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Research paper

Lepocreadiidae (Trematoda) associated with gelatinous zooplankton (Cnidaria and Ctenophora) and fishes in Australian and Japanese waters

Thomas H. Cribb [a,b,*], Scott C. Cutmore [b], Nicholas Q.-X. Wee [b], Joanna G. Browne [c,d], Pablo Diaz Morales [e], Kylie A. Pitt [c]

[a] School of the Environment, The University of Queensland, St Lucia, QLD 4072, Australia
[b] Queensland Museum, Biodiversity and Geosciences Program, South Brisbane, Queensland 4101, Australia
[c] School of Environment and Science and Australian Rivers Institute, Griffith University, Gold Coast Campus, Gold Coast, Queensland 4222, Australia
[d] Museums Victoria, GPO Box 666, Melbourne, Victoria 3001, Australia
[e] Sea Jellies Illuminated, Sea World, Main Beach, QLD 4217, Australia

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ABSTRACT

We examined gelatinous zooplankton from off eastern Australia for lepocreadiid trematode metacercariae. From 221 specimens of 17 species of cnidarian medusae and 218 specimens of four species of ctenophores, infections were found in seven cnidarian and two ctenophore species. Metacercariae were distinguished using *cox*1 mtDNA, ITS2 rDNA and morphology. We identified three species of *Prodistomum* Linton, 1910 [*P. keyam* Bray & Cribb, 1996, *P. orientale* (Layman, 1930), and *Prodistomum* Type 3], two species of *Opechona* Looss, 1907 [*O. kahawai* Bray & Cribb, 2003 and *O.* cf. *olssoni*], and *Cephalolepidapedon saba* Yamaguti, 1970. Two species were found in cnidarians and ctenophores, three only in cnidarians, and one only in a ctenophore. Three Australian fishes were identified as definitive hosts; four species were collected from *Scomber australasicus* and one each from *Arripis trutta* and *Monodactylus argenteus*. Transmission of trematodes to these fishes by ingestion of gelatinous zooplankton is plausible given their mid-water feeding habits, although such predation is rarely reported. Combined morphological and molecular analyses of adult trematodes identified two *cox*1 types for *C. saba*, three *cox*1 types and species of *Opechona*, and six *cox*1 types and five species of *Prodistomum* of which only two are identified to species. All three genera are widely distributed geographically and have unresolved taxonomic issues. Levels of distinction between the recognised species varied dramatically for morphology, the three molecular markers, and host distribution. Phylogenetic analysis of 28S rDNA data extends previous findings that species of *Opechona* and *Prodistomum* do not form monophyletic clades.

1. Introduction

A growing body of work over the last two decades has shown that, contrary to earlier thought [1], the gelatinous zooplankton comprising cnidarian medusae and ctenophores is important in the diet of wide range of marine fishes [2,3]. This dietary connection has potential importance for parasite transmission, especially as there are many records of larval parasites in gelatinous zooplankton [4]. Two superfamilies of trematodes dominate these reports – the Hemiuroidea and the Lepocreadioidea (specifically the Lepocreadiidae); transmission from the first intermediate hosts differ fundamentally for these two groups. For the Hemiuroidea, typically the cystophorous cercaria produced in the first intermediate host is consumed by a small crustacean which in turn may be consumed by a third intermediate host (including gelatinous zooplankton), which is followed by the eventual transmission to the definitive host. Lepocreadioids typically have their cercariae penetrate the second intermediate host (including gelatinous zooplankton), which is followed by the ingestion of that host by the definitive host; there is no suggestion of cycles requiring more than three hosts. Thus, hemiuroid life cycles are typically four-host whereas lepocreadioids are typically three-host. The differences in transmission likely have implications for the overall role of gelatinous zooplankton in the transmission of the two groups of trematodes.

This report considers trematodes of the family Lepocreadiidae. There is a surprisingly limited literature on the second intermediate hosts for this family; we have found reports for species of only seven of the 50

* Corresponding author at: School of the Environment, The University of Queensland, St Lucia, QLD 4072, Australia.
E-mail address: T.cribb@uq.edu.au (T.H. Cribb).

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recognised genera. As far as we can detect, the first convincing report of a lepocreadiid metacercaria was by Lebour [5], who studied metacercariae of a species of *Opechona* Looss, 1907 from four species of cnidarian medusae and one ctenophore, off Plymouth, England. Subsequent reports relate to *Cephalolepidapedon* Yamaguti, 1970, *Clavogalea* Bray, 1985, *Diploproctodaeum* La Rue, 1926, *Lepocreadium* Stossich, 1904, *Lepotrema* Ozaki, 1932, and *Opechonoides* Yamaguti, 1940. The report of the metacercaria for *Diploproctodaeum* is unique for the family in involving encystment in the open without a second intermediate host [6]. Reports for the other six genera are uniformly of unencysted metacercariae and for all include, but are not necessarily restricted to, cnidarians or ctenophores as intermediate hosts: *Cephalolepidapedon* [4,7]; *Clavogalea* [8]; *Lepocreadium* [9–16]; *Lepotrema* [7]; *Opechona* [4,5,7,17–29]; and *Opechonoides* [30]. Most reports of lepocreadiid metacercariae have based identification to species on experimental infections or morphological similarity between metacercariae and adults. However, recently, molecular approaches have been used to associate life cycle stages [4,8,13,30] and this approach shows great promise for definitive identification of these parasites.

In this study we use a combined molecular and morphological approach to associate and identify lepocreadiids from Australian gelatinous zooplankton and fishes. We demonstrate a small but distinctive fauna of lepocreadiids that can be inferred to be transmitted through gelatinous zooplankton to fishes that are plausible as predators of the gelatinous zooplankton involved, although not necessarily recognised as such. The patterns of host-specificity to second intermediate hosts vary, consistent with existing reports from the literature. Perhaps unsurprisingly, we find significant issues in species identification for all three of the genera that we report.

2. Materials and methods

2.1. Collection of jellyfish, fishes, and parasites

Gelatinous zooplankton (cnidarian medusae and ctenophores) were collected using scoop nets deployed from small boats within Moreton Bay, Queensland, and in offshore waters during two voyages on RV Investigator. The first voyage, during the austral spring (11–27 September 2019) sampled waters off southeast Queensland and the second voyage, during the austral autumn (14 May to 2 June 2021) sampled waters along Australia's east coast between Tasmania and southeast Queensland. During both voyages gelatinous zooplankton were collected from surface waters using a long-handled pool scoop lowered over the side of the ship and by bongo nets (mouth diameter: 0.7 m; mesh size: 500 μm) that were towed obliquely from the surface to ~35 m depth at night. Animals collected using scoop nets remained in good condition and could be easily identified but some individuals (particularly ctenophores and some hydrozoan medusae) collected by the bongo net were damaged and could not be identified to species. Identifications of cnidarian medusae and ctenophores were made principally with reference to Bouillon et al. [31] and Gershwin et al. [32,33]. Several identifications were problematic and reflect the fact that the taxonomy of this fauna cannot be considered settled. One species identified here, *Chrysaora kynthia* Gershwin & Zeidler, was consistent with the original description [34], but we note that this species is presently considered a *nomen dubium* in WoRMS [35].

Fishes were collected by the authors from multiple sites off Australia and a range of locations in the tropical Indo-west Pacific. Gastrointestinal trematodes were collected from freshly killed fishes, fixed by pipetting into nearly boiling saline solution, and preserved in 70–80% ethanol to enable parallel morphological and molecular analysis.

2.2. Morphological analysis

Specimens for morphological analysis were washed in distilled water, stained with Mayer's haematoxylin, destained in 1% HCl acid, neutralised in 1% ammonia solution, dehydrated through a graded ethanol series, cleared in methyl salicylate, and mounted on slides in Canada balsam. Measurements were made using an Olympus SC50 digital camera mounted on an Olympus BX-53 compound microscope using cellSens Standard imaging software and are given in micrometres (μm). Specimens were drawn using a *camera lucida* and digitized with Adobe Illustrator CC 2018. In the figures, tegumental spines are shown only around the oral sucker of specimens relating to the genus *Cephalolepidapedon* and on the tegument of specimens relating to *Opechona*. Although tegumental spines were detected on all the specimens relating to *Cephalolepidapedon* and *Prodistomum* Linton, 1910, they are too small to be drawn realistically. Voucher specimens of adult and metacercarial specimens were lodged in the Queensland Museum (QM), Brisbane, Queensland, Australia and the Meguro Parasitological Museum (MPM), Tokyo, Japan.

Table 1
Collection data and GenBank accession numbers for lepocreadiid ITS2 data included in phylogenetic analyses.

Species	Host species	GenBank accession #	Reference
Lepocreadiidae			
Cephalolepidapedon warehou	*Cyanea annaskala*	MT773345	[4]
Cephalolepidapedon warehou	*Seriolella brama*	MT773347	[4]
Clavogalea trachinoti	*Trachinotus coppingeri*	MH157057	[55]
Lepocreadium album	Unknown	MK418259	Unpublished
Lepocreadium oyabitcha	*Abudefduf sordidus*	OM777008	[30]
Lepocreadium trulla	*Lutjanus campechanus*	KU527433	[95]
Opechona austrobacillaris	*Pomatomus saltatrix*	MH157063	[55]
Opechona sp.	*Buccinanops cochlidium*	KF451939	[96]
Opechonoides opisthoporus	*Pomacentrus moluccensis*	OM777017	[30]
Prodistomum keyam	*Monodactylus argenteus*	MH157064	[55]
Prodistomum orientale	*Scomber australasicus*	MT773350	[4]
Prodistomum Type 3	*Scomber australasicus*	MT773352	[4]
Lepocreadiidae gen. sp. 1	*Crepipatella dilatata*	KF451933	[56]
Lepocreadiidae gen. sp. 2	*Pareuthria plumbea*	KF451935	[56]
Lepocreadiidae gen. sp. 3	*Scomber japonicus*	KF451937	Unpublished
Outgroup taxa			
Lepotrema adlardi	*Abudefduf bengalensis*	MH730000	[92]
Lepotrema amblyglyphidodonis	*Amblyglyphidodon curacao*	MH730003	[92]
Lepotrema melichthydis	*Melichthys vidua*	MH730008	[92]
Lepotrema moretonense	*Prionurus microlepidotus*	MH730013	[92]

2.3. Molecular analyses

Specimens for molecular analyses were processed according to the protocols used by Cribb et al. [36]. Three genetic markers were sequenced, the cytochrome *c* oxidase subunit 1 mitochondrial region (*cox*1 mtDNA), the second internal transcribed spacer region (ITS2 rDNA), and the large (28S) ribosomal subunit RNA coding region. *cox*1 and ITS2 sequence data were generated for all host/parasite localities, and 28S sequence data were generated for all *cox*1 genotypes. The partial *cox*1 region was amplified and sequenced using the primers Dig_cox1Fa [37] and Dig_cox1R [37], the complete ITS2 rDNA region using 3S [38] and ITS2.2 [39], and the partial D1-D3 28S rDNA region using LSU5 [40], 300F [41], ECD2 [42] and 1500R [43]. Geneious® version 10.2.3 [44] was used to assemble and edit contiguous sequences. Sequence data are lodged on GenBank under the accession numbers

Table 2
Collection data and GenBank accession numbers for lepocreadiid 28S data included in phylogenetic analyses.

Species	Host species	GenBank accession #	Reference
Lepocreadiidae			
Bianium arabicum	*Lagocephalus lunaris*	MH157076	[55]
Bianium plicitum	*Sphoeroides testudineus*	MZ345682	[81]
Clavogalea trachinoti	*Trachinotus coppingeri*	MH157067	[55]
Deraiotrema platacis	*Platax pinnatus*	MN073841	[97]
Diplocreadium tsontso	*Balistoides conspicillum*	FJ788472	[98]
Diploproctodaeum momoaafata	*Ostracion cubicus*	FJ788474	[98]
Diploproctodaeum monstrosum	*Arothron stellatus*	FJ788473	[98]
Echeneidocoelium indicum	*Echeneis naucrates*	FJ788475	[98]
Hypocreadium lamelliforme	*Balistes capriscus*	MZ345680	[81]
Hypocreadium myohelicatum	*Balistes polylepis*	MK648295	[99]
Hypocreadium patellare	*Balistoides viridescens*	FJ788478	[98]
Hypocreadium picasso	*Rhinecanthus aculeatus*	FJ788479	[98]
Hypocreadium toombo	*Pseudobalistes fuscus*	FJ788480	[98]
Lepidapedoides angustus	*Epinephelus cyanopodus*	FJ788482	[98]
Lepocreadium oyabitcha	*Abudefduf sordidus*	OM777006	[30]
Lepocreadium trulla	*Rhomboplites aurorubens*	KU527432	[95]
Lepotrema acanthochromidis	*Acanthochromis polyacanthus*	MH730014	[92]
Lepotrema adlardi	*Abudefduf bengalensis*	MH730015	[92]
Lepotrema amansis	*Amanses scopas*	MH730016	[92]
Lepotrema amblyglyphidodonis	*Amphiprion akindynos*	MH730017	[92]
Lepotrema cirripectis	*Cirripectes chelomatus*	MH730018	[92]
Lepotrema hemitaurichthydis	*Hemitaurichthys polylepis*	MH730020	[92]
Lepotrema melichthydis	*Melichthys vidua*	MH730021	[92]
Lepotrema monile	*Pomacentrus wardi*	MH730024	[92]
Lepotrema moretonense	*Prionurus microlepidotus*	MH730023	[92]
Lobatocreadium exiguum	*Pseudobalistes fuscus*	FJ788484	[98]
Mobahincia teirae	*Platax teira*	MH157068	[55]
Multitestis magnacetabulum	*Platax teira*	MH157071	[55]
Neohypocreadium dorsoporum	*Chaetodon flavirostris*	FJ788487	[98]
Neomultitestis aspidogastriformis	*Platax teira*	MH157072	[55]
Neopreptetos arusettae	*Pomacanthus sexstriatus*	FJ788490	[98]
Opechona austrobacillaris	*Pomatomus saltatrix*	MH157073	[55]
Opechona chloroscombri	*Chloroscombrus chrysurus*	MZ345679	[81]
Opechona corkumi	*Peprilus burti*	MZ345683	[81]
Opechona olssoni	*Scomber japonicus*	MT303947	[68]
Opechona pharyngodactyla	*Trachinotus rhodopus*	OQ676201	[100]
Opechona sp.	*Buccinanops cochlidium*	KF451939	[96]
Opechonoides opisthoporus	*Abudefduf whitleyi*	OM777005	[30]
Pelopscreadium spongiosum	*Ostracion cubicus*	FJ788469	[98]
Preptetos allocaballeroi	*Naso tonganus*	MZ702002	[53]
Preptetos cannoni	*Siganus lineatus*	MZ701993	[53]
Preptetos laguncula	*Naso lituratus*	MZ701986	[53]
Preptetos paracaballeroi	*Naso brevirostris*	MZ702004	[53]
Preptetos pearsoni	*Acanthurus mata*	MZ702007	[53]
Preptetos prudhoei	*Zebrasoma scopas*	MZ701995	[53]
Preptetos quandamooka	*Prionurus maculatus*	MZ702009	[53]
Preptetos zebravaranus	*Zebrasoma scopas*	MZ701999	[53]
Prodistomum alaskense	*Aptocyclus ventricosus*	MT303950	[68]
Prodistomum keyam	*Monodactylus argenteus*	MH157074	[55]
Prodistomum orientale	*Scomber japonicus*	MT299625–6	[68]
Prodistomum priedei	*Epigonus telescopus*	AJ405272	[101]
Lepocreadiidae gen. sp. 1	*Crepipatella dilatata*	KF451933	[56]
Lepocreadiidae gen. sp. 2	*Pareuthria plumbea*	KF451935	[56]
Lepocreadiidae gen. sp. 3	*Scomber japonicus*	KF451937	Unpublished
Lepocreadiidae sp.	*Rhizostoma pulmo*	OM910739	[8]
Outgroup taxa			
Aephnidiogenes major	*Diagramma pictum labiosum*	FJ788468	[98]
Austroholorchis sprenti	*Sillago maculata*	MH157075	[55]
Holorchis castex	*Diagramma pictum pictum*	FJ788476	[98]
Holorchis gigas	*Plectorhinchus chrysotaenia*	FJ788477	[98]

PP270064–121 and PP272965–988 (*cox*1), PP239516–551 (ITS2), and PP239552–561 (28S).

