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Molecular phylogeny and historical biogeography of *Nacella* (Patellogastropoda: Nacellidae) in the Southern Ocean

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ABSTRACT

The evolution and the historical biogeography of the Southern Ocean marine benthic fauna are closely related to major tectonic and climatic changes that occurred in this region during the last 55 million years (Ma). Several families, genera and even species of marine organisms are shared between distant biogeographic provinces in this region. This pattern of distribution in marine benthic invertebrates has been commonly explained by vicariant speciation due to plate tectonics. However, recent molecular studies have provided new evidence for long-distance dispersion as a plausible explanation of biogeographical patterns in the Southern Ocean. True limpets of the genus *Nacella* are currently distributed in different biogeographic regions of the Southern Ocean such as Antarctica, Kerguelen Province, southern New Zealand Antipodean Province, North-Central Chile and South American Magellanic Province. Here, we present phylogenetic reconstructions using two mitochondrial DNA markers (Cytochrome Oxidase I and Cytochrome *b*) to look into the relationships among *Nacella* species and to determine the origin and diversification of the genus. Phylogenies were reconstructed using two methods, Maximum Parsimony and Bayesian Inference, while divergence time among *Nacella* species was estimated following a relaxed Bayesian approach. For this purpose, we collected inter- and subtidal species belonging to four biogeographic regions in the Southern Ocean: Antarctica, Kerguelen Province, Central Chile, and Magellanic Province.

Our molecular results agree with previous morphological and molecular studies supporting the monophyly of *Nacella* and its sister relationship with *Cellana*. Two rounds of diversification are recognized in the evolution of *Nacella*. The first one occurred at the end of the Miocene and gave rise to the main lineages, currently distributed in Antarctica, South America or Kerguelen Province. Large genetic divergence was detected among *Nacella* species from these distant biogeographic provinces emphasizing the significance of trans-oceanic discontinuities and suggesting long-distance dispersal was relatively unimportant. The second diversification round consisted of a more recent Pleistocene radiation in the Magellanic region. In this province, different morphological species of *Nacella* exhibit extreme low levels of genetic divergence with absence of reciprocal monophyly among them.

According to our time estimation, the origin and diversification of *Nacella* in the Southern Ocean is more recent (<15 MY) than the expected under the hypothesis of vicariant speciation due to plate tectonics. The evolution of this genus seems to be closely related to drastic climatic and oceanographic changes in the Southern Ocean during the middle-Miocene climatic transition. In spite of the high number of species described for the Magellanic Province, molecular results indicate that these species are the most derived ones in the evolution of the genus and therefore that the Magellanic region does not need to correspond to the origin center of *Nacella*. The absence of genetic divergence among these species supports a very recent radiation process accompanied by rapid morphological and ecological diversification.

1. Introduction

The biogeography of the Southern Ocean is the result of major tectonic and extreme climatic changes since the Mesozoic (Lawver et al., 1992; Crame, 1999; Zachos et al., 2001; Clarke et al., 2004a). Reconstructions of paleocurrents of the past 55 Ma have shown that the opening of the Tasmanian gateway and the Drake Passage

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shaped the present oceanographic circulation in the Southern Ocean (Thomson, 2004; Clarke et al., 2005; Torsvik et al., 2008). With the opening of such gateways, the main current started to flow west–east around Antarctica, causing the thermal isolation of this continent, which experienced around 34 Ma a major continental glaciation associated with sea-ice formation (Kennet, 1977; Lawver and Gahagan, 2003; Mackensen, 2004). Mackensen (2004) recognized three periods in which the most dramatic changes occurred in the Southern Ocean: (1) the Eocene/Oligocene boundary ~34 Ma, when the Antarctic Circumpolar Current (ACC) significantly modified global oceanic circulation for the first time; (2) the Middle Miocene ~14 Ma, when the reformation of an East Antarctic ice sheet influenced mode and level of Antarctic bottom-water formation, generating the intensification of the ACC; (3) the late Pleistocene, characterized by alternation between glacial and interglacial periods that affected seasonality and intensity of sea-ice formation.

In spite of this oceanographic history which must have hindered contact between shallow benthic marine faunas of the southern continents, genera and even species are currently shared among them, especially among Antarctica and southern South America (Clarke et al., 2004a; Thatje et al., 2005; Zelaya, 2005). This Antarctic-Magellan connection is thought to represent the last contact between Antarctica and southern South America during the Oligocene (Brandt et al., 1999; Arntz, 2005), after which the Antarctic fauna adapted to colder conditions and evolved endemic evolutionary lineages (Clarke and Johnston, 1996, 2003; Barnes and Conlan, 2007). Several marine groups, such as *Euphausia* (Patarnello et al., 1996), *Afrolittorina* and *Austrolittorina* (Williams et al., 2003; Waters et al., 2007), show important levels of genetic differences between Southern Ocean provinces supporting the vicariant speciation hypothesis. However, recent molecular studies in other groups indicate more recent divergence time estimations than expected based on timing of the relevant continental drift events and therefore provide new evidence for long-distance dispersal underlying diversification events (Ó Foighil et al., 1999; Donald et al., 2005; Burridge et al., 2006; Waters, 2007). In this regard, several studies in Subantarctic marine groups like *Mytilus*, *Macrocystis* and *Durvillaea*, have shown a clear connection between the Magellanic biota and the Subantarctic islands of the Kerguelen Province (Coyer et al., 2001; Gérard et al., 2008; Fraser et al., 2009). These studies support the idea that the biogeography, in several Southern Ocean marine groups, especially those with high dispersive capacity is influenced by recent events of long-distance dispersion mediated by the West Wind Drift and the ACC (Waters, 2007).

The true limpets belonging to the Order Patellogastropoda are common inhabitants of rocky shores from the tropics to the polar regions (Powell, 1973; Branch, 1985a,b; Ponder and Lindberg, 1997). They differ from other gastropods in fundamental features like shell geometry and microstructure, radular form, gill morphology, and other anatomical characters (Ridgway et al., 1998; Ponder and Lindberg, 2008). The systematics of the group has been relatively neglected and most of the works have concentrated on the description and discrimination of the species. Historically, Patellogastropods have been classified based on the external form of the shell, but in many species this is highly variable, leading to taxonomic confusion (Ridgway et al., 1998). Recent molecular studies recognized at least seven families (Lottiidae, Acmaeidae, Pectinodontidae, Patellidae, Lepetidae, Eoacmaeidae, and Nacellidae) in the order (Nakano and Ozawa, 2007).

