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First molecular identification of the trematode *Maritrema bonaerense* Etchegoin & Martorelli, 1997 (Plagiorchiida, Microphallidae) from its intermediate hosts, the gastropod *Heleobia australis* (d'Orbigny, 1835) (Littorinimorpha, Cochliopidae) and the crab *Neohelice granulata* (Dana, 1851) (Decapoda, Varunidae) in Argentina

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Abstract

The genus *Maritrema* Nicoll, 1907 (Platyhelminthes, Trematoda, Plagiorchiida, Microphallidae) comprises cosmopolitan species that predominantly parasitize birds. Although approximately 65 species have been described worldwide, including 6 for Argentina, molecular data referring to *Maritrema* species are still scarce worldwide, especially in South America. Unfortunately, this lack of references for nucleotide sequences is an obstacle to understanding the taxonomy and life cycles of trematodes, and impedes advancing our studies on the phylogeny and geographical distribution of these parasites. For that reason, we performed the molecular study of developmental stages of *Maritrema bonaerense*: cercariae (collected from the snail first intermediate host *Heleobia australis*, inhabiting Mar Chiquita lagoon) and metacercariae (collected from the crab second intermediate host *Neohelice granulata*, inhabiting Mar Chiquita lagoon and San Antonio Oeste, Argentina). The accordance between the ITS2 sequence of *M. bonaerense* cercaria from the snail *H. australis* and the sequences of metacercariae from the crab *N. granulata* was 100%, supporting previous findings of the life cycle of *M. bonaerense* based on morphological data. All *Maritrema* species are included in a monophyletic and well-supported clade. *Maritrema bonaerense* grouped more closely with *Maritrema gratiosum*. These findings contribute to the knowledge of digeneans in coastal marine ecosystems.

Key Words

digeneans, ITS2 sequence, life cycle, South America

Introduction

The genus *Maritrema* Nicoll, 1907 (Platyhelminthes, Trematoda, Plagiorchiida, Microphallidae) comprises cosmopolitan species that predominantly parasitize birds in brackish, marine and to a lesser extent, freshwater ecosystems (Deblock 2008; Capasso et al. 2019). Their life cycles also involve gastropods and crustaceans as first

and second intermediate hosts, respectively (Yamaguti 1975). To date, approximately 65 species of this genus have been described worldwide (Presswell et al. 2014), including 6 species from Argentina: *Maritrema bonaerense* Etchegoin & Martorelli, 1997; *M. orensense* Cremonte & Martorelli, 1998; *M. madrynense* Diaz & Cremonte, 2010; *M. formicae* Diaz, Gilardoni & Cremonte, 2012; *M. patagonica* Rauque, Flores & Brugni, 2013, and *M. pichi* Capasso D´Ámico & Diaz, 2019.

As with other digeneans, molecular data referring to *Maritrema* species are still scarce in South America. For example, the only DNA sequences available in Argentina are from *M. madrynense* (Bagnato et al. 2015). Unfortunately, this lack of references for nucleotide sequences for South America is an obstacle to understanding the taxonomy and life cycles of trematodes (López-Hernández et al. 2019). Likewise, an increase in genetic data would be a significant step forward in our studies on the phylogeny and geographical distribution of these parasites in the region.

The life cycle and developmental stages of *M. bonaerense* were originally described by Etchegoin and Martorelli (1997) from Mar Chiquita lagoon (Buenos Aires province, Argentina). Later, Alda et al. (2013) redescribed the adult and metacercaria, and experimentally confirmed the life cycle of this species which includes the cochliopid snail *Heleobia australis* as first intermediate host and the crabs *Cyrtograpsus angulatus* and *Neohelice granulata* as second intermediate hosts, and the birds *Chroicocephalus maculipennis*, *Larus atlanticus* and *L. dominicanus* as definitive hosts. Both mentioned studies were conducted using only morphological analyses. For that reason, and taking into account the scarcity of genetic data related to the species of *Maritrema*, we performed the molecular study of cercariae and metacercariae of *M. bonaerense*, collected from the snail *H. australis* inhabiting Mar Chiquita lagoon and from the crab *N. granulata* inhabiting Mar Chiquita lagoon and San Antonio Oeste (Rio Negro province, Argentina).

