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Phylogeography and demographic inference in *Nacella* (*Patinigera*) *concinna* (Strebel, 1908) in the western Antarctic Peninsula

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A B S T R A C T

Endemic to Antarctic ecosystems, the limpet *Nacella* (*Patinigera*) *concinna* (Strebel, 1908) is an abundant and dominant marine benthic invertebrate of the intertidal and shallow subtidal zone. In order to examine the phylogeographic pattern and historical demography of the species along the western Antarctic Peninsula, we amplified 663 bp of the mitochondrial DNA cytochrome oxidase subunit I of 161 *N. concinna* specimens from five localities, as well as two specimens from South Georgia and Sub-Antarctic Marion Island. As two different morphotypes, one characterized by an elevated shell in the intertidal and the other by a flat one in the subtidal, have been recurrently reported for this species, we also compared intertidal and subtidal samples from two localities of King George Island (Admiralty and Fildes Bay) through geometric morphometric and genetic analyses. As a result, elliptic Fourier analyses on shell shape morphology detected highly significant differences between intertidal and subtidal morphotypes. In contrast, mtDNA analyses between these morphotypes did not detect statistical differences between them and support the hypothesis that subtidal and intertidal *N. concinna* forms correspond to be the same population unit.

Genetic analyses depicted low levels of haplotypic and nucleotide diversity in *N. concinna* in all localities. Among populations, comparisons did not detect any genetic structure, supporting the existence of a single genetic unit along the western Antarctic Peninsula. A marked L-shaped distribution of pairwise differences and significant negative Tajima's *D* and Fu's F_S indices suggest the existence of a recent demographic expansion of this species. Time estimations corrected by the "time dependency of molecular rate" hypothesis for this demographic event (7,500–22,000 years ago) fit well with the last glacial–interglacial transition period. Low levels of genetic diversity in *N. concinna* could reflect the dramatic effect of glacial periods on population sizes, especially in Antarctic species with narrow bathymetric ranges.

Genetic similarities between South Georgia and Antarctic samples, as well as between *Nacella delesserti* (Philippi, 1849) and *N. concinna* (Strebel, 1908) fell within the range of intraspecific variation. The genetic proximity between sub-Antarctic *N. delesserti* and the Antarctic limpet could be explained through north-eastward long-distance dispersion events during the late Pleistocene.

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1. Introduction

Antarctica is considered to be an island continent isolated from other regions of the Southern hemisphere by geographic distance, water currents and extreme environmental conditions such as low temperatures, the presence of ice and exceptional seasonality (Crame, 1999; Clarke et al., 2004, 2005; Strugnell and Linse, 2005). The current biodiversity in Antarctica has resulted from different biogeographic and evolutionary processes operating over this continent since the Mesozoic (Zachos et al., 2001; Brandt, 2005; Brandt et al., 2007; Rogers, 2007). Marginal and extreme environments are thought to enhance the evolution of novel species through habitat fragmentation and strong selective pressure, thus driving the appearance and establishment of new taxa. In the particular case of Antarctica, this situation may have been favored by the elimination of competitors and predators, as well as by the stimulation of adaptation and speciation processes in survivors (Crame, 1999; Strugnell and Linse, 2005).

It is also interesting to note how the progressive isolation of this continent has led to a drastic extinction of plants and animals in the terrestrial realm (Clarke and Crame, 1989; Convey et al., 2008). In the Antarctic Ocean, similar changes are marked by the reduction of several key benthic groups, which are abundant and dominant in adjacent sub-Antarctic regions, such as brachyuran

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crabs, lobsters, cartilaginous and non-notothenoid fishes. Nevertheless, other groups like sponges, bryozoans, sea spiders, echinoderms, amphipods and isopods are abounding and diverse, indicating that the continent's isolation and climatic changes have not impeded their success (Crame, 1999; Arntz and Ríos, 1999; Gray, 2001; Clarke et al., 2004). Nearshore benthic assemblages in Antarctica are diverse and with high standing stocks (Clarke and Johnston, 2003; Bowden, 2005). In fact, the modern Antarctic benthic fauna includes more than 4000 species and comprise a significant part of Earth's biodiversity (Clarke et al., 2004; Gutt et al., 2004; Peck et al., 2005). Many of these species are found only in this continent and endemism may reach over 90% for some groups of Antarctic invertebrates (Clarke and Johnston, 2003). The marked changes in the diversity of invertebrate groups over the last 40 Myr indicate that Antarctica offers good insight into macro-evolutionary processes, especially the relationship between speciation and extinction (Strugnell and Linse, 2005). At the same time, this particular region presents opportunities for studying life-history adaptations in slow growing benthic species that persist in habitats with high seasonality and frequent natural disturbance (Bowden, 2005).

Members of the genus *Nacella* Schumacher, 1817 (Nacellidae: Patellogastropoda) are exclusive inhabitants of the Antarctic–Sub-Antarctic ecosystems (Powell, 1973). Currently, the genus is composed of 15 nominal species distributed in several regions of the Southern Ocean, like the Antarctic Peninsula, the Magellan province, the Kerguelen province and the Antipodean province in Southern New Zealand (Powell, 1973; Valdovinos and Rüth, 2005). Recent phylogenetic studies indicate that the origin of *Nacella* relates to the Middle Miocene Climatic Transition ($\sim$14 Ma), long after the separation of the Southern Ocean Continents, like Antarctica and South America (González-Wevar et al., 2010). Molecular analyses using two mitochondrial markers (COI and CytB) suggest that the diversification of the genus can be divided into two main phases: a first gradual appearance of *Nacella* in different biogeographic regions like Antarctica, South America and the Kerguelen Province, between 9.0–5.0 Ma; a second diversification stage characterized by a rapid morphological and ecological radiation of the genus in the Magellanic Province during the Pleistocene ($\sim$2.0–0.4 Ma; González-Wevar et al., 2010).