*cox*1 sequence data generated during this study were aligned in MEGA 7 [45], with UPGMA clustering for iterations 1 and 2. Alignments were transferred to Mesquite v.3.31, translated (echinoderm/flatworm mitochondrial code) and inspected for internal stop codons. After the correct reading frame was determined, the first column was removed so that the reading frame began on position one, simplifying position-coding in downstream analyses. All trimmed *cox*1 sequences were 474 base positions (bp). All codon positions in the *cox*1 datasets were evaluated for substitution saturation using the "Test of substitution saturation by Xia et al." function [46,47] as implemented in DAMBE v. 7.2 [48]; substitution saturation was not detected in any *cox*1 dataset. Unrooted neighbour joining analyses were conducted using MEGA 7, with the following parameters: "Model/Method = No. of differences", "Substitutions to Include = d: Transitions + Transversions", "Rates among Sites = Gamma Distributed", and "Gaps/Missing Data Treatment = Pairwise deletion". Nodal support for all Neighbour joining analyses were estimated by performing 10,000 bootstrap replicates.

ITS2 data generated during this study were aligned with sequence data available on GenBank (Table 1) in MEGA 7, with UPGMA clustering for iterations 1 and 2. The ends of the 5.8S-ITS2-28S rDNA alignment were trimmed for a final dataset of 477 bp. Rooted neighbour joining analyses were conducted as described above for *cox*1 datasets, with species of *Lepotrema* designated as functional outgroup taxa.

The partial 28S rDNA data generated during this study were aligned with sequence data available on GenBank (Table 2) using MUSCLE version 3.7 [49] run on the CIPRES portal, with ClustalW sequence weighting and UPGMA clustering for iterations 1 and 2. The resultant alignment was refined by eye using Mesquite v.3.31; the ends of the alignment were trimmed, and indels constituting more than three bp and present in >5% of the sequences in the dataset were removed (leaving a final trimmed dataset of 1314 bp). Bayesian inference analysis was performed using MrBayes version 3.2.7 [50] and maximum likelihood analysis using RAxML version 8.2.12 [51], both run on the CIPRES portal. The best nucleotide substitution model was estimated using jModelTest version 2.1.10 [52]. Both the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) predicted the GTR + I + Γ as the best estimator; Bayesian inference and maximum likelihood

Table 3
Species and numbers of gelatinous zooplankton species sampled for lepocreadiid metacercariae from off the Queensland coast.

Taxon	Species	n
Cnidaria, Hydrozoa	*Aegina citrea*	19
	Aequorea sp.	12
	Aldersladia magnificus	45
	Eutima sp.	26
	Geryonia proboscidalis	6
	Hydrozoa 1	12
	Hydrozoa 2	20
	Hydrozoa 3	1
	Hydrozoa 4	3
	Liriope tetraphylla	21
	Solmissus sp.	1
Cnidaria, Scyphozoa	*Catostylus mosaicus*	10
	Chrysaora kynthia	8
	Chrysaora pentastoma	26
Cnidaria, Siphonophorae	*Chelophyes* sp.	1
	Diphyes sp.	7
	Eudoxoides spiralis	2
Ctenophora	*Bolinopsis* sp.	20
	Hormiphora sp.	53
	Ocyropsis sp.	32
	Pukia sp.	113

analyses were conducted using the closest approximation to this model. Nodal support in the maximum likelihood analysis was estimated by performing 1000 bootstrap pseudoreplicates. Bayesian inference 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. Bayesian inference analysis used the following parameters: "nst = 6", "rates = invgamma", "ngammacat = 4", and the priors parameters of the combined dataset were set to "ratepr = variable". Samples of substitution model parameters, and tree and branch lengths were summarised using the parameters "sump burnin = 3,000" and "sumt burnin = 3,000". Species of the Aephnidiogenidae were designated as functional outgroup taxa.

2.4. Species recognition

Species were distinguished using, as a starting point, the criteria for species delineation proposed by Bray et al. [53].

3. Results

3.1. General results

Table 3 lists the gelatinous zooplankton species and numbers (220 individuals of medusae of 17 cnidarian species and 218 individuals of four ctenophore species) examined during this study. Seven of the 17 cnidarian species and two of the ctenophore species were infected with lepocreadiid metacercariae (Table 4). Given the relatively small size and only weakly informative morphology of the metacercariae, our approach to identification was based principally on sequence data. In our view this approach was justified by the discovery that, although some of the species were abundant, some were exceptionally rare (including one found only once); for such a specimen a sequence is likely to be more informative than a tiny wholemount. Nonetheless, some specimens, including some hologenophores [sensu [54]] were stained and mounted for microscopical examination.

Metacercariae were initially genetically characterised systematically by ITS2 rDNA data, with a subset later characterised by *cox*1 mtDNA data; *cox*1 data proved more informative than the more conserved ITS2 data. Sequence data for both markers were generated for adult worms of interest. 28S rDNA data were generated for a reduced set of samples once initial identifications had been made. All sequences were compared against our unpublished sequence database of trematodes of Indo-Pacific fishes, as well as by BLAST analysis relative to sequences available on GenBank. Preliminary analyses demonstrated that all the metacercarial sequences, and those of related adults from fishes, were closely related to and in some cases identical to species morphologically consistent with three lepocreadiid genera: *Cephalolepidapedon*, *Opechona* and *Prodistomum*. Results relevant to each genus, for specimens from both plankton and fishes, are summarised separately below. Phylogenetic analysis of 28S sequence data suggests that *Opechona* is a weak concept, although all the species considered here cluster together. For *Prodistomum*, ITS2 and 28S data both indicate the presence of multiple independent lineages, including for those taxa considered here. However, based on morphology, the putative representatives of the three genera are strongly cohesive and are considered together on that basis.

3.2. Prodistomum Linton, 1910

We found new adult specimens consistent with this genus in *Scomber australasicus* from off southern Queensland, from *Scomber japonicus* from off Japan, and from *Monodactylus argenteus* from off southern Queensland. Metacercariae consistent with this genus were found in both cnidarians and ctenophores from off the southern Queensland coast.

3.2.1. cox1 sequence data

43 *cox*1 sequences were generated for samples from five species of gelatinous zooplankton and three species of fish. No previously reported *cox*1 sequences available on GenBank matched or were close to any of the sequences reported here. Analysis of *cox*1 sequences distinguished six clearly distinct, strongly supported lineages differing from each other at a minimum *p*-distance of 9.7% (46 bp) (Fig. 1). These six lineages are as follows:

Type 1 (Australia). Five sequences, including three from hologenophore specimens, from *S. australasicus* and 10 from metacercariae from four cnidarian species. Intra-type variation was at a *p*-distance of 0–0.4% (0–2 bp).

Type 2 (Japan). Six sequences from hologenophore specimens from *S. japonicus*. Apart from one specimen which differed from the others at a

Table 4
Distribution of lepocreadiid metacercariae in gelatinous plankton off the east Australian coast. New records ●; records from Browne et al. [4] ▮ and Duong et al. [30] ○.

Host	1	2	3	4	5	6	7	8	9	10	11	12	13
Cephalolepidapedon saba		●				●	●						
Cephalolepidapedon warehou								▮	▮				
Opechona cf. *olssoni*		●		●		●	●						●
Opechona kahawai		●				●							
Opechonoides opisthoporus										○		○	○
Prodistomum orientale (Type 1)	▮●	●	●	●	●		●					●	
Prodistomum Type 3	▮	●	●	●			●				●		
Prodistomum keyam												●	

Hosts: Cnidaria (1–9), Hydrozoa: 1. *Aequorea* sp.; 2. *Aldersladia magnificus;* 3. *Geryonia proboscidialis;* 4. Unknown Hydrozoa; 5. Unknown Hydrozoa; Scyphozoa: 6. *Chrysaora kynthia;* 7. *Chrysaora pentastoma;* 8. *Cyanea* sp.; 9. *Pseudorhiza* sp.; Ctenophora (10–14): 10. *Bolinopsis* sp.; 11. *Hormiphora* sp.; 12. *Ocyropsis* sp.; 13. *Pukia falcata*.

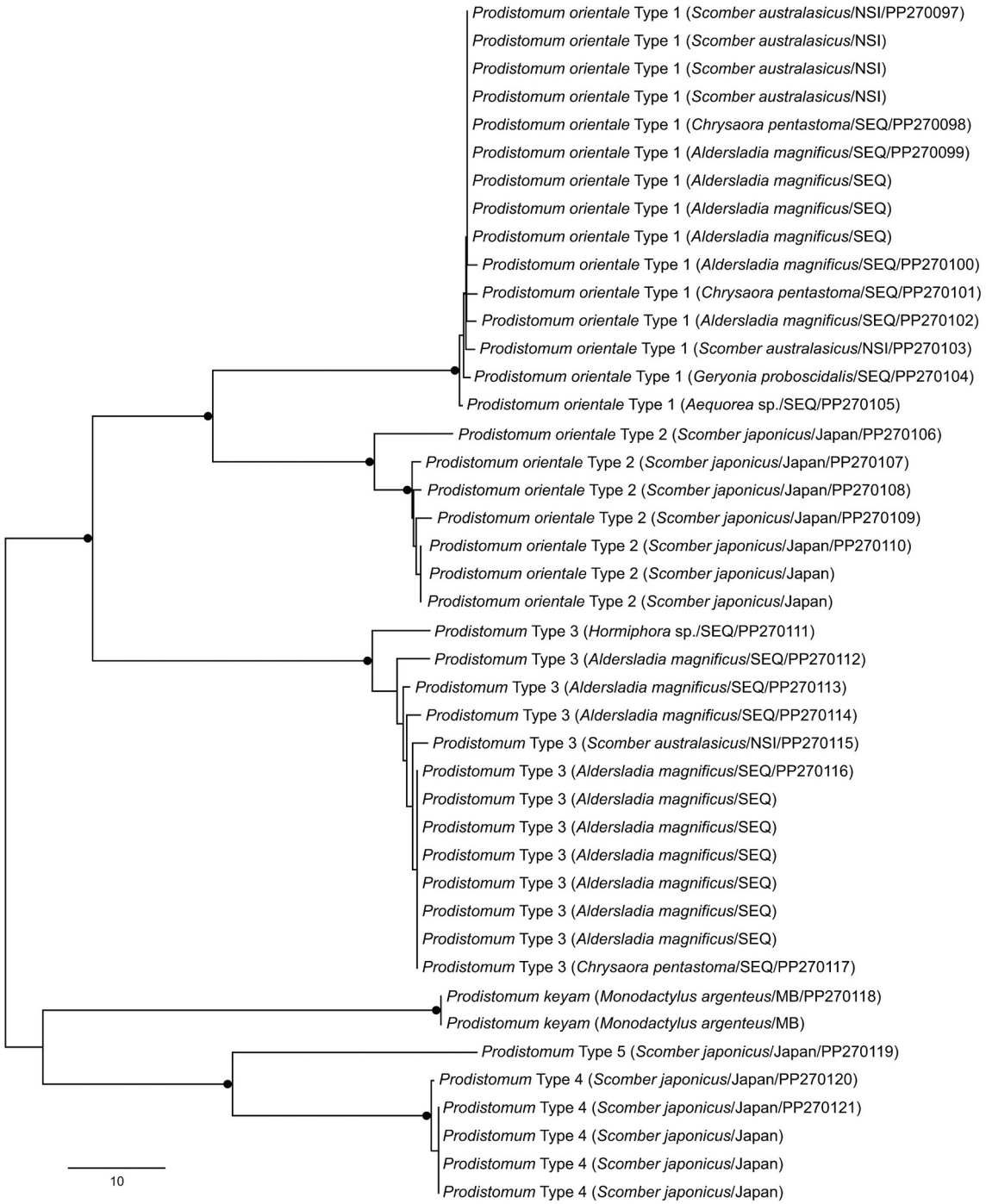

Fig. 1. Phylogram from the unrooted Neighbour-joining analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) mtDNA dataset for adult and metacercarial samples morphologically consistent with the genus *Prodistomum*. Strongly supported nodes (>80) are indicated by a filled circle. The scale bar indicates the number of base differences. *Abbreviations:* MB: Moreton Bay; NSI: North Stradbroke Island; SEQ: south-east Queensland.

p-distance of 2.5–3.2% (12–15 bp), intra-type variation was at a *p*-distance of 0–0.6% (0–3 bp). Type 2 differs from Type 1 at a *p*-distance of 9.7–11.1% (46–53 bp).

Type 3 (Australia). One sequence from a hologenophore specimen from *S. australasicus* and 12 metacercarial sequences from three cnidarian species. Intra-type variation was at a *p*-distance of 0–2.7% (0–13 bp); one sequence differed from all others at a *p*-distance of 2.1–2.7% (8–13 bp), the others at only 0–1.5% and 0–7 bp. Type 3 differs from Types 1 and 2 at a *p*-distance of 15.3–15.8% (73–75 bp) and 14.1–14.9% (67–71 bp), respectively.

Type 4 (Japan). Five sequences from hologenophore specimens from *S. japonicus*. Intra-type variation was at a *p*-distance of 0–0.2% (0–1 bp). This type differs from Types 1, 2 and 3 at a minimum *p*-distance of 17.9% (85 bp).

Type 5 (Japan). One sequence from a hologenophore specimen from *S. japonicus*. This type differs from Type 4 at a *p*-distance of 9.7–9.9% (46–47) bp and from Types 1, 2 and 3 at a minimum *p*-distances of 17.2% (82 bp).

Type 6 (Australia). Identified as *Prodistomum keyam* Bray & Cribb, 1996. Two identical sequences generated from specimens from *Monodactylus argenteus* previously reported by Bray et al. [55]. This type differs from Types 1–5 at a minimum *p*-distance of 18.1% (86 bp).

