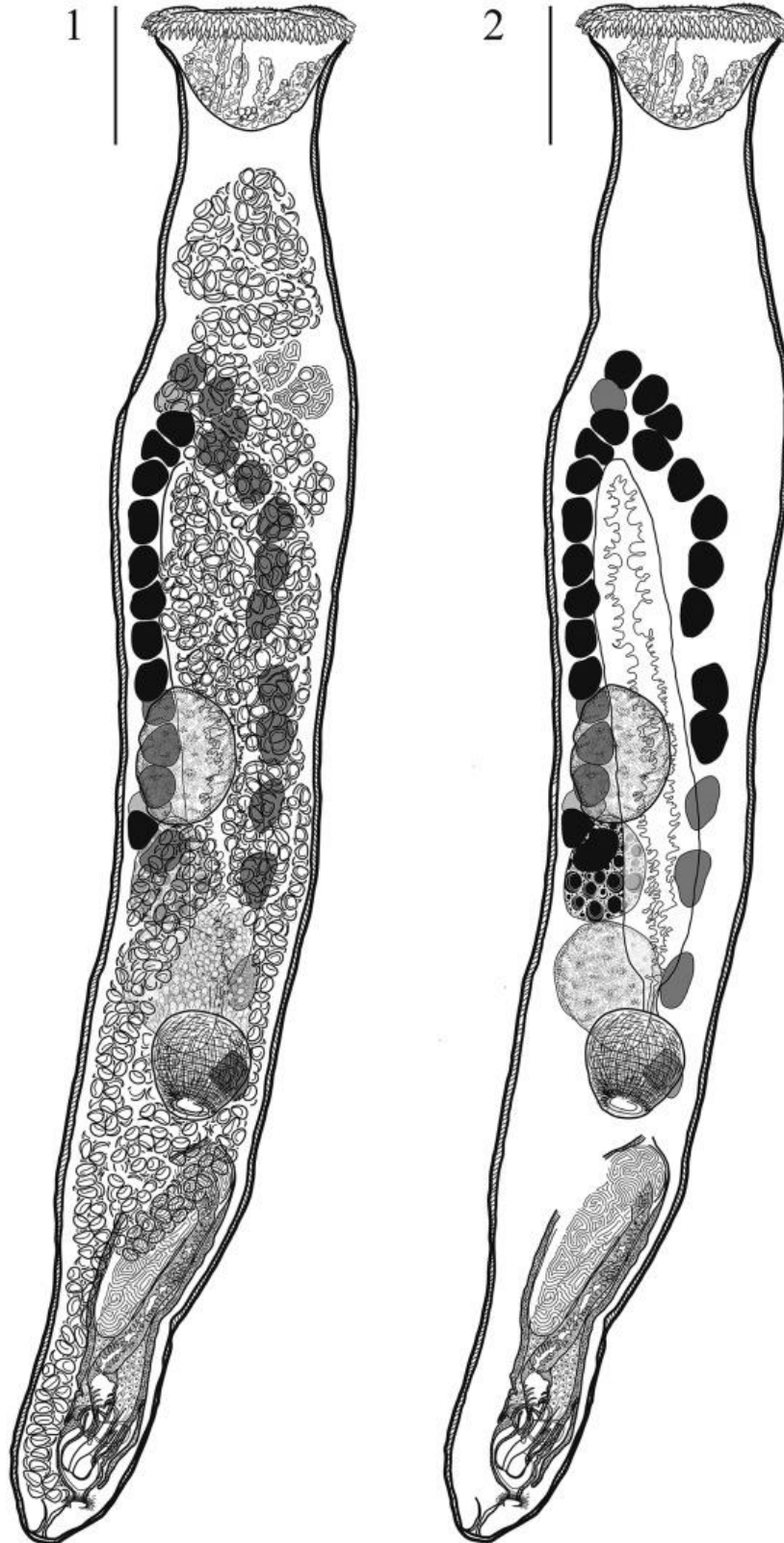
<sup>b</sup> Accession numbers for specimens lodged with the Smithsonian National Museum of Natural History, P.O. Box 37012 Washington D.C., 20013-7012, USA

<sup>c</sup> Liu is credited as the lone authority for *D. hefeiensis* in [49]

