



## *Hypanocotyle bullardi* n. gen. n. sp. (Monogenea: Hexabothriidae) from gill of the diamond stingray *Hypanus dipterurus* (Jordan et Gilbert) (Myliobatiformes: Dasyatidae) in the Southeastern Pacific Ocean off Peru

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### ABSTRACT

A new genus and species of monogenean belonging to Hexabothriidae, *Hypanocotyle bullardi* n. gen. n. sp., is described based on specimens collected from the gill filaments of the diamond stingray, *Hypanus dipterurus* (Jordan et Gilbert) (Myliobatiformes: Dasyatidae), a demersal chondrichthyan collected off the coast of Callao, Peru. *Hypanocotyle* n. gen. has the following combination of diagnostic features that differentiate it from other hexabothriid genera: haptor symmetrical; vasa efferentia having proximal (narrow, with thin glandular wall) and distal (expanded, interlaced, with thick glandular wall) portions, joining medially to form vas deferens; vas deferens having proximal (expanded, sinuous, with thick glandular wall) and distal (narrow, strongly sinuous, with thin glandular wall) portions; male copulatory organ unarmed, proximal portion slightly sinuous and tube-like, distal portion funnel-shaped; prostatic glands present, distributed around of the MCO; seminal receptacle present; ootype lacking longitudinal rows of large cells (no oötype côtelé); vaginae parallel, with well-differentiated proximal (glandular, narrow, tube-like, slightly sinuous) and distal (musculoglandular, convoluted) portions; gland cells surrounding the vaginal duct along the entire length of distal portion, densely clustered in middle portion; uterine eggs with 2 elongate filaments. Phylogenetic reconstructions by maximum-likelihood method, based on newly obtained partial 18S and 28S sequences, shows that *H. bullardi* n. gen. is included within the family Hexabothriidae, order Dicybothriidea. This is the second hexabothriid genus recorded from a diamond stingray (Dasyatidae), and the fourth hexabothriid species recorded from Peru. A key to hexabothriid genera is provided.

### 1. Introduction

The Hexabothriidae Price, 1942 (Monogenea) comprises species that infect the buccal cavity, gill arches, and respiratory surfaces of cartilaginous fish (sharks, stingray and chimaeras) [1,2,3]. Boeger and Kritsky [4] conducted a phylogenetic analysis of the Hexabothriidae using morphological characters and recognized 13 valid genera but considered many species as *incertae sedis*, pointing out the possibility that some of these species could represent new genera. Currently, the family contains > 60 species assigned to 16 genera, with *Mobulicola* Patella et Bullard, 2013 being the only genus that has been proposed in

the last 4 years [3].

The Dasyatidae (stingrays) are the most diverse group of myliobatiform fishes, having approximately 97 species assigned to 19 accepted genera [5]. They typically inhabit shallow coastal waters, lagoons, estuaries, and occasionally fresh waters, but a few species may range in deeper waters (beyond 100 m) [6]. To date, members of 14 monogenean genera have been reported from dasyatids: *Entobdella* Blainville in Lamarck, 1818; *Listrocephalos* Bullard, Payne et Braswell, 2004; *Neoentobdella* Kearn et Whittington, 2005; *Trimusculotrema* Whittington et Barton, 1990 (Capsalidae); *Dasyoncocotyle* Hargis, 1955 (Hexabothriidae); *Loimopapillosum* Hargis, 1955 (Loimoidae); *Calicotyle* Diesing,

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1850; *Dasybatotrema* Price, 1938; *Dendromonocotyle* Hargis, 1955; *Empruthotrema* Johnston et Tiegs, 1922; *Heterocotyle* Scott, 1904; *Merizocotyle* Cerfontaine, 1894; *Monocotyle* Taschenberg, 1879 and *Thaumacotyle* Odhner, 1910 (Monocotylidae) [7,8]. Thus far, the only hexabothriid genus having species that infect dasyatids only is *Dasyonchocotyle* Hargis, 1955 (2 spp.), which appears to be restricted to species of *Hypanus* Rafinesque, i.e. *Dasyonchocotyle dasyatis* (Yamaguti, 1968) and *D. spiniphallus* Hargis, 1955 [4,7,8,9].

Records of hexabothriids infecting marine chondrichthyans from Peru are scarce: *Callorhynchocotyle marplatensis* Suriano et Incorvaia, 1982 from the plownose chimaera *Callorhynchus callorhynchus* (Linnaeus); *Rhinobatonchocotyle cyclovaginatus* Doran, 1953 and *R. pacifica* Oliva et Luque, 1995 from the Pacific guitarfish *Pseudobatos planceps* (Garman) [10].

During a parasitological survey of Peruvian marine fishes during June 2015 through May 2016, several monogenean specimens were collected from the gill filaments of the diamond stingray *Hypanus dypoterurus* (Jordan et Gilbert) (Dasyatidae) off the coast of Callao, Peru. Detailed morphological analysis revealed them to represent a new species for which the proposal of a new genus was warranted. The following is a description of this taxon based on morphological and molecular sequence data. In addition, Bullard et Dippenaar's [1] key to the genera of Hexabothriidae is revised to include the new species.