In Antarctica, the true limpet *N. concinna* (Strebel, 1908) is one of the most conspicuous and dominant marine benthic macro-invertebrates (Walker, 1972; Picken, 1980; Picken and Allan, 1983; Peck and Veal, 2001). The species is distributed along the Antarctic Peninsula and its adjacent island systems, like the Palmer Archipelago, Seymour and Paulet Island, along the islands of the Scotia Arc (South Georgia Island, South Orkney Islands and South Shetland Islands) and Bouvet Island (Powell, 1973). It is a very common species in the nearshore of the West Antarctic environment with a mean population density of 124×21 m^{-2} on Signy Island (Picken, 1980). The species inhabits a bathymetric range from the intertidal zone down to 110 m, where it mainly grazes on microphytobenthos and microalgae (Picken, 1980; Davenport, 1988; Brêthes et al., 1994). Like many Antarctic marine organisms, *N. concinna* is also described as a long-lived organism, reaching shell lengths of $\sim$41 mm in 21 years, and some specimens have even lived up to more than 70 years (Picken, 1980). *N. concinna* is a dioecious species with external fertilization and broadcast spawners with pelagic larvae. Unusually for a patellogastropod, *N. concinna* forms spawning clusters of 3–35 individuals for up to seven days during the spring bloom period (Picken and Allan, 1983; Stanwell-Smith and Clarke, 1998).

The species was originally described as *Patella polaris* during the XVII century and Strebel (1908) distinguished two different forms, namely, a shallow water morph named *Patinella polaris* and a deeper water one called *P. polaris* var. *concinna*. Later, Powell (1973) recognized no differences between these forms and renamed both *N. concinna,* within the subgenus *Patinigera*. Walker (1972) and Picken (1980) recorded different bathymetric migration patterns between these intertidal and subtidal forms, with the first one migrating vertically on a seasonal mode as a response to decreasing temperatures and ice formation while the second one remains far below the low tide level all year. The elevated intertidal shape allows higher water volume retention offering an advantage towards avoiding desiccation, to extreme temperature ranges and hypoxia conditions. According to Nolan (1991) this form also favors colonizing the intertidal over the sublittoral form highly preferred by predators. Beaumont and Wei (1991) performed a morphological and genetic study on the species. In that study, morphological differences were corroborated and the subtidal group showed larger height/length ratios compared to the intertidal one. Morphological and genetic studies using five allozymic polymorphic loci (*Es*-1, *Icd*, *Gpi*, *Pgm*-1 and *Got*-1) in specimens of both sub-populations indicate that these forms are genetically identical, without evidence of any structure between them. On other hand, morphology showed significant differences in shell shape, suggesting that morphological variation in this species results from environmentally induced phenotypic plasticity (Beaumont and Wei, 1991). Recent morphometric and molecular studies using ISSR-PCR markers in three localities along the Antarctic Peninsula confirmed the morphological differences between intertidal and subtidal forms. At the same time, the molecular markers also detected significant genetic differences between these forms, indicating that they could correspond to different populations with low levels of gene flow (de Aranzamendi et al., 2008).

Despite the marked interest in the ecology, colonization, physiology, life history and evolution of Antarctic marine fauna, only a limited number of studies have examined genetic diversity patterns in these organisms. Molecular studies in euphausiids and nothotheniidae fishes have showed that these groups exhibit high levels of genetic differences in the Southern Ocean (Bargelloni et al., 2000). Nucleotide sequence data from mitochondrial genes have revealed high levels of genetic structure and cryptic speciation in the crinoid *Promachocrinus kerguelenensis* (Wilson et al., 2007). A recent molecular study in nudibranch *Doris kerguelenensis* showed high levels of genetic diversity in this species, pointing towards recent explosive radiation (Wilson et al., 2009). In a study on the Antarctic silverfish *Pleuragramma antarcticum*, Zane et al. (2006), we identified high levels of polymorphism. In spite of the high levels of genetic diversity, the authors detected no association between localities and a weak population structure in the species.

In order to examine the evolutionary history of *N. concinna* in the Antarctic Peninsula, we reconstructed intraspecific phylogeographic relationships along the Antarctic Peninsula and analyzed patterns of haplotype frequencies in the species. For this, we used DNA sequences from the mitochondrial cytochrome oxidase subunit I gene. Mitochondrial genetic markers, and especially COI, have been successfully used to obtain a first insight into populations' demographic histories (Zane et al., 2006; Wilson et al., 2007; Mahon et al., 2008). The distribution of *N. concinna* in Antarctica is restricted to ice-free zones; its narrow bathymetric range and reproduction mode with free-living larvae makes this species a suitable model to study the effect of Pleistocene glaciations on the demography of Antarctic species. We included subtidal and intertidal specimens from two localities, so as to determine the degree of genetic divergence among these different morphologies. In these samples, we also conducted geometric morphometric analyses to determine the presence of morphological differences between these groups.