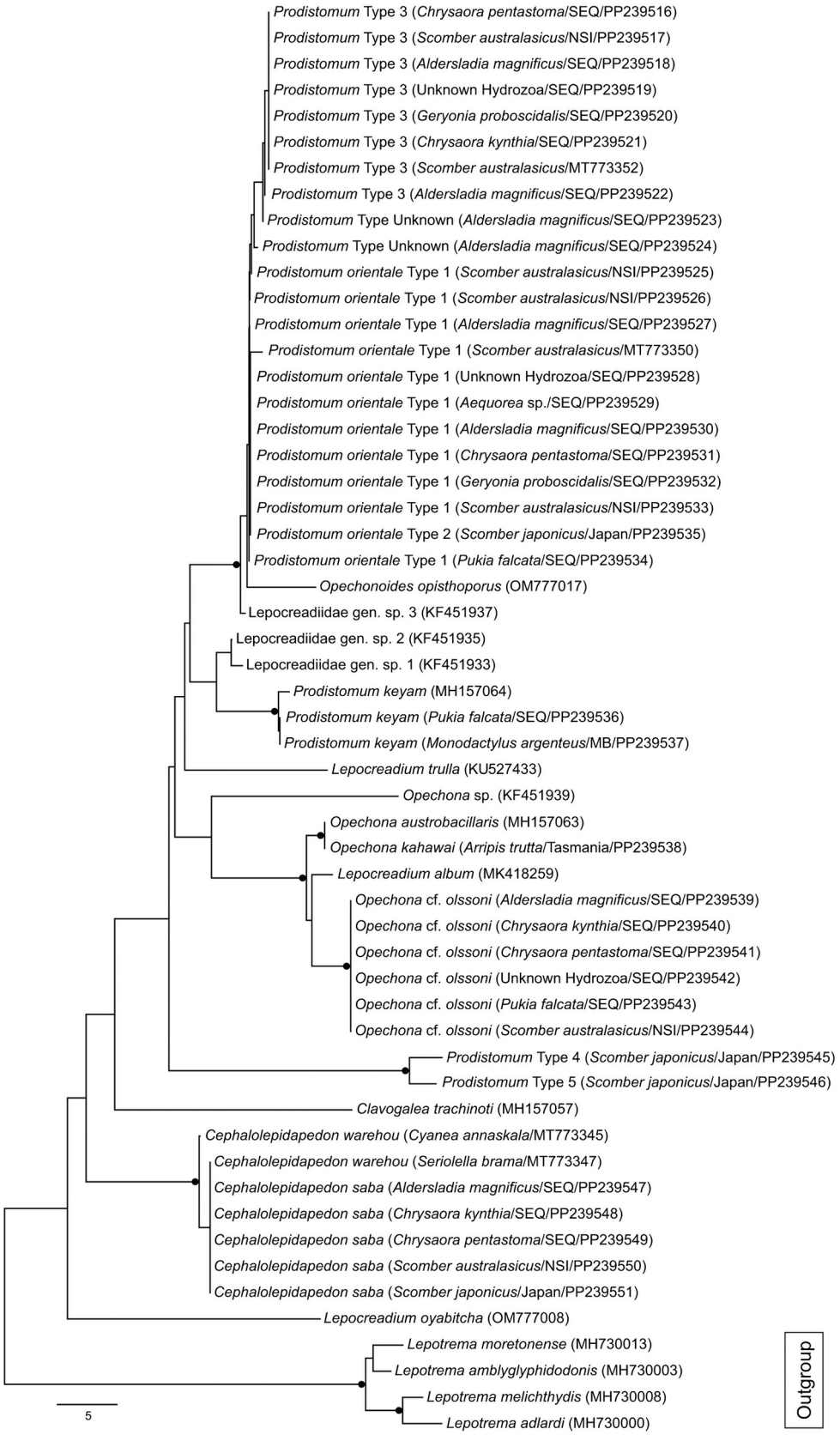

Fig. 2. Phylogram from the Neighbour-joining analysis of the ITS2 rDNA sequences of adult and metacercarial samples morphologically consistent with the genera *Cephalolepidapedon*, *Opechona* and *Prodistomum*, with relevant sequences from GenBank. Taxa of *Prodistomum* form paraphyletic clades in this analysis. Strongly supported nodes (>80) are indicated by a filled circle. The scale bar indicates the number of base differences. *Abbreviations*: MB: Moreton Bay; NSI: North Stradbroke Island; SEQ: south-east Queensland.

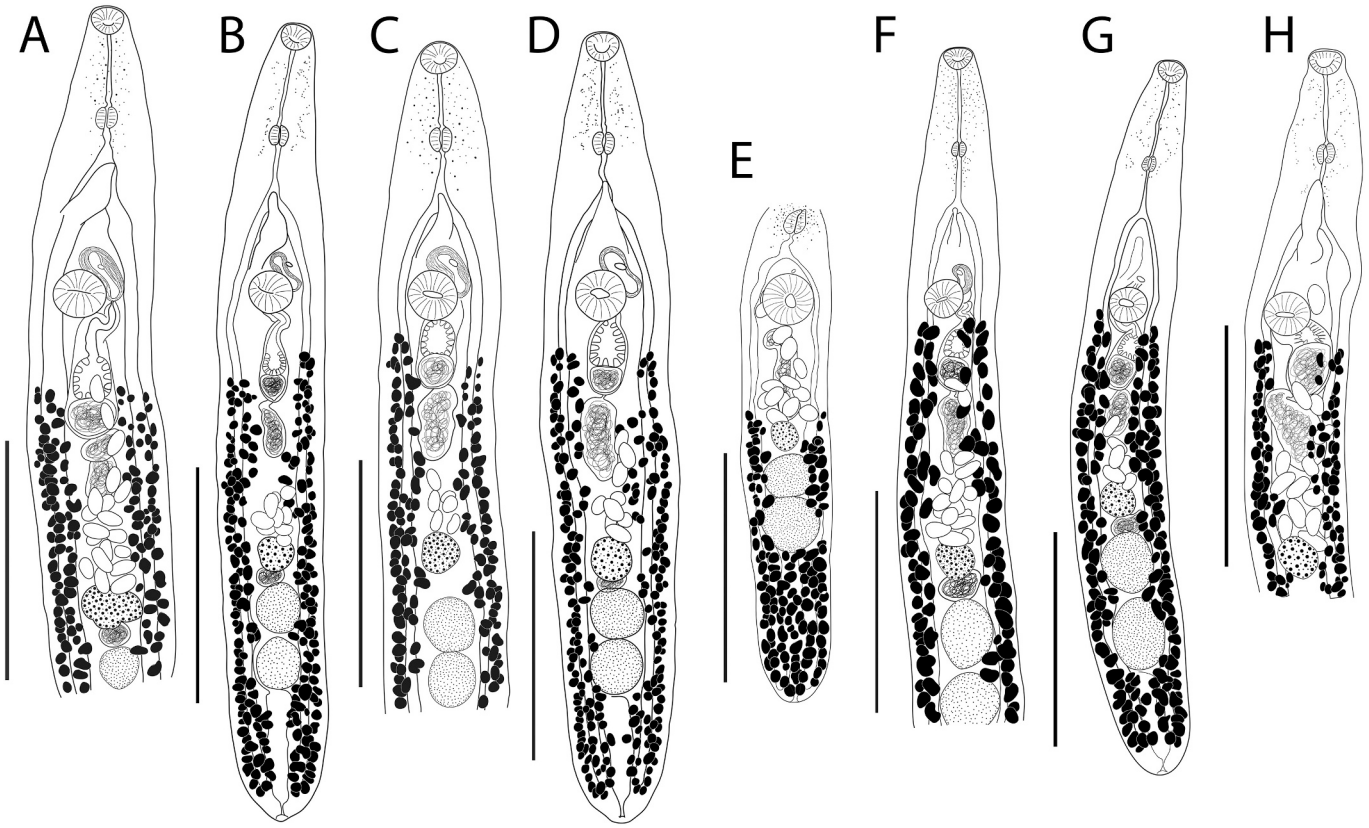

Fig. 3. Morphological specimens relating to five *cox*1 types of *Prodistomum*. A, B – hologenophore and paragenophore of Type 1 (interpreted as *P. orientale*) from *Scomber australasicus* off southeast Queensland; C, D hologenophore and paragenophore of Type 2 (interpreted as *P. orientale*) from *Scomber japonicus* from off Minabe, Japan; E hologenophore of Type 3 from *S. australasicus* off southeast Queensland; F, G hologenophore and paragenophore of Type 4 from *S. japonicus* from off Minabe, Japan; H hologenophore of Type 5 from *S. japonicus* from off Minabe, Japan. Scale bars: 500 μm.

3.2.2. ITS2 rDNA sequence data

ITS2 sequences formed three distinct groups; those for Types 1, 2 and 3 differed from Types 4 and 5 at a *p*-distance of 6.1–6.7% (29–32 bp) and from *P. keyam* at 2.5–2.9% (12–14 bp). Types 4 and 5 differed from *P. keyam* at a *p*-distance of 6.5% (31 bp). These levels of distinction are reflected by the fact that the three groups of sequences form independent clades in the overall analysis (Fig. 2).

Sequences for three adult specimens of Type 1 (Australia) and two adult specimens of Type 2 (Japan), from the two species of *Scomber* Linnaeus, were identical; thus, a *p*-distance of 9.7–11.1% (46–53 bp) in *cox*1 sequences between these two types (relating to a geographical distinction) were not reflected by any differences in ITS2 data. These sequences agreed (0–1 bp differences) with sequences of 22 metacercariae from gelatinous zooplankton from off southeast Queensland. The single sequence of Type 3 from *S. australasicus* from off southeast Queensland, together with 18 from metacercariae from gelatinous zooplankton from the same region, differed from Types 1 and 2 at a *p*-distance of 0.2–0.4% (1–2 bp); this negligible distinction was despite *cox*1 *p*-distances of 15.1–15.8% (72–75 bp). Browne et al. [4] reported four sequences as *Opechona* cf. *kahawai*, two from *S. australasicus* and two from the cnidarian *Aequorea* sp. However, one sequence from each of the two hosts is identical to that for *Prodistomum* Type 3; of the other two sequences, one is identical to, and one is a single base pair different from, *Prodistomum* Types 1 and 2. Sequences of Types 1, 2 and 3 formed a clade with *Opechonoides opisthoporus* Duong, Cutmore, Cribb, Pitt, Wee & Bray, 2022 (6–8 differences) from pomacentrid fishes and "Lepocreadiidae gen. sp. 3" (unpublished; 1–3 differences) from *S. japonicus* from off Argentina.

The single sequences of Types 4 and 5 (both from *S. japonicus* from off Minabe, Japan) differed from each other at a *p*-distance of 1.0% (5 bp).

Three sequences of *P. keyam* from *M. argenteus* (Type 6) varied at a single bp and differed from Types 1–5 at a *p*-distance of 2.3–7.3% (11–35 bp); these sequences were identical to or closely matched (0–1 bp difference) a single metacercaria collected from a ctenophore, *Pukia falcata*. These sequences formed a moderately supported clade with two Argentinian lepocreadiids reported as cercariae by Gilardoni et al. [56] which differed from each other at a single bp.

3.2.3. 28S rDNA sequence data

Partial 28S rDNA sequences were identical for Types 1 and 2 and differed from those of Type 3 at a *p*-distance of 0.38% (5 bp) (Fig. 10). Types 4 and 5 differed from each other at a *p*-distance of just 0.15–2.20% (2–3 bp). Types 1–3 differed from Types 4 and 5 at *p*-distances of 0.16–0.19% (21–25 bp). *Prodistomum keyam* differed from all other sequences at a *p*-distance of 0.11–0.14% (15–19 bp). Lepocreadiid relationships based on 28S rDNA sequences are reviewed following the genus-specific analyses.

3.2.4. Morphology

Figure 3 A, C, E, F and H represent the morphology of hologenophore specimens of adults of *Prodistomum* corresponding to Types 1–5. Once hologenophore specimens associated with the five types were distinguished, they were matched with additional intact paragenophore specimens for three of the types (Types 1, 2 and 4), always from the same host and locality (Figs. 3 B, D and G). Key measurements of the five types are given in Table 5. Measurements for the oesophagus and pseudoesophagus were combined because we found the two regions to be difficult to distinguish. All five types are highly morphologically similar, but we found potentially informative distinctions between them. The

Table 5
Measurements of five *cox*1 types of *Prodistomum* from Australian and Japanese species of *Scomber*.

Identity	T1	T2	T3	T4	T5
Host	*S. australasicus*	*S. japonicus*	*S. australasicus*	*S. japonicus*	*S. japonicus*
Locality	Off Qld	Off Minabe	Off Qld	Off Minabe	Off Minabe
n	12	10	1 (H)	9 (5H)	1 (H)
Body L	1360–1856 (1607)	1647–1885 (1741)		1464–1773 (1635)	
Body W	181–256 (219)	249–330 (282)	200	223–278 (250)	
Body L / W	6.4–8.03 (7.38)	5.3–6.79 (6.2)		6.39–7.44 (6.73)	
Forebody	425–527 (492)	469–559 (505.8)		449–617 (521)	510
Forebody % BL	27.3–33.7 (30.7)	26.3–30.7 (29.1)		29.4–32.1 (30.7)	
OS L	46–57 (51)	52–62 (57)		43–57 (49)	52
OS W	55–67 (62)	66–76 (70)		58–75 (67)	63
Prepharynx L	140–184 (157)	110–190 (145)		149–209 (176)	144
Pharynx L	33–50 (40)	46–54 (51)	63	34–44 (38)	39
Pharynx W	37–47 (41)	45–52 (48)	42	31–39 (34)	38
Oes. + pseud.	91–153 (130)	123–143 (131)	50	100–134 (115)	119
VS L	72–153 (113)	98–107 (102)	107	79–111 (87)	87
VS W	75–107 (90)	100–111 (106)	107	80–108 (88)	92
OS W / VS W	1.23–1.64 (1.46)	1.39–1.64 (1.51)		1.20–1.50 (1.33)	1.46
VS to ant. Testis	450–669 (541)	531–675 (584)	288	488–625 (550)	
Testis to post. End	188–313 (254)	269–338 (304)	306	213–281 (250)	
Ant. test. L	90–137 (110)	117–153 (132)	100	120–167 (143)	
Ant. test. W	87–178 (108)	113–143 (127)	107	100–137 (116)	
Post. test. L	110–110 (110)	130–163 (141)	133	140–183 (158)	
Post. test. W	90–127 (104)	113–133 (124)	123	90–133 (115)	
Cirrus-sac L	223–326 (263)	246–290 (269)		188–313 (241)	244
Cirrus-sac W	53–87 (68)	73–97 (82)		44–109 (71)	94
VS to Ovary	359–525 (427)	398–523 (453)	204	374–492 (422)	425
Ov to ant test	9–86 (42)	8–74 (41)	14	0–66 (39)	
Ovary L	53–87 (76)	83–107 (95)	73	43–97 (83)	93
Ovary W	53–100 (77)	83–107 (93)	67	53–103 (82)	90
VF to VS	58–182 (92)	62–142 (99)	181	−27–63 (1)	12
VF to VS as % Ov to VS	12.5–35.1 (21.5)	13.3–33.4 (22.1)	88.7	−7.2–15 (0.3)	2.8
Genital atrium L	67–107 (83)	96–125 (110)	67	58–95 (79)	
Genital atrium W	30–52 (38)	38–58 (47)	44	30–65 (47)	
EV to pharynx	5–127 (70)	53–132 (91)		69–169 (118)	35
% FB L	1.1–29.8 (14.3)	11–23.5 (18)		13.7–29.5 (22.5)	6.9
Egg L	51–60 (55)	47–57 (52)	52	44–62 (56)	65
Egg W	27–32 (30)	27–34 (30)	33	26–40 (32)	35

Abbreviations: BL, body length; EV, excretory vesicle; H, hologenophore; L, Length; Oes, oesophagus; OS, oral sucker; Ov, ovary; Pseud, pseudoesophagus; W, width; VF, vitelline follicles; VS, ventral sucker.

clearest distinctions detected are summarised in the following key:

1a. Vitelline follicles reach to just anterior to ovary……Type 3 (Australia).