The origin and diversification of Nacellidae are not well resolved but the monophyly and the sister relationship of the group members, *Nacella* and *Cellana*, are well supported by morphological and molecular data (Powell, 1973; Lindberg, 1998; Koufopanou et al., 1999; Nakano and Ozawa, 2007). High southern latitude *Nacella* is currently limited to Antarctic and Subantarctic waters, while *Cellana* is distributed around Australia, New Zealand, Japan, Hawaii, and the Juan Fernández Islands. *Nacella* is currently composed of only 15 nominal species, while *Cellana* includes more than 50 (Powell, 1973). The members of Nacellidae are common inhabitants of the intertidal and subtidal rocky shores, where they graze on algae, diatoms and bacterial films (Picken, 1980; Valdovinos and Rüth, 2005). Nacellidae species, especially *Cellana*, are characterized by free spawning of gametes, followed by a non-feeding trochophore stage of 1–2 days, and a lecithotrophic period of 7–11 days (Bird et al., 2007). This short period of larval dispersion, together with the homing behavior of adults, suggest low levels of continental interchange and high endemism for the family (Branch, 1985a). For example, *Cellana* larvae belonging to species from New Zealand and Hawaii spend around 3–11 and 4 days in the water column before settlement, respectively (Goldstien et al., 2006; Bird et al., 2007). No direct information exists about larval life spans of *Nacella* species, but on the one hand, because of its sister relationships with *Cellana*, members of the genus may present a comparable short larval duration. On the other hand, based on *Nacella*'s current distribution, time to settlement could be significantly longer as described for other Antarctic and Subantarctic marine invertebrates (Clarke, 1983; Pearse et al., 1991; Bowden, 2005; Peck et al., 2006). Along these lines, Picken (1980), studied the larval development of *N. concinna*, under controlled laboratory conditions (0 °C), and concluded that this species achieved settlement in one month.

Morphological taxonomy divides *Nacella* into two subgenera – *Nacella* (*Nacella*) with *mytilina* and *kerguelenensis* as only representatives, while *Nacella* (*Patinigera*) includes the other 13 species (Powell, 1973; Valdovinos and Rüth, 2005). The subgenus *Nacella* Schumacher 1817 and *Patinigera* Dall 1905 were defined on the base of type species *Patella mytilina* Helbling 1779 and *P. magellanica* Gmelin 1791, respectively. Main differences between subgenus consist in apex position, thickness and interior coloration of the shell (Powell, 1973). Based on the current distributions of the species, *Nacella* can also be divided into five biogeographical groups from Antarctica, Central Chile, the Magellanic Province, the Kerguelen Province, and the Antipodean Province (Hedgpeth, 1969; Powell, 1973; Valdovinos and Rüth, 2005). *N. concinna* is distributed along the Antarctic Peninsula, the Scotia Arc, including several Subantarctic islands like South Orkney and South Georgia (Picken, 1980; Beaumont and Wei, 1993; Clarke et al., 2004b). Four species have been described in the Subantarctic islands of the Kerguelen Province: *N. delesserti* for Marion Island, South Africa; *N. kerguelenensis*; and *N. edgari* from Kerguelen and Heard islands; and *N. macquariensis* for Heard and Macquarie islands. *Nacella terroris* is distributed exclusively in the Subantarctic Campbell Island in the Antipodean Province from southern New Zealand, where it coexists with *Cellana strigilis strigilis* (Powell, 1973). *Nacella clypeater* is distributed along the Central Chilean coast, from Puerto Montt to southern Peru (Alamo and Valdivieso, 1997; Lancellotti and Vasquez, 2000). Finally, at least eight nominal species have been described for the Magellanic Province. According to the recent revision of Nacellidae from southern South America (Valdovinos and Rüth, 2005), the species *N. magellanica*, *N. deaurata* and *N. delicatissima* are distributed from Chiloé Island through Cape Horn, Falkland Island, and Southern Patagonia. Other species have a narrower distribution, like *N. venosa* from Chiloé through Cape Horn, and *N. chiloensis* from the Chiloé Island (Valdovinos and Rüth, 2005). The species *N. flammea* and *N. fuegiensis* are distributed from Aysén (45°32′ LS; 72°04′ LW) to the Magellan Strait. Finally, *N. mytilina* exhibits the widest distribution including the Magellan Strait, Cape Horn, southern Patagonia, Falkland Island, and Kerguelen Island (Powell, 1973). As mentioned above, systematics of *Nacella* has been based on shell morphology and is still unclear, especially for the Magellanic Province species. For example, Powell (1973) considered *N. venosa* and *N. chiloensis* as subspecies of *N. magella-*

nica, and *N. delicatissima* as an ecotype of *N. deaurata*. Recent morphological studies by Valdovinos and Rüth (2005) concluded that all the species described for the Magellanic Region, with the exception of *N. fuegiensis* (considered as a synonym of *N. magellanica*), are true taxonomic units with intraspecific variation, especially in shell (shape, thickness and color) and radular teeth morphology.

The purpose of this study is to understand the phylogenetic relationships and the historical biogeography of the current *Nacella* species in the Southern Ocean, by using mitochondrial sequence data. On the one hand, based on the genetic differences between the species from Antarctica and southern South America, it will be possible to estimate the last contact time between *Nacella* from these continents. From these results, we will determine whether the general distribution and diversity patterns of *Nacella* conform to the hypothesis of plate tectonics vicariant processes in the Southern Ocean. On other hand, the comparison among species from South America, Antarctica and Kerguelen Province will give us a better insight into colonization and differentiation processes of the Subantarctic islands. Therefore, our results should shed light on tempo and modes of speciation in the limpet *Nacella* along its current distributions. Finally, this study will contribute to assessing the relative importance of vicariant and dispersal processes in the origin and evolution of marine benthic invertebrates along the Southern Ocean.

