Parasitology

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Three new species of *Helicometroides* Yamaguti, 1934 from Japan and Australia, with new molecular evidence of a widespread species

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Abstract

We report specimens of monorchiids infecting Haemulidae from the waters off Japan and Australia; these specimens represent five species of *Helicometroides* Yamaguti, 1934, three of which are unambiguously new. *Helicometroides murakamii* n. sp. infects *Diagramma pictum pictum* from off Minabe, Japan; *Helicometroides gabrieli* n. sp. infects *Plectorhinchus chrysotaenia* from off Lizard Island, Australia; and *Helicometroides wardae* n. sp. infects *Plectorhinchus flavomaculatus* and *Plectorhinchus multivittatus* from off Heron Island, Australia. *Helicometroides murakamii* n. sp. and *H. gabrieli* n. sp. conform to the most recent diagnosis of *Helicometroides* in lacking a terminal organ, but *H. wardae* n. sp. possesses a terminal organ with distinct, robust spines; despite this morphological distinction, the three form a strongly-supported clade in phylogenetic analyses. We also report specimens morphologically consistent with *Helicometroides longicollis* Yamaguti, 1934, from *D. pictum pictum* from off Minabe, Japan, and *Diagramma pictum labiosum* on the Great Barrier Reef, Australia. Genetic analyses of ITS2 rDNA, 28S rDNA and *cox*1 mtDNA sequence data for the Japanese specimens reveal the presence of two distinct genotypes. Specimens of the two genotypes were discovered in mixed infections and are morphologically indistinguishable; neither genotype can be associated definitively with *H. longicollis* as originally described. We thus identify them as *H. longicollis* lineage 1 and 2, pending study of further fresh material. Genetic analyses of specimens from the Great Barrier Reef are consistent with the presence of only *H. longicollis* lineage 1. This species thus has a range that incorporates at least Australia and Japan, localities separated by over 7000 km.

Introduction

The most recent taxonomic overview of the Monorchiidae Odhner, 1911 characterizes the family by the possession of a spined tegument, complex and spined terminal genitalia, and restricted fields of vitelline follicles (Madhavi, 2008). However, members of the family exhibit wide variation in the number of testes, body shape, structure of the terminal genitalia and distribution of vitelline follicles, which has led the position of some presently-recognized monorchiid genus concepts to be controversial. *Helicometroides* Yamaguti, 1934 was originally placed in the Allocreadiidae Looss, 1902. Yamaguti (1971) transferred it to the Opecoelidae Ozaki, 1925, before Gibson and Bray (1982) transferred it to the Enenteridae Yamaguti, 1958. Bray and Cribb (2001) commented that *Helicometroides* likely belonged in the Monorchiidae, but did not formally transfer it. Madhavi (2008) finally transferred *Helicometroides* to the Monorchiidae, and synonymized another monorchiid genus, *Hysterorchis* Durio and Manter, 1968, with the concept. Of the five currently recognized species of *Helicometroides*, only *H. longicollis* Yamaguti, 1934 is represented by genetic sequence data. Recent phylogenetic analyses have reported it as sister to all other monorchiid taxa (Wee *et al.*, 2020*a*, 2020*b*, 2020*c*), or to all except *Cableia pudica* Bray *et al.*, 1996 (Panyi *et al.*, 2020; Wee *et al.*, 2021*b*).

In this study, new collections from haemulid fishes from the waters off Japan and Australia revealed what we interpret as five species of *Helicometroides*, three of which are described as new here. The other two species are morphologically cryptic with respect to each other. Their relationship to the type-species, *H. longicollis*, is explored.

Materials and methods

Host and parasite collection

Fishes were collected from multiple localities across the Indo-west Pacific (see Table 1) *via* spear or line fishing, or purchased fresh from local fisheries. Live fish were euthanized *via* cranial pithing or an overdose of anaesthetic [AQUI-S® (AQUI-S, Melling, Lower Hutt, New Zealand)]. The gastrointestinal tract was removed and examined for parasites using the gut

Table 1. Haemulid fishes examined from the Indo-west Pacific during this study

Species	Location					Total
	Heron Island	Lizard Island	Moreton Bay	Japan	Western Australia	
Diagramma pictum labiosum Macleay [post Searle *et al*., 2014]	**5 (4)**	**2 (2)**	3	–	–	**10 (6)**
Diagramma pictum pictum (Thunberg)	–	–	–	**4 (3)**	–	**4 (3)**
Parapristipoma trilineatum (Thunberg)	–	–	–	9	–	**9**
Plectorhinchus albovittatus (Rüppell)	–	3	–	–	–	**3**
Plectorhinchus chaetodonoides Lacepède	2	4	–	–	–	**6**
Plectorhinchus chrysotaenia (Bleeker)	–	**18 (7)**	–	–	–	**18 (7)**
Plectorhinchus cinctus (Temminck and Schlegel)	–	–	–	1	–	**1**
Plectorhinchus flavomaculatus (Cuvier)	**5 (4)**	2	6	–	2	**15 (4)**
Plectorhinchus gibbosus (Lacepède)	5	5	1	–	–	**11**
Plectorhinchus multivittatus (Macleay)	**4 (1)**	1	–	–	–	**5 (1)**
Plectorhinchus lessonii (Cuvier)	–	1	–	–	–	**1**
Plectorhinchus lineatus (Linnaeus)	1	6	1	–	–	**8**
Plectorhinchus obscurus (Günther)	–	2	–	–	–	**2**
Plectorhinchus picus (Cuvier)	3	–	–	–	–	**3**
Plectorhinchus schotaf (Forsskål)	–	–	–	–	1	**1**
Plectorhinchus unicolor (Macleay)	1	–	–	–	–	**1**
Pomadasys argenteus (Forsskål)	–	–	6	–	–	**6**
Pomadasys kaakan (Cuvier)	1	–	–	–	–	**1**
Total	**27 (9)**	**44 (9)**	**17**	**14 (3)**	**3**	**105 (21)**

Number of examined specimens for fish species that harbour species of *Helicometroides* in bold, with number of infected specimens in parentheses.

wash method described by Cribb and Bray (2010). Trematodes were washed in vertebrate saline, fixed without pressure in near-boiling saline, and preserved in 80% ethanol for parallel morphological and molecular characterization. Hologenophores (*sensu* Pleijel *et al*., 2008) were prepared for several specimens of each species.

Morphological analysis

Specimens were washed in fresh water, stained in Mayer's haematoxylin, destained in dilute HCl (1.0%), neutralized in dilute ammonium hydroxide (1.0%), dehydrated in a graded ethanol series, cleared in methyl salicylate and mounted in Canada balsam. Measurements were made using an Olympus SC50 digital camera mounted on an Olympus BX-53 compound microscope with cellSens Standard imaging software (Olympus, Tokyo, Japan). Measurements are in micrometres (μm) and presented as the range followed by the mean in parentheses. Drawings were made using the same Olympus BX-53 compound microscope fitted with a drawing tube and digitized using Adobe Illustrator CC 2018 software (Adobe Corporation, San Jose, California, USA). Type- and voucher specimens collected from fishes from Australian waters are lodged in the Queensland Museum (QM), Brisbane, and those from fishes from Japanese waters are lodged in the Meguro Parasitological Museum, Japan (MPM). To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of all new taxa have been submitted to ZooBank; the Life Science Identifier (LSID) is reported in the taxonomic summary for each species.

Molecular sequencing

Total genomic DNA was extracted using standard phenol/chloroform extraction techniques (Sambrook and Russell, 2001). Molecular sequence data were generated for three ribosomal DNA (rDNA) markers: the second internal transcribed spacer region (ITS2), the partial D1–D3 region of the large ribosomal subunit (28S) rDNA coding region and the partial small ribosomal 18S rDNA region; and one mitochondrial DNA (mtDNA) region: the partial cytochrome c oxidase subunit (*cox*1) region. Amplification of the ITS2 and 28S regions was performed as per the protocols of Wee *et al*. (2017*b*) using the primers 3S (5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′; Morgan and Blair, 1995) and ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′; Cribb *et al*., 1998) for the ITS2 amplification, and LSU5 (5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′; Littlewood *et al*., 2000) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′; Snyder and Tkach, 2001) or 1200R (5′-GGG CAT CAC AGA CCT G-3′; Lockyer *et al*., 2003) for the 28S amplification. Amplification of the 18S region was performed as per the protocols of Martin *et al*. (2017) using the primers WormA (5′-GCG AAT GGC TCA TTA AAT CAG-3′; Littlewood and Olson, 2001) and WormB (5′-CCT GTT ACG ACT TTT ACT TCC-3′; Littlewood and Olson, 2001). Amplification of the *cox*1 region was performed following the protocols of Wee *et al*. (2017*a*) using the primers Dig_cox1Fa (5′-ATG ATW TTY TTY TTY YTD ATG CC-3′; Wee *et al*., 2017*a*) and Dig_cox1R (5′-TCN GGR TGH CCR AAR AAY CAA AA-3′; Wee *et al*., 2017*a*). Amplifications were conducted on a Takara TP-650 PCR Thermocycler (Takara Bio, Shiga, Japan). Sanger sequencing of the amplified DNA was conducted at the Australian Genome Research Facility. Sequencing of the

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ITS2 and *cox*1 regions was conducted with the same primers used for amplification. For the 28S region, the primers used for amplification were also used in sequencing, in addition to two internal primers: 300F (5′-CAA GTA CCG TGA GGG AAA GTT G-3′; Littlewood *et al*., 2000) and ECD2 (5′-CCT TGG TCC GTG TTT CAA GAC GGG-3′; Littlewood *et al*., 1997). For the 18S rDNA region, four internal primers were used: 300F_18S (5′-AGG GTT CGA TTC CGG AG-3′; Elwood *et al*., 1985), 600R (5′-ACC GCG GCK GCT GGC ACC-3′; Littlewood and Olson, 2001), 1100F (CAG AGG TTC GAA GAC GAT C-3′; Littlewood and Olson, 2001) and 1270R (5′-CCG TCA ATT CCT TTA AGT-3′; Littlewood and Olson, 2001). Geneious® version 11.0.5 (Kearse *et al*., 2012) was used for the assembly and editing of contiguous sequences. Collection data for taxa sequenced in the current study are presented in the taxonomic section of each species description.

Molecular analyses

Phylogenetic analyses were performed with the ITS2, 28S and *cox*1 sequences only, ITS2 and *cox*1 to determine species boundaries and 28S for testing relationships between the monorchiid taxa. 18S sequence data were generated specifically for future analyses as few comparable sequences are available, although analyses were still conducted on the sequences to determine species boundaries.