1b. Vitelline follicles reach to much closer to ventral sucker than to ovary……2.

2a. Pharynx relatively large (>46 um long) ……Type 2 (Japan).

2b. Pharynx relatively small (<44 um long – one exceptional specimen) ……3.

3a. Vitelline follicles well-separated from posterior margin of ventral sucker…… ……Type 1 (Australia).

3b. Vitelline follicles reach close to or anterior to posterior margin of ventral sucker…… ……4.

4a. Excretory vesicle terminates <13% of forebody length from pharynx…… ……Type 4 (Japan).

4b. Excretory vesicle terminates <7% of forebody length from pharynx…… ……Type 5 (Japan).

Figure 4A shows morphology of a *Prodistomum* species metacercaria from a hydrozoan medusa, *Aldersladia magnificus* Gershwin. This is representative of nine mounted specimens from *A. magnificus* and four from medusae of a scyphozoan, *Chrysaora pentastoma* Péron & Lesueur. Given the small size of these metacercariae (maximum length 478 μm), preparation of hologenophores leaving useful amounts of the body for morphological analysis was not attempted. The morphology is consistent with that of adult specimens of *Prodistomum* from species of *Scomber*, especially in the distinctively posterior ventral margin of the oral aperture. In addition, the other two lepocreadiid genera for which metacercarial types are characterised below (relating to species of *Cephalolepidapedon* and *Opechona*) were associated with sequences from adult worm hologenophores, meaning that the chance of false genus-level attribution of these metacercariae is remote. Specimens relating to Type 1 and Type 3 were found sympatrically in an overlapping range of jellyfish (both mainly *A. magnificus*). In the absence of hologenophores, we therefore cannot attribute the whole paragenophore morphological specimens to either lineage. Regardless of their lineage, these metacercariae clearly undergo dramatic allometric change as the forebody shrinks from as much as 55.4% of the body length to as little as 29.2% in gravid adults of Type 1 specimens.

Metacercariae from Port Phillip Bay jellyfish identified by Browne et al. [4] as *O.* cf. *kahawai* were evidently misidentified metacercariae of one or more *Prodistomum* species as indicated by their small size and especially their long forebodies. No specimens of metacercariae consistent with *Opechona* were found in the collection of Browne et al. [4] when it was re-examined.

3.2.5. Species recognition and identity

Identification of the adult types of *Prodistomum* reported from species of *Scomber* here is challenging, especially in view of the limited morphological samples for some types and difficulties with the existing taxonomic literature. We suspect that one key potential criterion for species recognition, host identity, may be uninformative in this system. Although three *cox*1 types were found only in *S. japonicus* and two only

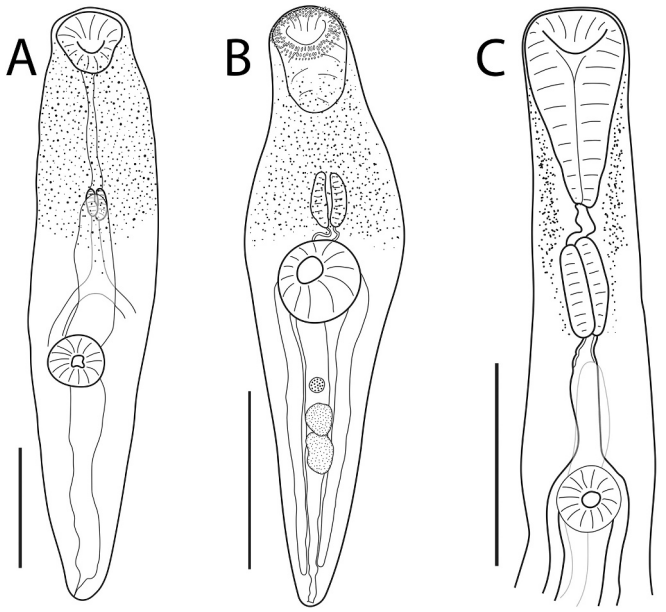

Fig. 4. Lepocreadiid metacercariae from medusae of *Aldersladia magnificus* from off southeast Queensland. A. *Prodistomum* sp. B. *Cephalolepidapedon saba.* C. *Opechona* cf. *olssoni*. Scale bars: A, 100 μm; B,C 200 μm.

in *S. australasicus*, these two fishes are so biologically and morphologically similar that the host identity distinction may well be insignificant. The distribution of *cox*1 types may reflect geographical rather than host distribution. Thus, here we consider only molecular and morphological data as potentially informative for the recognition of species in this system.

Analyses of *cox*1, ITS2 and 28S data all show strongly that the clades comprising Types 1–3 and Types 4 + 5 are deeply distinct from each other and we thus consider them separately. Based on these analyses, it is possible that, despite their morphological similarity, the two groups of species will ultimately require separate genera.

Types 4 and 5 were collected from the same fish individual (*S. japonicus*, Minabe Fish Market, Japan) so that neither host nor geographical variation can be invoked to explain the correlation between the molecular and morphological distinctions between them. Based on limited samples, the distinction between the anterior extent of the excretory vesicle is reliably discriminating. The *cox*1 molecular distinction is substantial (*p*-distance of 9.7–9.9%; 46–47 bp), and the ITS2 distinction (1.0%; 5 bp) and the 28S distinction (0.15–2.20%; 2–3 bp) are at least moderate (especially for samples collected in sympatry). We therefore hypothesise that these types represent separate species.

Types 1–3 form a clade in all molecular phylogenetic analyses. Notably, specimens of Types 1 and 3 were collected from the same individual of *S. australasicus* from off the Queensland coast. In terms of morphology, the restricted anterior vitelline follicle distribution for the single adult of Type 3 is distinctively different from that of the many specimens of Type 1. Although we have only a single sequence from a hologenophore adult of Type 3 and five from hologenophores of Type 1, the differences are strongly corroborated by the multiple metacercariae of both types that support the *p*-distance of 15.3–15.8% (73–75 bp) *cox*1 distinction between the two. Despite the clear morphological and *cox*1 differences, the ITS2 differences between Type 1 and Type 3 are minor, at just 1–2 bp. In this context we note the mounting evidence that ITS2 does not satisfactorily distinguish all combinations of species that are deemed convincing on other bases [36,57–59]. We therefore conclude that the low level of ITS2 distinction between Type 1 and Type 3 constitutes an "absence of evidence" rather than "evidence of identity". The distinction between these two types is supported by a small (five bp) difference in 28S data. We thus hypothesise that they represent distinct species. We conclude that Browne et al. [4] reported both these species from Queensland waters in *S. australasicus* and cnidarian medusae, both as *O.* cf. *kahawai.*

Type 1 (Australia) and Type 2 (Japan) form a clade in the *cox*1 analysis, differing at a *p*-distance of 9.7–11.1% (46–53 bp), but have identical ITS2 and 28S sequences. They differ slightly in the size of the pharynx [Type 1: 33–50 (40) long vs Type 2: 46–54 (51) long] but share the positive morphological character of highly similar and distinctively shaped muscular genital atria. We conclude that this combination of forms is best interpreted as a single species with geographical variation. Wee et al. [60] interpreted 33–36 bp differences in *cox*1 data as intraspecific geographical variation for the monorchiid *Helicometroides longicollis* Yamaguti, 1934 from different subspecies of *Diagramma pictum* (Haemulidae) from Australia and Japan. Similarly, Cutmore and Cribb [57] interpreted differences of 19–21 bp differences in *cox*1 for the aporocotylid *Elaphrobates chaetodontis* (Yamaguti, 1970) Yong, Cribb & Cutmore, 2021 from Australia and Japan as intraspecific. The level of difference here (*p*-distance of 9.7–11.1%; 46–53 bp) is significantly larger than for either of these species. However, other studies have demonstrated comparable or even higher levels of *cox*1 distinction over different combinations of localities for other Indo-Pacific trematodes: up to 75 bp for *Hurleytrematoides morandi* McNamara & Cribb, 2011 (Monorchiidae), up to 62 bp for *Gorgocephalus yaaji* Bray & Cribb, 2005 (Gorgocephalidae), up to 60 bp for *Transversotrema enceladi* Cutmore, Cribb & Corner, 2023 (Transversotrematidae), and up to 54 bp for *Preptetos laguncula* Bray & Cribb, 1996 (Lepocreadiidae) [53,61–63]. In those studies, as here, distinct *cox*1 populations have been interpreted as representing single, widespread species where the populations ultimately form clades, the hosts are the same (or very similar), and the parasites are actually or almost morphologically indistinguishable.

Above we conclude that our collections from species of *Scomber* and jellyfish are presently best interpreted as representing four species (Types 1 + 2, 3, 4 and 5) plus the previously described *P. keyam*. Here we consider the issue of the application of names to them. The genus *Prodistomum* is characterised within the Lepocreadiidae especially by lack of oral spines, having a short to long pseudoesophagus, blindly ending intestinal caeca, and the uterus being principally pretesticular [64]. There are currently 19 species recognised and another seven presently accepted as synonyms. The specimens of Types 1–5 all have the vitelline follicles restricted to the hindbody. This character distinguishes them from 13 species in which the follicles enter the forebody: *Prodistomum gracile* Linton, 1910 (type-species); *Prodistomum alaskense* (Ward & Fillingham, 1934) Bray & Merrett, 1998; *Prodistomum angelae* (Kruse, 1981) Bray & Cribb, 1996; *Prodistomum hynnodi* (Yamaguti, 1938) Bray & Gibson, 1990; *Prodistomum keyam*; *Prodistomum lichtenfelsi* Raychard, Blend & Dronen, 2008; *Prodistomum menidiae* (Manter, 1947) Bray & Gibson, 1990; *Prodistomum polonii* (Molin, 1859) Bray & Gibson, 1990; *Prodistomum priedei* Bray & Merrett, 1998; *Prodistomum siddiqi* (Ahmad, 1984) Madhavi & Bray, 2018; *Prodistomum travassosi* (Ahmad, 1984) Madhavi & Bray, 2018; *Prodistomum vinodae* (Ahmad, 1984) Madhavi & Bray, 2018; and *Prodistomum waltairense* (Madhavi, 1972) Bray & Gibson, 1990. *Prodistomum pomatomi* (Amato, 1983) Lopes, Mainenti, Knoff & Correa Gomes, 2017 has vitelline follicles extending only to the level of the ventral sucker but this species is distinct from the present forms in having an oesophagus that is far longer than the prepharynx. In five species the vitelline follicles are restricted to the hindbody: *Prodistomum gaevskayae* (Ahmad, 1991) Madhavi & Bray, 2018; *Prodistomum girellae* (Yamaguti, 1940) Bray & Gibson, 1990; *Prodistomum libyacum* (Al-Bassel, 2001); *Prodistomum mohsini* (Ahmad, 1984) Madhavi & Bray, 2018; and *Prodistomum orientale* (Layman, 1930) Bray & Gibson, 1990. Of these, *P. gaevskayae* (described from a fistulariid) and *P. girellae* (described from a girellid) are each distinguished from the present forms by the possession of a relatively enormous pharynx, which have lengths of 160–200 μm and 110–150 μm, respectively. *Prodistomum mohsini* (described from a carangid) has a large, funnel-shaped oral sucker and *P. libyacum* (described from a mullid) has a conspicuously tri-lobed

ovary and a cirrus-sac that does not extend posterior to the ventral sucker. These distinctions leave just *P. orientale*, described from *S. japonicus*, as the only presently recognised species with which the present forms might be recognised.

Prodistomum orientale was described by Layman [65] as *Pharyngora orientalis* Layman, 1930 from *S. japonicus* from Peter the Great Bay (within the Sea of Japan), and has been reported repeatedly since. Bray and Gibson [66] reviewed the genus *Prodistomum* in detail, recombined *Ph. orientalis* with *Prodistomum*, and attributed 44 additional reports to it including recognition of seven other nominal species as synonyms. Reported hosts, in addition to those belonging to the Scombridae, include members of the Carangidae, Chaetodontidae, Clupeidae, Cottidae, Engraulidae, Girellidae, Macrouridae, Nemipteridae, Polynemidae, Priacanthidae, Scorpaenidae, Serranidae, Tetraodontidae and Trichiuridae. Most of these reports provided little morphological data and no figures. Since the detailed work of Bray and Gibson [66], we are aware of only two further reports of the species, one from Tunisia [67] and one from the North-western Pacific by Sokolov et al. [68], the later report publishing 28S rDNA data for this species derived from specimens taken from *S. japonicus*. In our view it is highly unlikely that all the records attributed to *P. orientale* genuinely relate to that species. We find the wide range of hosts at best surprising and some of the images suggest the possibility of distinction; the wide reported geographic distribution is presently difficult to interpret but is possible given the wide distribution of some of the hosts. Most tellingly, however, we conclude that the type-host, in an area close to the type-locality, is infected with three morphologically similar but distinguishable forms (Types 2, 4 and 5) which are also clearly distinct in molecular analyses and thus should be considered distinct species. Determination of which (if any) of these types can be interpreted as *P. orientale* depends on comparison with the original description of the species. Although we consider all five of the types reported from *Scomber* species here to generally resemble *P. orientale* as originally described, it is noteworthy that the original description shows the oral sucker as distinctly longer than wide, unlike the condition of any of our specimens. However, the original figure also depicts the oral sucker as distinctly retracted which may well have led to its artificial elongation. We note a comparable shape and retraction of the oral sucker in the figure of *Acanthocolpoides israelensis* Fischthal, 1980, a species considered a synonym of *P. orientale* by Bray & Gibson [66].

Comparison of the present specimens with those of Layman [65] is made difficult by the fact that there is no scale-bar on the original figure and there is no other indication of the size of the figured specimen. Layman reported specimens from 2.1 to 3.278 mm long, all larger than all the specimens reported here (Type 1 max. 1.807 mm; Type 2 max. 1.885 mm; Type 3 max. approximately 1.5 mm; Type 4 max. 1.773 mm; Type 5 max. approximately 1.5 mm). Despite this discrepancy, we think it probable that one of the three types collected from Japanese waters characterised here relates to the original *P. orientale*. We cautiously infer that our Type 2 specimens from *S. japonicus* from Japan, together with Type 1 from Australia, can be identified as *P. orientale* on the basis that they share the distinct separation of the vitellarium from ventral sucker not seen in either Types 4 or 5. We infer that Types 4 and 5 from Japan and Type 3 from Australia represent distinct species. However, given the uncertainty about the status of the multiple synonyms of *P. orientale* (including five reported from species of *Scomber*), we cannot determine or predict whether Types 3, 4 and 5 constitute new species or if they correspond to one of the previously described synonyms. Our evidence shows that three species co-occur in (the same individual of) *S. japonicus* in Japanese waters. Thus, *P. orientale* might easily have been based on any one of these species and the type-material may even be a mixture of more than one. We suspect that the nature of the type-specimens of *P. orientale* (probable imperfect fixation and especially the absence of sequence data) means that we can never be fully confident of the nature of this species. However, perhaps this presents no real problem. If there are multiple comparable species in the type-host at the type-locality, then taxonomic stability requires only that, as proposed here, one of them is effectively nominated to be recognised as *P. orientale*.

3.2.6. Summary of samples reported, and identifications made here

Prodistomum orientale (Layman, 1930) Bray & Gibson, 1990.