2. Materials and methods

2.1. Sampling and identification

Nacella specimens used in this study were identified following Powell (1973) and Valdovinos and Rüth (2005). According to Valdovinos and Rüth (2005), Magellanic species of *Nacella* can be clearly distinguished by different morphological features such as shell shape, foot and mantle tentacle color pattern. However, field observations indicate that these characteristics are not so clear and easy to recognize in some specimens. In fact, intermediate shell forms and mixture of diagnostic characters are frequently detected in several specimens, suggesting the absence of reproductive isolation and the presence of hybrids in this region. For these reasons, we only considered in this study those Magellanic species that clearly constitute different morphological units (*N. deaurata*, *N. flammea*, *N. magellanica*, and *N. mytilina*, Fig. 1). Species from other biogeographic regions such *N. concinna* from the Antarctic Peninsula, *N. clypeater* from Central Chile, *N. kerguelenensis* and *N. macquariensis* from the Kerguelen Province (Fig. 2) were also included. Eleven species of *Cellana* from the Indo-West Pacific were used as sister groups, and different Patellidae genera (*Cymbula*, *Helcion*, *Scutellastra*, and *Patella*), were used as outgroups (Table 1).

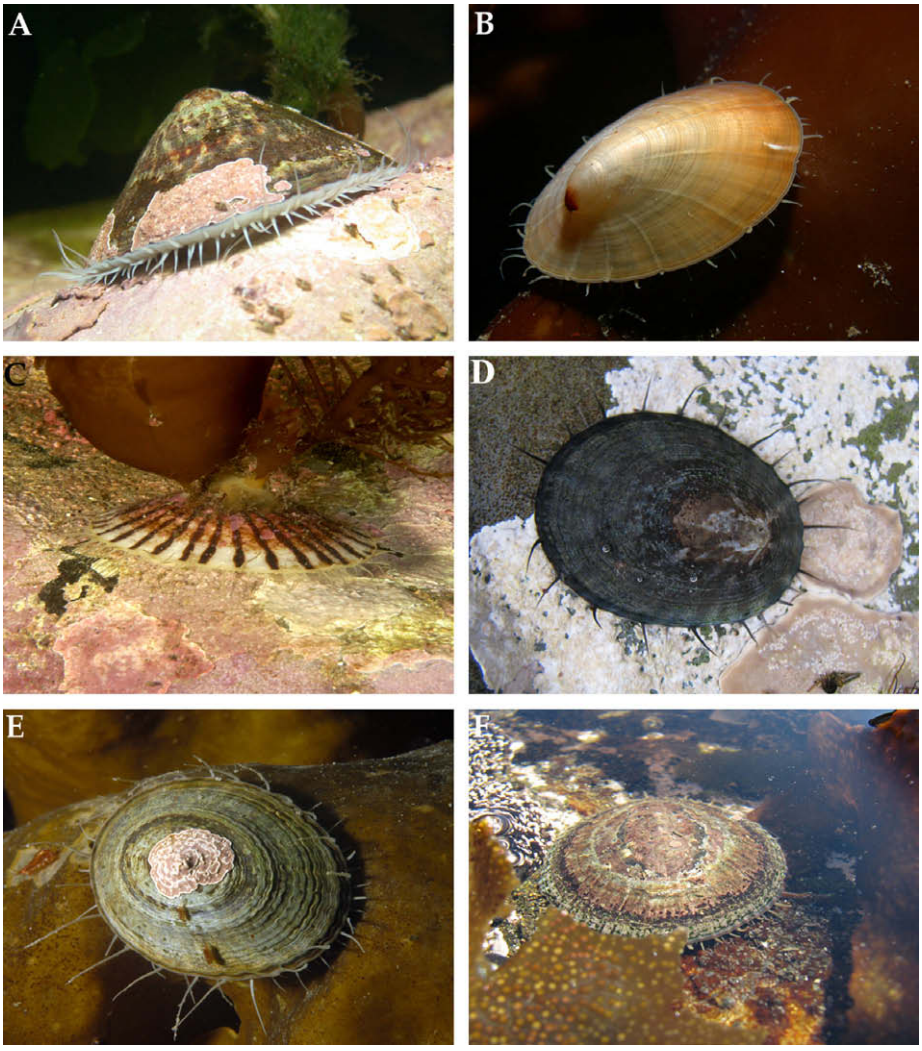

Fig. 1. *Nacella* species in their natural habitat emphasizing the morphological and habitat differences among them. (A–D) Magellanic Province species: (A) intertidal *N. magellanica*, (B) subtidal *N. mytilina* grazing over the macroalgae *Macrocystis pyrifera*, (C) subtidal *N. flammea*, (D) intertidal *N. deaurata*; (E) Antarctic limpet *N. concinna*; (F) Central Chile species *N. clypeater*. Photographs (A)–(C) and (E) courtesy of César Cárdenas (ccardenas.biosub@gmail.com; www.guiamarina.com).