It is important to mention here that although adult stages from the definitive hosts could not be obtained because Mar Chiquita is a Man and Biosphere Reserve (UNESCO) within which the birds are protected, *M. bonaerense* is the only species of *Maritrema* that parasitizes *H. australis* and *N. granulata* in this location (Etchegoin 2001; Parietti et al. 2013). Therefore, there was no possibility of misidentifications of developmental stages collected for this study.

Materials and methods

The specimens of *H. australis* were collected in Mar Chiquita lagoon, Buenos Aires province, Argentina (37°45'08"S, 57°26'18"W). In the laboratory, molluscs were isolated individually in 45 ml plastic cups and maintained under a 12–12 light-dark photoperiod for 48 h to stimulate shedding of cercariae. Crabs (*N. granulata*) collected in Mar Chiquita lagoon and in San Antonio Oeste (40°43'36"S, 64°54'49"W) (Fig. 1) were transported to

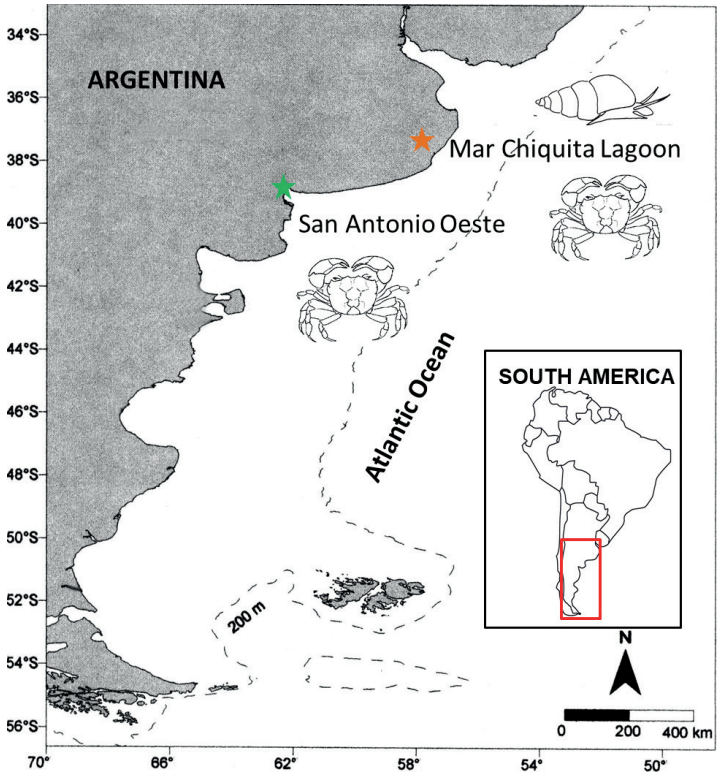

Figure 1. Map of sampling sites from Argentina: Mar Chiquita Lagoon (Buenos Aires province) where the snail *Heleobia australis* and the crab *Neohelice granulata* were collected and San Antonio Oeste (Rio Negro province) where *N. granulata* were collected. Invertebrate drafts extracted from Alda et al. (2013).

Table 1. Molecular data of *Maritrema* species considered in this study.

Species	Life stage	Host	Habitat type	Country	ITS2	p-distance	Reference
Maritrema bonaerense	*cercaria*	*Heleobia australis*	brackish	Argentina	ON833442		this study
Maritrema bonaerense	*metacercaria*	*Neohelice granulata*	brackish	Argentina	ON833466	0.00	this study
Maritrema bonaerense	*metacercaria*	*Neohelice granulata*	marine	Argentina	ON833467	0.00	this study
Maritrema gratiosum	*metacercaria*	*Semibalanus balanoides*	marine	Ireland	HM584171	0.04	Galaktionov et al. (2012)
Maritrema subdolum	*cercaria*	*Peringia ulvae*	brackish	Russia	HM584172	0.08	Galaktionov et al. (2012)
Maritrema eroliae	*cercaria*	*Clypeomorus bifasciata*	marine	Kuwait	HQ650132	0.11	Al-Kandari et al. (2011)
Maritrema novaezealandense	*cercaria*	*Zeacumantus subcarinatus*	marine	New Zealand	KJ540203	0.10	Born-Torrijos et al. (2014)
Maritrema madrynense	*adult*	*Larus dominicanus*	marine	Argentina	KF575167	0.10	Diaz and Cremonte (2010)
Maritrema brevisacciferum	*metacercaria*	*Caridina indistincta*	freshwater	Australia	KT355824	0.09	Kudlai et al. (2015)
Maritrema oocysta	*cercaria*	*Hydrobia ulvae*	marine	Ireland	HM584170	0.10	Galaktionov et al. (2012)
Microphallus similis	*metacercaria*	*Carcinus maenas*	marine	Russia	HM584180	0.14	Galaktionov et al. (2012)