(= *cox*1 Types 1 and 2, this study)

Definitive hosts: *Scomber japonicus* Houttuyn; *Scomber australasicus* Cuvier (Scombridae).

Definitive host localities: Minabe Fish Market, Wakayama Prefecture, Japan (33°44′N, 135°19′E), 30 specimens including seven hologenophores from *S. japonicus*. Off Flinders Beach, North Stradbroke Island, Queensland, Australia (27°24′S, 153°30′E), 18 specimens including three hologenophores from *S. australasicus*. Rainbow Channel, Moreton Bay, Queensland, Australia (27°25′S, 153°24′E), 76 specimens from *S. australasicus*.

Prevalence: *S. japonicus*, 1 of 1; *S. australasicus*, 5 of 5.

Deposition of specimens: Japanese samples - MPM 25273; Australian samples - QM G241025–38; G241159–238.

Second intermediate hosts: Ph. Cnidaria, Cl. Hydrozoa, Aequoreidae: *Aequorea* sp.; *Aldersladia magnificus* Gershwin; Geryoniidae: *Geryonia proboscidalis* (Forsskål); two unknown species; Cl. Scyphozoa, Pelagiidae: *Chrysaora pentastoma* Péron & Lesueur. Ph. Ctenophora, Pukiidae: *Pukia falcata* Gershwin, Zeidler & Davie.

Second intermediate hosts localities: Off southeast Queensland from Gold Coast Broadwater to off North Stradbroke Island.

Deposition of specimens: Australian samples - QM G241039–45.

Representative DNA sequences: *cox*1 (PP270097–110); ITS2 (PP239525–535); 28S (PP239553–554).

Prodistomum Type 3.

Definitive host: *Scomber australasicus* Cuvier (Scombridae).

Definitive host locality: Off Flinders Beach, North Stradbroke Island, Queensland Australia (27°24′S, 153°30′E), one hologenophore specimen.

Prevalence: 1 of 5.

Second intermediate hosts: Ph. Cnidaria, Cl. Hydrozoa, Aequoreidae: *Aldersladia magnificus* Gershwin; Geryoniidae: *Geryonia proboscidalis* (Forsskål); Hydrozoa: Unknown species; Cl. Scyphozoa, Pelagiidae: *Chrysaora kynthia* Gershwin & Zeidler (*nomen dubium*); *C. pentastoma* Péron & Lesueur, 1810; Ph. Ctenophora, Pleurobrachiidae: *Hormiphora* sp.

Second intermediate hosts localities: Off southeast Queensland from Gold Coast Broadwater to off North Stradbroke Island.

Deposition of specimens: QM G241046.

Representative DNA sequences: *cox*1 (PP270111–7); ITS2 (PP239516–522); 28S (PP239552).

Prodistomum Type 4

Definitive host: *Scomber japonicus* Houttuyn (Scombridae).

Definitive host locality: Minabe Fish Market, Wakayama Prefecture, Japan (33°44′N, 135°19′E), nine specimens including four hologenophores.

Prevalence: 1 of 1.

Deposition of specimens: MPM 25274.

Representative DNA sequences: *cox*1 (PP270120–121); ITS2 (PP239545); 28S (PP239558–559).

Prodistomum Type 5

Definitive host: *Scomber japonicus* Houttuyn (Scombridae).

Definitive host locality: Minabe Fish Market, Wakayama Prefecture, Japan (33°44′N, 135°19′E), one hologenophore specimen.

Prevalence: 1 of 1.

Deposition of specimens: MPM 25275.

Representative DNA sequences: *cox*1 (PP270119); ITS2 (PP239546); 28S (PP239557).

Prodistomum keyam Bray & Cribb, 1996

Definitive host: *Monodactylus argenteus* (Linnaeus) (Monodactylidae).

Definitive host locality: Amity Point, Moreton Bay, Queensland, Australia (27°24′S, 153°26′E).

Prevalence: 1 of 1.

Second intermediate host: Ph. Ctenophora, Pukiidae: *Pukia falcata* Gershwin, Zeidler & Davie.

Second intermediate host locality: Off North Stradbroke Island, southeast Queensland.

Deposition of specimens: QM G237271–4 (paragenophores) reported in Bray et al. (2018).

Representative DNA sequences: *cox*1 (PP270118); ITS2 (PP239536–537, MH157064).

3.3. *Cephalolepidapedon Yamaguti, 1970*

We found multiple adult and juvenile specimens consistent with *Cephalolepidapedon* in *S. australasicus* from off southern Queensland and a single specimen from *S. japonicus* from off Japan. Many metacercariae consistent with this genus were found in medusae of three cnidarian species from off the Queensland coast.

3.3.1. *Sequence data*

Analysis of *cox*1 sequence data distinguished two clearly distinct lineages (Fig. 5). The sequence of the single hologenophore specimen from *S. japonicus* from Japan differed from that of six specimens from *S. australasicus* and from 25 sequences from cnidarians from off Queensland at a *p*-distance of 10.5–11.8% (50–56 bp). Sequences from *S. australasicus* and Australian cnidarians differed at a *p*-distance of 0–2.5% (0–12 bp). We detected two marginally distinct groups of sequences among those from off Queensland. Four sequences of specimens from *S. australasicus* and 19 from medusae differed from two sequences of specimens from *S. australasicus* and eight from medusae at a *p*-distance of 1.7–2.7% (8–13 bp) whereas differences within each group were only 0–1.5% (0–7 bp). ITS2 rDNA sequences relating to three adult worms (one from Japan and two from Australia) and 61 metacercariae from Australian cnidarians were all identical (Fig. 2). 28S rDNA sequences from Australian and Japanese species of *Scomber* differed at 0.30% (4 bp).

No previously reported *cox*1 sequences available on GenBank matched or were close to any of the sequences reported here. Browne

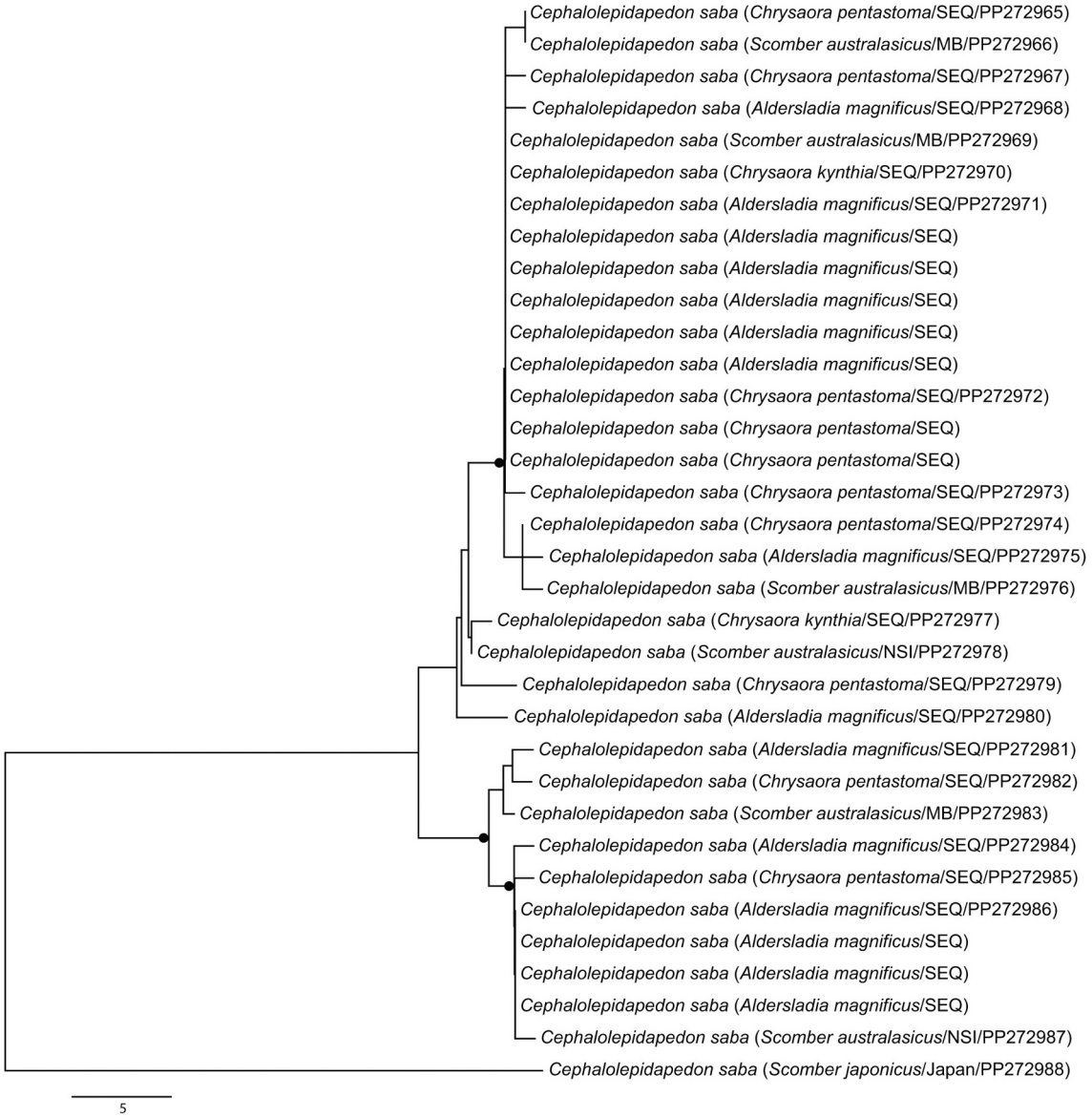

Fig. 5. Phylogram from the unrooted Neighbour-joining analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) mtDNA dataset for adult and metacercarial samples morphologically consistent with the genus *Cephalolepidapedon*. Strongly supported nodes (>80) are indicated by a filled circle. The scale bar indicates the number of base differences. *Abbreviations*: MB: Moreton Bay; NSI: North Stradbroke Island; SEQ: south-east Queensland.

et al. [4] reported five ITS2 rDNA sequences for *Cephalolepidapedon warehou* Bray & Cribb, 2003 from Port Phillip Bay, Victoria, three from two species of *Seriolella* Guichenot, including the type-host [*S. punctata* (Forster)] and two from cnidarian medusae. Four of these differed from all the Queensland and Japanese sequences mentioned above at a single consistent base position; the fifth sequence [from *S. brama* (Günther)] was identical to the new samples from off Queensland.

3.3.2. Morphology

Figure 6A shows the morphology of the single available hologenophore specimen of *Cephalolepidapedon* from *S. japonicus* from Japan and Figs. 6B–E hologenophore and paragenophore specimens from *S. australasicus* from Australia. Measurements for 13 new gravid specimens from *S. australasicus* from Moreton Bay and partial measurements of the single Japanese specimen are given in Table 6. Fig. 4B shows the morphology of a metacercaria from a medusa of *Aldersladia magnificus* from off southern Queensland.

We reviewed specimens of *C. warehou* collected by Browne et al. [4] from Port Phillip Bay, all adults from species of *Seriolella*. As reported, they are clearly consistent with *C. warehou* rather than *C. saba* Yamaguti, 1970 in that the vitelline follicles extend well into the forebody.

3.3.3. Species recognition and identity

The new specimens from species of *Scomber* reported here are clearly consistent with *Cephalolepidapedon*, especially in the possession of an infundibuliform oral sucker armed with multiple rows of relatively prominent spines [64]. This genus has just two recognised species, *C. saba*, described from *S. japonicus* from Hawaii [69] and *C. warehou* described from *Seriolella punctata* (Centrolophidae) from Tasmania [70]. The new Australian and Japanese specimens are clearly morphologically distinct from *C. warehou* in being relatively broader, having relatively larger suckers, and having the vitelline follicles restricted to the hindbody. All the new specimens are broadly consistent with *C. saba*. Following description of *C. saba*, Shimazu [71] described *Lepocreadium misakiense* Shimazu, 1986 from *S. japonicus* from Japan, but later [72] synonymised it with *C. saba*. *Stephanostomum scombri* Korotaeva, 1974 was described from *S. australasicus* from the Great Australian Bight by Korotaeva [73] but synonymised with *C. saba* by Bray and Gibson [66]. *Opechona acanthoris* Gaevskaya & Aljoshkina, 1985 was described from *S. japonicus* from the eastern Atlantic by Gaevskaya and Aljoshkina [74] but synonymised with *C. saba* by Bray and Gibson [66]. Thus, based on morphological studies, *C. saba* is presently considered a widespread species reported from two species of *Scomber*. In our view it is not certain that all these reports relate to a single species. As for *Prodistomum* as discussed above, we do not consider the distinction between the host

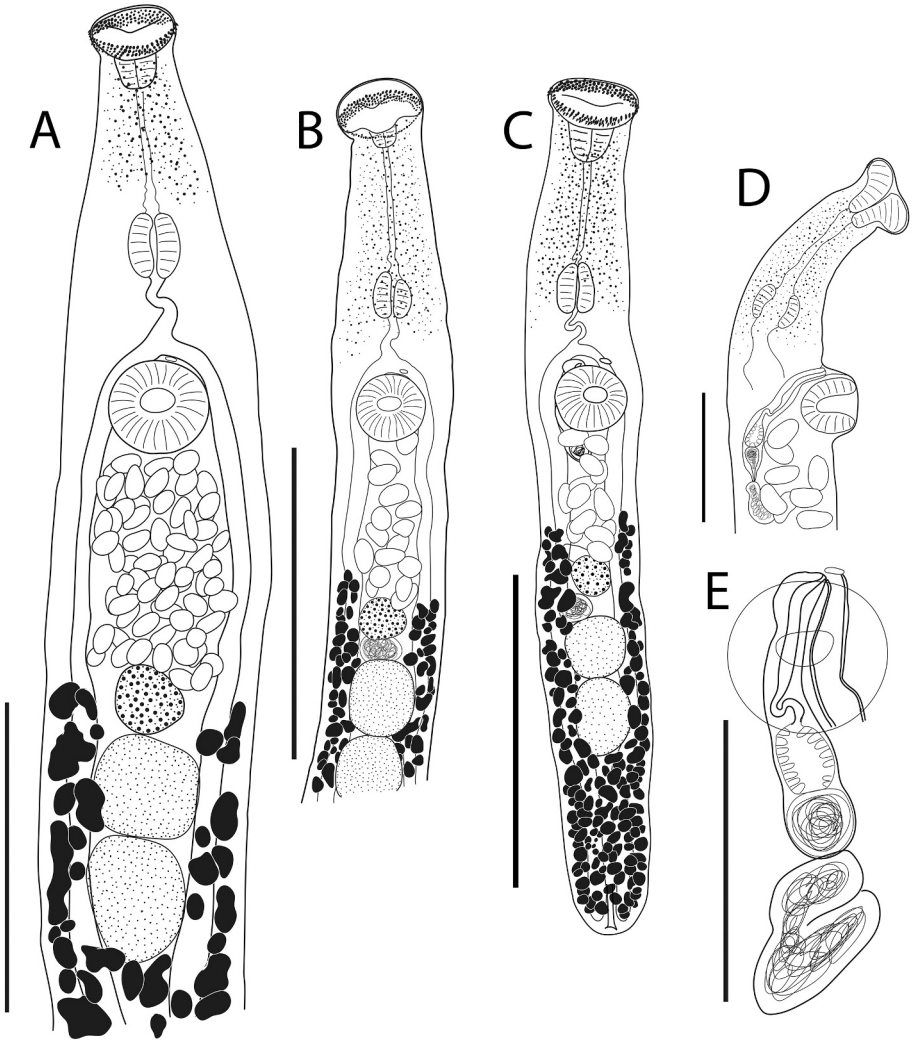

Fig. 6. *Cephalolepidapedon saba* adults. A. Hologenophore from *Scomber japonicus*, Japan; B–E from *Scomber australasicus*, southeast Queensland. B. Hologenophore; C. Paragenophore; D. Lateral anterior view; E. Terminal genitalia. Scale bars: A–C, 500 μm; D, E, 200 μm.