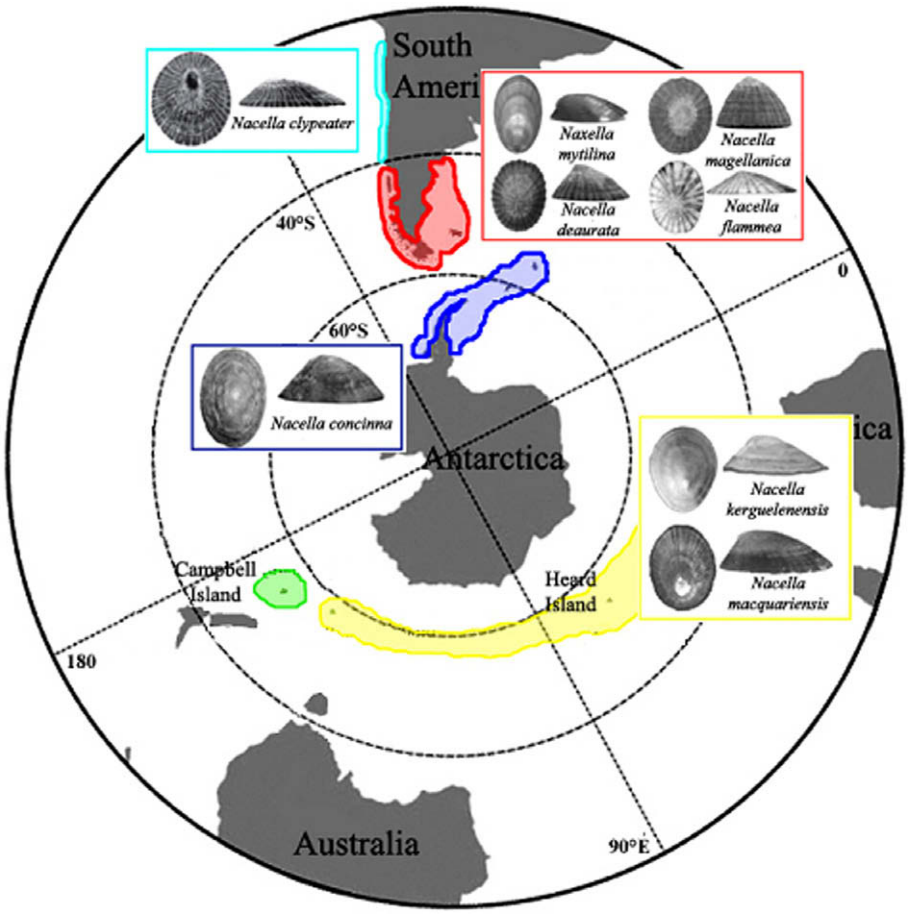

Fig. 2. *Nacella*'s groups distribution in different biogeographical regions along the Southern Temperate and Antarctic Regions (taken from Hedgpeth, 1969). Lateral and dorsal photographs correspond to the analyzed species in this study. Colors: Blue = Antarctica; Red = Magellanic Province; Yellow = Kerguelen Province; Green = Antipodean Province southern New Zealand; Light blue = Central Chile.

For phylogenetic purposes we analyzed two mitochondrial markers: Cytochrome Oxidase Subunit I (COI) and Cytochrome *b* (Cyt*b*). COI sequences of *Cellana* and Patellidae species were obtained from previous studies (Nakano and Ozawa, 2007), and we amplified this marker in all *Nacella* species. Cytochrome *b* was amplified in all the analyzed species (Table 1).

2.2. DNA extraction, PCR amplification and DNA sequencing

Animals were fixed in ethanol (95%), and total DNA was extracted from the mantle using the salting-out method described by Aljanabi and Martinez (1997). Partial fragments of COI and Cyt*b* were amplified by PCR, using universal primers published by Folmer et al. (1994) and Merritt et al. (1998), respectively. To amplify COI gene *N. concinna* and *N. mytilina*, it was necessary to designed specific internal primers, based on South American *Nacella* species sequences: COI-LEMF (5′-CTG-GGC-TTG-CTG-GGA-CTG-GTT-3′) and COI-LEMR (5′-AAT-AAA-TGC-TGA-TAA-AGA-ATA-3′).

Amplifications were done in a 25 μl reaction volume consisting of 2.5 μl 10× buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.0), 1.0 μl of 50 mM $MgCl_2$, 200 mM dNTPs, 0.5 μl of each primer (10 pg/μl), 1 U Taq (Invitrogen), 17.5 μl of double-distilled water plus 20 ng of DNA. Thermal cycling parameters included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 48 °C (COI) and 51.5 °C (Cyt*b*) for 45 s, and 72 °C for 1 min, which ended with a final 6 min extension at 72 °C. Amplification products were purified and sequenced in both directions. Finally, all sequences were deposited in GenBank under Accession Nos. GU901219–GU901257 (COI), and GU901258–GU901313 (Cyt*b*).

2.3. Phylogenetic analyses

Sequences were edited and aligned independently using Proseq 2.91 (Filatov, 2002), and reconstructions were performed using Maximum Parsimony (MP) and Bayesian Inference (MCMC) methods. Phylogenetic reconstructions were conducted from a matrix including the concatenated dataset (COI + Cyt*b*). For this purpose, we determined the congruence on the phylogenetic signal of the genes with the ILD test (Farris et al., 1995), implemented in the partition homogeneity test of PAUP* version 4.0b (Swofford, 2002). MP analyses were conducted using PAUP*, with the following assumptions: characters were treated as equally-weighed using a heuristic search and tree bisection reconnection (TBR), with the branch-swapping option. The steepest descent option was set off and MULTREES on with random-taxon addition sequences to search for optimal trees. Node supports values were computed using non-parametric bootstrapping with a full heuristic search option and 1000 pseudo-replicates (Felsenstein, 1981).

Bayesian reconstructions were performed using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003), with the GTR+I+G substitution model as determined by MrModeltest 2.2 (Nylander, 2004). All MCMC analyses were conducted twice, with random initial trees and for 5×10^6 generations. Trees were sampled every 100 generations and majority rule consensus phylograms, as well as poster-

Table 1
Patellogastropod species, specimens and sampling localities included in this study. Specimens identification was based on Powell (1973), Valdovinos and Rüth (2005), Nakano and Ozawa (2004, 2007). DNA sequences (COI and Cyt*b*) obtained in this study are marked with a • and GenBank Accession Nos. are still not available. * indicate additional sequences from Nakano and Ozawa (2007).