ITS2, *cox*1 and 18S sequences were aligned separately, with each dataset comprising only the species of *Helicometroides*; alignments were conducted in MEGA version X (Kumar *et al*., 2018) with the MUSCLE algorithm and UPGMA clustering for iterations 1 and 2. To determine the correct reading frame, the *cox*1 alignment was translated with the echinoderm/flatworm mitochondrial code and inspected for internal stop codons in MESQUITE v.3.6 (Maddison and Maddison, 2019). Once the correct reading frame was identified, the alignment was trimmed so that the reading frame began on position one; the final alignment comprised 464 base pairs. All codons in the alignment were then tested for non-stationarity and substitution saturation with a χ^2 test run on PAUP* (Swofford, 2002) and Xia's test as implemented in DAMBE7 (Xia *et al*., 2003; Xia and Lemey, 2009; Xia, 2018), respectively; no significant non-stationarity or substitution saturation was detected. Neighbour-joining (NJ) and pairwise distance analyses were conducted on all three datasets to explore the number of base pair differences, and to determine species boundaries, respectively. The parameters used for the NJ analyses were: 'test of phylogeny = bootstrap', 'no. of bootstrap replications = 10 000', 'model/method = no. of differences', 'substitutions to include = d: Transitions + Transversions' and 'rates among sites = Uniform rates'. The pairwise distance analyses used the 'no. of differences' model and gaps were treated as a character state.

Newly generated partial 28S sequences were aligned with comparable monorchiid sequences available on GenBank (Table 2) using MUSCLE version 3.7 (Edgar, 2004) with ClustalW sequence weighting and UPGMA clustering for iterations 1 and 2. The resulting alignment was trimmed manually, and indels three bases and larger, and affecting >5% of sequences were removed; the removed bases amounted to <2% of the original alignment. Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted using the implementations of RAxML version 8.2.6 (Stamatakis, 2014) and MrBayes version 3.2.7a (Ronquist *et al*., 2012), respectively, in the CIPRES portal (Miller *et al*., 2010). Both analyses were run with the closest estimation of the GTR + I + Γ model of evolution, based on implementations of both the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) in jModelTest version 2.1.10 (Darriba *et al*., 2012). Nodal support for the ML analysis was estimated by performing 1000 bootstrap pseudoreplicates. The BI analysis was run over 10 000 000 generations (ngen = 10 000 000) with two runs each containing four simultaneous Markov chain Monte Carlo (MCMC) chains (nchains = 4) and every 1000th tree saved (samplefreq = 1000). BI analysis used the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and the prior parameters of the combined dataset were set to ratepr = variable. Samples of substitution model parameters are 'sump burnin' = 3000 and 'sumt burnin' = 3000. Sequence data for the Lissorchiidae, the sister family to the Monorchiidae, were included, and *Skrjabinopsolus nudidorsalis* Sokolov *et al*., 2020, of the Deropristidae, was designated as the outgroup taxon, based on the phylogenetic findings of Sokolov *et al*. (2020).

Results

We examined 105 specimens of haemulids (17 species, four genera) from multiple Indo-west Pacific localities. Specimens consistent with the concept of *Helicometroides* were identified from five haemulid taxa – *Diagramma pictum pictum* (Thunberg), *D. pictum labiosum* Macleay, *Plectorhinchus chrysotaenia* (Bleeker), *P. flavomaculatus* (Cuvier) and *P. multivittatus* (Macleay) (Table 1). Based on the morphology, we can easily distinguish four species – *Helicometroides longicollis* and three new species. However, based on the genetic sequence data, we recognize five species; the specimens morphologically consistent with *H. longicollis* from Japan represent two distinct species, but which of the two truly represents *H. longicollis* cannot presently be determined given an absence of any basis of association. Specimens of one of the two cryptic Japanese species are genetically identical in the ITS2 region to specimens previously identified as *H. longicollis* infecting *D. pictum labiosum* from off Heron and Lizard Islands in Australia. The samples differ slightly in the 28S region, and significantly in the *cox*1 region, but at levels we consider best interpreted as representing intraspecific variation.

Species discrimination based on the molecular sequence data

The phylogram generated from the NJ analysis of the ITS2 dataset (Fig. 1A) suggests the presence of five operational taxonomic units (OTUs) which differ by 19–53 base pairs. Three of the OTUs correspond to morphologically distinctive forms that are formally described as new species below. The other two OTUs (represented by 41 sequences) correspond to specimens with morphology consistent with that of *H. longicollis*. Of the 41 sequences, 37 are nearly identical and relate to specimens from *D. pictum pictum* and *D. pictum labiosum* from Japan and Australia, respectively; the remaining four sequences differ from these by 19–21 base pairs and relate to samples from *D. pictum pictum* from Japan only.

The phylogram generated from the NJ analysis of the *cox*1 dataset (Fig. 1B) suggests the presence of six OTUs which differ by 51–81 base pairs. As with the NJ analysis of the ITS2 dataset, three of the OTUs correspond to new species. The remaining three OTUs (represented by 13 sequences) correspond to specimens morphologically consistent with *H. longicollis*. Of the 13 sequences, nine relate to specimens from *D. pictum labiosum* from Australia, and the remaining four specimens from *D. pictum pictum* from Japan (two sequences for each Japanese OTU). Sequences of the two OTUs from *D. pictum pictum* differ by 52 base pairs; sequences of these OTUs differ from those from *D. pictum labiosum* by 51–53 and 33–36 base pairs, respectively.

Phylograms generated from the ML and BI analyses of the 28S dataset (Fig. 2) suggest the presence of five OTUs which differ by

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Table 2. Collection data for 28S sequences from GenBank analysed in this study

Species	Host	Location	GenBank ID	Reference
Family Monorchiidae				
Allobacciger annulatus Wee *et al*., 2019	*Centropyge tibicen* (Cuvier)	Heron Island, Australia	MK955782	Wee *et al*. (2020*d*)
Allobacciger brevicirrus Wee *et al*., 2019	*Scolopsis bilineata* (Bloch)	Heron Island	MK955781	Wee *et al*. (2020*d*)
Allobacciger polynesiensis Wee *et al*., 2019	*Centropyge flavissima* (Cuvier)	Moorea, French Polynesia	MK955780	Wee *et al*. (2020*d*)
Ancylocoelium typicum Nicoll, 1912	*Trachurus trachurus* (Linnaeus)	North Sea, UK	AY222254	Olson *et al*. (2003)
Cableia pudica Bray *et al*., 1996	*Cantherhines pardalis* (Rüppell)	Heron Island	AY222251	Olson *et al*. (2003)
Diplomonorchis leiostomi Hopkins, 1941	*Leiostomus xanthurus* Lacepède	Ocean Springs, Mississippi, USA	AY222252	Olson *et al*. (2003)
Genolopa ampullacea Linton, 1910	*Haemulon macrostomum* (Günther)	Islamorada, Florida, USA	MN984474	Panyi *et al*. (2020)
Genolopa minuscula Panyi *et al*., 2020	*Anisotremus surinamensis* (Bloch)	Marathon, Florida, USA	MN984472	Panyi *et al*. (2020)
Genolopa vesca Panyi *et al*., 2020	*Haemulon sciurus* (Shaw)	Long Key, Florida, USA	MN984471	Panyi *et al*. (2020)
Gerricola queenslandensis Wee *et al*., 2021	*Gerres oyena* (Forsskål)	Heron Island	MZ271999	Wee *et al*. (2021*b*)
Helicometroides longicollis Yamaguti, 1934	*Diagramma pictum labiosum* Macleay	Heron Island	KJ658287	Searle *et al*. (2014)
Hurleytrematoides chaetodoni (Manter, 1942) Yamaguti, 1954	*Chaetodon striatus* Linnaeus	Mona Passage, Puerto Rica	MH244116	Andres *et al*. (2018)
Hurleytrematoides galzini McNamara and Cribb, 2011	*Forcipiger flavissimus* Jordan and McGregor	Heron Island	MK501988	Wee *et al*. (2019)
Hurleytrematoides loi McNamara and Cribb, 2011	*Chelmon rostratus* (Linnaeus)	Moreton Bay, Australia	MK501989	Wee *et al*. (2019)
Hurleytrematoides morandi McNamara and Cribb, 2011	*Chaetodon lunula* Lacepède	Heron Island	MZ323087	Wee *et al*. (2021*a*)
Infundiburictus arrhichostoma (Searle *et al*., 2014) Wee *et al*., 2020	*Diagramma pictum labiosum*	Heron Island	KJ658289	Searle *et al*. (2014)
'*Lasiotocus* sp.'	*Menidia menidia* (Linnaeus)	Great Bay Estuary, New Jersey, USA	MN984477	Panyi *et al*. (2020)
Lasiotocus mulli (Stossich, 1883) Odhner, 1911	*Mullus surmuletus* Linnaeus	Santa Pola, Mediterranean Sea, Spain	MT669011	Wee *et al*. (2020*c*)
Lasiotocus trachinoti Overstreet and Brown, 1970	*Trachinotus carolinus* Linnaeus	Jacksonville, Florida, USA	MN984478	Panyi *et al*. (2020)
Madhavia fellaminuta Wee *et al*., 2018	*Upeneus tragula* Richardson	Moreton Bay	MG920219	Wee *et al*. (2018)
Monorchiidae sp.	*Jactellina clathrata* (Deshayes)	Heron Island	MZ272001	Wee *et al*. (2021*b*)
Monorchis lewisi Cribb *et al*., 2017	*Acanthopagrus australis* (Günther)	Moreton Bay	MF503309	Cribb *et al*. (2018)
Monorchis monorchis (Stossich, 1890) Monticelli, 1893	*Diplodus vulgaris* (Geoffroy Saint-Hilaire)	Near Corsica, France	AF184257	Tkach *et al*. (2001)
Ovipusillus geminus Wee *et al*., 2019	*Gnathanodon speciosus* (Forsskål)	Moreton Bay	MF501987	Wee *et al*. (2019)
Ovipusillus mayu Dove and Cribb, 1998	*Gnathanodon speciosus*	Moreton Bay	MF503310	Cribb *et al*. (2018)
Parachrisomon delicatus (Manter and Prichard, 1964) Madhavi, 2008	*Upeneus tragula*	Moreton Bay	MG920218	Wee *et al*. (2018)
Postmonorchis orthopristis Hopkins, 1941	*Haemulon flavolineatum* Desmarest	Upper Matecumbe Key, Florida, USA	MN984475	Panyi *et al*. (2020)

(*Continued*)

Table 2. (*Continued.*)

