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Identification assisted by molecular markers of larval parasites in two limpet species (Patellogastropoda: *Nacella*) inhabiting Antarctic and Magellan coastal systems

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Abstract

In the Southern Ocean, many parasites of vertebrates (mainly helminth groups) have been recognized as endemic species, but parasites of marine invertebrates remain almost unknown. It is reasonable to assume that digenean larvae will parasitize gastropods, bivalves, amphipods, and annelids, the usual first and second intermediate hosts for those parasites. Here, using an identification assisted by molecular markers, we report the Digenea species parasitizing the most abundant limpet species inhabiting ice-free rocky intertidal and subtidal zones of the Southern Ocean, viz. *Nacella concinna* from the Antarctic and *Nacella deaurata* from the Magellan region. The limpets harbored larval Digenea (two metacercariae and one sporocyst). Phylogenetic analysis based on the multilocus tree supported the hypothesis that *N. concinna* is parasitized by a species of Gymnophallidae, whereas the limpet *N. deaurata* is parasitized by *Gymnophalloides nacellae* and a species of Renicolidae. In addition, differences in prevalence and intensity were also recorded between the two compared host species and also from other congeneric species. This new knowledge in parasite species in marine invertebrates from the Southern Ocean reveals the presence of a particular parasite fauna and confirms the utility of molecular tools to identify biodiversity still scarcely known.

Keywords *Nacella concinna* · *Nacella deaurata* · Digenean parasites · Molecular markers · Southern Ocean

Introduction

Nowadays, there is general agreement that parasite diversity is key to fully understanding population dynamics, interspecific competition, energy flow, and the mechanisms that shape biodiversity (Hudson et al. 2006). Parasites are a "biological magnifying glass" that can help to understand the evolutionary processes that shape extant population patterns (Froeschke and von der Heyden 2014). Parasites can decrease the survival or reproductive output of their host (Mouritsen and Poulin 2002). In addition, there is evidence that the outcome of interspecific competition between hosts (Hudson and Greenman 1998) and the interaction between predators and prey (Lafferty 1999) can be modified by

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parasites. Despite the relevance of parasites in the community, parasite biodiversity is still scarcely known in places such as Antarctica and Patagonia, and strongly underrepresented in most marine biodiversity lists (Klimpel et al. 2017).

Parasites in the Southern Ocean have been documented parasitizing fishes, birds, and mammals (Barbosa and Palacios 2009; Diaz et al. 2017; MacKenzie 2017). In contrast, the parasites of marine invertebrates remain widely unknown. Parasitological studies on marine invertebrates are important and necessary for understanding the life cycles of parasites, especially digeneans (Gibson et al. 2002). It is reasonable to assume that digenean parasites, with complex life cycles, are bound to gastropods, bivalves, amphipods, and annelids, which are used as first and second intermediate hosts before moving to their definite vertebrate hosts (Zdzitowiecki 1988). Due to this strict interdependence with hosts from different trophic levels, trophic interactions and host species distributions could be the main factors determining the presence of a given parasite in a particular environment (Rohde 2005).

The patellogastropod genus *Nacella* are true limpet species that are abundant in Antarctic and Magellan regions, inhabiting ice-free rocky intertidal and shallow subtidal zones of these ecosystems (Picken 1980; Morriconi 1999; Valdovinos and Ruth 2005; Rosenfeld et al. 2018). From a biogeographic point of view, these closely related species constitute evidence of the Antarctic–Magellan connection (González-Wevar et al. 2011). In the Antarctic Peninsula, *Nacella concinna* is the most abundant invertebrate, whereas in Patagonia, *Nacella deaurata* is one of the most abundant species of the group with a broad distribution including Atlantic and Pacific coasts (Morriconi 1999; Ríos and Mutschke 1999). The digenean *Gymnophalloides nacellae* has been reported parasitizing *N. deaurata* from Atlantic Patagonian (Puerto Deseado, 47°S, 65°W) and also *N. magellanica*, a true limpet that has a sympatric distribution with *N. deaurata* (Cremonte et al. 2013), suggesting a close historical relationship between these host species and their high ecological similarity. Given the close historical connection between *N. deaurata* and *N. concinna*, some similarity between their parasite fauna could be expected, unless their environments (including the presence of suitable hosts) limit their distribution. Thus, analyzing the parasite fauna in *N. concinna* and *N. deaurata* could provide insights into the historical connection between Antarctica and southern South America.