Species	Localities	No.	DNA ID	Cytb	COI
Nacellidae					
Nacella concinna (Strebel, 1908)	South Bay, Antarctic Peninsula, Chile	1	NCON-05	•	•
	Elephant Island, Antarctic Peninsula, Chile	2	NCON-44; NCON-46	•	•
	Covadonga Bay, Antarctic Peninsula, Chile	2	NCON-63; NCON-69	•	•
Nacella clypeater (Lesson, 1831)	La Misión, Valdivia, Chile	2	NCLY-15; NCLY-37	•	•
	Lenga, San Vicente Bay, Chile	2	NCLY-23; NCLY-26	•	•
	Coquimbo Bay, Chile	1	NCLY-45	•	•
Nacella deaurata (Gmelin, 1791)	Laredo Bay, Strait of Magellant, Chile	3	NDEA-09; NDEA-21; NDEA-27	•	•
	Aguila Bay, Strait of Magellant, Chile	2	NDEA-34; NDEA-35	•	•
Nacella magellanica (Gmelin, 1791)	Laredo Bay, Strait of Magellant, Chile	3	NMAG-04; NMAG-10; NMAG-22	•	•
	Orange Bay, Chile	2	NMAG-28; NMAG-44	•	•
Nacella mytilina (Helbling, 1779)	Punta Santa Ana, Strait of Magellant, Chile	2	NMYT-05; NMYT-24	•	•
	Isla Carlos III, Strait of Magellant, Chile	2	NMYT-35; NMYT-41	•	•
Nacella flammea (Gmelin, 1791)	Punta Santa Ana, Strait of Magellant, Chile	5	NFLA-01; NFLA-04; NFLA-06; NFLA-09; NFLA-15	•	•
Nacella kerguelenensis (E. A. Smith, 1877)	Heard Island	5	NKER-01; NKER-02; NKER-03; NKER-04; NKER-05	•	•
Nacella macquariensis (Finlay, 1927)	Heard Island	3	NMAC-01; NMAC-02; NMAC-03; NMAC-04; NMAC-05	•	•
Cellana eucosmia (Pilsbry, 1891)	Hurghada, Egypt	1	NUGB-L396	•	AB238543*
Cellana flava (Hutton, 1873)	Kaikoura, South Island, New Zealand	1	NUGB-L576	•	AB238545*
Cellana radians (Gmelin, 1791)	Omaha Beach, North Island, New Zealand	1	NUGB-L580	•	AB238551*
Cellana radiata enneagona (Reeve, 1854)	Madagascar	1	NUGB-L490	•	AB238553*
Cellana radiata orientalis (Pilsbry, 1891)	Okinawa, Japan	1	NUGB-L27	•	AB238554*
Cellana solida (Brainville, 1825)	Orford, Tasmania, Australia	1	NUGB-L401	•	AB238561*
Cellana taitensis (Röding, 1798)	Taihoae Bay, Nuku Hiva, Marquesas Islands	1	NUGB-L402	•	AB238562*
Cellana tesutudinaria (Linnaeus, 1758)	Okinawa, Japan	1	NUGB-L42	•	AB238563*
Cellana toreuma (Reeve, 1854)	Oga, Akita, Japan	1	NUGB-L3	•	AB238564*
Cellana tramoserica (Holten, 1802)	Botany Bay, NSW, Australia	1	NUGB-L647	•	AB238566*
Patellidae					
Cymbula oculus (Born, 1778)	West Bank, East London, South Africa	1	NHM	•	AB238572*
Helcion concolor (Krauss, 1848)	West Bank, East London, South Africa	1	NHM	•	AB238574*
Helcion dunkeri (Krauss, 1848)	Bloubergstrand, Cape Town, South Africa	1	NHM	•	AB238575*
Patella caerulea (Linnaeus, 1758)	Ceuta, Spain	1	NUGB-L653	–	AB238577*
Patella ferruginea (Gmelin, 1791)	Ceuta, Spain	1	NUGB-L655	•	AB238578*
Patella rustica (Linnaeus, 1758)	Ceuta, Spain	1	NUGB-L651	•	AB238579*
Scutellastra barbara (Linnaeus, 1758)	Kommetjie, Cape Town, South Africa	1	NHM	•	AB238581*
Scutellastra laticostata (Blainville, 1825)	Albany, WA, Australia	1	NUGB-L659	•	AB238584

ior probability for nodes, were assembled. MCMC scores were graphed against generations using Tracer v.1.5 (Drummond and Rambaut, 2007) to identify stationary, and thus to determine the number of generations that must be discarded as burn-in. Consensus phylograms with nodal posterior probability support were estimated per analysis.

2.4. Divergence time estimations

Divergence times in molecular evolution have been traditionally estimated under the Molecular Clock Hypothesis (MCH) that assumes a constancy of evolutionary rate among lineages in a genealogy or phylogeny. Nevertheless, before assuming the MCH it is necessary to determine whether the evolutionary rate is constant along the reconstructed genealogy. For this, we performed a likelihood ratio test (Felsenstein, 1981) using DAMBE (Xia and Xie, 2001). Another possibility is to use another method that relaxes the assumption of the MCH.

In this study we used a relaxed Bayesian approach for multilocus data as implemented in MULTIDIVTIME package (Thorne et al., 1998; Kishino et al., 2001; Thorne and Kishino, 2002). This method for divergence time estimations relies on a stochastic model for changes in the evolutionary rate over time (Kishino et al., 2001). It obtains time estimates by combining sequence data with external information like constraints on particular nodes based in the fossil record (Thorne and Kishino, 2002; Wiegmann et al., 2003). This method requires an assumed topology and the tree shown in Fig. 3 was considered as the best one because it corresponds to the most complete hypothesis on the evolution of Nacellidae.

To estimate branch lengths on the topology, a discretized gamma distribution with five rate heterogeneity categories (Yang, 1994), was used in conjunction with the F84 model of nucleotide change. The module PAML (Yang, 1997), generated a Maximum Likelihood estimates of different parameters like the transition/transversion ratios and unequal nucleotide frequencies. Once these parameters were obtained, we estimated the branch lengths for the rooted tree and the variance–covariance matrices with the module ESTBRANCHES. To estimate divergence times, Markov chain was sampled 200,000 times every five cycles, after a burning of 1,000,000 cycles. The prior for the "separation time between the root of ingroup and the present" parameter (rttm), which is not a calibration point and do not have a major effect on the posteriors, was set to 38 Ma as the maximum time of separation between *Nacella* and *Cellana*. The selection of this date was based on the oldest Nacellidae fossil (*Cellana ampla*) from the Upper Eocene of Oregon (Lindberg and Hickman, 1986). The prior rttmsd was set at one-half of rttm based on recommendations accompanying the software. The rt-rate (rtratesd) value was set by calculating the median value of the amount of evolution from the root to the tip of the tree. For time estimations, we used two internal constraints, based on *Nacella* fossil record. The first constraint was the appearance in the fossil record of *N. concinna* from Cockburn Island ~5.0 Ma (Jonkers and Kelley, 1998) and the second constraint