Species	Host	Location	GenBank ID	Reference
Proctotrema addisoni Searle *et al*., 2014	*Diagramma labiosum*	Heron Island	KJ658291	Searle *et al*. (2014)
Provitellus chaometra Wee *et al*., 2019	*Gnathanodon speciosus*	Moreton Bay	MK501984	Wee *et al*. (2019)
Provitellus infibrova Wee *et al*., 2019	*Gnathanodon speciosus*	Moreton Bay	MK501986	Wee *et al*. (2019)
Provitellus infrequens Wee *et al*., 2019	*Gnathanodon speciosus*	Moreton Bay	MK501985	Wee *et al*. (2019)
Provitellus turrum Dove and Cribb, 1998	*Pseudocaranx dentex* (Bloch and Schneider)	Heron Island	AY222253	Olson *et al*. (2003)
Pseudohurleytrema yolandae Wee *et al*., 2020	*Tripodichthys angustifrons* (Hollard)	Moreton Bay	MT649300	Wee *et al*. (2020*b*)
Retroporomonorchis pansho Wee *et al*., 2020	*Lutjanus fulvus* (Forster)	Lizard Island, Australia	MT672340	Wee *et al*. (2020*a*)
Sinistroporomonorchis glebulentus (Overstreet, 1971) Wee *et al*., 2020	*Mugil curema* Valenciennes	Beaufort, North Carolina, USA	MN984476	Panyi *et al*. (2020)
Sinistroporomonorchis lizae (Liu, 2002) Wee *et al*., 2020	*Moolgarda perusii* (Valenciennes)	Tonkin Bay, Vietnam	LN831724	Atopkin *et al*. (2017)
Family Lissorchiidae				
Asaccotrema vietnamiense Sokolov and Gordeev, 2019	*Rasbora paviana* Tirant	Cat Tien National Park, Vietnam	MK863409	Sokolov and Gordeev (2019)
Asymphylodora perccotti Besprozvannykh *et al*., 2012	*Perccottus glenii* Dybowski	Bolshaya Ussurka River Basin, Russia	FR822715	Besprozvannykh *et al*. (2012)
Lissorchis kritskyi Barnhart and Powell, 1979	*Carpiodes cyprinus* (Lesueur)	Pascagoula River, Mississippi, USA	AY222250	Olson *et al*. (2003)
Family Deropristidae				
Skrjabinopsolus nudidorsalis Sokolov *et al*., 2020	*Acipenser ruthenus* Linnaeus	River Volga Basin, Russia	MN700996	Sokolov *et al*. (2020)

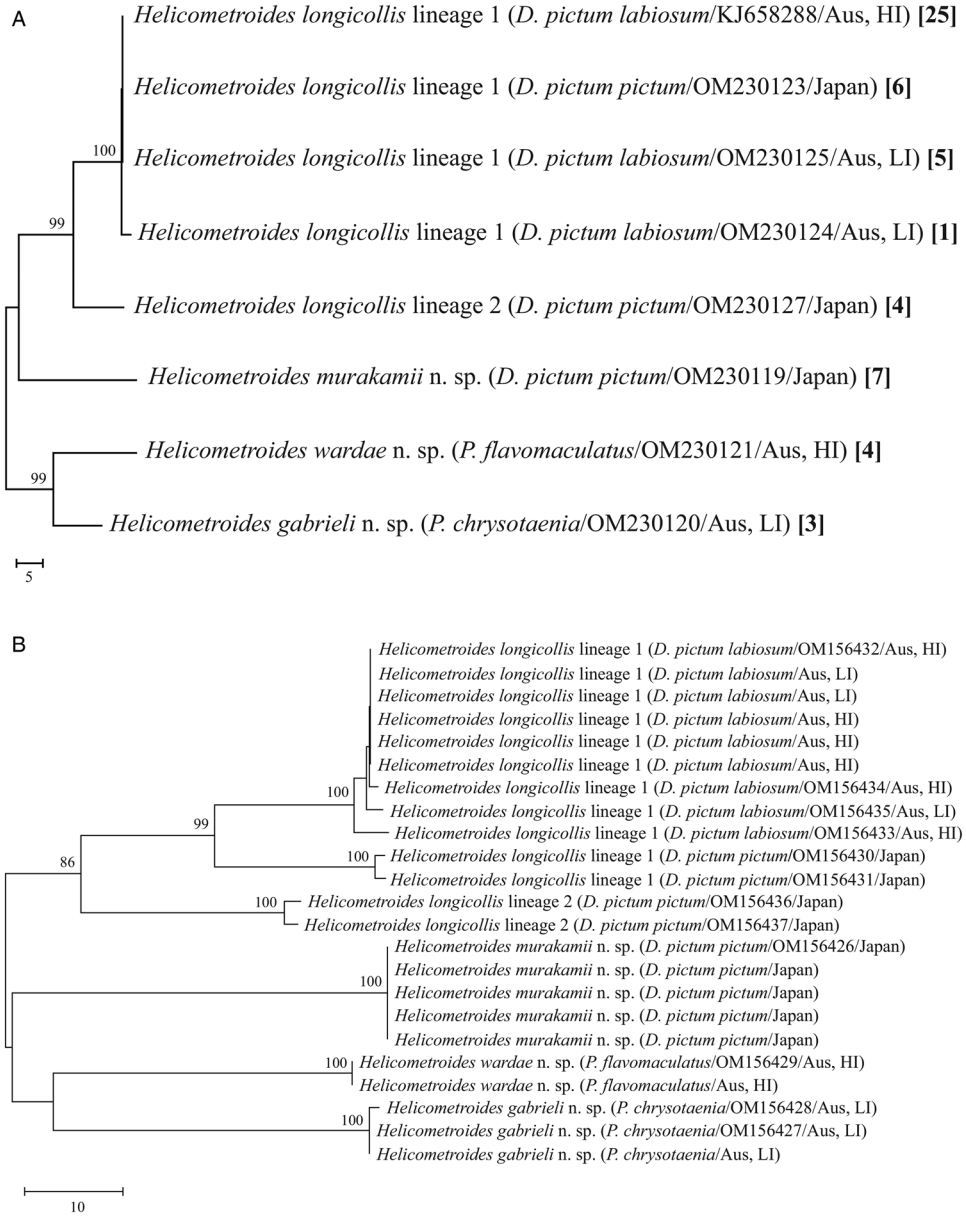

Fig. 1. Unrooted neighbour-joining analyses for the species of *Helicometroides*. (A) Phylogram generated for the ITS2 rDNA region; (B) phylogram generated for the *cox*1 mtDNA region. Scale bars indicate the number of base pair differences. Numbers in bold and brackets in (A) represent the number of identical replicates for each genotype. Aus, Australia; HI, Heron Island; LI, Lizard Island.

10–70 base pairs. As with the ITS2 and *cox*1 phylograms, three OTUs correspond to new species and the remaining OTUs (represented by three sequences) correspond to specimens morphologically consistent with *H. longicollis*. Of these three sequences, two (which differ by three base pairs) belong to an OTU that relates to specimens from *D. pictum pictum* and *D. pictum labiosum* from Japan and Australia, respectively; the third differs from the other two by 10–13 base pairs and relates to specimens from *D. pictum pictum* from Japan.

The phylogram generated from the NJ analysis of the 18S dataset (not shown) suggests the presence of five OTUs which differ by 11–37 base pairs. As with the ITS, *cox*1 and 28S phylograms, three OTUs correspond to new species and the remaining OTUs (represented by four sequences) correspond to specimens morphologically consistent with *H. longicollis*. Of these four sequences, three (which differ by 1–3 base pairs) relate to an OTU with specimens from *D. pictum pictum* and *D. pictum labiosum* from Japan and Australia, respectively; the fourth differs from the other three by 11–12 base pairs and relates to specimens from *D. pictum pictum* from Japan only.

Based on the combined morphological and molecular data, we interpret the sequences to represent five species. These five putative species are characterized below. Three species are morphologically and genetically distinct, and are formally described as new below. In contrast, the *H. longicollis* morphotype is interpreted as a complex of two cryptic species which occur together in Japan. One of these two species has been found in Australia where it shows intraspecific variation relative to the Japanese specimens. As it is not presently possible to determine which of the two species relates to the true *H. longicollis*, we distinguish them below as *H. longicollis* lineage 1 and *H. longicollis* lineage 2.

Genus concept and species descriptions

Some morphological features exhibited by the species of *Helicometroides* in this study mean that the existing diagnosis of the genus no longer captures its full variability; an amended diagnosis is presented to reflect these features.

Family Monorchiidae Odhner, 1911

Genus *Helicometroides* Yamaguti, 1934

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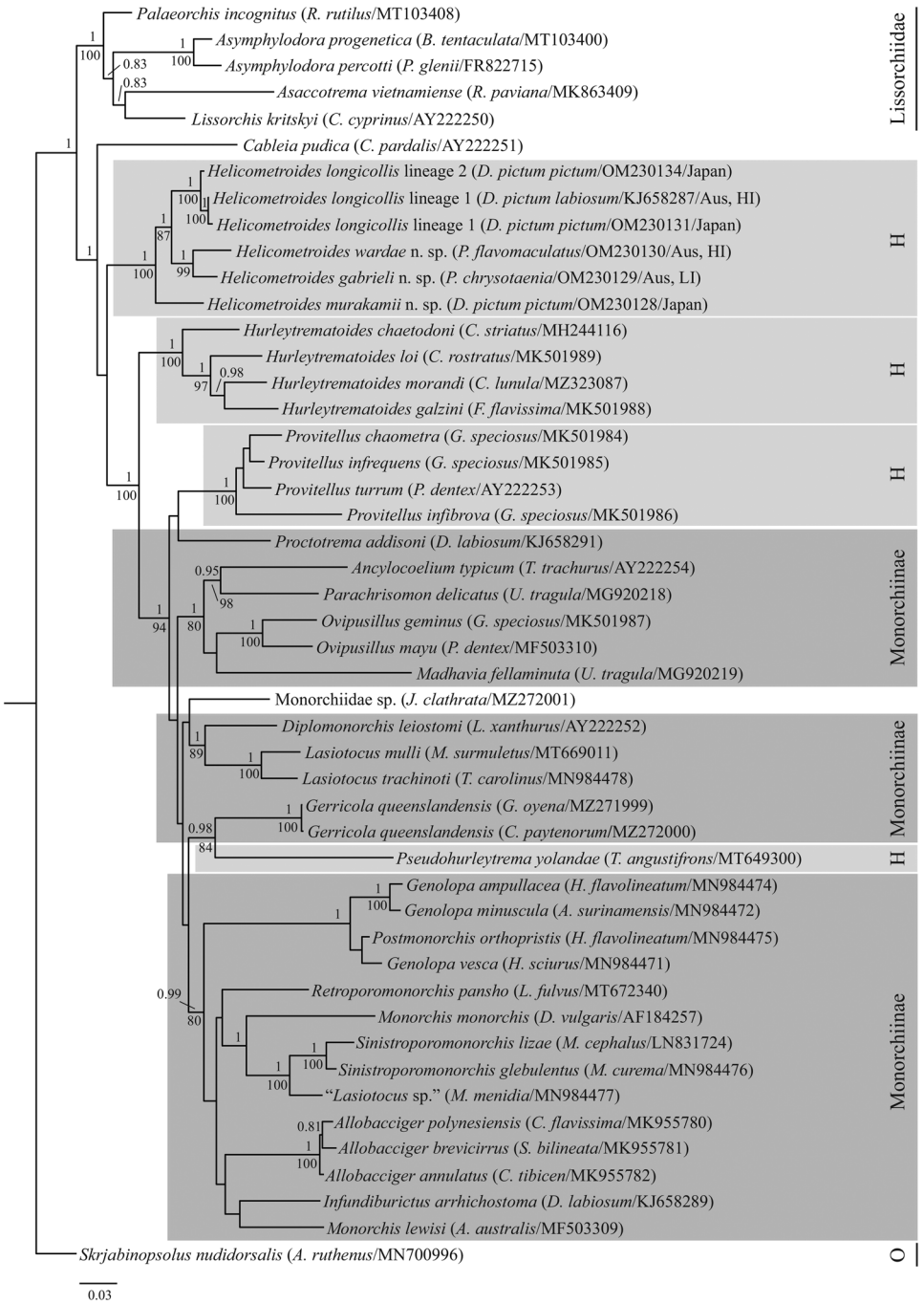

Fig. 2. Relationships of monorchiid taxa based on Bayesian inference analysis of the 28S rDNA dataset. Subfamilies are marked with a grey box, with the Hurleytrematinae in light grey and the Monorchiinae in dark grey; *Cableia pudica* and Monorchiidae sp. are unassigned. Posterior probabilities and bootstrap values are shown above and below the nodes. Nodal support below 0.80/80 not shown. Scale bar indicates expected number of substitutions per site. H, Hurleytrematinae; O, outgroup taxon.