2. Material and methods

2.1. Sampling, DNA extraction, PCR amplifications and alignment

Nacella concinna specimens from five different localities along the Antarctic Peninsula were collected by scuba diving during the years 2002–2008, namely, Elephant Island, Admiralty and Fildes Bays in King George Island, and Covadonga and South Bays along the west Antarctic Peninsula (Table 1). We obtained subtidal and intertidal specimens from two localities: Admiralty Bay (AB) and Fildes Bay (FB). We also included one specimen from South Georgia Island and one specimen of *N. delesserti* (Philippi, 1849) from sub-Antarctic Marion Island in the analyses (Fig. 1A and B). Animals were fixed in 95% ethanol and DNA was extracted from the mantle tissue, using the salting-out method described by Aljanabi and Martinez (1997). A partial fragment (663 bp) of the mtDNA gene cytochrome oxidase subunit i (COI) was amplified using specific primers: COI-NacF (5′-CTG GGC TTG CTG GGA CTG GTT-3′) and COI-NacR (5′-AAT AAA TGC TGA TAA AGA ATA-3′; González-Wevar et al., 2010). Amplifications were done in a 25 μl reaction volume consisting of 17.5 μl of double-distilled water, 200 mM dNTPs, 0.5 μl of each primer, 1U Taq (Promega), 2.5 μl 10× buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.0), 1.0 μl of 50 mM $MgCl_2$ and 50 ng of DNA. Thermal cycling parameters included an initial denaturation step at 94 °C for 3 min, followed by 40 cycles at 94 °C for 1 min, 48 °C for 45 s and 72 °C for 1 min, which ended with a final 6 min extension at 72 °C. Double stranded PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN). Purified products were sequenced in both directions using an Automatic Sequencer 3730 × l (Macrogen). All *N. concinna* haplotypes sequences will be deposited in GenBank.

Sequences were edited using Proseq 2.91 (Filatov, 2002) and aligned with ClustalW (Thompson et al., 1992). After sequence editing, COI data were translated into amino acids to check for premature stop codons, which are evidence of sequencing errors or the presence of nuclear pseudogenes. We performed a DNA saturation analysis following Roe and Sperling (2007) to examine how levels of saturation changes across the COI.

2.2. Population genetic structure

Levels of genetic polymorphism in *N. concinna* were determined by standard diversity indices, such as the number of haplotypes (k), segregating sites (S) and haplotype diversity (H), for each locality from the COI aligned sequences with DnaSP 5.00.07 (Librado and Rozas, 2009). We also estimated average pairwise sequence differences (Π) and nucleotide diversity (π), according to Nei (1987).

Levels of genetic differentiation between localities of *N. concinna* were estimated by pairwise Φ_{ST} using Arlequin v.3.11 (Excoffier et al., 2005). This method simply counts the number of different alleles between two haplotypes using the following formulae: $\delta_{xy}=\Sigma\delta_{xy}(i)$, where δ_{xy} is the Kronecker function, equal to 1 if the alleles of the i-th locus are identical for both haplotypes, and equal to 0 otherwise (Excoffier et al., 2005). This parameter is analagous to F_{ST} and is the correlation between alleles within individuals relative to the combined population (Holsinger and Weir, 2009).

First, we performed the comparisons between the intertidal and subtidal forms from both Admiralty Bay (AB) and Fildes Bay (FB). The resulting comparisons are useful to determine whether these

Table 1
Genetic diversity indices and neutrality test in *N. concinna*.

Locality	N	k	H	S	Π	π	Tajima's D	Fu's F_S
South Bay	31	5	0.688	5	0.888	0.00149	−0.576	−0.64
Elephant Island	29	8	0.729	7	0.985	0.00148	−1.34	−4.14*
Admiralty Bay	33	7	0.471	6	0.981	0.00089	−1.68	−4.68**
Fildes Bay	38	6	0.565	6	0.587	0.00104	−1.38	−2.48
Covadonga Bay	29	9	0.741	7	0.704	0.00163	−1.17	−5.08**
COI Total	160	16	0.630	18	1.079	0.00128	−1.77*	−12.38***

n: number of sampled specimens; ***k***: number of haplotypes detected; ***S***: polymorphic sites; ***H***: haplotype diversity; ***Π***: average number of nucleotide difference; ***π***: nucleotide diversity.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

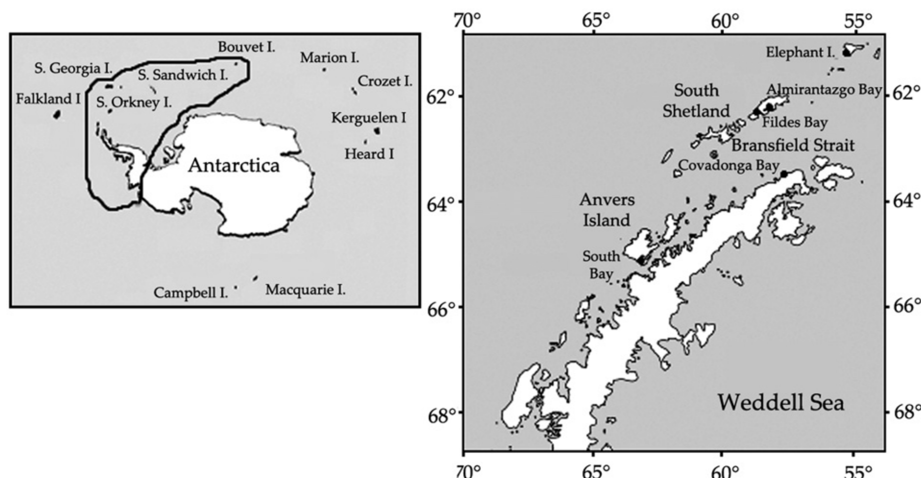

Fig. 1. (A) *N. concinna* distribution range in the Southern Ocean. (B) Sampling localities of *N. concinna* in the western Antarctic Peninsula.

morphologies correspond to identical genetic units or if they should be considered in further analyses as separate sub-samples. Second, we performed pairwise Φ_{ST} comparisons among all sampled localities. In parallel to this analysis we determined the average number of nucleotide differences between localities using DnaSP.

We used the program SAMOVA v.1 (SAMOVA, Spatial Analysis of Molecular Variance, Dupanloup et al., 2002) to define the number and composition of geographically homogeneous, maximally differentiated groups of populations. This method is based on a simulated annealing procedure that aims to maximize the proportion of total genetic variance due to differences among groups of populations and minimizing the variance portion among populations within groups. Differentiation indices Φ_{ST} (among populations), Φ_{SC} (among populations within groups) and Φ_{CT} (among groups) were tested through haplotype permutation (Excoffier et al., 2005).