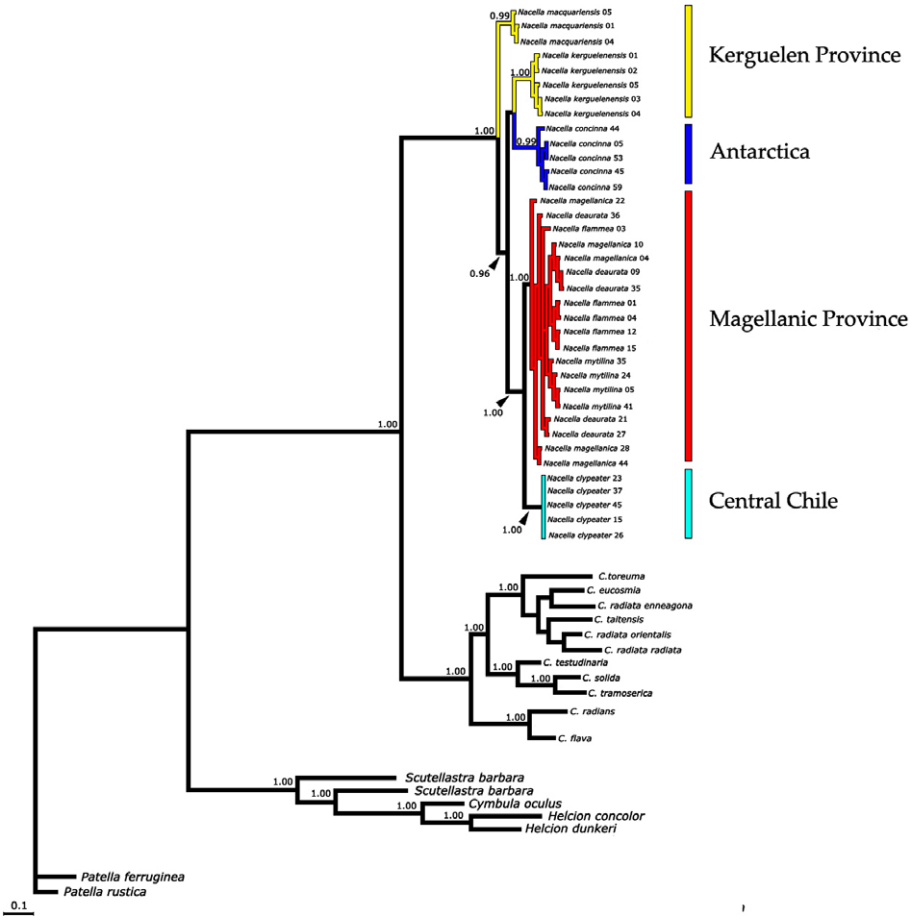

Fig. 3. Molecular phylogeny in *Nacella* produced by Bayesian analyses of the concatenated data set (COI + Cyt*b*). Color of the nodes indicate the origin as indicated in Fig. 2. Support values are Bayesian posterior probabilities (above the diagonal) and parsimony bootstrap support (below the diagonal). The trees were rooted using members of the family Patellidae.

was a *N. clypeater*-like forms from southern Peru from ~5.0 Ma (DeVries, 2009).

3. Results

Alignment lengths for COI and Cyt*b* fragments were 648 bp and 410 bp, respectively. Partition homogeneity test did not show significant differences between these molecular markers ($P = 0.65$), and sequences were combined and analyzed as a single data set. Concatenated genes included 1058 bp with 514 variable and 473 parsimonious informative sites. For individual and combined analyses, MP and Bayesian methods recovered nearly identical topologies. Both markers clearly discriminated between major taxonomic groupings of the Patellogastropoda included in the analyses, with high bootstrap (bs) and posterior probability (pp) supports. The sister relationship and the monophylies of *Nacella* and *Cellana* are strongly supported (pp = 1.0; bs = 100%). Parsimony trees (data not shown) were in basic agreement with the respective Bayesian reconstructions (Fig. 3).

Our findings indicate that the ancestor of *N. macquariensis* from Heard Island separated from the ancestor of all the other *Nacella* species considered in this study, inhabiting Antarctica, Kerguelen Province, Central Chile and Magellanic Region. Then, we observed a clade containing the species *N. concinna* and *N. kerguelenensis* from Antarctica and Heard Island, respectively. The next clade to appear included the common ancestor of *N. clypeater* from Chile and all the extant Magellanic species. Finally, the most derived clade is composed by the four Magellanic species: *N. flammea*, *N. magellanica*, *N. deaurata* and *N. mytilina*. COI and Cyt*b* sequences obtained from intermediate forms (data not shown), generally assigned to other morphospecies, were always clustered within the Magellanic clade.

Our results show that *Nacella* species from different regions have marked genetic differences. Although specimens of *N. kerguelenensis* and *N. macquariensis* were collected in the same locality, the genetic differences between them (~7.5%) are comparable to those observed between *Nacella* species from Antarctica and South America. The four species of the Magellanic Province included in the analyses have very low levels of genetic divergence among them. In fact, Magellanic species of *Nacella*, with the exception of *N. mytilina*, do not exhibit reciprocal monophyly at the resolution level of the chosen markers.

3.1. Time divergence estimations

The absence of a molecular clock for both genes was confirmed by the likelihood ratio test (COI twice the log likelihood difference = 3310, df = 57, $P = 0.000$; Cyt*b* twice the log likelihood difference = 496, df = 57, $P = 0.000$). According to this result, the Molecular Clock Hypothesis was rejected for further divergence time estimation analyses. The divergence time estimations using MULTIDIVTIME indicate that the separation between *Nacella* and *Cellana* (Nacellidae) took place during the middle Miocene (~14 ± 1.8 Ma). According to our phylogenetic reconstruction, the ancestor of *N. macquariensis* branched off after the separation of

both genera (~9.3 ± 1.5 Ma). A second clade formed by the ancestors of the Antarctic *N. concinna*, the Kerguelen Province *N. kerguelenensis* and the South American species appeared ~8.7 ± 1.4 Ma. Then, a third clade formed by *N. kerguelenensis–N. concinna* was originated ~8.1 ± 1.4 Ma, while the next clade containing *N. clypeater* and the common ancestor of the Magellanic species appeared ~5.4 ± 1.1 Ma. Finally, the radiation in the Magellanic Region occurred very recently (between 2.0 and 0.4 Ma) during the Plio-Pleistocene (Fig. 4). This sequence of speciation events is generally well supported by posterior probabilities along phylogenetic tree, except for the *N. concinna–N. kerguelenensis* clade, where the order of divergence is poorly resolved (0.74 pp). These results indicate that the origin and the diversification of the current species of *Nacella* took place long after the separation of the continents and occured in two main stages: first, the appearance between 9 and 5 Ma of the most genetically distant species of *Nacella*, including *N. macquariensis*, *N. kerguelenensis*, *N. concinna*, *N. clypeater*; and then a second rapid and recent diversification of the Magellanic species.