Table 6
Measurements of samples of *Cephalolepidapedon saba*.

Host	*S. australasicus*	*S. japonicus*	*S. japonicus*	*S. japonicus*	*S. japonicus*
Locality	southern Queensland	Hawaii	Japan	Japan	Japan
n	13	7	6	8	1 (H)
Source	Present study	Yamaguti (1970)	Shimazu (1986)	Shimazu (1989)	Present study
Body L	1096–1664 (1329)	1700–3200	1030–1460	1120–1900	
Body W	163–225 (187)	300–530	250–320	340–560	352
Body L / W	5.71–8.47 (7.15)				
Forebody	350–491 (431)			480–600	556
Forebody % BL	29.5–36.6 (32.5)		38–46	29–43	
OS L	107–153 (124)	130–180	120–150	120–180	143
OS W	122–157 (135)	140–190	130–160	120–170	143
Prepharynx L	140–223 (174)	60–150	80–160	80–130	210
Pharynx L	63–97 (76)	110–140	60–90	90–130	107
Pharynx W	55–73 (65)	60–95	70–80	70–90	90
Oesophagus	33–70 (51)	50–100	40–60	40–50	103
VS L	93–133 (107)	130–180	120–140	140–170	160
VS W	100–138 (111)	140–200		150–180	173
OS W / VS W	1.13–1.33 (1.21)		0.82–1	1.04–1.19	0.83
VS to ant. Test.	233–380 (285)				450
Test. to post. End	243–393 (302)				
Ant. test. L	97–137 (107)		70–120	80–190	163
Ant. test. W	77–113 (94)	120–280	80–140	130–240	169
Post. test. L	103–140 (123)	120–220			194
Post. test. W	77–137 (97)				163
Cirrus-Sac L	160–236 (194)	280–360	100–140	160–270	
Cirrus-Sac W	33–70 (47)	60–80		40–70	
VS to Ov	144–256 (188)				338
Ov to ant. Test.	5–43 (24)				0
Ov L	52–75 (62)	130–250	50–80	60–120	112
Ov W	47–77 (63)	110–200	40–100	70–160	113
VF to VS	150–188 (166)				375
% Body length					
Egg L	53–60 (57)	51–60	50–60	50–58	53–58 (56) [$n = 5$]
Egg W	28–37 (32)	30–40	30–40	32–36	32–37 (35) [n = 5]

Abbreviations: BL, body length; H, hologenophore; L, Length; Oes, oesophagus; OS, oral sucker; Ov, ovary; W, width; VF, vitelline follicles; VS, ventral sucker; W, width.

species of *Scomber* as reliably informative in distinguishing these forms (whereas the distinct host of *C. warehou*, from a different family of fishes, can probably be considered informative and consistent with the specific distinction of that species).

The morphological basis for considering the identity of the new collections is inconclusive (Table 6, Figs. 6A–E). Although there is some overlap, we note two distinct size groups for specimens broadly consistent with *C. saba*. Yamaguti's original description and that for *O. acanthoris* report lengths of 1.7–3.2 mm and 2.7–3.2 mm, respectively. The size of *O. acanthoris* is consistent with the original measurements for *C. saba*, but its reporting from the Atlantic makes it arguably the most biogeographically distinct of all the records interpreted as *C. saba*. In our view, this species is too little known to allow much confidence in its identification as *C. saba.* The specimens reported by Korotaeva [73] from the Great Australian Bight (1.15 mm), by Shimazu [71], Shimazu [72] from Japan (1.0–1.9 mm), and most of those reported here from Australia (1.1–1.7 mm), are significantly smaller than those in the other reports. It seems likely that flattening of Yamaguti's Hawaiian material accounts for some of this discrepancy. We suspect that there is evidence of flattening in the rather simply conical shape of the oral sucker in Yamaguti's figure and those of Korotaeva [73], Gaevskaya and Aljoshkina [74] and Shimazu [71]; in our Australian and Japanese samples (as well as for *C. warehou*) the posterior portion of the oral sucker tends to be distinctly constricted and plug-like. Beyond these observations, we detect no differences sufficiently clear (in the context of differing handling and standards and styles of description), to suggest the presence of more than one morpho-species. As shown in our figures, the single specimen we collected from Japan is substantially larger than any of the 43 we have examined from Australia.

The sequence data available for the discrimination of species in this genus is limited and ambiguous. Most importantly, there are no data from the type-locality of *C. saba*, Hawaii, only for Australian and Japanese samples which differ at a *p*-distance of 10.5–11.8% (50–56 bp), 0, and 0.30% (4 bp) for *cox*1, ITS2 and 28S respectively. These levels of difference in the *cox*1 and ITS2 datasets are comparable to the *p*-distance of 9.7–11.1% (46–53 bp). and 0 (ITS2) bp we reported above between *Prodistomum* Types 1 and 2, which we interpreted as relating to a single species. Notably, the difference of four bp in the 28S dataset contrasts with the identical sequences for the *Prodistomum* types. However, such a difference in 28S sequences over range is not without precedent; Cutmore et al. [75] reported an eight bp difference in 28S data for samples of *Ankistromeces olsoni* Nolan & Cribb, 2006 from Australia and Japan that differed at just 9–20 base pairs in the *cox*1 dataset, had overlapping morphometrics and infected the same host species. Below we argue for the continued recognition of distinction between *Opechona austrobacillaris* Bray & Cribb, 1998 and *O. kahawai* Bray & Cribb, 2003, based on morphological and host distinctions, despite molecular distinctions of only 23–28, 0 and 1 bp differences in *cox*1, ITS2 and 28S sequences in sympatry. In this context, it is again noteworthy that a lack of difference in ITS2 sequences in this genus is not necessarily inconsistent with the presence of separate species. ITS2 sequences for *C. warehou* from Victorian *Seriolella* species and medusae differ from those from Queensland *S. australasicus* and medusae at 0–1 bp, but the two species are morphologically clearly distinct and infect fishes of different families. Application of our criteria for species recognition [53] leads us to recognise all the new samples as *C. saba.* That is, despite the moderate differences in *cox*1 sequences, in the absence of compelling morphological or host distinctions, we consider interpretation of a single widespread species in similarly widespread species of *Scomber* to be plausible and presently the most conservative option. However, we certainly think that the status of this species over range and between hosts requires further investigation.

In Australian waters, *C. saba* has been found only from off southern Queensland and *C. warehou* has only been found to the south, from off Tasmania and Victoria. Although the morphological distinction between the two species is clear, the only molecular evidence available relates to ITS2 sequences. The consistent single bp difference between four of the Victorian sequences and those from off Queensland probably corresponds to the distinction between the two species. As discussed above, ITS2 rDNA sequences are not infrequently proving to differ only slightly or not at all between what appear to be clearly different species based on other evidence (in this case host and morphology). Interpretation of this system is made more difficult by the single Victorian sequence which is identical to that of all Queensland samples. We conclude that, in Australian waters, this genus is best interpreted as represented by *C. warehou* in southern waters and by *C. saba* in more northerly waters, but this hypothesis needs testing based on fresh sampling and further sequencing.

3.3.4. Summary of samples reported, and identifications made here

Cephalolepidapedon saba Yamaguti, 1970

Definitive hosts: *Scomber japonicus* Houttuyn; *Scomber australasicus* Cuvier (Scombridae).

Definitive host localities: Minabe Fish Market, Wakayama Prefecture, Japan (33°44′N, 135°19′E), one hologenophore specimen from *S. japonicus*. Off Flinders Beach, North Stradbroke Island, Queensland,

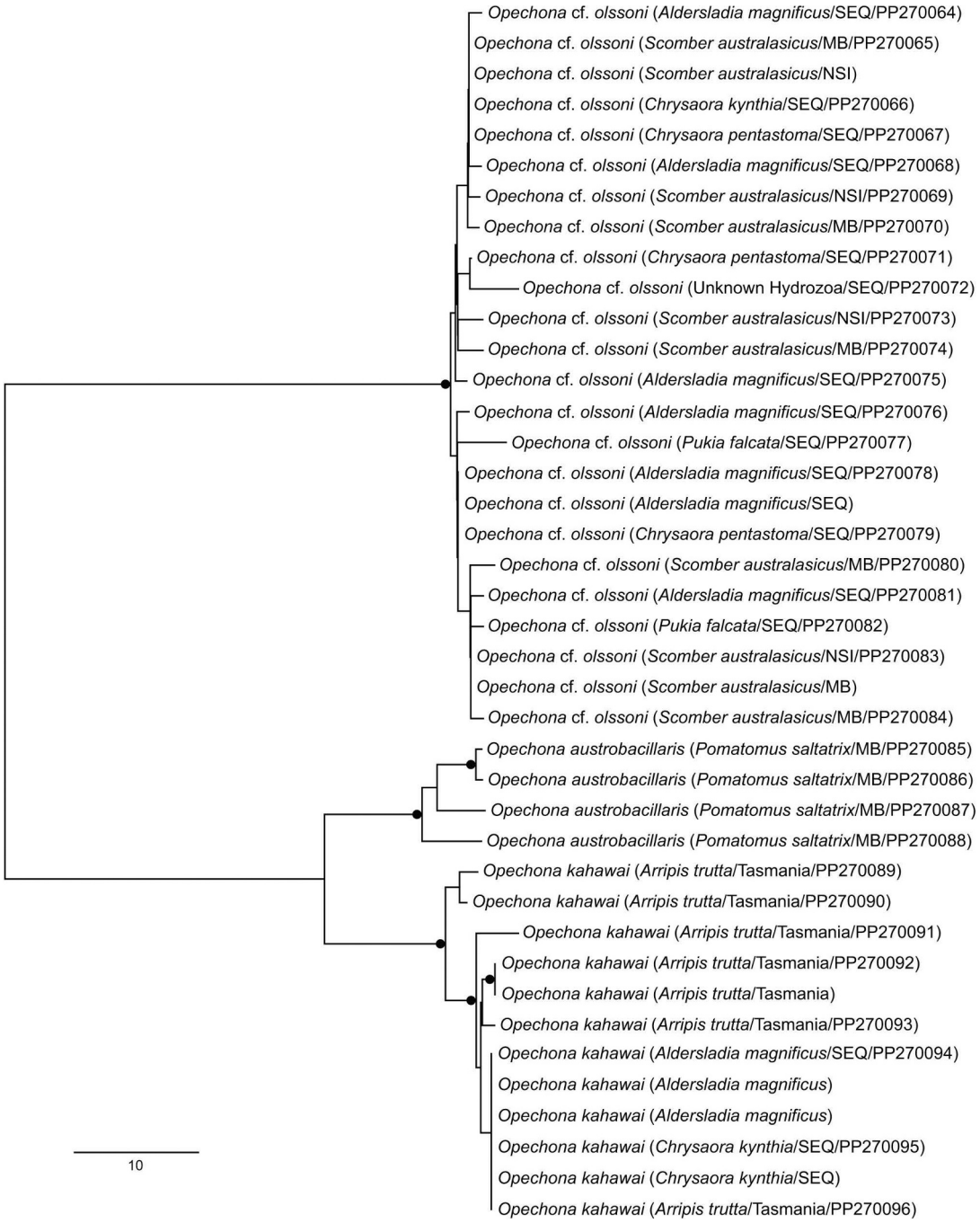

Fig. 7. Phylogram from the unrooted Neighbour-joining analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) mtDNA dataset for adult and metacercarial samples morphologically consistent with the genus *Opechona*. Strongly supported nodes (>80) are indicated by a filled circle. The scale bar indicates the number of base differences. *Abbreviations*: MB: Moreton Bay; NSI: North Stradbroke Island; SEQ: south-east Queensland.

Australia (27°24′S, 153°30′E), one hologenophore specimen from *S. australasicus*. Rainbow Channel, Moreton Bay, Queensland, Australia (27°25′S, 153°24′E), 42 specimens including four hologenophores from *S. australasicus*.

Prevalence: *S. japonicus*, 1 of 1; *S. australasicus*, 5 of 5.

Deposition of specimens: Japanese sample - MPM 25272; Australian samples - QM G241047–60, G241239–67.

Second intermediate hosts: Ph. Cnidaria, Cl. Hydrozoa, Aequoreidae: *Aldersladia magnificus* Gershwin; Cl. Scyphozoa, Pelagiidae: *Chrysaora kynthia* Gershwin & Zeidler (*nomen dubium*); *C. pentastoma* Péron & Lesueur, 1810.

Second intermediate hosts localities: Off southeast Queensland from Gold Coast Broadwater to off North Stradbroke Island.

Deposition of specimens: Australian samples - QM G241061–74; G241268–77.

Representative DNA sequences: *cox*1 (PP272965–988); ITS2 (PP239547–551); 28S (PP239560–561).

3.4. *Opechona Looss, 1907*

Adult specimens consistent with *Opechona* were found in Australian waters in three fish species and metacercariae were found in medusae of three cnidarians and one ctenophore from off the Queensland coast. No relevant material was collected from Japan.

3.4.1. *Sequence data*

A total of 40 *cox*1 sequences were generated (Fig. 7), revealing the presence of three distinct lineages. Three sequences from hologenophore specimens and one paragenophore specimen consistent with *O. austrobacillaris* from *Pomatomus saltatrix* (the type-host) from Moreton Bay varied at a *p*-distance of 0.21–2.332% (1–11 bp). Seven sequences from hologenophore specimens consistent with *O. kahawai* from *Arripis trutta* (the type-host is an unidentified species of *Arripis*) from close to the type-locality and five metacercariae from two species of cnidarian medusae varied at a *p*-distance of 0–1.48% (0–7 bp) and differed from those of *O. austrobacillaris* at 4.85–5.91% (23–28 bp). Nine sequences (eight from hologenophores) from specimens from *S. australasicus* and 12 from metacercariae from cnidarians and ctenophores differed from each other at just 0–1.69% (0–8 bp) and from those of *O. austrobacillaris* and *O. kahawai* at 15.4–17.3% (73–82 bp). No comparable *cox*1 sequences for other species of *Opechona* are available on GenBank.

A total of 24 ITS2 sequences were generated. Two sequences of *O. austrobacillaris* from *P. saltatrix* and one of *O. kahawai* from *A. trutta* were identical and identical to that previously reported for *O. austrobacillaris* by Bray et al. [55]. These four sequences differ at a *p*-distance of 1.26% (6 consistent bp) from two sequences from adults from *S. australasicus* and 20 from metacercariae from cnidarians and ctenophores.

In 28S rDNA analysis, two new sequences from *S. australasicus* and one identified as *O kahawai* from *A. trutta* were compared with existing sequences of *O. kahawai* and *O. austrobacillaris*. The two *O. kahawai* sequences were identical and differed from the single *O. austrobacillaris* sequence at a single bp. Samples from *S. australasicus* differed from *O. kahawai* and *O. austrobacillaris* at a *p*-distance of 0.61% (8 bp) and 0.68% (9 bp), respectively.