Amended diagnosis

Body pyriform to elongate. Forebody long. Tegument with minute spines throughout. Oral sucker unspecialized. Pharynx spherical or ellipsoidal. Ventral sucker unspecialized. Intestine bifurcates in forebody; caeca terminate in hindbody. Testes two, spherical to elongate, opposite or diagonal. Cirrus-sac contains bipartite seminal vesicle, tubular pars prostatica and spined cirrus. Genital pore median or slightly sinistral, anterior to or level with ventral sucker. Ovary pre-testicular, with three to four distinct lobes. Uterus mainly in hindbody, distinctly helical. Metraterm present, may contain spines. Terminal organ absent or present; if present, unipartite, with robust spines. Vitellarium dense, in two lateral fields of follicles, reaches anteriorly and posteriorly beyond ventral sucker. Eggs with single polar filament at anopercular end. Excretory vesicle variable, tubular, saccular or Y-shaped. In digestive tract of marine fishes; Indo-west Pacific and Atlantic Oceans.

Type-species: *Helicometroides longicollis* Yamaguti, 1934.

Other species: *Helicometroides atlanticus* (Gaevskaya and Aleshkina, 1983) Searle *et al.*, 2014 (syn: *Hysterorchis atlanticus* Gaevskaya and Aleshkina, 1983); *Helicometroides gabrieli* n. sp. (present study); *H. leiperi* (Ahmad, 1982) Madhavi, 2008 (syn: *Hysterorchis leiperi* Ahmad, 1982); *Helicometroides murakamii* n. sp. (present study); *H. pseudovitellosus* (Madhavi, 1974) Searle *et al.*, 2014 (syn: *Hysterorchis pseudovitellosus* Madhavi, 1974); *H. vitellosus* (Durio and Manter, 1968) Madhavi, 2008

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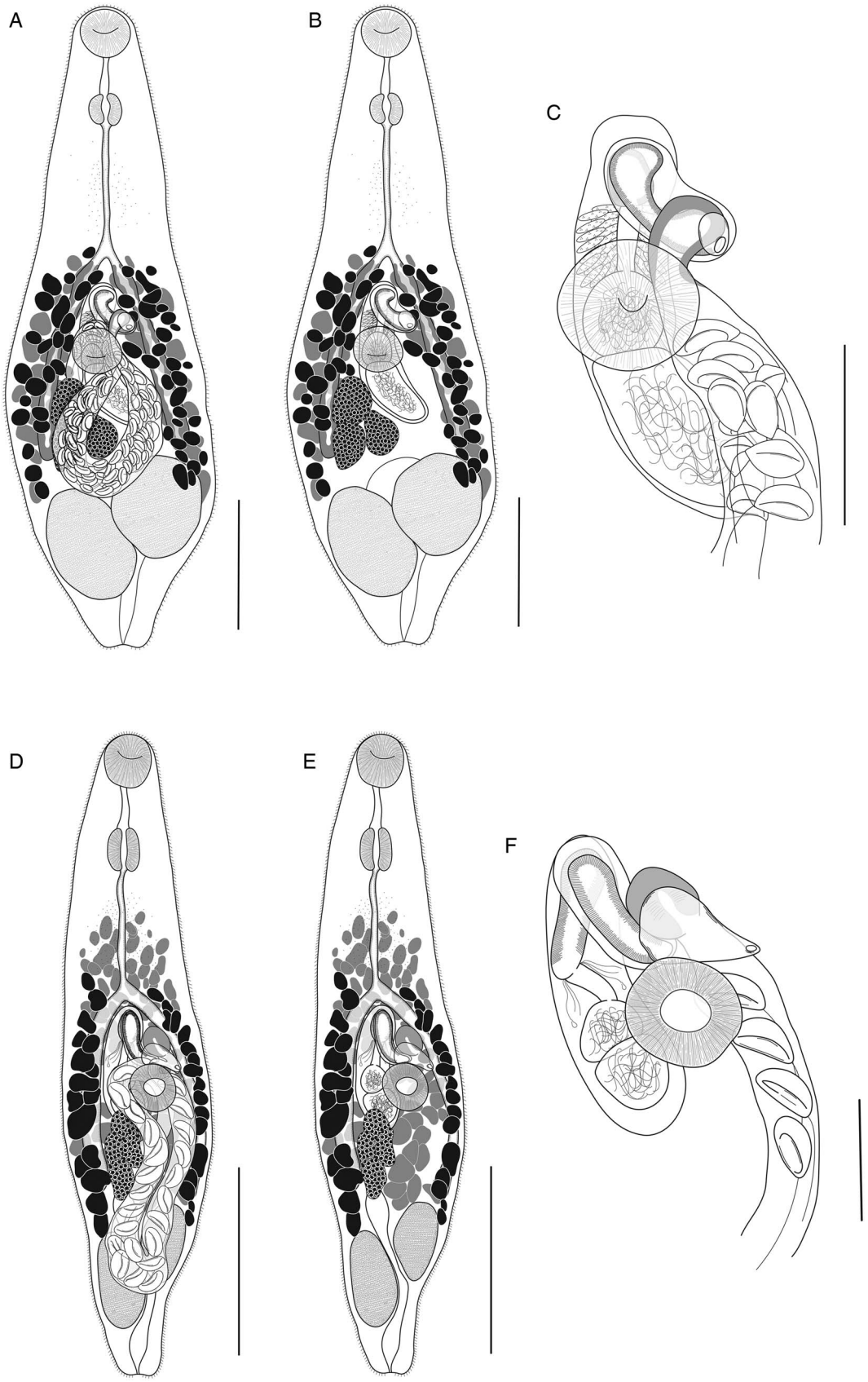

Fig. 3. *Helicometroides murakamii* n. sp. from *Diagramma pictum pictum* from Japan and *Helicometroides gabrieli* n. sp. from *Plectorhinchus chrysotaenia* from Australia. (A) Ventral view of *H. murakamii* n. sp.; (B) ventral view of *H. murakamii* n. sp., uterus not shown; (C) terminal genitalia of *H. murakamii* n. sp.; (D) ventral view of *H. gabrieli* n. sp.; (E) ventral view of *H. gabrieli* n. sp., uterus not shown; (F) terminal genitalia of *H. gabrieli* n. sp. Scale bars: A, B, D and E: 200 μm; C: 100 μm; F: 50 μm.

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Table 3. Measurements of three new species of *Helicometroides*

	Helicometroides murakamii n. sp.	*Helicometroides gabrieli* n. sp.	*Helicometroides wardae* n. sp.
n	12 (4 hologenophores)	20 (5 hologenophores)	19 (5 hologenophores)
B L	954–1047 (989)	633–952 (754)	1315–1569 (1428)
B W	256–393 (320)	158–271 (213)	339–467 (417)
FB L	461–527 (491)	312–497 (384)	626–762 (703)
FB L % of B L	49–51 (50)%	38–54 (50)%	46–53 (50)%
OS L	63–87 (77)	49–83 (61)	69–111 (80)
OS W	70–87 (79)	49–75 (59)	77–107 (86)
VS L	66–79 (73)	42–69 (56)	84–117 (97)
VS W	71–87 (78)	44–76 (58)	93–124 (104)
OS W/VS W	0.97–1.61 (1.17)	0.93–1.43 (1.12)	0.75–0.93 (0.83)
Prepharynx	28–65 (52)	25–57 (45)	43–107 (82)
Pharynx L	49–65 (57)	42–78 (57)	56–92 (68)
Pharynx W	46–54 (50)	33–56 (43)	47–65 (55)
Oesophagus	152–199 (179)	110–181 (138)	238–390 (328)
RT L	159–202 (177)	82–175 (115)	242–393 (311)
RT W	99–152 (130)	46–98 (68)	88–132 (112)
LT L	133–179 (160)	68–144 (103)	247–387 (291)
LT W	87–156 (126)	44–104 (66)	71–139 (107)
Post T % of B L	5.3–12.7 (7.9)%	5.3–11.8 (7.9)%	6.5–11.6 (8.7)%
CS L	231–320 (272)	167–282 (233)	222–331 (284)
CS W	47–77 (61)	39–83 (60)	83–143 (108)
ASV L	35–74 (49)	33–71 (49)	48–103 (78)
ASV W	35–54 (43)	24–65 (43)	54–105 (79)
PSV L	72–108 (90)	38–91 (64)	36–95 (60)
PSV W	38–71 (54)	20–75 (50)	47–105 (70)
PP	16–42 (34)	26–65 (39)	16–59 (43)
Cirrus L	85–149 (116)	51–133 (82)	65–124 (90)
Cirrus W	22–43 (29)	12–32 (19)	61–85 (69)
Ovary L	111–153 (133)	70–122 (95)	101–210 (173)
Ovary W	70–107 (88)	27–78 (46)	74–163 (106)
Egg cap L	31–43 (35)	27–35 (31)	30–39 (34)
Egg cap W	14–21 (17)	14–20 (17)	13–20 (17)

B, body; L, length; W, width; FB, forebody; OS, oral sucker; VS, ventral sucker; RT, right testis; LT, left testis; Post T, post-testicular region; CS, cirrus-sac; ASV, anterior seminal vesicle; PSV, posterior seminal vesicle; PP, pars prostatica; Egg cap, egg capsule.
Measurements are in micrometres or percentages, means in parentheses.

(syn: *Hysterorchis vitellosus* Durio and Manter, 1968); *Helicometroides wardae* n. sp. (present study)

Remarks: The use of the terms 'metraterm' and 'terminal organ' in the taxonomic descriptions of monorchiids is confused. The two terms have been used interchangeably in the descriptions of *Helicometroides* species, with some studies reporting a metraterm with no mention of a terminal organ (Yamaguti, 1934; Madhavi, 1974; Gaevskaya and Aleshkina, 1983), others reporting a terminal organ with no mention of a metraterm (Ahmad, 1982; Searle *et al*., 2014), and in one, the terminal organ being described as undifferentiated from the metraterm (Durio and Manter, 1968). In each of these cases, a metraterm is the best descriptor, as the structure typically appears to be no more than a thickening of the walls of the anterior end of the uterus and is a direct continuation of the uterus. We consider a terminal organ to be a more specialized structure, strongly differentiated from the uterus (and metraterm). Under this interpretation, four of the five species reported here, *H. longicollis* lineage 1 and 2, *H. murakamii*, and *H. gabrieli*, have a simple metraterm and the fifth, *H. wardae*, has a metraterm and a terminal organ.