Unfortunately, little is known about larval stages of parasites in general, and identifying parasite species taxonomically at larval stages based on morphology is extremely challenging. Nevertheless, use of molecular markers has been shown to be an appropriate tool to identify parasite genera and species and to reveal parasite biodiversity (Criscione et al. 2005). In this study, we used the most common genes for parasite DNA barcoding to assist in the identification of parasites present in congeneric limpet species inhabiting the Southern Ocean, in particular in Magellan and Antarctic coastal environments. In addition, we describe the larval stages of digenean parasites present in both gastropod species, and quantify common parasite ecological parameters in order to document novel information about host–parasite associations in the Southern Ocean.

Materials and methods

A total of 403 individuals of *N. concinna* and 214 individuals of *N. deaurata* were obtained during January of 2016 and 2017 (Fig. 1). All samples were collected in shallow waters, most of them intertidal; those from the subtidal zone (237 individuals of *N. concinna*) were collected at a maximum depth of 10 m. Samples of *N. concinna* were obtained on the Antarctic Peninsula at Doumer Island, Palmer Archipelago (A1, 64°52′33″S; 63° 35′1″W) and Kopaitik Island (A2, 63°19′15″S; 57°53′55″W). Samples of *N. deaurata* were collected from two sites in the Magellan region, viz. Santa Ana Point (SA1, 53°37′52″S; 70°54′49″W) and the Beagle Channel (SA2, 54°51′34″S; 68°45′49″W). Most samples were processed in situ, except when logistic limitations made this impossible (e.g., vessel limitations or short sampling time allowed). Each specimen was sexed and its maximum valvar length measured using a vernier device with accuracy of 0.5 mm. The valves, mantle, gills, gonad, and digestive gland of each specimen were examined under a stereoscopic microscope after careful dissection. All recorded endoparasites were fixed in 70% alcohol for taxonomic identification. Preliminary identification was based on specialized literature (Gibson et al. 2002; Bray et al. 2008), followed by molecular identification.

The collected parasites were isolated, and each morphotype was transferred to a 1.5-ml microcentrifuge tube. DNA extraction was performed using the commercial kit E.Z.N.A (Omega Bio-tek) using the manufacturer's protocol. Following the recommendation of Blasco-Costa et al. (2016), we used a set of most common genes used in DNA barcoding of parasites: the small ribosomal subunit (18S rRNA) using the primers described by Hall et al. (1999), the large ribosomal subunit (28S rRNA) using the primers described by Hassouna et al. (1984), and the ribosomal internal transcribed spacer subunit 2 (ITS2) using primers described by Tkach et al. (2003). All polymerase chain reactions (PCRs) were performed in final volume of 25 µl containing 1 × PCR buffer, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.4 pM of each primer, 1× bovine serum albumin (BSA), 0.025 units of GoTaq DNA polymerase (Promega), and 2.5 µl of concentrated DNA. The amount of $MgCl_2$ used