4. Discussion

4.1. Molecular phylogeny of Nacellidae

Since the last revision of the Patellogastropoda (Lindberg, 1998), several morphological and molecular studies have attempted to resolve evolutionary relationships in this order (e.g. Ridgway et al., 1998; Sasaki, 1998; Koufopanou et al., 1999; Harasewych and McArthur, 2000; Nakano and Ozawa, 2004, 2007; Yoon and Kim, 2007). Most of them considered a limited number of *Nacella* species or included animals from one specific region, such as the Magellanic Province (*N. magellanica*, *N. deaurata* and *N. mytilina*; Harasewych and McArthur, 2000; Nakano and Ozawa, 2007; Yoon and Kim, 2007) or Antarctica (*N. concinna*; Koufonapou et al., 1999). This restricted sampling in previous molecular studies has not allowed for good resolution of the evolutionary history of Nacellidae, especially inside the clade of *Nacella*. Here, we present new information about phylogenetic relationships among *Nacella* species from at least four biogeographic regions in the Southern Ocean.

Our results support the monophyly of both *Nacella* (pp = 1.00; bs = 100%) and *Cellana* (pp = 1.00; bs = 100%) and the sister relationship of these genera, corroborating previous morphological and molecular studies (Powell, 1973; Lindberg, 1998; Koufonapou et al., 1999; Harasewych and McArthur, 2000; Nakano and Ozawa, 2007). The existence of *Nacella* and *Patinigera* subgeneric division as evolutionary units is not supported, since our reconstruction did not recover their monophyly (Fig. 3). The species *N. mytilina* and *N. kerguelenensis* (belonging to the subgenus *Nacella*) are included in complete different clades. In fact, both species are more closely related to members of the subgenus *Patinigera*. These findings put forward the need of a deep revision of the systematic and taxonomy of the genus.

Our molecular dating of Nacellidae suggests that the separation of the current genera (*Nacella* and *Cellana*) can be traced back to the middle Miocene (Fig. 4). An important increase in benthic $\delta^{18}O$ values is described during this period, often referred to the middle-Miocene climatic transition (MMCT), associated with polar cooling, major growth of the East Antarctic ice sheet, a large drop in global sea level and major changes in ocean circulation (Woodruff and Savin, 1989; Mackensen, 2004). Crame (1999) suggests that, during the late Miocene (12–10 Ma), an intense pattern of thermal zonation occurred in the oceans (horizontal and vertical). According to the author, this event might be responsible for an intensification of the ACC, resulting in the differentiation of Antarctic and Subantarctic fauna. Recently, Lewis et al. (2008), based on paleoecological $^{40}Ar/^{39}Ar$ analyses of associated ash fall, and climate inference from

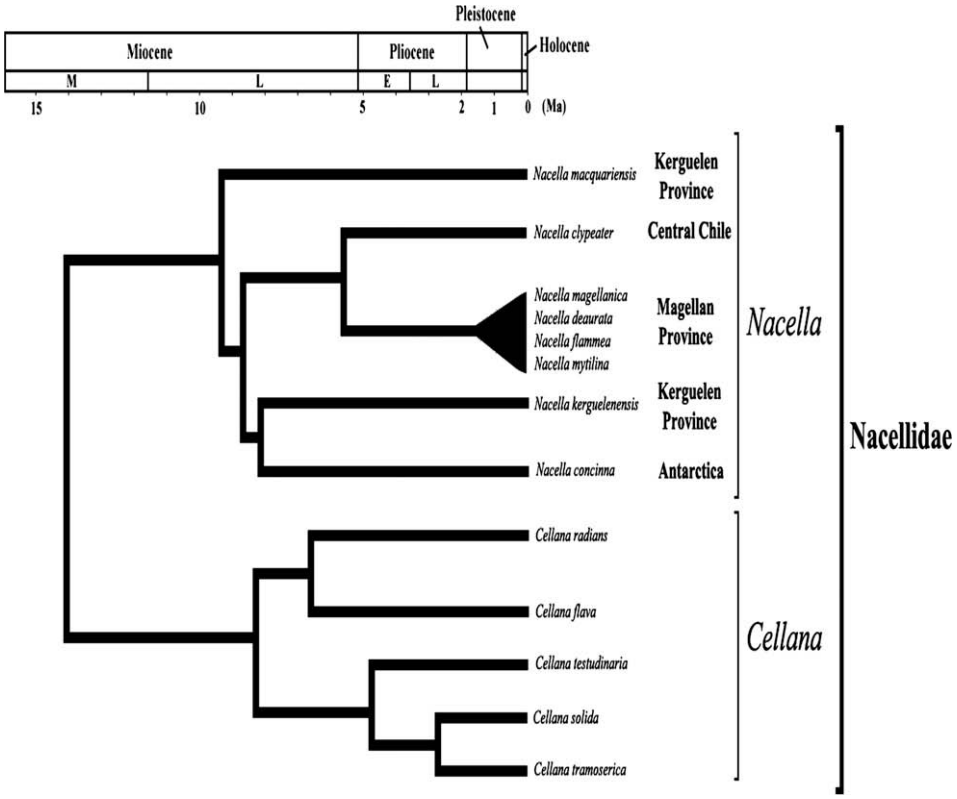

Fig. 4. Linearized tree showing the estimated divergence times of the Nacellidae with species emphasis in *Nacella* groups. Outgroups (Patellidae) and some species of *Cellana* were included in the computational analyses but are not shown.

glaciological modeling, suggested that mean summer temperatures in Antarctica cooled by at least 8 °C between 14.07 ± 0.05 and 13.85 ± 0.03 Ma, supporting the idea of major climatic changes in the Southern Ocean during this period.

At the specific level, our analyses clearly distinguish five major groups of *Nacella*: (i) *N. concinna* from Antarctica; (ii) *N. macquariensis*; (iii) *N. kerguelenensis*, both from Heard Island, Kerguelen Province; (iv) *N. clypeater* from Central Chile; and (v) the last one including all the Magellanic species. The large genetic distances among these groups supports the division of *Nacella* according to previous biogeographic regions described for the Southern Ocean (Hedgpeth, 1969; see Fig. 2). However, the two species *N. kerguelenensis* and *N. macquariensis* from the Kerguelen Province, exhibited high level of genetic divergence, similar to those obtained among *Nacella* species from different biogeographic regions.