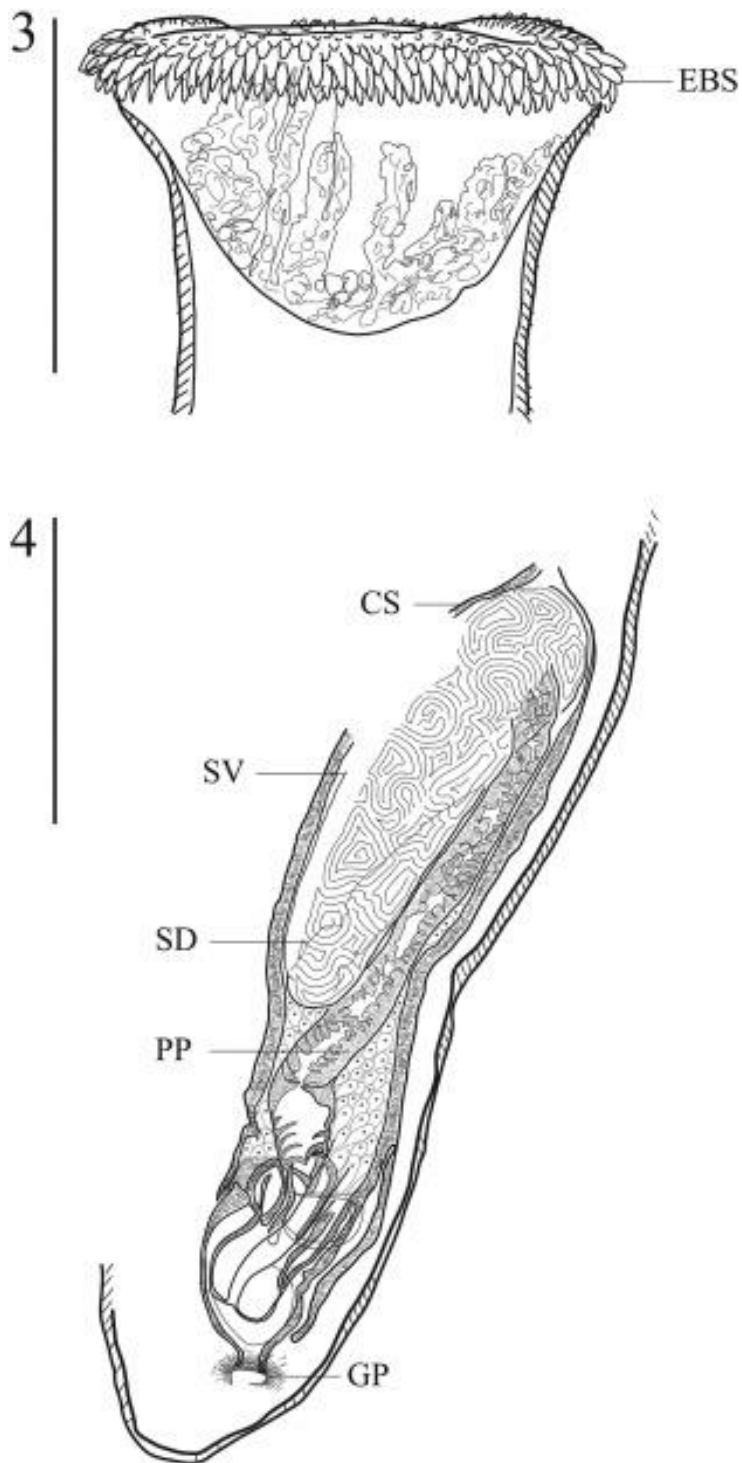
<sup>d</sup> Bivalve intermediate host

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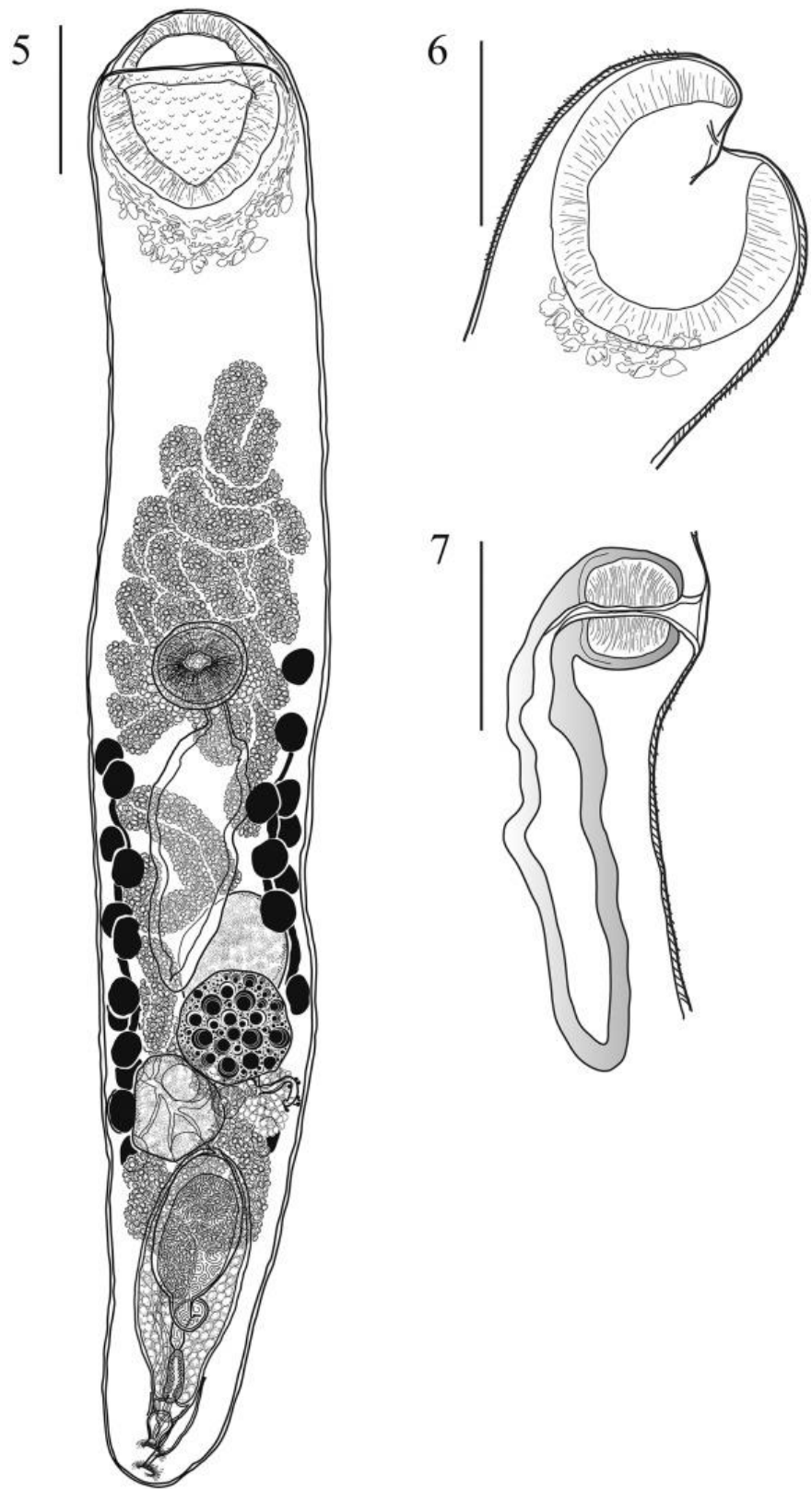


**Figs. 3–4.** *Dollfustrema dura* n. sp. from *G. javanicus* from off Lizard Island. 3. Holotype, rhynchus, ventral view. 4. Holotype, male terminal genitalia, ventral view. Abbreviations: CS, cirrus-sac; EBS, enlarged body spines; GP, genital pore; PP, pars prostatica; SD, seminal duct; SV, seminal vesicle. Scale bars: 200  $\mu$ m.

