

## Determining Intermediate Hosts for Opecoelidae and Microphallidae Species (Platyhelminthes: Trematoda) in the Southeastern Pacific Coast, Using Molecular Markers

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**ABSTRACT:** Metacercarial stages of digeneans were collected from decapod crustaceans inhabiting intertidal rocky zones in central Chile. The digeneans were identified through a molecular analysis based on the V4 region of the 18S ribosomal RNA gene. We analyzed 356 crustaceans belonging to 10 species, 115 intertidal fish belonging to 6 species, and 4 specimens of 1 coastal bird species. In total, 74.1% of crustaceans were parasitized with metacercariae. We found 1 species of Opecoelidae. This species showed low genetic divergence (0% and 0.1%) with adult digeneans found in intertidal fish and with the species *Helicometrina labrisomi* infesting a subtidal fish from northern Chile (*Labrisomus philippii*). Additionally, we found 2 species of Microphallidae, 1 closely related to *Maritrema* (1.3% genetic distance) and the other related to *Microphallus* (5% genetic distance). Therefore, our findings showed that the decapod crustaceans are relevant hosts in food webs from the southeastern Pacific coast. Furthermore, we found 5 species of crustaceans as second intermediate hosts for *H. labrisomi* and 2 species as secondary intermediate hosts for 2 Microphallidae, which contribute to elucidate parts of their life cycles through molecular markers and extended the host distribution of *H. labrisomi* in the southeastern Pacific coast.

Digeneans undergo several morphological changes from the first larval stage to the adult, such as increased body size, loss of locomotion structures, and development of external organs (e.g., suckers) and internal organs (e.g., reproductive system) (Cribb, 2005). To identify digenean species, most taxonomical keys are focused on the reproductive system, which is present only in the adults (Jousson et al., 1999). Therefore, larval stages of a digenean are difficult to identify at the species level. Identification may only be possible when the larvae and the life cycle are well known for a certain parasite species (Muñoz et al., 2012).

In Chile, the intermediate hosts for many parasites are completely unknown. Only a few digeneans' life cycles have been elucidated so far (e.g., Muñoz et al., 2014). Recently, genetic markers have been used to determine whether parasites of different development stages (e.g., Muñoz et al., 2012, 2014; López et al., 2014) and adult parasites with similar morphologies (Sepúlveda and González, 2014; Sepúlveda et al., 2014) are the same species. Therefore, the objectives of this study are (1) to determine whether decapod crustaceans are intermediate hosts for several digenean species found in coastal fish and birds in central Chile, and (2) to identify digeneans of decapod crustaceans from

the intertidal rocky zone of Chile, by sequencing part of the ribosomal 18S gene.

We collected 356 decapod crustaceans belonging to 10 species (Table I) from intertidal rocky pools in 2 localities of the central Chilean coast: Montemar (32°57'20"S, 71°33'03"W) and Las Cruces (33°29'22"S, 71°38'26"W), between July and September 2013. The crustaceans were placed in plastic containers with sea water and carried to the laboratory (PARALAB, Universidad de Valparaíso) to be dissected. The crustacean species were identified through morphological distinctions indicated by Viviani (1969). We captured 115 fish belonging to 6 species (Table I), through the use of anesthesia (0.1% clove oil solution) and a hand net, from the rocky intertidal in the central and south Chilean coast, Las Cruces (33°29'22"S, 71°38'26"W) and Lebu (37°37'07"S, 73°40'37"W), between July 2013 and January 2014. The fish were identified using the descriptions of Reyes and Hüne (2012). Additionally, 4 dead birds corresponding to 1 species, *Larus dominicanus*, were found in the rocky intertidal of Montemar (32°57'S, 71°33'W) during May and August 2014.

In the laboratory, some crustaceans (~50) were anesthetized with an overdose (1%) of a clove oil solution (AQUI-S, Bayer S.A., Santiago, Chile) before dissection; while others were frozen and thawed for posterior analysis. All specimens were examined using the same dissecting technique, which consisted in placing each individual in a Petri dish with saline (8%), and observed under a stereomicroscope (Leica M80, Leica Microsystem, Wetzlar, Germany). The pereopods, cephalothorax, and abdomen were removed with dissecting needles, and digenean metacercariae were collected mainly from the muscles. The metacercariae were counted and measured in length and width using an eyepiece reticle of a light microscope (Leica DMLS2, Leica Microsystem). The metacercariae were categorized following Leiva et al. (2015). The fish and birds were dissected, the digestive tracts were removed to collect the parasites under the stereomicroscope, digeneans were sorted, and metacercariae and adult digeneans were fixed in absolute ethanol in individual tubes for posterior molecular analyses.