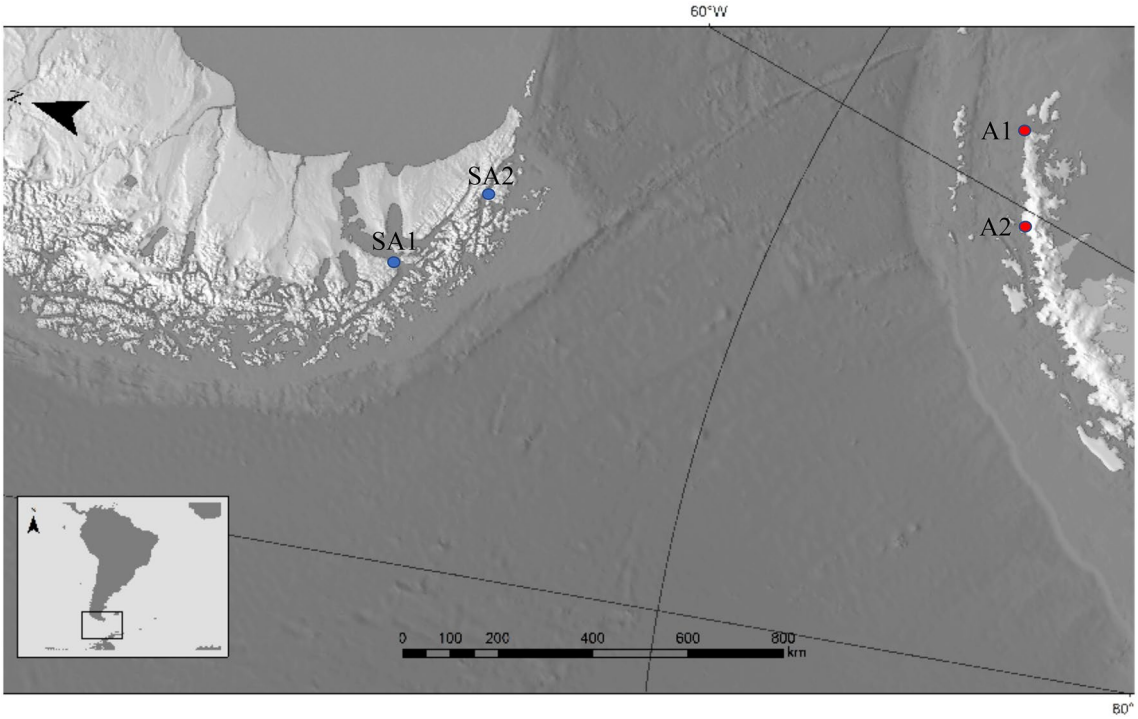

Fig. 1 Map depicting Sub-Antarctic and Antarctic zones relevant to this study. *SA1* Santa Ana Point, *SA2* Beagle Channel, *A1* Doumer Island, *A2* Kopaitik Island

was different for each gene, being 3 mM for the 18S rRNA and a total of 2 mM in the PCR for 28S rRNA and ITS2. The thermocycling included an initial denaturation step (95 °C, 5 min), 35 cycles of amplification with 94 °C for 45 s, 45 °C (18S rRNA), 50 °C (28S rRNA) or 55 °C (ITS2) for 45 s, and 72 °C for 1 min, and a final extension step of 72 °C for 5 min.

The PCR products for each specimen were sequenced using an automated capillary electrophoresis sequencer (ABI 3500) available in the core facility Austral-*omics* (https://www.austral-omics.com). We sequenced the three mentioned genes for each individual included in the analyses, including at least one individual per parasite morphotype. Sequences were edited and aligned using Geneious 10.0.9 (Kearse et al. 2012). The sequences obtained from the parasites in this study were compared with sequences obtained from the public database GenBank (hosted at www.ncbi.nlm.nih.gov) (Sayers et al. 2018). The information about the sequences obtained from GenBank is shown in Online Resource 1. To maximize the chances of correct species identification, we used two phylogeny-based approaches. The first consisted of analysis of a matrix with the three genes 18S rRNA, 28S rRNA, and ITS2 concatenated, while the second consisted of phylogenetic analysis of each gene separately. The advantage of the latter approach is that it allows incorporation of a larger set of species from GenBank, as not all species in GenBank have data for all three genes analyzed in this study. Regardless of the approach used to construct the genetic matrix, phylogenetic relationships were reconstructed under maximum likelihood (ML) in raxmlGUI v1.5 (Silvestro and Michalak 2012), and Bayesian inference (BI) trees were reconstructed using BEAUti and the BEAST v2.4.6 (Bouckaert et al. 2014). The best evolutionary model for the whole dataset was determined using jModelTest2 (Miller et al. 2010). The Bayesian information criterion (BIC) was used for model selection, resulting in GTR + G as the best model for all data analysis. Rapid bootstrap analysis (Stamatakis et al. 2008) was carried out with 1000 (ML) replications and 30,000,000 Markov chain Monte Carlo (MCMC) generations for the Bayesian analysis. Maximum-likelihood bootstrap proportions above 70% were considered to show strong support (Hillis and Bull 1993; Wilcox et al. 2002).

The prevalence (i.e., the percentage of examined hosts infested with one or more individuals of a determined parasite species) and mean intensity (i.e., the mean number of a particular parasite species per host, considering only infested hosts) were calculated according to Bush et al. (1997). To test whether values of these parameters differed between regions (Magellan and Antarctic), we conducted a randomization of the data to generate a null distribution of parameters, enabling the null hypothesis of similarity to be tested. For each region, 10,000 random values of prevalence and mean intensity were estimated by randomizing the full

dataset, and for each random sample, the difference between regions (e.g., $\text{Prevalence}_{\text{Magellan}} - \text{Prevalence}_{\text{Antarctica}}$) was calculated to build a null distribution of these differences. Consequently, statistically significant differences between regions were only supported if the observed (empirical) difference of a parameter was greater than 95% of the random values. All these analyses were conducted in R (R Development Core Team 2013). Further details about the implementation of these tests are included in an annotated R script in Online Resource 2. The data used with this script are given in Online Resource 3.