the laboratory and maintained in aerated water. Posteriorly, infected snails and crabs were necropsied, and the developmental stages (sporocyst, cercariae and metacercariae) were stored in 96% ethanol for molecular studies. Cercariae and metacercariae of *M. bonaerense* were identified according to Etchegoin and Martorelli (1997) and Alda et al. (2013).

The molecular characterization of the developmental stages of *M. bonaerense* was made using rRNA ITS2 sequences. The DNA extraction, PCR amplification, and sequencing were performed using the protocol described in Gilardoni et al. (2020). Newly generated ITS2 sequences were deposited in GenBank and aligned using MAFFT (Katoh et al. 2019) together with available *Maritrema* spp. and with *Microphallus similis* as outgroup (Table 1). Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted in MEGA X (Kumar et al. 2018) and MrBayes version 3.2.7a (Ronquist et al. 2012) respectively. Genetic divergences amongst taxa were calculated as uncorrected p-distances using MEGA X.

For both, ML and BI, to determine the nucleotide substitution model that gave the best fit to our data set, the program MEGAX which held the JModel test analysis was employed, with model selection based on the Akaike information criterion (AIC). Results indicated that the general time reversible model with an estimate of gamma distributed among-site rate variation (GTR+G) was the most appropriate. For the ML tree, the percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 35 nucleotide sequences. There are a total of 771 positions in the final dataset. For the BI tree, GTR was selected as the substitution model (command lset nst=6). We ran four independent chains of 100 million generations each, sampling every 5000 generations, with the first 1000 trees discarded as "burn-in". Chain convergence was confirmed using Tracer v.1.6 (Rambaut et al. 2018). Finally, a 50% majority rule consensus tree was constructed.

Results and discussion

The PCR amplification of the ITS2 rRNA from cercaria from Mar Chiquita Lagoon and metacercariae from Mar Chiquita Lagoon and San Antonio Oeste gave products of 540 bp, 557 bp and 543 bp respectively. The accordance between the ITS2 sequence of *M. bonaerense* cercaria from *H. australis* and the sequences of metacercariae from *N. granulata* was 100% (Fig. 2). This result supports previous findings of the life cycle of *M. bonaerense* based on morphological data (Etchegoin and Martorelli 1997; Alda et al. 2013).

The genus *Maritrema* constitutes a monophyletic and well-supported clade. Among all the species of *Maritrema* compared in this work, *M. bonaerense* seems to be more closely related to *M. gratiosum* Nicoll, 1907. Both species constitute a well-supported clade separated from the other *Maritrema* spp. The genetic divergence (p-distance) revealed *M. bonaerense* presents 0.04 variation with *M. gratiosum*, 0.08–0.11 with the other *Maritrema* spp. and 0.14 with *Microphallus similis* (outgroup). The molecular data support the morphological taxonomy of the genus *Maritrema*, which is distinguished by the vitellarium in symmetrical ribbons reaching close to margin of hindbody, surrounding uterine coils and testes, horseshoe-shaped with posteriorly directed opening or complete ring (Deblock 2008). Despite the high number of *Maritrema* species morphologically described, molecular data are very scarce: rRNA 18S (6 spp sequenced), 28S (10 spp.), ITS1 (5 spp.), ITS2 (7 spp.), mitochondrial DNA cox1 (1 sp.). To date, most available sequences belong to species infecting marine or brackish hosts (Table 1). However, *Maritrema* species are present in freshwater habitat as *M. brevisacciferum* (Kudlai et al. 2015). Our findings contribute to the development of molecular database that may be used in future studies about these common and widespread parasites infecting birds worldwide.