For the molecular analyses, each digenean (metacercariae and adults) was isolated and transferred to a 1.5 ml microcentrifuge tube (1 in each tube). DNA extraction was performed according to the modified technique of Miller et al. (1988), involving treatment with sodium dodecyl sulphate and digestion with Proteinase K. Proteins were removed by precipitation with NaCl, and the DNA was precipitated with isopropanol. The V4 region of the ribosomal 18S gene was amplified by polymerase chain reaction (PCR) using the primers and protocols described by Hall et al. (1999). The PCR products were visualized on a 1.5% agarose gel and sequenced using an automated capillary electrophoresis sequencer (ABI 3730XL, Macrogen Inc., Seoul,

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TABLE I. Hosts parasitized with digeneans (Microphallidae and Opecoelidae). The number (n) of hosts collected, and abundance (ABU) and prevalence (PRE, %) of digeneans found in each host species are shown. Digeneans identified by molecular analysis (MA) based on the 18S RNA gene are indicated per host species, represented by X.

Hosts	n	Microphallidae (several species)			Opecoelidae ( <i>Helicometrina labrisomi</i> )		
		ABU	PRE	MA	ABU	PRE	MA
<b>Decapod crustaceans</b>							
<i>Allopetrolisthes angulosus</i>	162	0.7 ± 2.6	16.7		3.4 ± 5.0	70.4	
<i>Allopetrolisthes punctatus</i>	34	0.7 ± 2.0	14.7	X	2.8 ± 2.6	82.4	X
<i>Paraxanthus barbiger</i>	6	4.5 ± 7.0	33.3	X			
<i>Petrolisthes granulosus</i>	12				3.3 ± 4.6	50	
<i>Petrolisthes laevigatus</i>	4	2.3 ± 3.3	50		3.8 ± 2.5	75	
<i>Petrolisthes tuberculatus</i>	55	2.4 ± 6.4	38.2		10.3 ± 13.7	83.6	
<i>Petrolisthes tuberculatus</i>	26	4.9 ± 11.1	42.3	X	10.1 ± 10.1	88.5	X
<i>Petrolisthes violaceus</i>	32				13.6 ± 17.5	96.9	X
<i>Rhynchocinetes typus</i>	18				0.2 ± 0.4	22.2	X
<i>Taliepus dentatus</i>	7				0.9 ± 1.1	42.9	X
<b>Fish</b>							
<i>Auchenionchus microcirrhis</i>	6				8.2 ± 4.2	42.8	X
<i>Auchenionchus variolosus</i>	2				0.5 ± 0.7	50	X
<i>Auchenionchus crinitus</i>	4				2.7 ± 3.8	50	X
<i>Calliclinus geniguttatus</i> †	34				0.24 ± 0.89	8.8	X
<i>Gobiesox marmoratus</i>	59				0.11 ± 0.64	3.4	X
<i>Helcogrammoides chilensis</i>	10				0.1 ± 0.3	10	
<b>Birds</b>							
<i>Larus dominicanus</i>	4	0.3 ± 0.5	25				

South Korea). The sequences were submitted to Genbank under accession numbers KX179596–KX179626.

Phylogenetic trees were generated with the neighbor-joining (NJ), the maximum composite likelihood (ML) and maximum parsimony (MP) algorithms. The Mega v6 software (Tamura et al., 2013) was used for NJ and ML algorithms, and the Akaike Information Criterion was used to determine the best evolution model. GTR + I was used for ML and TN93 + G for NJ. The MP analysis was performed in the PAUP\* 4.0b10 program (Swofford, 2001) using heuristic search with the tree bisection-reconnection and branch-swapping options. Statistical support for the nodes was estimated for each algorithm used by a bootstrap with 1,000 pseudoreplicates (Felsenstein, 1985). The individuals were classified into groups according to species, and the distances were computed with the average number of mutations (substitutions). The divergence between groups was determined by applying the K2P and number of mutations in base pairs (bp).