3.4.2. *Morphology*

Figure 8 shows the morphology of hologenophore or paragenophore adult specimens relating to the three genetic lineages of *Opechona* collected from *A. trutta, P. saltatrix* and *S. australasicus*. Figs. 9A–C show measurements that distinguish the three combinations of the lineages. Table 7 summarises key measurements for specimens, both adult and immature, from *S. australasicus*. Fig. 4C shows the morphology of a hologenophore metacercaria from a cnidarian medusa relating to the lineage found as adults in *S. australasicus*.

3.4.3. *Species recognition and identity*

The new specimens from fishes reported here are clearly consistent with the concept of *Opechona*, especially in the possession of an infundibuliform oral sucker which lacks prominent spines, a well-developed pseudoesophagus and a uroproct [64]. Our studies support the recognition of three species of *Opechona* in the new collections, two known, *O. kahawai* and *O. austrobacillaris*, and one not previously reported from Australia, that from *S. australasicus*. In contrast to the taxa of *Prodistomum* discussed above, the combination of host and morphology serves to distinguish the three species relatively easily whereas the molecular distinctions are much less robust. The form from *S. australasicus* is genetically clearly distinguished from the other two for all three markers analysed. In contrast, in terms of molecular data, the two recognised species (*O. kahawai* and *O. austrobacillaris*) are distinguished convincingly only by *cox*1 sequence data.

Opechona austrobacillaris was described from Western Australia from *P. saltatrix* by Bray & Cribb [76] and then reported from Moreton Bay off Queensland from the same fish species by Bray et al. [55] who sequenced specimens for both 28S and ITS2 data. The four new *cox*1 sequences relate to specimens from *P. saltatrix* collected from Moreton Bay as part of that study. The *cox*1 sequences do not match or form a clade with those of any of the metacercariae from cnidarians or ctenophores.

Opechona kahawai was described from an unidentified species of *Arripis* from off Stanley, Tasmania by Cribb & Bray [70]. It was later

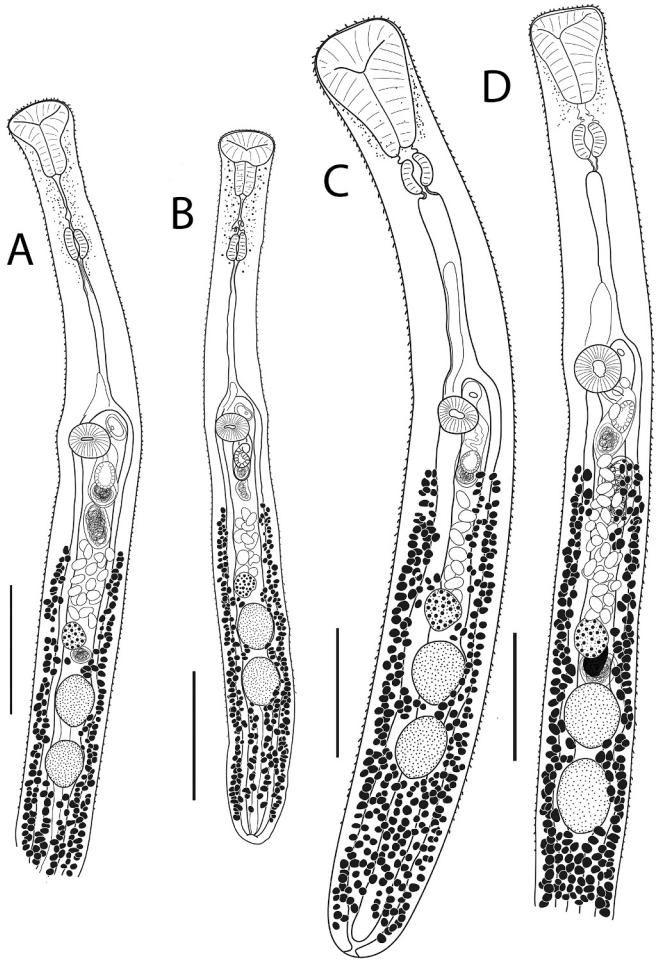

Fig. 8. Morphological specimens relating to three species of *Opechona*. A, B. *O.* cf. *olssoni*, hologenophore and paragenophore from *Scomber australasicus* off southeast Queensland; C. *O. kahawai from Arripis trutta* off Tasmania; D. *O. austrobacillaris* from *Pomatomus saltatrix* off southeast Queensland. Scale bars: 500 μm.

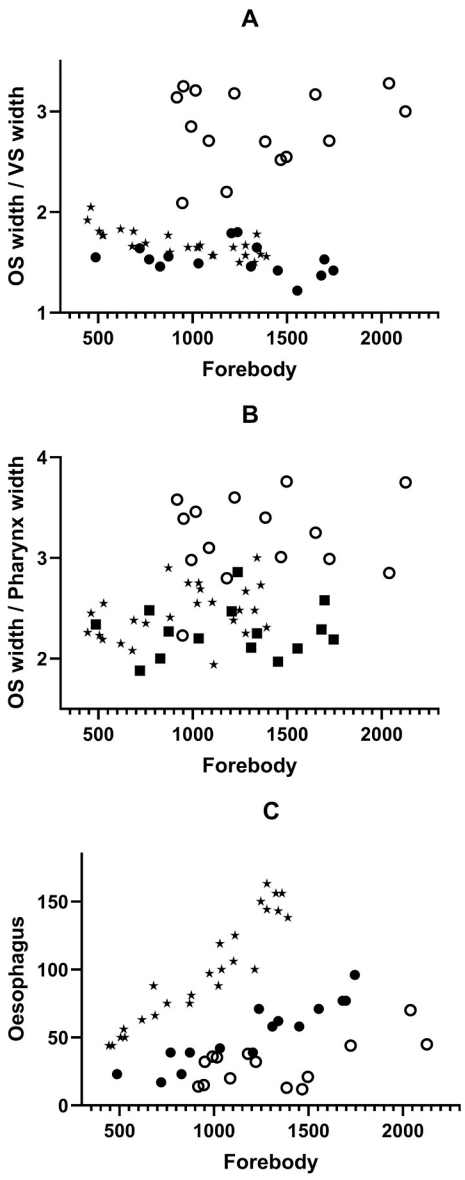

Fig. 9. Morphometric discrimination of three species of *Opechona* from Australian waters. A. Forebody length versus oral sucker to ventral sucker width ratio; B. Forebody length versus oral sucker width to pharynx width ratio; C. Forebody length versus oesophagus length. *O. austrobacillaris* ●; *O. kahawai* ○; *O.* cf. *olssoni* *.

reported from *Seriola lalandi* Valenciennes from Victoria by Hutson et al. [77] but without a figure or description. Bray et al. [55] reported that 28S sequences for *O. austrobacillaris* and *O. kahawai* differ at only a single bp, leading to the comment that "Given the minor genetic differences, the relationship between these two morphologically distinct forms warrants further study." Cribb & Bray [70] stated that *O. austrobacillaris* and *O. kahawai* differ in the sucker-ratio and in the length of the pseudoesophagus relative to that of the oesophagus. In specimens newly examined here, together with specimens previously deposited in the QM, we found no reliable interspecific distinction in the length of the pseudoesophagus relative to that of the oesophagus. However, the new specimens are strongly consistent with the distinction in sucker width ratio (Fig. 9A); oral suckers of *O. kahawai* tend to be relatively larger than those of *O. austrobacillaris* whereas the proportions are reversed for the ventral sucker, leading to a clear distinction in sucker width ratio. A difference is also evident in the oral sucker width to pharynx width ratio (Fig. 9B) and to a lesser extent in the ventral sucker width to pharynx width ratio (Fig. 9C). We now have *cox*1, ITS2 and 28S sequence data for both putative species and the differences are low: a *p*-distance of 4.85–5.91% (23–28 bp) for *cox*1, 0 bp for ITS2 and 1 bp for 28S. However, the combination of consistent although low molecular distinction correlating with distinct host distribution and reliable morphological distinction in the context of distribution in broad sympatry leads us to continue to recognise *O. kahawai* and *O. austrobacillaris* as distinct. It is noteworthy that metacercariae of *O. kahawai* have been sequenced from medusae of two cnidarian species from southeast Queensland. Although the adult of the species has not been detected in these waters, we note that *A. trutta* is known from the region [78] but is yet to be surveyed there for its parasites.

The third species distinguished here is from *S. australasicus* from southern Queensland. Sequences of this species form a strongly supported clade with those generated from 12 metacercariae collected from both cnidarian medusae and ctenophores. *cox*1 sequences of this species differ from those of *O. austrobacillaris* and *O. kahawai* at a *p*-distance of 15.4–16.9% (73–80 bp). Corresponding morphological specimens have a proportionally longer oesophagus than those of *O. austrobacillaris* and *O. kahawai*, specimens of *O. kahawai* have a consistently greater sucker width ratio, and specimens of *O. austrobacillaris* have a consistently smaller oral sucker width to pharynx width ratio. We conclude that the form from *S. australasicus* is unambiguously distinct from *O. austrobacillaris* and *O. kahawai*.

The genus *Opechona* currently comprises 14 valid species of which just five [*O. austrobacillaris*, *O. bacillaris* (Molin, 1859) Dollfus, 1927, *O. kahawai*, *O. occidentalis* Montgomery, 1957 and *O. olssoni* (Yamaguti, 1934) Yamaguti, 1938], have a strikingly infundibuliform oral sucker and an elongate body, as seen for the three species reported here. Our combined molecular and morphological results allow for convincing identification of new specimens of *O. austrobacillaris* and *O. kahawai*, as discussed above. The third species, from *S. australasicus*, might relate to any of the other three species or could be new. All three described species are broadly like the Australian specimens subject to the limitations of differences in handling and limited sample sizes. Of the three, *O. olssoni* is immediately the most plausible given that it was described by Yamaguti [79] from *S. japonicus* from Japan. Sokolov et al. [68] summarised other records, mainly from the North-western Pacific, and provided molecular data (ITS1 rDNA) that suggest that the species is not conspecific with *O. bacillaris*. They concluded, however, that more work is needed to distinguish the two species. The concept of *O. bacillaris*, the type-species for the genus, is partly problematic. It was described from a centrolophid fish, but subsequent records summarised by Bray and Gibson [66] are overwhelmingly from *Scomber scombrus* and *Merlangius merlangus* (Gadidae), but also from species of *Spinachia* (Gasterosteidae), *Capros* (Caproidae) and *Pomatomus* (Pomatomidae); it seems possible that these reports relate to multiple species as is often the case for the first-named species of older genera. If *O. bacillaris* does indeed show host-specificity to the Centrolophidae, then this would create a basis for distinction from the form in Australian *S. australasicus*. The most recently proposed of the three species, *O. occidentalis* was described from a sebastid from the west coast of North America [80], a host and geographical distribution probably distinguishing it from the Australian form.

In our 28S analyses, sequences relating to samples of *Opechona* from *S. australasicus* formed a clade with that reported by Sokolov et al. [68] for *O. olssoni* from *S. japonicus*, but differ at four bp. Despite their morphological similarity and closely related hosts, it is noticeable that these two forms show greater distinction for this marker than is seen between *O. austrobacillaris* and *O. kahawai* which infect unrelated hosts and are morphologically distinguishable. The limited available non-identical molecular data in the context of the conflicting emerging evidence (see *Cephalolepidapedon* and *Prodistomum* above) that the lepocreadiid fauna of *S. japonicus* in the north-western Pacific and that of *S. australasicus* in Australian waters are similar but not identical, both suggest caution in this identification. We conclude that the evidence is

Table 7
Measurements of 13 gravid specimens (incl. 1 hologenophore) of *Opechona* cf. *olssoni* from *Scomber australasicus* from off southeast Queensland.

Body L	2688–4080 (3461)
Body W	272–384 (331)
Body L / W	9.6–11.6 (10.4)
Forebody	976–1392 (1207)
Forebody % BL	30.6–39.7 (34.8)
OS L	250–350 (296)
OS W	206–276 (234)
Prepharynx L	100–231 (163)
Pharynx L	100–141 (128)
Pharynx W	75–109 (93)
OS W / Ph W	1.94–3 (2.53)
Oesophagus	88–163 (130)
Pseudoesophagus	356–625 (448)
Pseudoesophagus % FB	32.1–44.9 (37)
Pseudoesophagus L / Oesophagus L	3–4.7 (3.5)
VS L	106–156 (135)
VS W	125–163 (146)
OS W / VS W	1.5–1.78 (1.61)
VS W / Ph W	1.24–1.73 (1.57)
VS to ant. Test.	648–1104 (875)
Test. to post. End	536–1104 (790)
Distance between testes	0–94 (43)
Ant. test. L	144–253 (193)
Ant. test. W	125–206 (157)
Post. test. L	172–253 (199)
Post. test. W	131–213 (165)
Cirrus-sac L	181–381 (278)
Cirrus-sac W	63–116 (91)
Ovary to VS	512–896 (699)
Ovary to ant. Test.	19–94 (61)
Ovary L	84–119 (103)
Ovary W	84–125 (107)
VF to VS	250–400 (331)
EV to ant. Extremity	775–1263 (1055)
% Forebody L	75.7–94.1 (87.3)
Egg L	76–86 (80)
Egg W	37–56 (45)

Abbreviations: BL, body length; L, Length; Oes, oesophagus; OS, oral sucker; Ov, ovary; Pseud, pseudoesophagus; W, width; VF, vitelline follicles; VS, ventral sucker.

insufficient to allow a positive identification of the Australian samples as any previously proposed species, and that it is presently best identified as *O.* cf. *olssoni*. Finally, we note that Korotaeva [73] reported *O. bacillaris* from *S. australasicus* from Australia (Great Australian Bight). This report is likely to represent the same species as reported here, but this requires further study.

3.4.4. Summary of samples reported, and identifications made here

Opechona austrobacillaris Bray & Cribb, 1998

Definitive host: *Pomatomus saltatrix* (Linnaeus) (Pomatomidae).

Definitive host localities: Off Iluka, New South Wales, Australia (29°24′S, 153°20′E), eight specimens. Moreton Bay, Queensland, Australia (27°24′S, 153°12′E), four specimens.

Prevalence: 7 of 8.

Deposition of specimens: QM G241075–86.

Representative DNA sequences: *cox*1 (PP270085–088).

Opechona kahawai Bray & Cribb, 2003

Definitive host: *Arripis trutta* (Forster) (Arripidae).

Definitive host localities: Off Stanley, Tasmania (40°46′S, 145°18′E), five specimens. Gypsy Bay, Tasmania (42°54′S, 147°41′E), 13 specimens including 4 hologenophores.

Prevalence: 3 of 4.

Deposition of specimens: QM G241087–99.

Second intermediate host: Ph. Cnidaria, Cl. Hydrozoa, Aequoreidae: *Aldersladia magnificus* Gershwin; Cl. Scyphozoa, Pelagiidae: *Chrysaora kynthia* Gershwin & Zeidler (*nomen dubium*).

Second intermediate host localities: Off southeast Queensland from Gold Coast Broadwater to off North Stradbroke Island.

Representative DNA sequences: *cox*1 (PP270089–096); ITS2 (PP239538); 28S (PP239556).

Opechona cf. ***olssoni***

Definitive host: *Scomber australasicus* Cuvier (Scombridae).