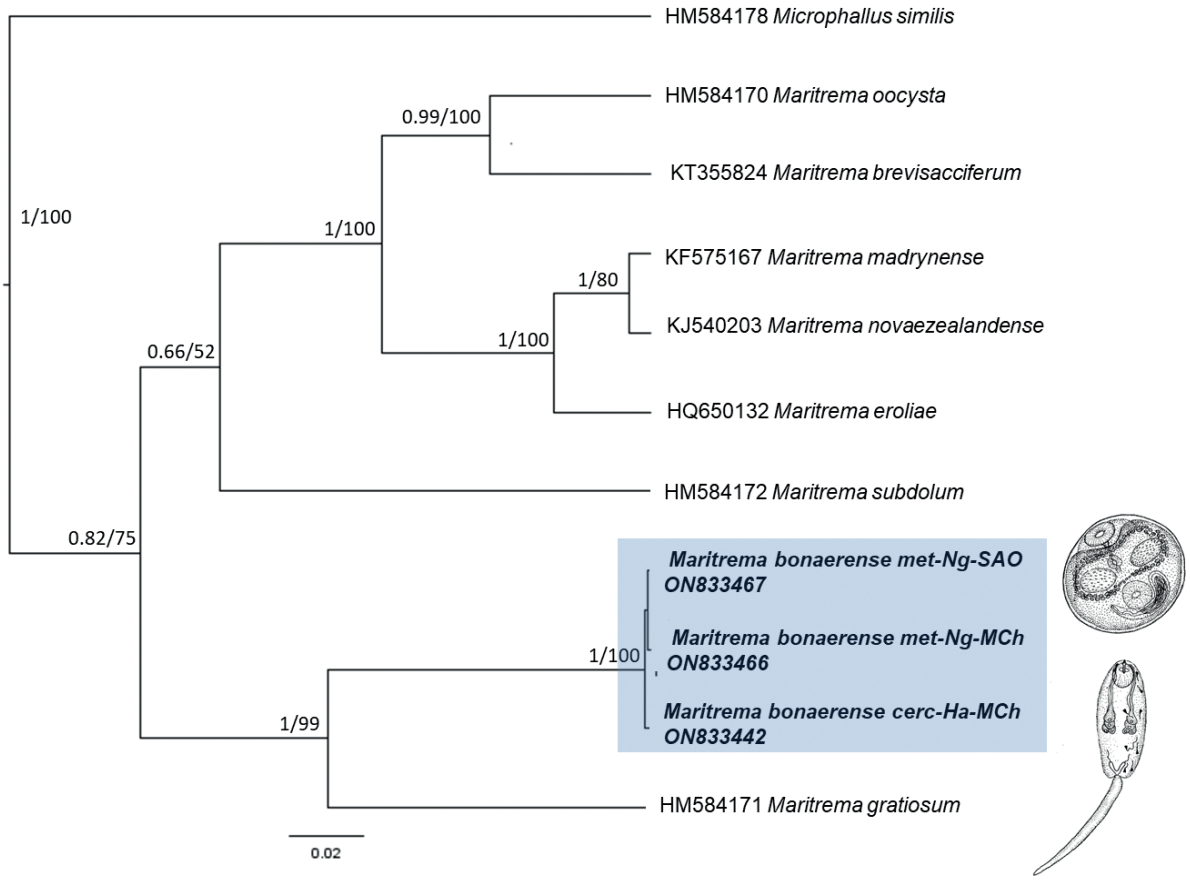

Figure 2. Phylogram for *Maritrema* species (*Microphallus similis* as outgroup), inferred by ML/BI of sequence data for ITS2 of the rRNA genes. The newly generated sequences are indicated in bold. Values on the branches correspond to posterior probabilities > 0.85 followed by bootstrap support > 60. Values below these thresholds were not reported. Abbreviations: cerc-cercaria, met-metacercaria, Ng-*Neohelice granulta*, Ha-*Heleobia australis*, MCh-Mar Chiquita Lagoon, SAO-San Antonio Oeste. Drafts of life stages extracted from Etchegoin and Martorelli (1997).

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