Genetic sequences of species of Microphallidae (*Microphallus fusiformis*, *Microphallus primas*, and *Maritrema oocysta*) were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>), and our sequences of *Helicometrina nimia*, *H. labrisomi*, and *Helicometra fasciata* collected from fish in northern Chile (24°S) were also included (published in Genbank by González et al., 2013).

Of the whole sample (n = 356 crustaceans), 74.1% were parasitized, harboring 1–66 metacercariae. Opecoelid metacercariae were characterized by an oval form, 150–240 µm wide by 175–350 µm long, with a very thin brown cover and a thin (15–19 µm) cyst wall. Microphallid metacercariae were characterized by their roundish shape, 160–330 µm wide by 210–368 µm long, with no cover layer and a thick (20–25 µm) cyst wall. Opecoelid

metacercariae were more abundant and prevalent than microphallids (Table I). Metacercariae of both opecoelids and microphallids showed different abundances and prevalences in the decapod crustaceans, being more common in *Petrolisthes* spp. than in other genera of crustacean examined (Table I). Adult opecoelid digeneans of *Helicometrina* sp. were found in 6 intertidal fish species (Table I; Fig. 1). Only 1 adult microphallid species was found in a seagull, but there was not enough DNA extraction for further analysis.

According to their genetic sequences, opecoelid metacercariae were grouped into 1 clade with adult specimens (Table II; Fig. 2). This clade included metacercariae collected from 4 crustaceans (*Allopetrolisthes punctatus*, *Petrolisthes tuberculatus*, *Taliepus*

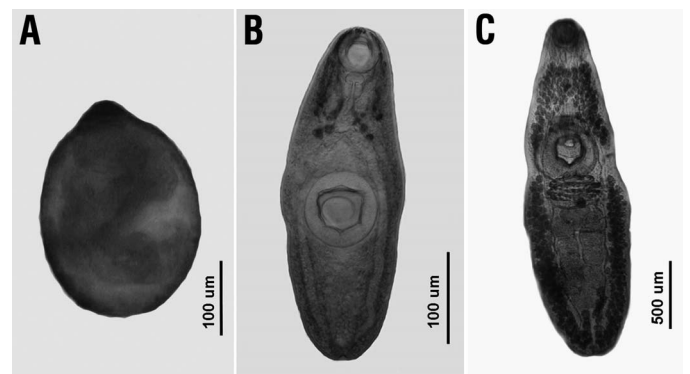


FIGURE 1. Specimens of *Helicometrina labrisomi* at different development stages. (A) Cyst of a metacercaria; (B) excysted metacercaria from *Petrolisthes tuberculatus*, and (C) adult digenean from *Gobiesox marmoratus*.

TABLE II. Pairwise sequence divergences for genetic distance in the V4 region of the ribosomal 18S gene. Metacercariae were from decapod crustaceans and adult worms were from fish. Genetic divergences (below the diagonal) were calculated using the neighbor-joining (NJ) algorithm (K2P model). The mean number of mutations between pairwise comparisons is shown for each clade (above the diagonal). Group numbers: (1) *Helicometrina labrisomi* (in decapod crustaceans); (2, 3) *H. labrisomi* (in fish); (4) *Helicometrina nimia* (in fish); (5) *Helicometra fasciata* (in fish); (6, 7) Microphallidae (in decapod crustaceans); (8) *Maritrema oocysta* (in barnacles); (9) *Microphallus fusiformis*; (10) *Microphallus primas* (in decapod crustaceans); (11) *Lobatostoma anisotremus* (outgroup).

		1	2	3	4	5	6	7	8	9	10	11
Opcoelidae												
1	<i>Helicometrina labrisomi</i> (metacercariae), this study	—	0	0.3	9	18	55	57	55	56	57	66
2	<i>Helicometrina labrisomi</i> (adults), this study	0	—	0.3	9	18	55	57	55	56	57	66
3	<i>Helicometrina nimia</i> (GenBank)*	0.1	0.1	—	9.3	18.3	55.3	57.3	55.3	56.3	57.3	66.3
4	<i>Helicometrina nimia</i> , this study	2.3	2.3	2.4	—	19	55	53	53	51	55	65
5	<i>Helicometra fasciata</i> (GenBank)	4.8	4.8	4.9	5	—	55	51	51	55	51	65
Microphallidae												
6	Microphallidae sp.1 (metacercariae), this study	15.8	15.8	15.9	15.7	15.9	—	23	6	25	25	67
7	Microphallidae sp. 2 (metacercariae), this study	16.6	16.6	16.7	15.2	14.8	6.1	—	20	19	22	66
8	<i>Maritrema oocysta</i> (GenBank)	15.8	15.8	15.9	15.1	14.6	1.5	5.3	—	24	24	64
9	<i>Microphallus fusiformis</i> (GenBank)	16.2	16.2	16.3	14.5	16.1	6.7	5	6.4	—	27	67
10	<i>Microphallus primas</i> (GenBank)	16.5	16.5	16.6	15.8	14.6	6.6	5.8	6.4	7.2	—	70
11	Outgroup	19.5	19.5	19.6	19.2	19.2	19.7	19.4	18.7	19.8	20.7	—