New species

Helicometroides murakamii n. sp. (Fig. 3A–C)

Type-host: *Diagramma pictum pictum* (Thunberg), painted sweetlips (Perciformes: Haemulidae).

Type-locality: off Minabe, Wakayama Prefecture, Japan (33° 44′N, 135°19′E).

Site of infection: Intestine.

Prevalence: 3/4 (75%).

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Deposition of specimens: Holotype and 11 paratypes, including hologenophores (MPM Coll. No. 21817a & 21817b).

Molecular sequence data: ITS2 rDNA, seven identical replicates, one submitted to GenBank (GB OM230119); 28S rDNA, seven sequences, six identical replicates, other sequence differs by one base position, one of the six submitted to GenBank (GB OM230128); 18S rDNA, three identical replicates, one submitted to GenBank (GB OM230113); *cox*1 mtDNA, five identical replicates, one submitted to GenBank (GB OM156426).

ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The LSID for *H. murakamii* n. sp. is urn:lsid:zoobank.org:act:2D0DB23C-2D33-4FBB-875A-A31C838997F0.

Etymology: This species is named in honour of the famous Japanese author, Haruki Murakami, in recognition of his many inspirational books.

Description (Based on 12 gravid, unflattened specimens; measurements in Table 3). Body fusiform, widest just behind mid-hindbody. Forebody long. Tegument thin, covered with minute spines throughout, with prominence decreasing slightly in hindbody. Eye-spot pigment granules obvious, restricted to forebody. Oral sucker terminal, roughly round, with opening subterminal. Ventral sucker round, approximately same size as oral sucker. Prepharynx distinct. Pharynx almost round to oval, smaller than oral sucker. Oesophagus simple, long. Intestinal bifurcation in posterior forebody, distinctly anterior to ventral sucker; caeca with thick gastrodermis, terminate in mid-hindbody just anterior to testes.

Testes subspherical to slightly ellipsoidal, in posterior hindbody, slightly oblique, contiguous; left testis overlaps right testis ventrally, both overlap excretory vesicle ventrally. Post-testicular region short. Vas deferens not detected. Cirrus-sac in mid-body, reaches anteriorly and posteriorly beyond ventral sucker; anterior third of cirrus-sac recurves postero-sinistrally. Seminal vesicle bipartite; posterior chamber much larger than anterior chamber. Pars prostatica short, distinct from seminal vesicle and cirrus, surrounded by distinct prostatic cells. Cirrus follows recurved portion of cirrus-sac, armed with minute spines. Genital atrium distinct, unspined. Common genital pore small, immediately anterior to and sinistro-lateral to ventral sucker.

Ovary with three or four distinct lobes, just postero-dextral to ventral sucker, ventrally overlaps cirrus-sac, excretory vesicle, right caecum, and one or both testes in some specimens. Egg-forming complex not traced. Uterine seminal receptacle present. Vitellarium comprising dense lateral follicles extending from level of intestinal bifurcation to level of anterior margin of testes, confluent only in forebody, dorsally overlaps ovary; posterior follicles dorsally and ventrally overlap anterior margins of one or both testes in some specimens. Uterine coils mostly in hindbody, never in post-testicular space, extending from anterior half of testes to just anterior to ventral sucker, usually with two broadly spiral coils, ventrally overlap ovary, posterior end of cirrus-sac and testes in some specimens; ascending loop always partially overlaps ventral sucker, opens into genital atrium. Metraterm distinct, thick-walled, at anterior end of ascending loop of uterus. Terminal organ absent. Egg capsules with single long filament at anopercular end.

Excretory vesicle I-shaped, saccular anteriorly, tapering into long tube posteriorly, extends to level of anterior margin of testes, dorsal to uterus and testes. Excretory pore terminal.

Helicometroides gabrieli n. sp. (Fig. 3D–F)

Type-host: *Plectorhinchus chrysotaenia* (Bleeker), yellow-striped sweetlips (Perciformes: Haemulidae).

Type-locality: off Lizard Island, northern Great Barrier Reef, Queensland, Australia (14°40′S, 145°27′E).

Site of infection: Intestine.

Prevalence: 7/18 (39%).

Deposition of specimens: Holotype and 19 paratypes, including hologenophores (QM G239463–G239482).

Molecular sequence data: ITS2 rDNA, three identical replicates, one submitted to GenBank (GB OM230120); 28S rDNA, three sequences, one submitted to Genbank (GB OM230129); 18S rDNA, two identical replicates, one submitted to GenBank (GB OM230114); *cox*1 mtDNA, three replicates representing two genotypes, one of each genotype submitted to GenBank (GB OM156427–OM156428).

ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The LSID for *H. gabrieli* n. sp. is urn:lsid:zoobank.org:act:0BE05236-59ED-4C12-B7F0-716802EFD14A.

Etymology: This species is named in memory of Gabriel Debono, brother-in-law of NW.

Description (Measurements based on 20 gravid, unflattened specimens; measurements in Table 3). Body elongate, weakly fusiform, widest approximately at level of ventral sucker. Forebody long. Tegument thin, covered with minute spines, with prominence decreasing slightly in hindbody. Eye-spot pigment granules obvious, restricted to forebody. Oral sucker terminal, roughly round, with opening immediately subterminal. Ventral sucker round, roughly same size as oral sucker. Prepharynx distinct. Pharynx oval, smaller than oral sucker. Oesophagus simple, long. Intestinal bifurcation in posterior forebody, distinctly anterior to ventral sucker; caeca lack thick gastrodermis, terminate in mid-hindbody at level of or just anterior to testes.

Testes typically ellipsoidal, almost globular in some specimens, in posterior hindbody, oblique, separated by excretory vesicle in some specimens. Post-testicular region short. Vas deferens not detected. Cirrus-sac in mid-body, reaches anteriorly and posteriorly beyond ventral sucker; anterior third of cirrus-sac recurves postero-sinistrally. Seminal vesicle bipartite; chambers typically approximately same size. Pars prostatica short, distinct from seminal vesicle and cirrus, surrounded by distinct prostatic cells. Cirrus follows recurved portion of cirrus-sac, armed with minute spines. Genital atrium distinct, unspined. Common genital pore small, immediately anterior to and sinistro-lateral to ventral sucker.

Ovary with three or four distinct lobes, just postero-dextral to ventral sucker, ventrally overlaps cirrus-sac, excretory vesicle, and right caecum in most specimens. Egg-forming complex not traced. Uterine seminal receptacle present. Vitellarium comprising dense lateral follicles extending from level of middle of oesophagus to level of anterior margin of testes, confluent only at anterior end, dorsally and ventrally overlap ovary, dorsally or ventrally overlap one or both testes. Uterine coils mainly in hindbody, never in post-testicular space, extending from middle of testes to just anterior to ventral sucker, usually with two broadly spiral coils, ventrally overlap ovary, testes and posterior end of cirrus-sac; ascending loop always partially overlaps ventral sucker, opens into genital atrium. Metraterm distinct, thick-walled, at anterior end of ascending loop of uterus. Terminal organ absent. Egg capsules with single long filament at anopercular end.

Excretory vesicle I-shaped, extends anteriorly between testes, reaches just posterior to posterior margin of cirrus-sac; anterior margin indiscernible in most specimens. Excretory pore terminal.

Helicometroides wardae n. sp. (Fig. 4A–C)

Type-host: *Plectorhinchus flavomaculatus* (Cuvier), goldspotted sweetlips (Perciformes: Haemulidae).

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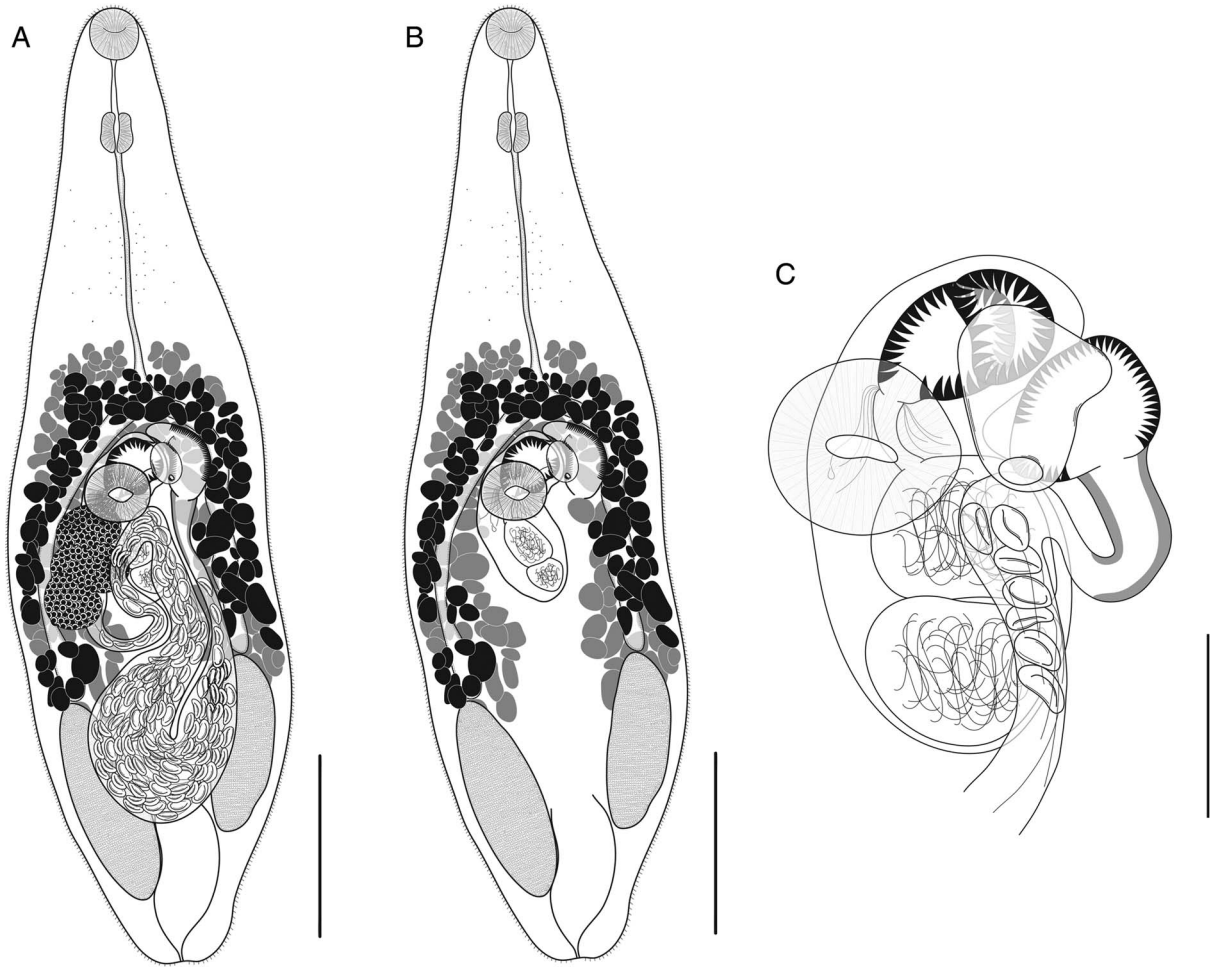

Fig. 4. *Helicometroides wardae* n. sp. from *Plectorhinchus flavomaculatus* from Australia. (A) Ventral view of *H. wardae* n. sp.; (B) ventral view of *H. wardae* n. sp., ovary and uterus not shown; (C) terminal genitalia of *H. wardae* n. sp. Scale bars: A and B: 300 μm; C: 100 μm.