## 2. Materials and methods

### 2.1. Specimens collection and morphological analyses

Stingrays were collected during June 2015 through May 2016 off the coast of Callao, Peru (12°5'S, 78°11'W) using gillnets, euthanized immediately after capture, and dissected. The gills were excised and placed in Petri dishes with sea water and examined for monogeneans with the aid of a stereomicroscope. Monogeneans were collected alive, washed in sea water, slight coverslip pressure, fixed in hot 4% formalin, stained with Semichon's carmine or Ehrlich's hematoxylin, cleared in Eugenol, and mounted in Canada balsam to study internal organs. Two whole specimens and the haptor appendix dissected from 2 specimens were individually mounted in Gray and Wess medium for study of sclerotized structures [11]. Specimens were examined using a compound Olympus™ BX51 light photomicroscope equipped with Nomarski Differential Interference Contrast (DIC) optics, and drawings were made with the aid of a drawing tube. In morphological descriptions, measurements are in micrometers, representing straight-line distances between extreme points of the structures measured, and are expressed as the range followed by the mean and number (n) of structures measured in parentheses. Scientific names, including taxonomic authorities and dates, for fishes follow Eschmeyer et al. [12]. Nomenclature and classification of Myliobatiformes, Dasyatidae and *Hypanus* spp. follow Last et al. [5]. Anatomical terms for hexabothriids follow Patella and Bullard [1]. Prevalence and mean intensity of infection were calculated according to Bush et al. [13]. Type material was deposited in the Helminthological Collection of the Museum of Natural History at the San Marcos University (MUSM), Peru and in the Helminthological Collection of Instituto Oswaldo Cruz (CHIOC), Brazil.

### 2.2. DNA extraction, PCR amplification and DNA sequencing

The genomic DNA was extracted using the phenol-chloroform method [14]. The partial region spanning 28S rDNA was amplified by PCR using the primers C1 (5'-ACCCGCTGAATTTAAGCAT-3') and D2 (5'-TGGTCCGTGTTTCAAGAC-3') [15,16]. The partial region spanning the 18S rDNA was amplified by PCR using the primers S1 (5'-TTCCGATAAC GAACG AGACT -3') and IR5 (5'-TACG GAAA CCTT GTTA CGAC) [17]. PCR assays were carried out in a total volume of 15 µl containing 7.5 µl of 2× GoTaq® Colorless Master Mix (Promega), 0.5 µl Mg<sup>2+</sup> (50 mM concentration), 1.5 µl of each primers with final

concentration at 0.5 µM, 2.0 µl of cDNA sample and ultrapure water to complete, using cycling parameters as previously described by these authors. The PCR products were analysed by electrophoresis in 1.5% agarose in Tris-borate EDTA gels, stained with SyberGreen (Invitrogen, Eugene, Oregon, USA) and photographed under UV transillumination. Amplified PCR products were purified with ExoSap-IT (USB® Products Affymetrix Inc., Cleveland, Ohio, USA). DNA cycle sequencing reactions were performed using BigDye Terminator v.3.1 (Applied Biosystems, Foster City, CA, USA) and automated sequencing was done using the Sequencing Platform at the Fundação Oswaldo Cruz-PDTIS/FIOCRUZ in Brazil. Sequences of both strands were generated, edited and aligned by using the MEGA version 7.0 software [18]. Sequences were compared to others available in the GenBank database using the BLASTN program from the National Center for Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov/BLAST>) [19].

### 2.3. Molecular analyses

Sequences were aligned with Clustal W in MEGA 7.0 [20]. The evolutionary model automatically selected by MEGA 7 for reconstruction of Maximum likelihood (ML) phylogenetic trees [21] were the generalized time-reversible (GTR G + I) for the partial 18S rDNA tree and Tamura-Nei (G) for partial 28S rDNA. The trees were resampled by 5000 bootstrap replicates to evaluate the reliability of the groups. Sequences of the new species were deposited in GenBank under the accession numbers MG591251 for the partial 18S rDNA gene with 526 bp and MG591249 and MG591250 for the partial 28S rDNA gene with 892 and 771 bp, respectively. The sequences from GenBank that were used for the phylogenetic analysis are listed in Table 1.

## 3. Results

### 3.1. Morphological characterization of the new genus

#### 3.1.1. *Hypanocotyle n. gen*

Diagnosis. Monogenea, Hexabothriidae. Body elongate. Haptor symmetrical, parallel with midline of body; sucker complexes equally sized, distributing in 2 bilateral columns of 3 sucker complexes each; sclerites equally sized, having C-shaped shaft and sharp distal hook. Appendix armed (= having anchors), lying on midline of haptor, originating from dorsal surface of haptor, with forked end comprising

**Table 1**

List of the species of Monogenea and outgroups used in the phylogenetic analyses of *Hypanocotyle bullardi* n. gen. n. sp. using sequences of the partial 18S and partial 28S rDNA genes.

Species	Family	18S rDNA	28S rDNA
<i>Diclybothrium armatum</i> Leuckart, 1835	Diclybothriidae	KP796254	
<i>Hexabothrium appendiculatum</i> (Kuhn, 1829)	Hexabothriidae		AF131724
<i>Hypanocotyle bullardi</i> n.gen. n.sp.	Hexabothriidae	MG591251	MG591249 MG591250
<i>Kuhnia scomбри</i> (Kuhn, 1829)	Mazocraeidae	AJ228783	AF382044
<i>Leptomazocraes orientalis</i> Mamaev, 1975	Mazocraeidae	KX094554	KU872036
<i>Mazocraeoides dussumieri</i> Mamaev, 1975	Mazocraeidae		KF306257
<i>Mazocraeoides prashadi</i> Chauhan, 1950	Mazocraeidae	KT267182	
<i>Paradiclybothrium pacificum</i> Bychowsky & Gusev, 1950	Diclybothriidae	KP796243	
<i>Paramazocraes thrissocles</i> Tripathi, 1959	Mazocraeidae	KU872031	KU872039
<i>Polystomoides oris</i> Paul 1938	Polystomatidae	FM992698	FM992705
<i>Pseudohexabothrium taeniurae</i> Agrawal, Chisholm & Whittington, 1996	Hexabothriidae	AJ228791	AF382035

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