\* Misidentification of the species.

*dentatus*, and *Rhynchocinetes typus*), adult digeneans of 6 intertidal fish (*Auchenionchus* spp., *Calliclinus geniguttatus* (Labrisomidae), and *Gobiesox marmoratus* (Gobiesocidae)), and adult digeneans of 1 subtidal fish (*Labrisomus philippii* [Labrisomidae]). This clade showed low genetic divergence (0–0.1%) and a support bootstrap over 93% (Fig. 2); therefore, it was represented by 1 digenean species, *Helicometrina labrisomi*. The adults of *H. nimia* collected from 2 subtidal fish species (*Acanthictius pictus* and *Paralabrax humeralis* [Serranidae]) were grouped in another clade (Table II; Fig. 2), which had a genetic distance of 2.2–2.3% with the clade of *H. labrisomi*.

The sequences of Microphallidae metacercariae shaped 2 clades; the first clade, compound by metacercariae collected from 2 crustaceans: *A. punctatus*, *P. tuberculosis* (designated as Microphallidae sp. 1 in Fig. 2), was closely related with *Maritrema* supported by 99% and 98% bootstraps, using ML and MP algorithms, respectively, and with a genetic distance of 1.5%. The other clade, compound by metacercariae of 3 crustacean species: *A. punctatus*, *P. tuberculosis*, and *Paraxanthus barbiger* (designated as Microphallidae sp. 2) was close to *M. primas*, but with a large genetic divergence (5.8%) and number of mutations (22 bp). Also, this clade did not show a significant bootstrap support. Therefore, Microphallidae sp. 2 does not belong to *Microphallus* (Table II; Fig. 2).

In Chile, 2 species of *Helicometrina*, *H. nimia* and *H. labrisomi*, had been previously reported in marine fish (Table I). *Helicometrina labrisomi* was recently described by Oliva et al. (2015) from the fish *Labrisomus philippii*; however, for several years this species was confused with *H. nimia*. In this study, *H. labrisomi* was the only *Helicometrina* species found, as metacercaria stage, in all the decapod crustaceans parasitized. However, adult *H. labrisomi* was found in several intertidal fish from central Chile: *Auchenionchus* spp., *C. geniguttatus* (Labrisomidae), and the clingfish *G. marmoratus* (Gobiesocidae), meaning that, for this digenean, decapod crustaceans are secondary intermediate hosts and intertidal fish are definitive hosts. According to other records, *H. labrisomi* (also recorded as *Helicometrina* cf. *nimia* or

*Helicometrina* sp.) is a generalist species that has been found in other fish from the intertidal zone of central and central-south Chile, including *Sicyases sanguineus* (Gobiesocidae) (Muñoz and Delorme, 2011), *Calliclinus nudiventris* (Labrisomidae) (Inzunza et al., 1989), *Girella laevisfrons* (Kyphosidae) (Muñoz and Delorme, 2011), and *Scartichthys viridis* (Blenniidae) (Muñoz-Muga and Muñoz, 2010; Muñoz and Delorme, 2011). However, because of the high abundance and prevalence of *H. labrisomi* in the fish of Labrisomidae (Muñoz and Castro, 2012), these are the main definitive hosts.

When identifying the intermediate hosts for a trophically transmitted parasite, it is important to demonstrate the existence of trophic links between the intermediate hosts and definitive hosts. In this regard, all intertidal fish parasitized with *H. labrisomi*, such as Labrisomidae and Gobiesocidae, prey on decapod crustaceans (Muñoz and Ojeda, 1998; Pardo-Gandarillas et al., 2004). The wide distribution of *H. labrisomi*, from the north (23°S) in *L. philippii* to the central-south (37°S) in *C. geniguttatus*, suggests that this parasite is quite versatile in its use of hosts. All these facts indicate that *H. labrisomi* is a generalist parasite in both secondary and definitive hosts.