2.3. Population historical inference

Genealogical relationships among *N. concinna* localities were determined using haplotype networks constructed with the median-joining algorithm in Network v.4.5.1.0 (Röhl, 2002). This method allows simple reconstruction of phylogenies, based on intraspecific molecular data like mitochondrial DNA variation, which often are complicated to analyze, especially when dealing with large sample sizes and with small genetic divergence among individuals (Bandelt et al., 1999; Posada and Crandall, 2001). To determine past demographic changes in *N. concinna*, Tajima's D and Fu's F_S tests were calculated using DnaSP to evaluate the assumption of selective neutrality of mtDNA sequences and population mutation-drift equilibrium. Significant negative values for these tests are evidence of excess of rare polymorphisms in a population, indicating either recent demographic expansion or positive selection. We constructed a mismatch distribution of the COI sequence data to compare it to the Poisson expectation for constant and varying population sizes (Slatkin and Hudson, 1991). The rapid population growth model proposed by Rogers and Harpending (1992) rests on the estimation of three parameters: τ the date of growth/decline measured in units of mutational time ($\tau=2\,\mu t$ where t=time in years and μ=mutation rate per sequence per year); initial theta $\theta_i=2N_i u$ (before the population growth/decline); and final theta $\theta_f=2N_f u$ (after population growth/decline). The demographic expansion parameters were estimated using the nonlinear least square approach, described by Schneider and Excoffier (1999) implemented in Arlequin. Finally, by using Fluctuate v.1.44 (Kuhner et al., 1998) we co-estimated the θ parameter and population growth rate g from *N. concinna* COI sequences through Metropolis-Hastings sampling. This method takes a set of aligned DNA sequences as input and uses them to make maximum likelihood estimates of θ and g. Theta is defined as 4 times the effective population size of the mutational rate in a diploid organism, or 2 times the effective population size of the mutational rate on a haploid at present time. Parameter g corresponds to the exponential growth or decline rate of the population. Positive values for this parameter indicate growth while negative ones imply decline (Kuhner et al., 1998).

2.4. Geometric morphometrics analyses

Shell shape variation between subtidal and intertidal *N. concinna* specimens from AB and FB was measured using outline analyses based on the elliptic Fourier analysis (EFA). Outlines were drawn from digital photographs and corresponded to a two dimensional projection of the lateral shape of the shells. We included adult specimens (>4 cm) in all morphometric analyses. Elliptic Fourier transformations were done using the SHAPE software (Iwata and Ukai, 2002). Elliptic Fourier descriptors (EFDs) can be used to delineate any kind of form and have been effectively applied to the evaluation of various biological shapes in plants and animals (Iwata and Ukai, 2002). This method is based on the separate Fourier decompositions of the incremental changes of the x- and y-coordinates, as a function of the cumulative length along the outline (Renaud and Michaux, 2003, 2007). The ChainCoder module extracted the contours of the objects from digital images and stores the relevant information as chain codes. Then, the module Chc2Nef provided the normalized EFD from the chain-coded contour, and coefficients of EFD were calculated by discrete Fourier transformation following Kuhl and Giardina (1982). These coefficients were subsequently normalized to be invariant with respect to size, rotation and starting point, with a procedure based on the ellipse of the first harmonic. With the PrinComp module, we performed the principal components analyses on the variance–covariance matrix of the EFDs coefficients. Principal components' analysis is effective for summarizing the information regarding the variation contained in these coefficients (Rohlf and Archie, 1984), which were estimated using PAST v.1.77 (Hammer et al., 2001). Finally, multivariate analyses of variance (MANOVA) were performed with PAST in order to evaluate the importance of between-group differentiation relative to within-group variation. A test for significance of morphology differences (Wilk's lambda test) is also provided and Hotteling pairwise comparisons, Bonferroni corrected and uncorrected, were also performed using PAST to determine differences between morphologies and localities.

3. Results

3.1. Molecular genetics

The *N. concinna* COI sequence data set comprised 161 specimens and consisted of 663 nucleotide positions. A single specimen of *N. delesserti* from Marion Island was added to the data set for comparisons. No indels or stop codons were detected, as expected for coding regions, and they also were not saturated in the third codon position. Only one amino acid substitution was detected, out of a possibility of 220, in the entire COI data set (translated using the invertebrate mitochondrial table; Kumar et al., 2004). A third position transversion (T–G) generated an amino acid change from isoleucine to methonine (both amino acids are type D with hydrophobic side chain). The Antarctic limpet exhibited low levels of genetic diversity along the Antarctic Peninsula; only 15 characters (2.2%) were variable and 8 of them (1.2%) were parsimoniously informative. Sequences were adenine and thymine (A–T) rich (61.6%), compared to mean guanine and cytocine (G–C) content (39.4%). Genetic diversity indices, like the number of polymorphic sites, haplotype diversity and nucleotide diversity (Table 1), were low but comparable to other molecular studies in Antarctic marine invertebrates (Díaz, 2008; Hunter and Halanych, 2008; Mahon et al., 2008; Thornhill et al., 2008; Díaz et al., 2011).

Pairwise Φ_{ST} comparisons between intertidal and subtidal *N. concinna* from the Admiralty and Fildes Bays (Table 2) showed no significant difference. From this point, for both Fildes and Admiralty, we considered intertidal and subtidal samples as similar and pooled these samples for further genetic analyses.

The new pairwise Φ_{ST} calculated among all five localities along South Shetland and the western Antarctic Peninsula showed no significant difference, except for the comparison between Elephant Island and Admiralty Bay (P=0.049). In general, Admiralty Bay exhibited higher values for Φ_{ST}. General Φ_{ST}

comparisons evidenced an absence of genetic structure among *N. concinna* along 800 km in the South Shetland region and Western Antarctic Peninsula.