Other hosts: *Plectorhinchus multivittatus* (MacLeay), many-lined sweetlips (Perciformes: Haemulidae).

Type-locality: off Heron Island, southern Great Barrier Reef, Queensland, Australia (23°27′S, 151°55′E).

Site of infection: Intestine.

Prevalence: 4/15 (26%) ex *P. flavomaculatus*; 1/5 (20%) ex *P. multivittatus.*

Deposition of specimens: Holotype and 18 paratypes, including hologenophores (QM G239483–G239501).

Molecular sequence data: ITS2 rDNA, four identical replicates, three from *P. flavomaculatus* and one from *P. multivittatus*, one from each host submitted to GenBank (GB OM230121–OM230122); 28S rDNA, three identical replicates, two from *P. flavomaculatus* and one from *P. multivittatus*, one from each host submitted to Genbank (GB OM230130 & OM260310); 18S rDNA, four identical replicates, all from *P. flavomaculatus*, one submitted to GenBank (GB OM230115); *cox*1 mtDNA, two identical replicates, both from *P. flavomaculatus*, one submitted to GenBank (GB OM156429).

ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The LSID for *H. wardae* n. sp. is urn:lsid:zoobank.org:act:11BEBFDF-F34B-4BC6-A87B-DCE677E70F57.

Etymology: The species is named in honour of Dr Selina Ward, in recognition of her constant encouragement, support and guidance.

Description (Measurements based on 19 gravid, unflattened specimens; measurements in Table 3). Body fusiform, widest in middle third of body. Forebody long. Tegument thin, covered with minute spines, with prominence decreasing slightly in hindbody. Eye-spot pigment granules obvious, restricted to forebody. Oral sucker terminal, roughly round, with opening immediately subterminal. Ventral sucker round, slightly larger than oral sucker. Prepharynx distinct. Pharynx oval, smaller than oral sucker. Oesophagus simple, long. Intestinal bifurcation in posterior forebody, distinctly anterior to ventral sucker; caeca lack thick gastrodermis, terminate in mid-hindbody just anterior to testes.

Testes noticeably elongate, in posterior hindbody, slightly oblique or symmetrical, never contiguous or overlapping, slightly overlap excretory vesicle dorsally. Post-testicular region short. Vas deferens not detected. Cirrus-sac in mid-body, reaches anteriorly and posteriorly beyond ventral sucker; anterior third not recurving posteriorly. Seminal vesicle bipartite; chambers approximately same size. Pars prostatica short, distinct from seminal vesicle and cirrus, surrounded by distinct prostatic cells. Cirrus armed with robust spines. Genital atrium distinct, unspined. Common genital pore small, immediately anterior to and sinistro-lateral to ventral sucker, or level with middle of and sinistro-lateral to ventral sucker.

Ovary with three or four distinct lobes, just postero-dextral to ventral sucker, ventrally overlaps cirrus-sac, dorsally overlaps ventral sucker and right caecum. Egg-forming complex not traced. Uterine seminal receptacle present. Vitellarium comprising dense lateral follicles extending from level of anterior margin of testes to

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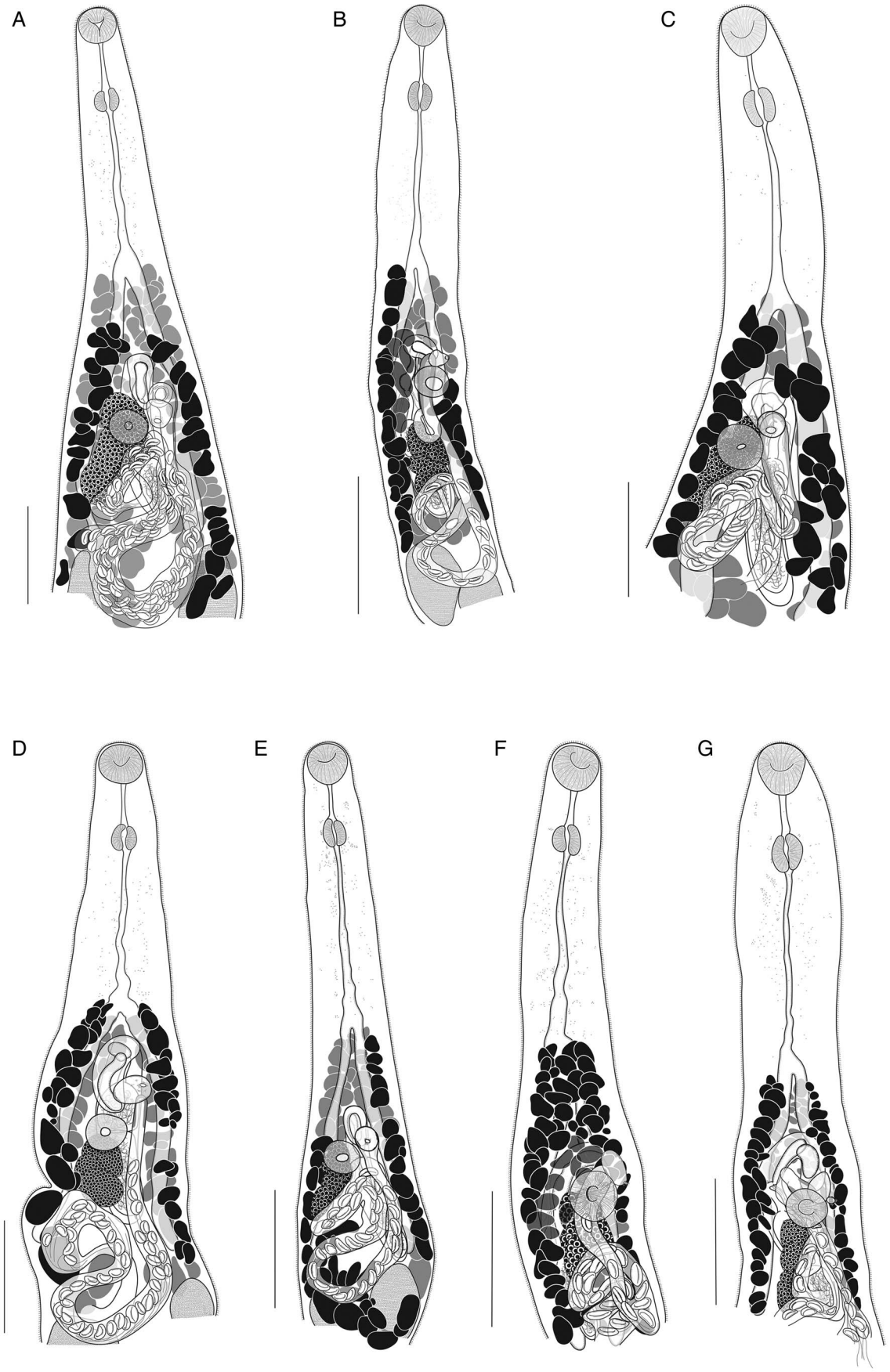

Fig. 5. Hologenophores of *Helicometroides longicollis* lineage 1 and 2 from Japan and Australia, illustrating the morphological similarity and variation between and within each species; variation can be exaggerated by the mounted position of a specimen (fully dorsoventral or semi-lateral). (A and B) *H. longicollis* lineage 1 from Japan; (C–E) *H. longicollis* lineage 1 from Australia; (F and G) *H. longicollis* lineage 2 from Japan. Scale bars: A–G: 200 μm.

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Table 4. Measurements of *Helicometroides longicollis* from present (both lineages) and previous studies

	H. longicollis (Yamaguti, 1934)	*H. longicollis* lineage 1 (Searle *et al.*, 2014)	*H. longicollis* lineage 1 (Japan)	*H. longicollis* lineage 1 (Australia, new material)	*H. longicollis* lineage 2
n	5 (holotype and 4 paratypes)	59	6 (all hologenophores)	17 (10 hologenophores)	3 (all hologenophores)
B L	1600–2000	685–1805 (1251)	–	1142–1569 (1338)	–
B W	390–630	100–454 (221)	–	152–308 (237)	–
FB L	–	381–1101 (719)	658–898 (800)	572–966 (702)	626–731 (665)
FB L/B L	–	51–67 (60)%	–	57–62 (59)%	–
OS L	73–95	30–76 (63)	70–79 (75)	57–83 (69)	72–85 (80)
OS W	120	38–83 (63)	74–82 (78)	59–75 (68)	75–79 (78)
VS L	80	–	62–80 (74)	51–84 (64)	73–78 (76)
VS W	63–95	40–92 (63)	65–83 (76)	51–92 (68)	75–82 (79)
OS W/VS W	1.26	0.76–1.39 (0.95)	0.93–1.14 (1.03)	0.74–1.28 (0.99)	0.95–1.05 (0.99)
Prepharynx	42–120	85–297 (136)	66–93 (76)	38–94 (62)	57–67 (63)
Pharynx L	53–70	31–64 (44)	46–56 (51)	40–62 (47)	45–67 (56)
Pharynx W	42–53	27–61 (37)	44–49 (48)	31–47 (38)	43–53 (48)
Oesophagus	320–380	221–485 (343)	258–399 (321)	169–422 (286)	252–290 (271)
RT L	210–300	88–225 (155)	–	140–219 (168)	–
RT W	130–200	32–114 (68)	–	51–101 (70)	–
LT L	220	–	–	128–194 (163)	–
LT W	180	–	–	46–86 (69)	–
Post T/B L	–	3.0–10.0 (6.0)%	–	4.2–9.7 (6.1)%	–
CS L	400	189–384 (272)	378–514 (434)	233–436 (347)	410–462 (436)
CS W	–	–	54–68 (60)	41–78 (58)	71–79 (76)
ASV L	–	–	72–88 (82)	38–105 (65)	61–84 (73)
ASV W	–	–	39–42 (41)	28–52 (38)	51–61 (57)
PSV L	–	–	108–122 (113)	46–123 (81)	150–182 (166)
PSV W	–	–	36–47 (40)	25–56 (41)	60–66 (64)
PP	–	–	49–84 (68)	42–88 (60)	38–60 (49)
Cirrus L	–	46–101 (62)	115–153 (137)	79–164 (115)	107–136 (117)
Cirrus W	–	–	20–31 (26)	14–27 (22)	20–26 (23)
Ovary L	170–290	108–215 (149)	188–241 (221)	119–212 (151)	122–208 (154)
Ovary W	110–170	39–123 (72)	74–123 (98)	44–80 (66)	84–140 (104)
Egg cap L	36.0–36.8	23–35 (29)	29–38 (32)	26–36 (31)	29–38 (33)
Egg cap W	18.4	11–20 (16)	12–19 (16)	14–23 (16)	14–20 (17)

B, body; L, length; W, width; FB, forebody; OS, oral sucker; VS, ventral sucker; RT, right testis; LT, left testis; Post T, post-testicular region; CS, cirrus-sac; ASV, anterior seminal vesicle; PSV, posterior seminal vesicle; PP, pars prostatica; Egg cap, egg capsule.
Measurements are in micrometres or percentages, means in parentheses

just anterior to intestinal bifurcation, confluent only anteriorly, separate in middle and posterior sections, dorsally overlaps ovary and testes. Uterine coils restricted to hindbody, never in post-testicular space, extending from middle of testes to ventral sucker, usually with two broadly spiral coils, ventrally overlap ovary, testes and posterior end of cirrus-sac; ascending loop partially overlaps ventral sucker in some specimens (not in drawn specimen), opens into posterior end of terminal organ. Metraterm distinct, thick-walled, at anterior end of ascending loop of uterus; contains large spines in some specimens (not in drawn specimen). Terminal organ unipartite, clearly differentiated from metraterm, short, with robust spines. Egg capsules with single long filament at anopercular end.