None of the metacercariae from the decapod crustaceans corresponded to *H. nimia*. Serranid fish, which harbor *H. nimia*, have distinct trophic habitats from those of Labrisomid fish or other intertidal fish. Just a few species of crustaceans are common prey for these 2 groups of fish (Muñoz and Ojeda, 1998; Vargas et al., 1999). Therefore, the life cycle of *H. nimia* might include development in organisms from the subtidal zone in northern Chile.

The other group of parasites found in the decapod crustaceans belonged to the Microphallidae. At least 2 species were identified, including an undetermined species of *Maritrema*. The other species was related to *Microphallus*, but the genetic differences were too high to consider the species to belong to this genus. Unfortunately, there is not enough information about the molecular sequences of adult Microphallidae to match the species with another genus. Coastal birds are the definitive hosts for most microphallid digeneans, and several records have been made in



FIGURE 2. Phylogenetic tree based on the V4 region of the ribosomal 18S gene, using a Maximum Likelihood algorithm (ML) and a GTR + I model. Percentages of supporting values from different analyses are given in the tree, next to the branches, in the following order: ML/NJ/MP. Abbreviations for parasites: Hna (*Helicometrina*), adul (adult) and met (metacercariae). Abbreviations for intermediate hosts: Ap (*Allopetrolisthes punctatus*), Pt (*Petrolisthes tuberculatus*), Td (*Talipeus dentatus*), Pv (*Petrolisthes violaceus*), Pb (*Paraxanthus barbiger*), Rt (*Rhynchocinetes typus*). Abbreviations for definitive hosts: Asp (*Auchenionchus crinitus*), Cg (*Calliclinus geniguttatus*), Am (*Auchenionchus microcirrhis*), Av (*Auchenionchus variolosus*), Api (*Acanthistius pictus*), Ph (*Paralabrax humeralis*), Lp (*Labrisomus philippii*), Gm (*Gobiesox marmoratus*). \*Misidentification of the species.

seagulls (e.g., Cremonte and Martorelli, 1998; González-Acuña et al., 2009; Díaz et al., 2012; this study). There are few parasitological analyses in coastal birds of Chile, and we do not know the potential spectrum of definitive host species for microphallids.

In the intertidal rocky coast of Chile there is a great diversity of snails, and the first intermediate hosts for many digenean species are currently unknown (Muñoz et al., 2014). Therefore, for *H. labrisomi*, *Maritrema* sp., and the unidentified microphallid found in the decapod crustaceans of this study, there is no knowledge about their first intermediate hosts. A snail species of Columbellidae and a shrimp are the first and secondary intermediate hosts, respectively, for *Helicometra gibsoni*, which is a close relative of *Helicometrina* spp. (Meenakshi et al., 1993). Several species of Columbellidae inhabit the coast of Chile and may be first hosts for opecoelids. Likewise, microphallids usually use different species of snails as first intermediate hosts (Saville and Irwin, 1991; Martorelli et al., 2004; Muñoz, 2005). For example, Ching (1963) found that the snails *Littorina* spp. are first hosts for *Maritrema laricola*, whereas Martorelli et al. (2004) found that

*Zeacumantus subcarinatus* (Batillaridae) are first hosts for *Maritrema novaezealandensis*. For both *Maritrema* species, the secondary intermediate hosts include several species of decapod crustaceans. This result indicates that each digenean species uses several host species, likely depending on the availability of host species in the digenean's habitat. Seagulls (*Larus* spp.) prey on a wide spectrum of food (Bahamondes and Castilla, 1986), such as mollusks, fish, and crustaceans; the latter group includes several species of *Petrolisthes*. The relatively high prevalence of metacercariae Microphallidae in intertidal decapod crustaceans, mainly in *Petrolisthes* and *Allopetrolisthes* (Leiva et al., 2015), confirm that these decapods can be important secondary intermediate hosts for these digeneans.

This study demonstrated that the genetic marker was useful in recognizing intertidal crustacean decapods from central Chile as secondary intermediate hosts for *H. labrisomi* and microphallids. Furthermore, our findings suggest that the decapod crustaceans are relevant hosts in south Pacific food webs. Future studies should be focused on the first intermediate hosts for these

digeneans and the digeneans in birds in order to advance in the full knowledge of their life cycles.

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