The average number of nucleotide differences between populations was very low and ranged between 0.647 and 1.039. The highest value was recorded between the southernmost locality South Bay and Covadonga Bay in Continental Antarctic Peninsula. The average differences between the most distant localities (South Bay/Elephant Island) was 0.973, while the lowest value (0.581) was recorded between intertidal and subtidal subsamples from Admiralty Bay (Tables 2 and 3). SAMOVA analysis corroborated Φ_{ST} pairwise estimations and did not recover spatial structure in *N. concinna*. However, the first partition separated Admiralty Bay from the rest of the localities, even though variances (Φ_{CT}) among groups explained a small amount of the variance (0.90%), while differences (Φ_{ST}) within the populations explained 99.11% of such.

The median-joining network resulted in a typical star-like haplotype network and a very short genealogy (Fig. 2). Network analysis recovered 18 different haplotypes, including the single

Table 2
Pairwise Φ_{ST} values (below diagonal) and average number of nucleotide differences (above diagonal) in *N. concinna* along western Antarctic Peninsula.

	ABs	ABi	FBi	FBs
Admiralty Bay subtidal (ABs)	–	0.581	0.645	0.563
Admiralty Bay intertidal (ABi)	−0.04175	–	0.749	0.632
Fildes Bay intertidal (FBi)	−0.02655	−0.02453	–	0.703
Fildes Bay subtidal (FBs)	0.02636	−0.00607	−0.00189	–

1023 permutations, no significant values ($P < 0.05$) were detected.

Table 3
Pairwise Φ_{ST} values (below diagonal) and average number of nucleotide differences between localities (above diagonal) in *N. concinna*.

	SB	EI	AB	FB	CB
South Bay (SB)	–	0.973	0.795	0.837	1.039
Elephant island (EI)	−0.0208	–	0.796	0.827	1.026
Admiralty Bay (AB)	0.0296	**0.0389**	–	0.647	0.843
Fildes Bay (FB)	0.0030	0.0027	0.0236	–	0.879
Covadonga Bay (CB)	−0.0166	−0.0186	0.0355	0.0014	–

1023 permutations, significant values ($P < 0.05$) are in bold.

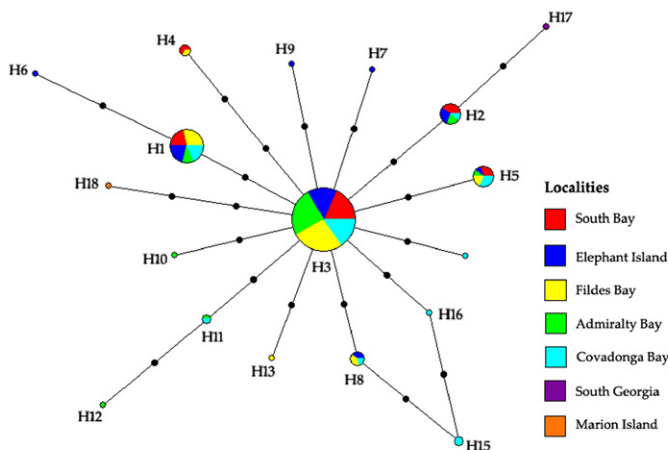

Fig. 2. Haplotype network of 162 *N. concinna* and one *N. delesserti* mtDNA sequences. Each haplotype is represented by a colored circle whose size is proportional to it frequency in the whole sampling effort. ● correspond to a specific mutation.

specimens from South Georgia (*N. concinna*) and Marion Island (*N. delesserti*). The central haplotype (H3) is the most frequent (58%) and most broadly distributed one, occurring at all localities along the Antarctic Peninsula. As proposed by Posada and Crandall (2001), H3 should correspond to the most ancestral haplotype, whereas the most derived ones are linked to it with a maximum branch length of two mutational steps. Three haplotypes (H1, H2 and H5), each located at one mutational step away from H3, were present in most of the localities and exhibited intermediate frequency (6.2–17.4%). The remaining 12 haplotypes occurred at low frequency, with no more than five representatives of each detected in the data set. The single haplotype from South Georgia stood two mutational steps away from H3 and only one step from H2 present in Covadonga, Fildes, Admiralty Bays and Elephant Island. In the case of *N. delesserti*, the unique haplotype from sub-Antarctic Marion Island (H18) was separated by only two mutational steps from the dominant H3 haplotype in *N. concinna* (H3; Fig. 2).

As expected from a star-like network, Tajima's *D* and Fu's F_S neutrality tests were both significantly negative for the whole data set, indicating that this species may have experienced a recent demographic expansion event under a neutral model.

Distribution of pairwise differences among sequences was L-shaped (Fig. 4) due to the fact that the majority of the individuals shared the same haplotype (H3). The mismatch distribution did not differ significantly from the expected stepwise expansion model ($P_{SSD}=0.31$). Considering the divergence rate estimated for *Cellana* (1.0% per million year, González-Wevar et al., unpublished data), the start of the expansion was estimated to be around 75,000 and 220,000 years before the present, for sudden and continuous exponential growth models, respectively (Fig. 5).

3.2. Morphometrics

Principal components and multivariate analyses of the morphology of the shells detected significant differences between subtidal and intertidal *N. concinna* morphotypes in both localities AB and FB. Principal component analyses indicated that PC1 and PC2 together explained the 91.6% of the variance (Fig. 3). PC1 explained 85.6% of the variance and mainly consisted of height shell change, while PC2 (6.4%) corresponded to small changes in shell length (Fig. 3). Most of the variance (72%) in shell shape morphology was explained by bathymetric variation in *N. concinna* (subtidal and intertidal forms). Differences between the localities (AB and FB) explained only a 12% of the variance in the data set. Hotteling pairwise comparisons detected significant levels of differentiation between subtidal and intertidal shells in both localities (same Wilks lambda$=3.96\times10^{-26}$; Table 4). In general, pairwise comparison results indicated that the levels of subtidal–intertidal differences were higher in several orders of magnitude than the subtidal–subtidal and intertidal–intertidal ones (Table 4).