4.2. Biogeography of Nacella

Based on our results, we propose that the modes and tempo of speciation in *Nacella* can be separated in two different stages. The first one includes the appearance of *Nacella* lineages between 9.3 ± 1.5 and 5.4 ± 1.1 Ma in Antarctica, Central Chile, Magellanic and the Kerguelen Province. This first round of gradual speciation processes among different biogeographic regions took place long after the separation of the Southern Ocean continents or the formation of the Antarctic Polar Front. Our divergence time estimations among these lineages are in agreement with other molecular studies in Southern Ocean marine benthic organisms. For example, Stankovic et al. (2002) estimated a divergence time between Antarctic and Subantarctic notothenioid fishes of 6.6–7.0 Ma. Díaz (2008) working on different species of the echinoid *Sterechinus* from Antarctica and South America placed the separation time between *S. neumayeri and S. agassizi* at 4.2–5.9 Ma. According to our divergence time estimation, the beginning of divergence between Antarctic and South American *Nacella* does not coincide with plate tectonic dynamics, but with a latter processes, like the drastic middle Miocene oceanographic change in the Southern Ocean (Crame, 1999; Mackensen, 2004; Lewis et al., 2008). During this period, as suggested by Crame (1999), the ACC could have turned into an effective dispersive barrier between Antarctic and Subantarctic waters. In the case of Subantarctic Provinces, the lack of connection among *Nacella* species through the ACC, specifically between the Magellanic and the Kerguelen Provinces, contrasts with previous molecular studies in other marine benthic organisms that clearly show recurrent and modern gene flow among Subantarctic regions. For example, phylogeographic studies in *Ostrea chilensis* (Ó Foighil et al., 1999), *Macrocystis pyrifera* (Coyer et al., 2001), *Mytilus* spp. (Gérard et al., 2008), and *Durvillaea antarctica* (Fraser et al., 2009) hypothesized long-distance dispersal, probably by rafting, as the main mechanism to explain the absence of genetic discontinuity in these species. Because most *Nacella* species live directly on rocks and boulders, dispersal by rafting might be restricted and uncommon in this group. In addition, and despite the proposal that planktonic stage could reach 3–4 weeks in *Nacella*, large geographic distances among shallow habitats in the Southern Ocean may prevent recurrent long-distance dispersal among these regions. From a biogeographical point of view, *Nacella* distribution encompasses a very wide geographic area including several Subantarctic islands, usually separated from each other by hundreds to thousands of kilometers (Chown et al., 2008). In this context, we propose that the main lineages of *Nacella* were originated early after the MMCT, through erratic and exceptional long distance colonization events, followed by speciation.

The absence of reciprocal monophyly and the presence of short branches among the Magellanic species of *Nacella* could suggest that described species in this area just correspond to a single morphologically variable species. Moreover, Magellanic morphospecies could be even interpreted as ecotypes when considering that they occur in sympatry together with ecological differences in terms of substrate preference and subtle differences in their distribution in the intertidal and shallow subtidal zone (Powell, 1973; Valdovinos and Rüth, 2005). Absence of genetic isolation in previous morphological described species have been reported in Patellogastropod like in the genera *Helcion* and *Notoacmea* (Nakano and Spencer, 2007; Ponder and Lindberg, 2008). An alternative explanation would correspond to a very recent diversification of the genus in this region followed by rapid morphological differentiation. High endemic species diversity has been described in other groups of molluscs in the fjords of the Magellanic Province (Valdovinos et al., 2003). According to these authors, the presence of multiple refugia along this Province during the last glacial cycles may have enabled these groups to survive repeated ice advances and retreats. At the same time, the fragmentation and isolation of these areas could have favored speciation in this region (Valdovinos et al., 2003). As regards of rapid morphological diversification, limpet shell shape and thickness are known to vary strongly between species that occupy different habitats (Nakano and Ozawa, 2005; Nakano and Spencer, 2007). For instance, species inhabiting seaweeds, such as *N. mytilina*, have marked shell differences (thickness, shape and color pattern), in comparison to those living on rocky substrates, such as *N. magellanica* or *N. deaurata*. Non-coupled rate of morphological and neutral molecular evolution is a common feature in several groups of Patellogastropods, such as *Notoacmea* and *Helcion* (Nakano and Spencer, 2007; Ponder and Lindberg, 2008). Further studies using fast evolving nuclear markers, like AFLPs and EPICs (Exon Priming Intron Crossing) should give us new and better insights into the contemporary evolutionary processes of *Nacella* in this region and determine whether Magellanic Province harbor a single morphologically variable species or several recently diversified ones.

The fossil record of the genus *Nacella* is relatively scarce with Pliocenic *N. concinna* described for the Cockburn Island, Antarctic Peninsula (Jonkers and Kelley, 1998), and *N. aff. terroris* from the Chiloé Island in southern Chile (Watters and Flemming, 1972). Magellanic fossil of *Nacella*, includes only Holocene specimens identified as *N. deaurata* and *N. magellanica* from Patagonia, Argentina (Gordillo, 1999; Gordillo et al., 2005). These fossil records in southern South America, further support our divergence time estimation in the evolution of *Nacella* in this region. Recently, DeVries (2009) described at least five Pliocenic species of *Nacella*: *N.* (*Nacella*) *lacrima*; *N.* (*Patinigera*) *oblea*; *N.* (*P.*) *chalaensis*; *N.* (*P.*) *intiforma*; and *N.* (*P.*) *oconaensis*, from tropical latitudes of southern Peru. These fossils could possibly correspond to an unknown *Nacella* diversification in southern Peru associated in morphology and geography to the extant *N. clypeater* from central and northern Chile. Nevertheless, DeVries (2009) also described other species like *Nacella reicheae* from the Late Oligocene of Peru as well as *N. nielseni* from the late early Miocene of southern Chile (DeVries, 2009).

Finally, our datings of the evolutionary events in the evolution of the genus *Nacella* are in agreement with most of the fossil records of the living representatives, with the exception of the Oligocene an Early Miocene species from Peru and Chiloé (DeVries, 2009). If these fossils truly represent *Nacella* species, they could correspond to low latitude ancestors of the modern Subantarctic and Antarctic taxa. However, this scenario is not compatible with our phylogenetic reconstructions, where the South American clade of *Nacella* appears as the most derived one. Alternatively, considering that shell morphology and even microstructure are highly homoplasious in Patellogastropods, these fossils could rather belong to an extinct Nacellid lineage that predates the split into *Nacella* and *Cellana*.

In this respect, the inclusion of *N. terroris* (Antipodean Province), *N. delesserti* and *N. edgari* (Kerguelen Province) will give us more detailed information and a better understanding about the complete phylogeny and biogeography of *Nacella* in the Southern Ocean.

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