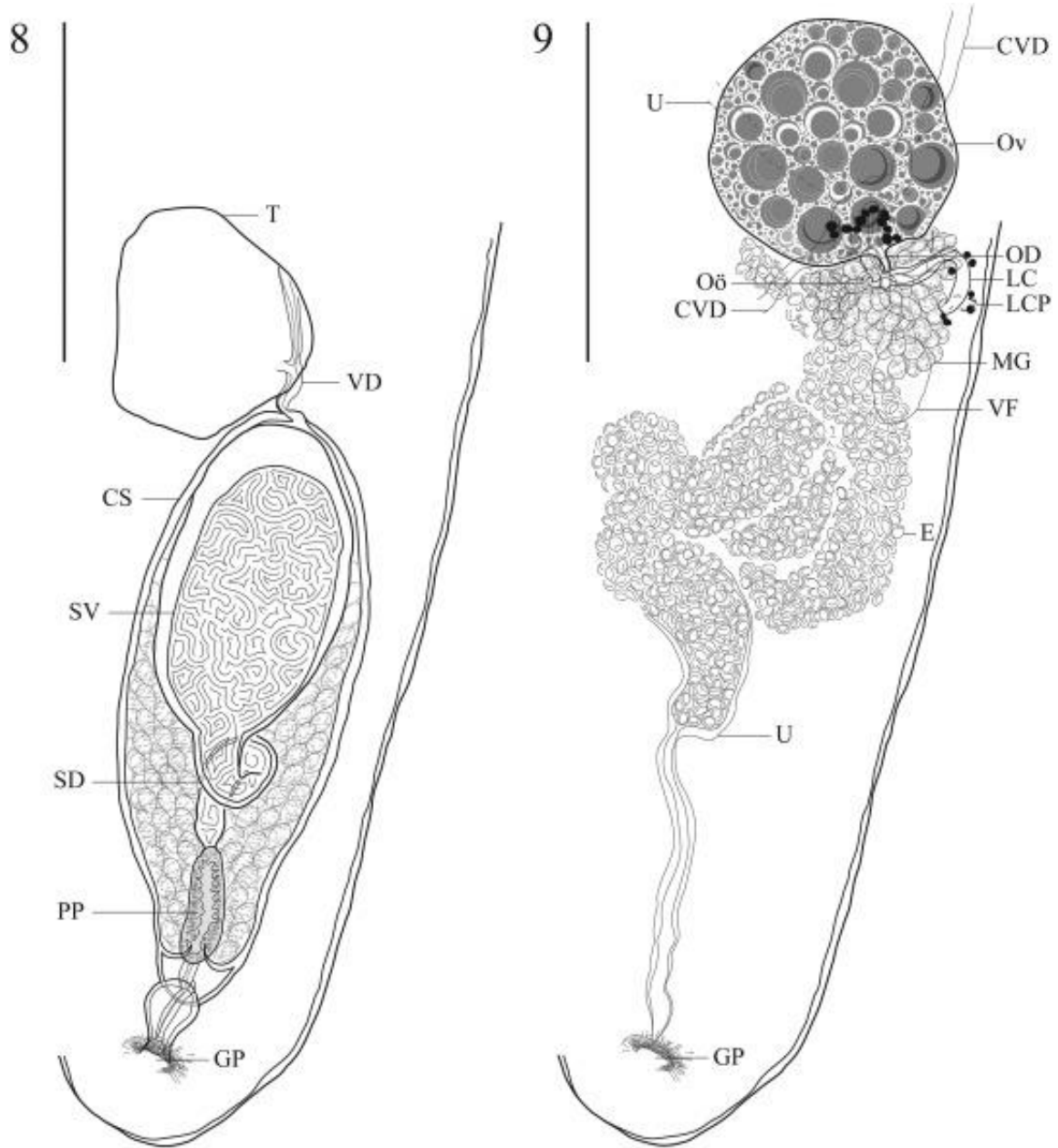




**Figs. 5–7.** *Heterobucephalopsis perarduum* n. sp. from *G. javanicus* from off Lizard Island. 5. Holotype, adult, whole mount, ventral view. 6. Paratype, rhynchus, lateral view. 7. Paratype, pharynx and caecum, lateral view. Scale-bars: 250  $\mu$ m.



**Fig. 8–9.** *Heterobucephalopsis perarduum* n. sp. from *G. javanicus* from off Lizard Island. 8. Holotype, male terminal genitalia. 9. Holotype, female terminal genitalia, ventral view. Abbreviations: CS, cirrus-sac; CVD, common vitelline duct; E, egg; GP, genital pore; LC, Laurer's canal; LCP, Laurer's canal pore; MG, Mehlis' gland; OD, oviduct; Oö, oötype; Ov, ovary; PP, pars prostatica; SD, seminal duct; SV, seminal vesicle; T, testis; U, uterus; VD, vas deferens; VF, vitelline follicle. Scale-bars: 250 µm.



**Fig. 10.** The genetic relationships among species of bucephalid inferred from the D1–D3 region of the large subunit (28S) of ribosomal DNA (rDNA) locus following analysis using Bayesian inference. Posterior probabilities are indicated for all major nodes. \* indicate sequence data representing bucephalid intermediate life cycle stages obtained from bivalve hosts.