4. Discussion

4.1. Absence of genetic differentiation between intertidal and subtidal forms of N. concinna, in spite of significant morphological differences

Geometric morphometric analyses are sensitive to morphological variations and, according to some authors, this approach has proved to be sensitive enough to detect a similar pattern of population structure as molecular markers (D'Anatro and Lessa, 2006). Geometric morphometric analyses in *N. concinna* detected

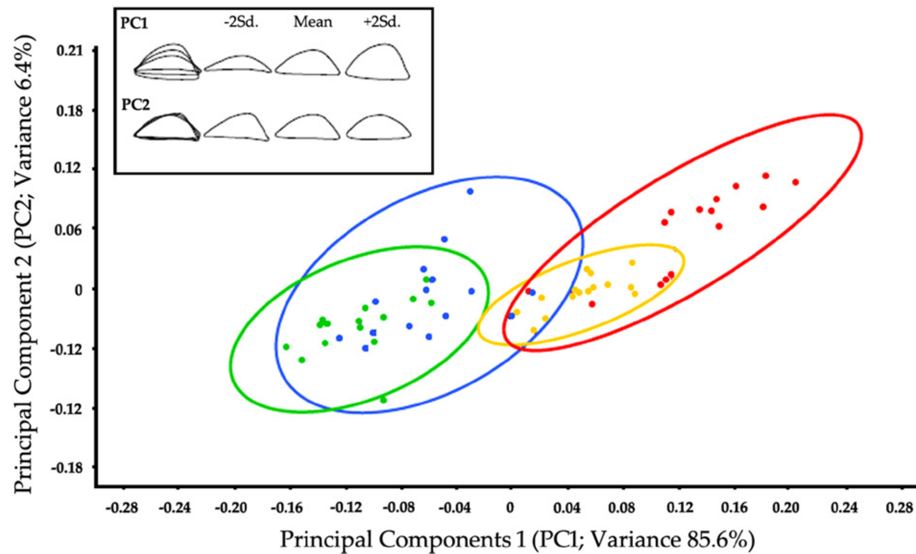

Fig. 3. Multivariate principal components analysis of the shell shape variation of *N. concinna* in Admiralty and Fildes Bay, where: Orange = Fildes Bay intertidal; Green = Fildes Bay subtidal; Red = Admiralty Bay intertidal; and Blue = Admiralty Bay subtidal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

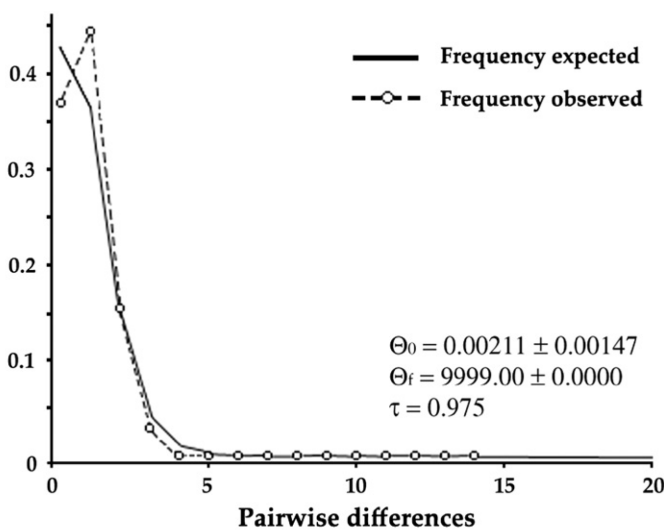

Fig. 4. Pairwise difference distribution for the cytochrome oxidase subunit I (COI) in *N. concinna* along the western Antarctic Peninsula.

significant differences between subtidal and intertidal morphologies from both Admiralty and Fildes Bays and are in total agreement with previous studies (Picken, 1980; Beaumont and Wei, 1991; Nolan, 1991; Brêthes et al., 1994; de Aranzamendi et al., 2008; Hoffman et al., 2010). At the same time, morphometric differences were also detected between AB/FB subtidal and between AB/FB intertidal morphotypes. Marked morphological differences between intertidal and subtidal specimens of *N. concinna* were also recorded in a recent study in the species in Adelaide Island, Antarctic Peninsula (Hoffman et al., 2010). These authors concluded that their morphological analyses revealed not only marked differences between these two morphotypes but also a continuous cline in shell shape from the intertidal zone down to 25 m depth, suggesting that the distinction between morphotypes may be artificial.

Mitochondrial DNA genetic analyses between these morphotypes from AB and FB specimens of *N. concinna* did not detect statistical differences between these different morphological units. Our molecular results support the hypothesis that both subtidal and intertidal forms belong to a single *N. concinna*