Definitive host: Off Flinders Beach, North Stradbroke Island, Queensland, Australia (27°24′S, 153°30′E), three specimens including two hologenophore specimen. Rainbow Channel, Moreton Bay, Queensland, Australia (27°25′S, 153°24′E), 35 specimens including six hologenophores.

Prevalence: 5 of 5.

Deposition of specimens: QM G241100–13; G241128–58.

Second Intermediate host: Ph. Cnidaria, Cl. Hydrozoa, Aequoreidae: *Aldersladia magnificus* Gershwin; Hydrozoa, Unknown species; Cl. Scyphozoa, Pelagiidae: *Chrysaora kynthia* Gershwin & Zeidler (*nomen dubium*); *Chrysaora pentastoma*; Ph. Ctenophora, *Pukia falcata* Gershwin, Zeidler & Davie.

Second Intermediate host localities: Off southeast Queensland from Gold Coast Broadwater to off North Stradbroke Island.

Deposition of specimens: QM G241114–21.

Representative DNA sequences: *cox*1 (PP270064–084); ITS2 (PP239539–544); 28S(PP239555).

3.5. 28S phylogenetic analysis

New partial 28S rDNA sequences relating to the taxa considered above were analysed relative to previously available lepocreadiid sequences. Fig. 10 shows the topology inferred from the Maximum Likelihood analysis; the BI analysis differed only slightly and one point of distinction relevant to this study is noted below. As found repeatedly recently [53,58,68,81,82], the Lepocreadiidae formed two major but only moderately well-supported clades. The first clade is here recognised as the "*Lepocreadium* Clade" on the basis that it contains two species of the type-genus of the family (although they do not themselves form a clade). The second clade is recognised as the "*Diploproctodaeum* Clade" based on its inclusion of two species of *Diploproctodaeum*, the oldest of the 15 genera represented in it. All the taxa arising from this study resolve in the *Lepocreadium* clade. Notably, taxa identified as belonging to *Opechona* and *Prodistomum* failed to form clades. For *Opechona*, four taxa (*O. kahawai*, *O. austrobacillaris*, *O. olssoni* and *O.* cf. *olssoni*) form a well-supported clade distinct from *O. chloroscombri* and *O. corkumi* + *Opechona* sp. which are also isolated from each other. For *Prodistomum*, *P. alaskense*, *P. priedei*, *P. orientale* and *Prodistomum* Type 3 (this study) formed a weakly supported clade sister to *Opechonoides opisthoporus*. This clade was well-separated from one comprising *Prodistomum* Types 4 + 5 of this study together with *O. chloroscombri*; *P. keyam* was weakly associated with that clade. These poorly supported genera contrast with high support for some genera within the *Diploproctodaeum* Clade: nine species of *Lepotrema*; five species of *Hypocreadium*; and two species of *Bianium*. The eight species of *Preptetos* form a weakly supported clade in ML analysis but two strongly supported paraphyletic clades in BI analysis. Only the two species of *Diploproctodaeum* never form a clade. Our analysis includes sequences of three unidentified lepocreadiids lodged on GenBank (KF451933, KF451935 and KF451937). Of these, KF451937 from *Scomber japonicus* from Argentina is clearly associated with the clade comprising *Prodistomum orientale* + *Prodistomum* Type 3. The two sequences from unassociated cercariae from Argentinian gastropods (KF451933 and KF451935) resolve within the *Lepocreadium* Clade but are not close to any other taxa.

4. Discussion

4.1. Infection of gelatinous zooplankton

The starting point for this study was to explore the role of gelatinous zooplankton in the transmission of lepocreadiid trematodes to fishes. Our studies have identified three species of *Prodistomum*, two species of

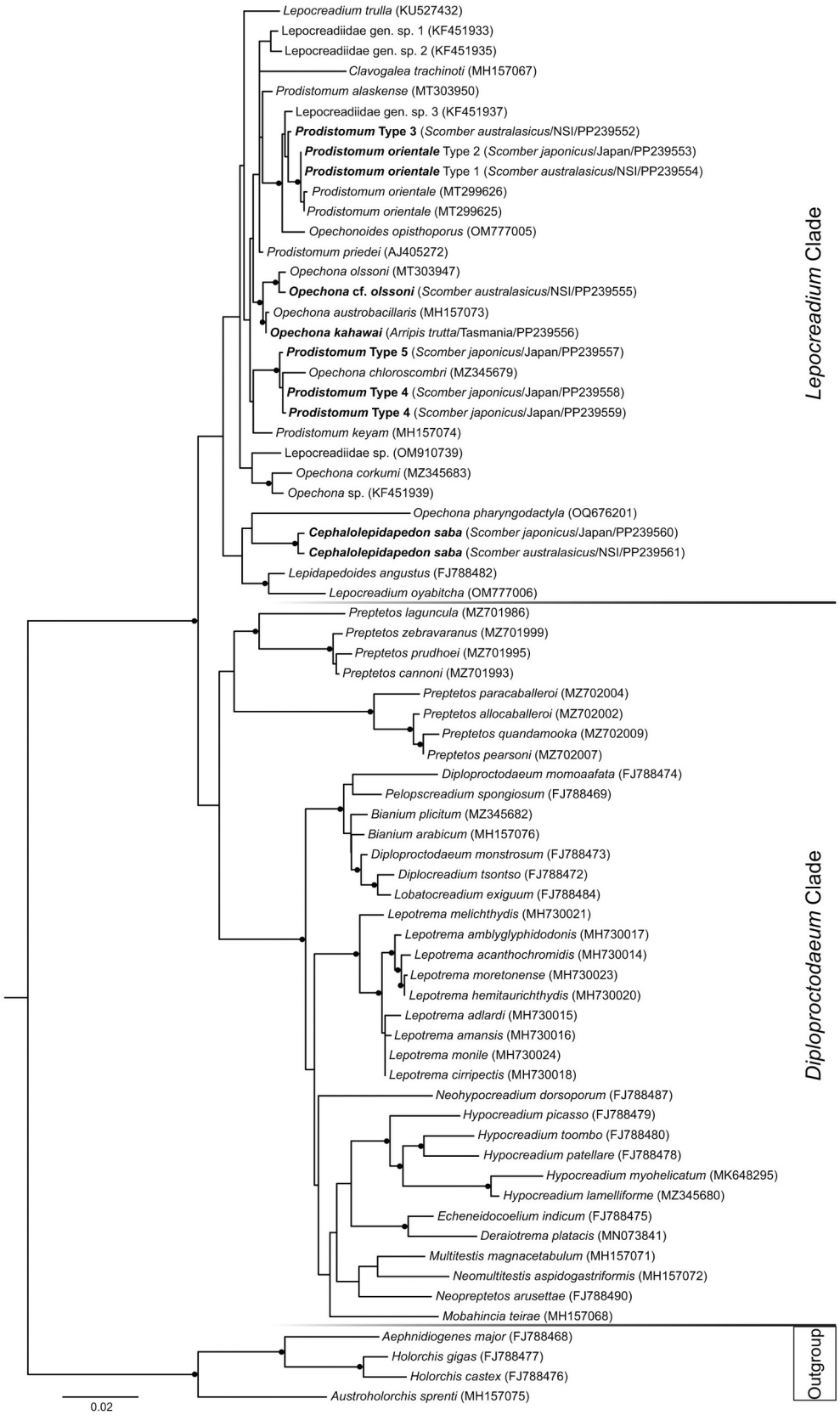

Fig. 10. Relationships between species of the Lepocreadiidae based on Bayesian inference phylogenetic analysis of the 28S dataset. Strongly supported nodes (Bayesian posterior probabilities >0.8 and maximum likelihood bootstrap values >80) are indicated by a filled circle. The scale-bar indicates expected number of substitutions per site. *Abbreviation*: NSI: North Stradbroke Island.

Opechona, and one of *Cephalolepidapedon* present as metacercariae in Australian gelatinous zooplankton. All six species were linked to adult trematodes in Australian marine fishes – four in *S. australasicus*, one in *A. trutta* and one in *M. argenteus*. The presence of two of these species in *S. japonicus* in Japanese waters is consistent with consumption of gelatinous zooplankton by that species as well. However, importantly, the presence of metacercariae in an animal is not proof that it leads to transmission; the animal must be known to be eaten by the definitive host for this to be considered demonstrated. If the animal is not eaten by the appropriate definitive host it should be considered a "dead-end". Demonstration or inference of dead-end hosts for metacercariae is rare [e.g. [83]], requiring negative evidence that is rarely obtained. The four fish species considered here (*M. argenteus, S. australasicus, S. japonicus*, and *A. trutta*) are mid-water feeders that mainly consume copepods, euphausiids and pelagic baitfish [84–86]. *Scomber japonicus*, however, also consumes some gelatinous zooplankton, including appendicularians, which have gelatinous feeding houses [84,85] and salps [87], but they have never been reported to consume cnidarian jellyfish or ctenophores. *Monodactylus argenteus* consumes zooplankton (e.g. copepods) but also [2] filamentous algae and epibenthic invertebrates such as amphipods, barnacle cirri and bryozoan lophophores [88,89] but has not been recorded consuming gelatinous zooplankton. The frequent occurrence of metacercaria in gelatinous zooplankton and of adult lepocreadiids of the same species in fishes, in this study leads us to infer, cautiously, that the infections from gelatinous zooplankton are indeed transmitted and that the four implicated fishes consume cnidarian jellyfish and/or ctenophores. We suspect that the lack of reports of cnidarian jellyfish and ctenophores in the diets of these (and many other) fishes relates to gelatinous zooplankton being consumed at least partly opportunistically and because the bodies of gelatinous zooplankton are rapidly digested so that these animals are difficult to identify in the gut contents of fishes given the absence of persistent hard-parts [2,90]. The dietary records for the three fishes reported as hosts here demonstrate clearly that none are obligate or perhaps even specialist consumers of jellyfish. Metabarcoding of gut contents of another scombrid, *S. scombrus*, revealed that it frequently feeds on hydrozoan medusae and ephyrae (i.e. juvenile medusae) of the scyphozoan jellyfish *Aurelia aurita* [91], so predation on cnidarian jellyfish and ctenophores is plausible for the scombrids considered in this study. Indeed, we predict that the importance of jellyfish in the diets of zooplanktivorous and mid-water feeding fishes is significantly underappreciated. We also note, however, the possibility that other animals are involved in the transmission of these lepocreadiids. Early studies based on infection experiments [10,14,18], showed that metacercariae of some lepocreadiids can infect a range of phyla and our work has not assessed other potential hosts to test this possibility.

4.2. Lepocreadiidae systematics

The 28S phylogenetic analyses here leads to two significant conclusions. First, the systematics of the group evidently still requires significant work. As found recently [53,68,81,82], the Lepocreadiidae here divides into two well-supported clades. These two clades may ultimately deserve subfamily status; available family group names have been proposed previously. All the taxa newly sequenced here fall clearly into the *Lepocreadium* Clade, but the internal relationships are chaotic. The two best-represented genera considered here, *Opechona* and *Prodistomum*, are both polyphyletic. If we assume that the 28S analysis gives broadly reliable results, as is suggested by the strong clustering of species of some genera (esp. *Hypocreadium*, *Lepotrema* and *Preptetos*), then this means that characters such as the uroproct and pseudoesophagus that help define these genera are more homoplastic than is presently appreciated. In this context, we note that we find the wide phylogenetic separation of the *Prodistomum* types considered in this study especially surprising given their close morphological similarity. Resolution of these discrepancies will depend on the sequencing and restudy of more species, especially the type-species of *Opechona* and *Prodistomum*.

The 28S rDNA molecular phylogenetic analysis is also potentially informative with respect to the distribution of the use of gelatinous zooplankton as intermediate hosts for lepocreadiids. All such reports where the lepocreadiid genus has been identified resolved in the *Lepocreadium* Clade, except for the report of *Lepotrema clavatum* Ozaki, 1932 by Kondo et al. [7]. Although this work is generally convincing, it is desirable for the identification of these metacercariae to be confirmed with sequence data. Notably, although multiple *Lepotrema* species occur in our study area [92], none were found in any of the examined zooplankton. The remaining taxa reported from jellyfish (species of *Cephalolepidapedon*, *Clavogalea, Lepocreadium, Opechona*, *Opechonoides* and *Prodistomum*), all belong to the *Lepocreadium* Clade. These genera are scattered throughout the clade (especially given the polyphyly of *Opechona* and *Prodistomum*), suggesting that use of gelatinous zooplankton as second intermediate hosts may be general, or nearly so, though certainly not exclusive for this clade. In this context we note that the present study is the first to report species of *Prodistomum* as metacercariae in gelatinous zooplankton, so that it seems plausible that use of these hosts by further genera may yet be reported.

The literature relating to *Lepocreadium* Clade metacercariae in gelatinous zooplankton and the observations here suggests substantial uniformity across the group. The metacercariae are apparently always unencysted, they undergo some to quite significant growth as metacercariae, and the host-specificity is often low. Low host-specificity may be unsurprising in the context of a permissive site of infection such as the mesoglea of their hosts. A striking feature of all the metacercariae reported here, and seemingly typically in the literature as well, is the heavy development of pigment in the forebody. With growth to sexual adults, the pigment becomes strongly dispersed in the forebody. Although it is clear from the descriptions of cercariae of some of these forms [e.g. 15, 18] that the pigment arises from the disintegration of the cercarial eye-spots, the volume of pigment is exceptional relative to that seen in most other trematode metacercariae that develop from oculate cercariae.

4.3. Identification and a global fauna

The widespread records of lepocreadiid metacercariae in gelatinous zooplankton and the developing evidence from sequence data (at the species level, at the level of genera, and for *Lepocreadium* Clade as a whole) combine to strongly suggest that trematodes comparable to those reported here occur globally. A global fauna points to the potential wide and general importance of gelatinous zooplankton in fish diets. Most certainly the emerging evidence suggests that highly similar species or even the same species of lepocreadiids occur widely in comparable fish species. The findings presented here suggest, however, that there remains an enormous task to tease apart the species and populations of trematodes so that the names applied to them give a realistic representation of the underlying biology. We see this process as requiring not only extensive sampling followed by morphological and molecular analyses, but further consideration of the underlying species concepts and subsequent approaches to application of species recognition criteria. This is no easy task. We are of the view that essentially global distributions, as are often suggested by the older literature, are generally unlikely but certainly not impossible [e.g. [93,94]]. Morphological analysis requires numerous samples of well-fixed specimens from multiple localities (a requirement not always met in the past or comprehensively in this study). It seems clear that genetic data are essential to unravelling these complexes, but with two important considerations. First, molecular data, or at least the specific markers used here, cannot be relied upon to behave consistently; in the present study we have seen an especially weak correlation between inter- and intra-specific variation among ITS2 and 28S rDNA sequences. This problem leads to the second consideration, that satisfactory biological understanding of the system is not possible based on molecular data alone; understanding and

satisfying taxonomic hypotheses require knowledge of host-specificity, morphology, distribution, and life history. On this basis, here we have proposed conservative identifications of taxa (i.e., several not formally named) for which the evidence falls short of being convincing. Resolution of the status of the taxa involved will require a global effort.

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Ethical approval

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

CRediT authorship contribution statement

Thomas H. Cribb: Writing – original draft, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Scott C. Cutmore:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Nicholas Q.-X. Wee:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Joanna G. Browne:** Writing – review & editing, Investigation, Conceptualization. **Pablo Diaz Morales:** Resources, Investigation, Data curation, Conceptualization. **Kylie A. Pitt:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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