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 using the descriptions of Reyes and Hüne (2012). Additionally, we found 2 species of Microphallus, 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.

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 southern 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 Microsystems, Wetzlar, Germany). The pereiopods, 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 Microsystems). 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, 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, 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).

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 labrisomi, 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...
Table II. Pairwise sequence divergences for genetic distance in the V4 region of the ribosomal 18S gene. Mectarcariae 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 labrissomi (in decapod crustaceans); (2, 3) H. labrissomi (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).

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* Misidentification of the species.

dentatus, and Rhyynchocinetes 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 of 93% (Fig. 2); therefore, it was represented by 1 digenean species, Helicometrina labrissomi. The adults of H. nimia collected from 2 subtidal fish species (Acanthictius pectus and Paralabrax humerals [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. labrissomi.

The sequences of Microphallidae mectarcariae shaped 2 clades; the first clade, composed by mectarcariae collected from 2 crustaceans: A. punctatus, P. tuberculosus (designated as Microphalidae sp. 1 in Fig. 2), was closely related with Maritrema supported by 99% and 98% bootstrap, using ML and MP algorithms, respectively, and with a genetic distance of 1.5%. The other clade, composed by mectarcariae of 3 crustacean species: A. punctatus, P. tuberculosus, 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 Microphallidae (Table II; Fig. 2).

In Chile, 2 species of Helicometrina, H. nimia and H. labrissomi, had been previously reported in marine fish (Table I). Helicometrina labrissomi 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. labrissomi was the only Helicometrina species found, as mectarcarcia stage, in all the decapod crustaceans parasitized. However, adult H. labrissomi 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. labrissomi (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 laevisrons (Kyphosidae) (Muñoz and Delorme, 2011), and Sartichthys viridis (Benniidae) (Munoz-Muga and Muñoz, 2010; Muñoz and Delorme, 2011). However, because of the high abundance and prevalence of H. labrissomi in the fish of Labrisomidae (Munoz 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. labrissomi, such as Labrisomidae and Gobiesocidae, prey on decapod crustaceans (Muñoz and Ojeda, 1998; Pardo-Gandarillas et al., 2004). The wide distribution of H. labrissomi, 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. labrissomi is a generalist parasite in both secondary and definitive hosts.

None of the mectarcariae 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 microphalid digeneans, and several records have been made in...
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 novacezalandensis*. 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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LITERATURE CITED


SEPÚLVEDA, V. A., M. T. GONZÁLEZ, AND M. E. OLIVA. 2014. Two new species of Encoytlallye (Monogenea: Capsalidae) based