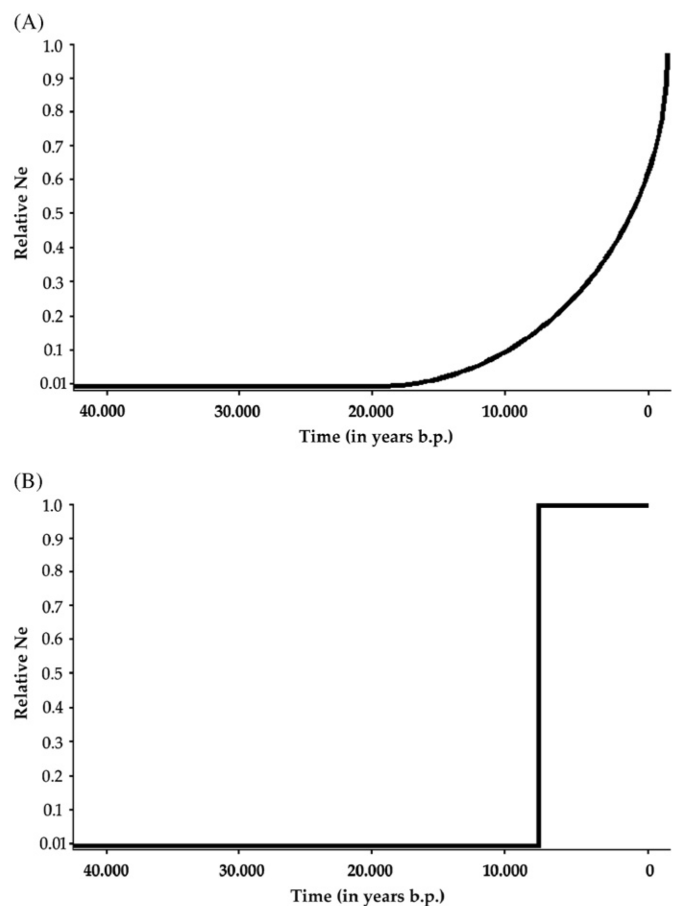

Fig. 5. Demographic expansion time estimations in *N. concinna* based on instantaneous (A) and exponential (B) growth models. Estimations incorporate "time dependency of molecular rate" correction.

Table 4
P-values of Hotelling pairwise comparisons between intertidal and subtidal morphotypes of Admiralty and Fildes Bays, Bonferroni corrected (above diagonal) and uncorrected (below diagonal).

	ABi	ABs	FBi	FBs
Admiralty Bay intertidal (ABs)	–	3.99×10^{-12}	4.73×10^{-5}	2.41×10^{-15}
Almirantazgo Bay subtidal (ABi)	2.40×10^{-11}	–	5.54×10^{-10}	0.00252
Fildes Bay intertidal (FBi)	0.000284	3.32×10^{-9}	–	2.78×10^{-14}
Fildes Bay subtisdal (FBs)	1.45×10^{-14}	0.01512	1.66×10^{-13}	–

population unit. These results agree with Beaumont and Wei (1991), who concluded that these different morphotypes are genetically homogeneous and only represent environmentally induced phenotypic plasticity in the species. In general, shell shape, thickness and even coloration of patellogastropods is strongly affected by microhabitats and diet (Lindberg, 1998; Nakano and Ozawa, 2005,2007; Nakano and Spencer, 2007; Lindberg, 2008; de Aranzamendi et al., 2009). Phenotypic plasticity has been commonly described in several genera of the order such as *Patella* Linnaeus, 1758 (Côrte-Real et al., 1992; Pagarete et al., 2005), *Helcion* Montfort, 1810 and *Nacella* (Morriconi and Calvo, 1993; de Aranzamendi et al., 2008, 2009).

Taking into account the distribution of *N. concinna* in different bathymetric levels of the coast, it is possible that non-assortative mating between the morphotypes could be operating in this species. Nevertheless, the particular mode of reproduction of *N. concinna* through external fertilization, forming stacks of several individuals during the reproductive period, and its indirect development with free-living larvae (Picken, 1980) suggests that non-random mating is unlikely to occur in this species. In fact, reproductive studies indicate that intertidal morphotype migration to lower depths occur before fertilization and spawning, indicating the mixture of both morphologies at the same level of reproduction (Picken and Allan, 1983; Brêthes et al., 1994). How the segregation of this morphotypes is maintained in Antarctica is still unclear and further reproductive mating choice experiments are needed. Moreover, low differences between intertidal and subtidal forms have been recently detected by de Aranzamendi et al. (2008) working with fast evolving markers (ISSR). The existence of such genetic differences suggests that *N. concinna* would not correspond to a panmictic population along a bethymetric gradient. However, recent studies using AFLP in the species found no evidence for differentiation either between the two bathymetric morphotypes or by depths. Furthermore, a Bayesian cluster analysis did not detect any evidence for cryptic genetic structure (Hoffman et al., 2010). These findings, along with the sequence homogeneity of mitochondrial markers, support the idea that the Antarctic limpet would be just phenotypically plastic, although further studies are needed to estimate unequivocally if some degree of genetic difference do exist, besides the observed morphological variability. In any case, such differences would not challenge the existence of a single *Nacella* species in the shallow Antarctic realm.

4.2. Genetic homogeneity in N. concinna along the western Antarctic Peninsula

An interesting result of the present study is the extremely low level of genetic diversity of mtDNA COI in *N. concinna*. The Antarctic limpet represents a single genetic unit with very low levels of haplotypic and nucleotide diversity (Table 1) along the western coast of the Antarctic Peninsula. Only 16 haplotypes were found in 162 Antarctic limpets across ~800 km (Fig. 2). Levels of genetic diversity observed in *N. concinna* are low compared to the ones observed in *Nacella* species from the Magellan Province (de Aranzamendi et al., 2009; González-Wevar et al., 2010). For 139 individuals of *N. magellanica*, belonging to seven localities along the Magellan Strait, we found 49 haplotypes (unpublished data). The most common haplotype in the species (H3) was shared by more than 58% of the individuals and distributed in all the analyzed localities along the Antarctic Peninsula and South Shetland Islands. Mitochondrial sequence diversity in *N. concinna* failed to recognize any statistically significant genetic structure in the species among the five localities examined in the Antarctic Peninsula. The only locality exhibiting a small degree of differentiation is Admiralty Bay with pairwise Φ_{ST} values one order of magnitude higher than what was observed by comparing between other localities (Table 3). Admiralty Bay is a well-sheltered bay with a maximum depth of 600 m and a surface of about 120 km^2 (Jazdzweski et al., 1986). In fact, Arnaud et al. (1998) concluded that communities of the South Shetlands appear to have distinctive features, as compared to those of continental Antarctica. The difference in pairwise comparisons between this locality and the rest of the studied sites may be a result of local oceanographic characteristics, or due to the comparatively sheltered situation of Admiralty Bay as a whole.