Excretory vesicle I-shaped, extends anteriorly between testes; anterior termination indiscernible. Excretory pore terminal.

Helicometroides longicollis species complex

Helicometroides longicollis Yamaguti, 1934

Type-host: Listed as '*Plectorhinchus pictus*' (authority not given).
Type-locality: off Tarumi, Japan.

Helicometroides longicollis lineage 1

Previous reports [see Searle *et al.* (2014)]

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Host: *Diagramma pictum labiosum* Macleay (Perciformes: Haemulidae).

Locality: off Heron Island, southern Great Barrier Reef, Queensland, Australia.

Representative DNA sequences: ITS2 rDNA (KJ658288); 28S rDNA (KJ658287).

New material from Japan (Fig. 5A and B)

Host: *Diagramma pictum pictum* (Thunberg).

Locality: off Minabe, Wakayama Prefecture, Japan (33°44′N, 135°19′E).

Site of infection: Intestine.

Prevalence: Unknown; sequences derived from three of four individual *D. pictum pictum* examined.

Deposition of specimens: Six vouchers, all hologenophores (MPM Coll. No. 21818).

Representative DNA sequences: ITS2 rDNA, six identical replicates, one submitted to GenBank (GB OM230123); 28S rDNA, three identical replicates, one submitted to GenBank (GB OM230131); 18S rDNA, two sequences, both submitted to GenBank (GB OM230116 & OM260311); *cox*1 mtDNA, two replicates, both submitted to GenBank (GB OM156430–OM156431).

Remarks: The specimens from Japan were collected from near the type-locality of *H. longicollis* (distance between the localities, <100 km), and broadly conform to the concept of the species. However, the true identity of these specimens remains uncertain, as a second morphologically indistinguishable species of *Helicometroides* infected the same individual fish. Measurements for these specimens are given in Table 4. The differentiation between the two species is considered in the discussion.

New material from Australia (Fig. 5C–E)

Host: *Diagramma pictum labiosum* Macleay.

Locality: off Heron Island, southern Great Barrier Reef, Queensland, Australia (23°27′S, 151°55′E); off Lizard Island, northern Great Barrier Reef, Queensland, Australia (14°40′S, 145°27′E).

Site of infection: Intestine.

Prevalence: 4/5 (80%), Heron Island; 4/30 (13%), Lizard Island.

New DNA sequences: ITS2 rDNA, 30 replicates, 29 identical, 25 from Heron Island, four from Lizard Island, one sequence from Lizard Island differs by two base pairs, an identical sequence from each location and the other sequence submitted to GenBank (identical sequences: GB OM230125–OM230126, other sequence: GB OM230124); 28S rDNA, 11 replicates, six from Heron Island, five from Lizard Island, one from each location submitted to GenBank (GB OM230132–OM230133); 18S rDNA, two identical replicates, both from Heron Island, one submitted to GenBank (GB OM230117); *cox*1 mtDNA, nine replicates, six from Heron Island, three from Lizard Island, a replicate of each genotype (four replicates) submitted to GenBank (GB OM156432–OM156435).

Remarks: All but one of the 30 ITS2 sequences of this species generated in this study (from both Australia and Japan) are identical to the sequence generated by Searle *et al.* (2014), with one sequence (from Lizard Island) differing at a single base position. Similarly, all but two 28S sequences generated in this study are identical to that generated by Searle *et al.* (2014), with two sequences (from Heron Island) differing at three base positions. Morphological measurements for 17 of these specimens, including 10 hologenophores, are given in Table 4.

Helicometroides longicollis lineage 2 (Fig. 5F and G)

Host: *Diagramma pictum pictum* (Thunberg).

Locality: off Minabe, Wakayama Prefecture, Japan (33°44′N, 135°19′E).

Site of infection: Intestine.

Prevalence: Unknown; sequences derived from two of four individual *D. pictum pictum* examined.

Deposition of specimens: Three vouchers, all hologenophores (MPM Coll. No. 21819).

Representative DNA sequences: ITS2 rDNA, four identical replicates, one submitted to GenBank (GB OM230127); 28S rDNA, one sequence, submitted to GenBank (GB OM230134); 18S rDNA, one sequence, submitted to GenBank (GB OM230118); *cox*1 mtDNA, two sequences, both submitted to GenBank (GB OM156436–OM156437).

Remarks: Hologenophores morphologically indistinguishable from those of *H. longicollis* lineage 1. Measurements for these specimens are given in Table 4.

Molecular phylogenetic analyses

ML and BI analyses of the 28S dataset produced phylograms with similar topologies (Fig. 2). The species of *Helicometroides* form a well-supported clade with identical strongly supported internal nodes in both analyses. There are three key differences between the two analyses. First, the relative phylogenetic positions of *C. pudica* and *Helicometroides* spp. are swapped in the two analyses, with the most basal monorchiid in the BI analysis being *C. pudica*, and in the ML analysis, *Helicometroides* spp. This instability has been observed previously in recent phylogenetic analyses (Wee *et al.*, 2021*b*). The second difference involves the phylogenetic position of *Postmonorchis orthopristis* Hopkins, 1941. In the BI analysis, it resolves as sister to *Genolopa vesca* Panyi *et al.*, 2020, and this clade is sister to the other represented species of *Genolopa* Linton, 1910; the position of *P. orthopristis* in this analysis is weakly supported. In the ML analysis, *P. orthopristis* resolves sister to all the species of *Genolopa* with strong nodal support. Finally, in the BI analysis, *Retroporomonorchis pansho* Wee *et al.*, 2020 is sister to a clade comprising *Monorchis monorchis* (Stossich, 1890) Looss, 1902, species of *Sinistroporomonorchis* Wee *et al.*, 2020, and a species identified only as '*Lasiotocus* sp'. In the ML analysis, *R. pansho* resolves sister to a clade comprising the aforementioned species, as well as *Infundibulurictus arrhichostoma* (Searle *et al.*, 2014) Wee *et al.*, 2020, *Monorchis lewisi* Cribb *et al.*, 2018, and the species of *Allobacciger* Hafeezullah and Siddiqi, 1970. The position of *R. pansho* in both arrangements is weakly supported.

Discussion

Genus concept

In both analyses of the 28S dataset presented in this study, the five species of *Helicometroides* recognized form a strongly-supported clade. Given the strength of this clade, it is inferred that the presence of a spined terminal organ is a labile character for *Helicometroides*. Four species, *H. longicollis* lineage 1 and 2, *H. murakamii* n. sp., and *H. gabrieli* n. sp., lack terminal organs but possess metraterms, which conforms to the previous generic diagnosis of *Helicometroides*. However, *H. wardae* n. sp. possesses a distinct terminal organ that has robust spines. In this, it is consistent with typical monorchiids; species from 49 of 54 other monorchiid genera are reported to possess a spined terminal organ. Of the five remaining genera, the species of *Cableia* Sogandares-Bernal, 1959, *Opisthomonorchis* Yamaguti, 1952 and *Pseudopisthomonorchis* Madhavi, 1974 do not possess a terminal organ, while some species of *Hurleytrematoides* and all species of *Pseudoproctotrema* Yamaguti, 1942 have unspined terminal

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organs. Given that *Helicometroides* comprises species with or without a spined terminal organ, and *Hurleytrematoides* species have spined or unspined terminal organs, the form of the terminal organ is clearly labile for some groups of monorchiids. It will be necessary to establish the phylogenetic position of the species of *Pseudoproctotrema*, *Opisthomonorchis* and *Pseudopisthomonorchis* to comprehend the evolution of terminal organ forms within the Monorchiidae. Notably, symmetrical testes have been previously used as a uniting morphological feature for *Helicometroides* (see Madhavi, 2008), despite *H. atlanticus* possessing somewhat oblique testes (Gaevskaya and Aleshkina, 1983); *H. murakamii* n. sp. and *H. gabrieli* n. sp. characterized here also possess distinctly oblique testes, as do some specimens of *H. wardae* n. sp. and *H. longicollis*. With the loss of a lack of a terminal organ and symmetrical testes as features common to all *Helicometroides* species, the genus is now best united by the possession of a distinctly helical uterus, filamented eggs, and well-developed and extensive vitelline follicles. The distinctly helical uterus easily distinguishes *Helicometroides* from all other monorchiid genera. Filamented eggs are relatively common in the Monorchiidae, with species from 10 genera (of the Anamonorchiinae Yamaguti, 1970, Hurleytrematinae Yamaguti, 1958 and Opisthomonorchiinae Yamaguti, 1952) reported to possess filamented eggs, but notably, the feature is labile in some genera (see *Provitellus* in Wee *et al*., 2019). Extensive vitelline follicles are uncommon in the Monorchiidae. Only species of three other genera, *Cableia*, *Hurleytrema* Srivastava, 1939, and *Octotestis* Yamaguti, 1951, have a comparable vitelline follicle range (ranging from at least the level of the ventral sucker to the testes).

Recognition of species

The conclusion is that the present samples represent four morphospecies, of which one, *H. longicollis*, is a complex of two cryptic species. Below, the validity of the three morphologically distinct forms, and the issues associated with *H. longicollis*, is considered.

Helicometroides murakamii n. sp.