Results

We found three different morphospecies of larval digeneans, one in *N. concinna* and two in *N. deaurata* (Table 1). In total, we sequenced one metacercariae from the Antarctic region, and three samples of metacercariae and between five and seven samples of cercariae from the Magellan region. Sequences are available in the GenBank database with accession numbers MK496658–MK496667 (18S rRNA), MK496669–MK496675 (28S rRNA), and MK487808–MK487817 (ITS-2).

The phylogenetic analysis (Fig. 2) based on the multigene matrix confirmed that *N. concinna* was parasitized by a species of Gymnophallidae, closely related to members of the genus *Gymnophallus* with bootstrap support of 99% and with genetic divergence of 4.7% from *Gymnophallus choledochus* (accession number KM268112). The limpet *N. deaurata* was host for *Gymnophalloides nacellae* and a species of Renicolidae (Fig. 2). The analyses of the single-gene matrices were concordant (Online Resource 4).

The prevalence and mean intensity per locality and parasite are presented in Table 1. The parasite *Gymnophallus* sp., the only parasite species found in the limpet *N. concinna*, was found in the mantle of the host, as metacercariae, and only at locality A1. The total prevalence of this parasite at locality A1 in Antarctica was 2.5%, whereas the mean intensity was 1 (7 hosts infested from a total of 277 analyzed). *Gymnophalloides nacellae*, one of the two parasite species found in the limpet *N. deaurata*, was found as metacercariae, encysted in both the mantle and digestive gland of the host and exclusively at locality SA1. The prevalence of this parasite at locality SA1 was 12.1% with mean intensity of 16.05 (22 hosts infested from a total of 182 analyzed). A second parasite species reported in *N. deaurata* was *Renicola* sp., found as a sporocyst containing cercariae in the digestive gland and gonad of their host from both Magellan localities (SA1 and SA2). The prevalence of *Renicola* sp. at SA1 was 1.1% (2 hosts infested from a total of 182 analyzed), with mean intensity of 300. *Renicola* sp. at SA2 showed

Table 1 Shell length (mean and standard deviation) and number of samples (*N*) for *Nacella concinna* and *Nacella deaurata* from each locality

Host	*Nacella deaurata*					*Nacella concinna*				
Location	SA1			SA2		A1			A2	
N samples	182			32		277			126	
Mean length (mm)	42.7 ± 6.7			33.4 ± 5.5		463 ± 5.9			45.6 ± 8.1	
Parasites	P	MI		P	MI	P	MI		P	MI
Gymnophallus sp.	0	0		0	0	2.5	1		0	0
Gymnophalloides nacellae	12.1	16.1		0	0	0	0		0	0
Renicola sp.	1.10	300		3.1	227	0	0		0	0

The main parasitological descriptors are also shown for each parasite: *P* prevalence, *MI* mean intensity

SA1 Santa Ana Point, *SA2* Beagle Channel, *A1* Doumer Island, *A2* Kopaitik Island