In theory, large population sizes should maintain high levels of genetic variability because genetic drift is low and the rate of mutation accumulation is high. Molecular diversity indices estimated in the Antarctic limpet (θ_k=4.9; θ_S=1.34; θ_H=3.15; θ_π=0.88) would be generated by small effective sizes (Ne) between 66.500 and 376.500 individuals. This is by far smaller than the expected population sizes found for this species, considering the high densities reported in several studies (Hedgpeth, 1969; Picken, 1980; Brêthes et al., 1994). In addition to low haplotype diversity, *N. concinna* exhibits a "star-like" genealogy, characterized by very short branches (Fig. 2), and a marked L-shaped distribution of pairwise differences (Fig. 4). Overall significant negative Tajima's *D* and Fu's F_S indices are also evidences of excess low frequency haplotypes relative to neutral mutation-drift equilibrium. All of these results strongly support the existence of a recent demographic expansion of this species. Under this scenario, our time estimations for this historical process range between 75,000 and 220,000 years ago. These time ranges do not fit with our hypothesis that *N. concinna* was able to (re-)expand during the last glacial–interglacial transition, after the last glacial maximum (LGM; ~20,000 years). However, a time dependency of molecular evolution rates has been recently described (Ho et al., 2005). As suggested by these authors, molecular studies at population levels have estimated much higher mutation rates than the substitution rates inferred from phylogenetic (species-level) analyses. In three cases (mtDNA of avian and primate taxa) they showed that short term (1–2 Myr) mutation rates could be ten folds higher than long-term substitution rates (Ho et al., 2005, 2007). Here, we used a substitution rate estimated specifically for the limpet genus *Cellana* (data not published), and our estimations may be biased due to the time dependency of molecular evolution rates. Under this perspective and incorporating a simple ten-fold correction,

our time estimations (7,500–22,000 years ago) would fit to the last glacial–interglacial transition period.

Low levels of genetic diversity detected in *N. concinna* could reflect a dramatic effect of glacial periods on population size and even on the persistence of the species along the Antarctic Peninsula. If we consider that *N. concinna* has a narrow bathymetric range (0–110 m), the extension of an ice sheet over the main part of the Antarctic continental shelf during glacial periods should have drastically reduced its habitat to isolated refugia in ice-free areas (Poulin et al., 2002; Thatje et al., 2005). Another possibility could be that *N. concinna* migrated northward during glaciations and returned to a southern distribution during interglacial periods. This is suggested by genetic closeness among *Nacella* samples from the Antarctic Peninsula, South Georgia and Marion Island. In this case, bottleneck and further founder effects may be plausible explanations for low diversity and genetic homogeneity detected in the Antarctic limpet. Our results contrasts with other studies on Antarctic marine benthic invertebrates that shows high levels of genetic diversity (Mahon et al., 2008; Thornhill et al., 2008; Krabbe et al., 2009; Wilson et al., 2009). However, most of these species exhibit a large bathymetric range that could prevent such drastic demographic impact during the recurrent Pleistocene glacial and interglacial cycles (Brey et al., 1996). Within this scenario, we could expect that the degree of genetic diversity in Antarctic benthic invertebrates would be positively related to their bathymetric range.

4.3. Genetic cohesiveness between sub-Antarctic N. delesserti and Antarctic N. concinna

The low levels of genetic differences between *N. delesserti* and *N. concinna* do not agree with other phylogenetic studies in the genus. Genetic similarities detected between these different taxonomic units are very low and within the range of intraspecific variation of other nacellid species (Goldstien et al., 2006; Bird et al., 2007). Recently, González-Wevar et al. (2010) have analyzed different *Nacella* species from Antarctica, South America and Kerguelen Province and have recovered deep molecular divergences among sub-Antarctic and Antarctic species without evidence of recent long-distance gene flow events. For example, Antarctic *N. concinna* and the Magellanic species of the genus exhibited much higher levels of genetic divergence (8.5%) than that observed between the former and Marion Island's *N. delesserti* (0.32%). At the same time, the species *N. macquariensis* and *N. kerguelenensis* collected from the Sub-Antarctic Heard Island (Kerguelen Province) also showed similar levels of genetic divergence (7.70%) between them (González-Wevar et al., 2010). As mentioned above, *N. delesserti* is a sub-Antarctic endemic species from Marion Island. Interestingly, the origin of this island, located ~5500 km away from the Antarctic Peninsula, is very recent (450,000 years; Chown et al., 2008). Therefore, the genetic proximity between *N. delesserti* and *N. concinna* could be explained through north-eastward long-distance dispersal events during the late Pleistocene, through the Circumpolar Antarctic Current. According to Beaumont and Wei (1991), *N. concinna* could be transported by drifting masses of algae, which supports the hypothesis that poses long-distance dispersion between Antarctica and Marion Island hypothesis. Phenotypic plasticity, as described in this and other studies, on *N. concinna* and other *Nacella* species from the Magellan Strait (Powell, 1973; Morriconi and Calvo, 1993; Valdovinos and Rüth, 2005; de Aranzamendi et al., 2008, 2009; Hoffman et al., 2010; González-Wevar et al., 2010) could have enhanced morphological differentiation of *N. delesserti* in Marion Island, and this could explain the described differences between this species and the Antarctic limpet (Powell, 1973). Because of the late Pleistocene origin of Marion Island, COI sequences as well as other mitochondrial and nuclear DNA genes (data not published) could not provide adequate resolutions to distinguish whether these two morphotypes correspond to different species. More individuals and localities of Marion Island's *N. delesserti* and faster evolving molecular markers are needed in order to offer a better explanation of this last finding.

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