Helicometroides murakamii n. sp. most closely resembles *H. pseudovitellosus* (from *Lutjanus* sp. [Lutjanidae], Bay of Bengal, India) in body size, range of the vitelline follicles and position of the ovary, cirrus-sac and testes. However, it differs in the possession of a much longer oesophagus (at least twice as long as the prepharynx *vs* about as long as the prepharynx), and oblique, contiguous testes (*vs* symmetrical, non-contiguous testes). It also resembles *H. vitellosus* (from *Plectorhinchus* sp. [Haemulidae], New Caledonia) in body shape and size, cirrus-sac shape, position and size, and shape and position of the ovary. The two species differ in the anterior extent of the vitelline follicles (level of intestinal bifurcation in *H. murakamii* n. sp. *vs* distinctly anterior to the intestinal bifurcation in *H. vitellosus*), the shape of the testes (globular in *H. murakamii* n. sp. *vs* distinctly elongate in *H. vitellosus*), the posterior extent of the testes (distinctly anterior to the posterior margin of the body in *H. murakamii* n. sp. *vs* immediately anterior to the posterior margin of the body in *H. vitellosus*) and cirral spines (spines lining the entire length of the cirrus in *H. murakamii* n. sp. *vs* absent from the middle third of the cirrus in *H. vitellosus*). *Helicometroides murakamii* n. sp. is easily differentiated from *H. atlanticus* [from *Parapristipoma octolineatum* (Valenciennes) [Haemulidae], from the coast of Angola (see Gaevskaya and Aleshkina, 1983), and from Dakar (see Diagne *et al*., 2015), east Atlantic Ocean] by the presence of a long, distinct prepharynx (*vs* very short), the anterior extent of the vitelline follicles (reaches the level of the intestinal bifurcation *vs* reaches distinctly anterior to the intestinal bifurcation) and the position of the ovary (anterior to the right testis *vs* anterior to the left testis). It differs from *H. longicollis* in the morphology of the vitelline follicles (not contiguous at the posterior end *vs* contiguous at the posterior end), length of prepharynx (28–65 *vs* 85–294), position of the ovary (just anterior to the left testis *vs* distinctly anterior to the left testis) and shape of the testis (globular *vs* elongate). It is most easily differentiated from *H. leiperi* [from *Otolithes ruber* (Bloch and Schneider) [Sciaenidae], Puri Coast, Bay of Bengal, India] in the range and extent of the vitelline follicles (from the level of the intestinal bifurcation to the level of the testes *vs* from just posterior to the intestinal bifurcation to the anterior region of ovary), position of the ovary (just anterior to the right testis *vs* in the middle of the body, distinctly pre-testicular), extent of caeca (terminate at the middle of the hindbody *vs* terminate near the posterior end of the hindbody) and size of the eggs (31–38 × 14–21 *vs* 55–61 × 26–29).

Helicometroides gabrieli n. sp.

In the possession of vitelline follicles that extend well-anteriorly beyond the intestinal bifurcation, reaching approximately the mid-oesophagus level, *H. gabrieli* n. sp. closely resembles *H. atlanticus* and *H. vitellosus*; all other species have a much more restricted vitelline distribution, with the follicles not extending anterior to the intestinal bifurcation, or only just anterior to it. *Helicometroides gabrieli* n. sp. is easily differentiated from *H. atlanticus* in the possession of a bipartite seminal vesicle (*vs* unipartite seminal vesicle) as well as a distinctly longer prepharynx, and from *H. vitellosus* in the lack of a gap in the cirral spines (*vs* middle third lacking spines) and oblique testes (*vs* symmetrical testes).

Helicometroides wardae n. sp.

Helicometroides wardae n. sp. is easily differentiated from all other species of *Helicometroides* in the possession of a spined terminal organ. It should be noted that it is also the only species in which some specimens have been observed to possess large spines in the metraterm; the drivers behind this variation remain unknown. Additionally, it has an ovoid and wide cirrus with large, robust spines, which further separates it from all other species of *Helicometroides*; *H. atlanticus*, *H. longicollis* and *H. vitellosus* each has a thin, elongate cirrus with small spines, and *H. leiperi* has an elongate cirrus with both long and short spines. While the description of *H. pseudovitellosus* lacks detail about the cirrus and cirral spines, the illustration of an elongated cirrus leads us to think that the cirral spines are small and thin. The other new species, *H. murakamii* n sp. and *H. gabrieli* n. sp., each also possesses a narrow cirrus with small spines.

Helicometroides longicollis lineage 1 and lineage 2

The interpretation of genetic variation in marine fish trematodes for the recognition of species is receiving substantial attention, particularly in the light of ITS2 and *cox*1 sequence data (Bray *et al*., 2021). Recent studies in the tropical Indo-west Pacific using the *cox*1 region have frequently identified morphologically indistinguishable trematodes in the same or comparable fishes forming distinct clades based on the geographical distribution (allopatric populations) as reflecting intraspecific variation rather than distinct species. Such findings have been reported for six species of the Aporocotylidae Odhner, 1912 (Cutmore *et al*., 2021), three species of the Gorgocephalidae Manter, 1966 (Huston *et al*., 2021), three species of the Lepocreadiidae Odhner, 1905 (Bray *et al*., 2018, 2021) and seven species of the Monorchiidae Odhner, 1911 (McNamara *et al*., 2014; Wee *et al*., 2021*a*). These intraspecific variations are much reduced in the ITS2 region (in some cases, the differences in the ITS2 region disappear entirely), resulting in the interpretation of a single population

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(one clade) rather than multiple populations (distinct clades). For example, *Preptetos paracaballeroi* Bray *et al.*, 2021 occurs at Heron and Lizard Islands where the populations differ by 27–31 base pairs in the *cox*1 region while there are no differences at all for the ITS2 region. Such dramatic conflict in genetic variation between *cox*1 and ITS2 sequences has presented a significant obstacle in the interpretation of the status of some populations. These issues led Bray *et al.* (2021) to propose a model for the recognition of species. Specifically, they proposed that, where appropriate data are available, a species should be recognized as distinct only if it comprises a monophyletic lineage in at least the most discriminating available molecular marker (in most recent cases, *cox*1), and has consistent morphological differences relative to other species or a host distribution distinct from that of closely related species. By these criteria, all the cases mentioned above can be interpreted as involving single, widespread species showing intraspecific geographical population structures. However, the nature of the *H. longicollis* complex presents components not accounted for by the criteria proposed by Bray *et al.* (2021). Specifically in this system, cryptic morphology is paired with sympatric lineages identified from a recombinant region (ITS2) in addition to a non-recombining region (*cox*1). In none of the cases described above was there sympatric co-occurrence of morphologically indistinguishable ITS2 rDNA lineages. The analyses of McNamara *et al.* (2014) hint at such a possibility for *Hurleytrematoides loi* McNamara and Cribb, 2011 and *H. sasali* McNamara and Cribb, 2011, but there is insufficient replication for the result to be compelling.

Sequencing of specimens collected from *D. pictum pictum* from Japan and *D. pictum labiosum* from Australia that corresponded to the morphotype of *H. longicollis* revealed two genotypes in the ITS2 and 28S datasets, and three genotypes in the *cox*1 dataset. In the first two datasets, the genotypes were separated into two forms, one that occurred in both *D. pictum pictum* and *D. pictum labiosum* from Japan and Australia, respectively, and another restricted to *D. pictum pictum* from Japan. In the *cox*1 dataset, the genotypes were separated into three forms, one from Australia, and two from Japan, with the genotype occurring in both Japan and Australia in the ITS2 and 28S datasets separated into distinct genotypes. Importantly, the genetic differences between the Japanese and Australian genotypes that were the same genotype in the ITS2 and 28S datasets (33–36 base pairs) are significantly less than their differences from the other genotype (51–53 base pairs). Considering the condition of the ITS2 and 28S datasets, the two genotypes with smaller genetic difference in the *cox*1 dataset are interpreted as representing a single species, and the other genotype as a second species. The key aspect that affects the interpretation of these data is that the two distinct genotypes from Japan occur sympatrically, including within the same individual host fish. As such, the two genotypes are interpreted as representing two truly cryptic species that correspond morphologically to *H. longicollis*. These circumstances contrast to the findings of Bray *et al.* (2021) who showed that *Preptetos laguncula* Bray and Cribb, 1996 and *P. zebravaranus* Bray *et al.*, 2021 are each represented by two *cox*1 clades which may co-occur in the same fish individual. However, those *cox*1 clades had identical ITS2 sequences, consistent with the interpretation of one species.

At present, there is no way to determine which of the two species is the true *H. longicollis*, given that no genetic sequence data exist for the original collection of the species. Thus, the two species can only be recognized as *H. longicollis* lineage 1 and lineage 2, with the former occurring in both Japan and Australia, and the latter so far restricted to Japan. It is hoped that new morphological material may reveal a reliable morphological basis for the distinction of these species and that the distinction will be recognizable in the holotype of *H. longicollis*.

Key to the species of Helicometroides

The inclusion of the three new species brings the number of formally characterized *Helicometroides* spp. to eight and introduces some important morphological differences between them. A dichotomous key is provided here to differentiate them.

1a. Terminal organ present, armed with robust spines ..... *Helicometroides wardae* n. sp.
1b. Terminal organ absent 2

2a. Distinct gap in cirral spines, with middle third of cirrus lacking spines ..... *H. vitellosus*
2b. No gap in cirral spines 3

3a. Vitelline follicles extend posteriorly only to middle of body, well anterior to testes; follicles not confluent anteriorly or posteriorly ..... *H. leiperi*
3b. Vitelline follicles extend posteriorly to level of testes, does not extend into post-testicular zone; follicles confluent at one or both ends 4

4a. Oesophagus shorter than prepharynx .... *H. pseudovitellosus*
4b. Oesophagus distinctly longer than prepharynx 5

5a. Seminal vesicle unipartite ..... *H. atlanticus*
5b. Seminal vesicle bipartite 6

6a. Vitelline follicles extend well anterior to intestinal bifurcation *Helicometroides gabrieli* n. sp.
6b. Vitelline follicles extend to level of or just posterior to intestinal bifurcation 7

7a. Testes globular; oesophagus <4× pharynx length ..... *Helicometroides murakamii* n. sp.
7b. Testes elongate; oesophagus >4× pharynx length *H. longicollis* (species complex)

Host exploitation

Species of *Helicometroides* have been reported from three families of marine bony fishes: Haemulidae, Lutjanidae and Sciaenidae. The haemulids are the most heavily parasitized group; four species from three genera harbour six species of *Helicometroides*. Haemulid fishes as a major host group for *Helicometroides* is consistent with the Monorchiidae as a whole; to our knowledge, 47 species of monorchiids from 18 genera infect a wide range of haemulids. Sciaenid fishes are significantly less parasitized by monorchiids; to our knowledge, 10 species of monorchiids from five genera have been reported from the family, with a single species of *Helicometroides*, *H. leiperi*, reported only from *O. ruber* (see Ahmad, 1982). Wee *et al.* (2020*a*) observed that most reports of monorchiid infections in lutjanid fishes are unconvincing; just two are well-supported with evidence. Wee *et al.* (2020*a*) suggested that the two lutjanids reported as hosts of *H. pseudovitellosus* (see Madhavi, 1974) and *H. vitellosus* (see Durio and Manter, 1968) were likely haemulids as neither host was identified to species, lutjanids are easily confusable with haemulids, and as species of *Helicometroides* are known overwhelmingly from haemulids.

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