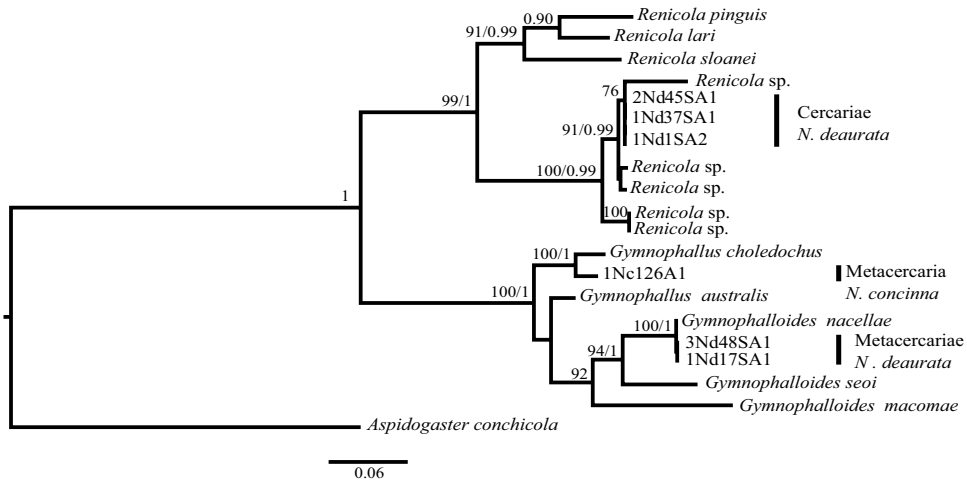

Fig. 2 Phylogenetic reconstruction based on concatenated genes 18S rRNA, 28S rRNA, and ITS2. Numbers along branches indicate bootstrap support values for ML and posterior probability for BI analysis. Nc, *Nacella concinna*; Nd, *Nacella deaurata*. *SA1* Santa Ana Point, *SA2* Beagle Channel, *A1* Doumer Island, *A2* Kopaitik Island

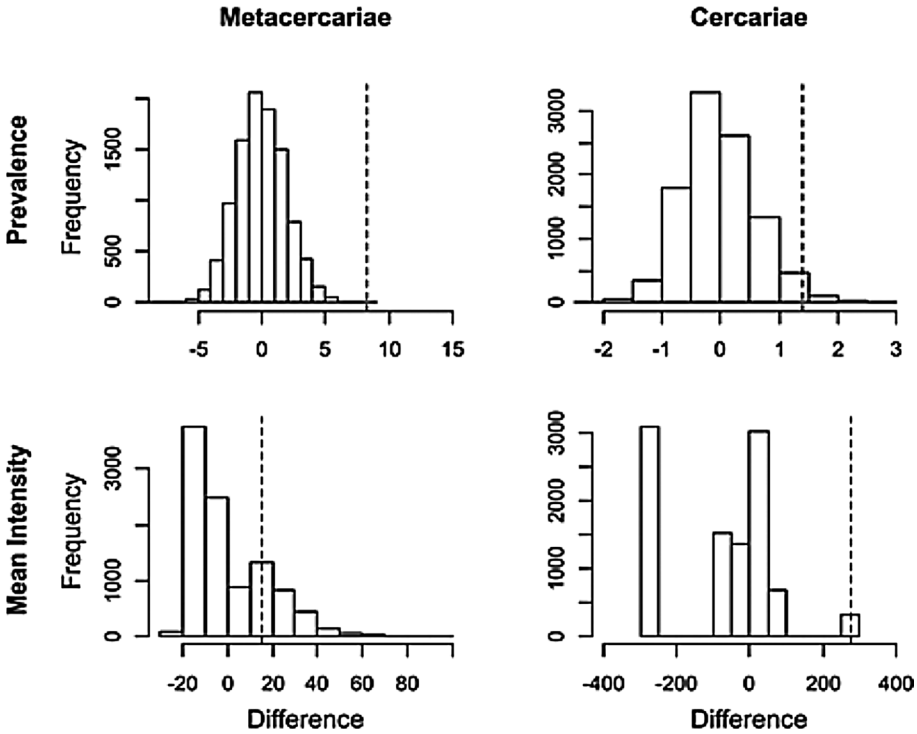

Fig. 3 Null distributions for expected differences between regions (Magellan and Antarctic) for prevalence and mean intensity. Each plot represents the distribution of 10,000 results for the difference between the Magellan and Antarctic region using permutations of the original dataset to calculate random parameter values

prevalence of 3.1% (1 host infested from a total of 32 analyzed) with mean intensity of 227.

Comparing these statistics between regions using randomization analysis (Fig. 3), the prevalence was significantly higher in the Magellan than Antarctic region for metacercariae ($p < 0.0001$) and cercariae ($p = 0.002$). The mean intensity, on the other hand, was not significantly different between the regions for metacercariae ($p = 0.13$), but was significant for cercariae ($p = 0.018$).

Discussion

We found different digenean species parasitizing the two limpet species examined in this study. *Gymnophallus* sp. was only found in the Antarctic limpet *Nacella concinna*, whereas *Gymnophalloides nacellae* and *Renicola* sp. were only detected in the Magellan limpet *N. deaurata*.

Gymnophallus sp. is a small homogeneous group of marine digeneans (Scholz 2002) that mainly uses charadriiform and anseriform birds as definitive hosts (Bartoli 1974). The high dispersal ability of the definitive host, likely a bird, could be the main factor allowing the arrival of this parasite

to Antarctic waters and its establishment as a new component of the trophic web in the area. Representatives of the genus *Gymnophallus*, specifically adults of *G. deliciosus*, have been recorded as parasites in the following Antarctic birds (Barbosa and Palacios 2009): *Phalacrocorax atriceps* (as *Phalacrocorax albiventer* and *P. purpurascens*), *Chionis albus* (as *C. alba*), *Stercorarius antarcticus* (as *Catharacta lonnbergi*), *Stercorarius maccomicki* (as *Catharacta maccormicki*), and *Larus dominicanus*. The closest species in GenBank is *Gymnophallus choledochus*, which has the particular characteristic in its life cycle of modulating the number of hosts in relation with the season—in summer this parasite uses a normal three-host life cycle, while in winter its life cycle includes only two hosts (Loos-Frank 1969, 1971). Such a reduction of the number hosts in the life cycle has been reported in other marine digenean parasite species (Poulin and Cribb 2002), and could be an adaptation to extreme or colder environments, such as those found in the Antarctic region. However, more parasites and molluskan hosts must be analyzed to increase our understanding of the life cycle evolution of digenean parasites in this region.

The parasite *G. nacellae* documented here as a metacercariae in *N. deaurata* was first described from *N. magellanica* and from *N. deaurata* by Cremonte et al. (2013) in the Argentinean Patagonia. The prevalence of this metacercaria in its second host *N. magellanica* from Puerto Deseado was 83.67%, but it was absent from Puerto Madryn in Argentinean Patagonia (see Gilardoni et al. 2018). Here, we expand the distribution of this metacercaria to the Magellan Strait (SA1), and although we were unable to find the metacercaria in samples of *N. deaurata* from the Beagle Channel (SA2), probably due to the low number of hosts examined, this species was reported at Conejo Island, Beagle Channel, Argentina by Cremonte et al. (2013).

Renicolidae gen. sp. has been reported previously in *N. magellanica* with prevalence of 0.98% in samples from Puerto Deseado (Bagnato et al. 2015). In addition, *Renicola williamsi* has been recorded as a parasite of *Stercorarius maccormicki*, an abundant bird in the Patagonian realm (see Barbosa and Palacios 2009).

From a biogeographic viewpoint, evidence of a recent connection between *Nacella* species from the Antarctic and the Magellan regions (Gonzalez-Wevar et al. 2012) led us to hypothesize potential similarities in their associated digenean fauna. However, the differences in the number and composition of digenean species between these regions suggest that little is still known about the processes underlying their distribution. As the most likely definitive hosts would be birds, the potential for dispersal across large geographical regions seems reasonable likely. A total of 49 out of the 63 Antarctic bird species are also present in the Magellan region according to eBird (www.ebird.org). These include species that have been recorded as hosts for *Gymnophallus* and *Renicola* sp., such as *Stercorarius maccormicki*. Thus, the dissimilarities in digenean fauna found between these regions are difficult to explain based on differences in definitive host distributions, because these regions are well connected by marine birds. However, based on the available data, we cannot rule out that other (nonmigratory) bird species could be serving as definitive hosts of the digenean found in this study. On the other hand, strong differences in abiotic conditions between the Antarctic and Magellan regions could be better hypotheses to explain the dissimilarities in digeneans associated with *Nacella* species (Poulin and Cribb 2002; Blakeslee et al. 2012; Byers et al. 2015). Perhaps, the abundance rather than the presence of a bird species could be more important in establishing the probability of migration and establishment of a parasite species. However, until more data are collected, the factors influencing parasite distributions will remain poorly understood.

In this study, we successfully used molecular data to narrow down the taxonomic identity of three parasite species (one to species level and two to generic level) present in two *Nacella* limpets inhabiting Antarctic and Sub-Antarctic regions. There is an obvious gap in the parasitological DNA data available in GenBank that currently prevents us from obtaining a more precise taxonomic determination. Nevertheless, this problem will soon be overcome as more parasitological studies are conducted and data are collected from poorly represented regions such as Antarctica and southern South America. These advances, complemented with detailed morphological descriptions of immature stages (eggs and larvae), will facilitate identification of cryptic parasite species (Criscione and Blouin 2004; Falk and Perkins 2013; López et al. 2015) and completion of parasite life cycles (Oliva et al. 2010; Peribañez et al. 2011; Muñoz et al. 2013), which will increase our knowledge of global patterns of biodiversity (Poulin and Morand 2004) and improve understanding of the underlying processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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