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LIMPETS OF THE GENUS *NACELLA* (PATELLOGASTROPODA) FROM THE SOUTHWESTERN ATLANTIC: SPECIES IDENTIFICATION BASED ON MOLECULAR DATA

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ABSTRACT

Several species of the genus *Nacella* show high phenotypic intraspecific variability and only a few morphological characters can be used to identify them, so that their taxonomic status is unclear. In this work molecular markers were used to determine if the most frequent forms of the genus observed along the coast of Patagonia and Tierra del Fuego in Argentina (*Nacella magellanica*, *Nacella deaurata*, *Nacella delicatissima* and *Nacella mytilina*) are true species, or if some are morphotypes of a single species. Maximum Parsimony and Bayesian phylogenetic analyses of the partial sequence of the mitochondrial cytochrome oxidase I and cytochrome *b* genes showed a polytomy comprising most of the individuals of the genus *Nacella* analysed. Using cytochrome *b*, among all species tested, only *N. mytilina* formed a well-supported clade. Interspecific genetic distances among the four *Nacella* species were very low and similar to the intraspecific ones. On the contrary, intersimple sequence repeats (ISSRs) fingerprint analyses confirmed the differentiation of *N. magellanica*, *N. deaurata* and *N. mytilina*. The specimens of *N. delicatissima* were intermixed with the individuals of *N. magellanica* and *N. deaurata*, suggesting that *N. delicatissima* is not a separate species but an infrequent morphotype of each of the other two species. ISSR–PCR was more useful in revealing genetic differences among closely related species than mitochondrial DNA sequences. These results could be explained by recent speciation processes in these limpets of the Southwestern Atlantic.

INTRODUCTION

The limpets of the order Patellogastropoda have a particular evolutionary interest since this group may be sister (the 'basal branch') to all the other living Gastropoda, according to morphological and molecular analyses (Powell, 1973; Haszprunar, 1988a, b; Lindberg, 1988; Ponder & Lindberg, 1997; Koufopanou *et al*., 1999; Harasewych & McArthur, 2000; Nakano & Ozawa, 2004, 2007).

Patellogastropoda are common members of many marine intertidal rocky communities around the world, and play important ecological roles in the structure and succession processes of coastal ecosystems. The extant species of Patellogastropoda are currently classified into five families: Lottiidae, Lepetidae, Acmaeidae, Patellidae and Nacellidae. The species of the genus *Nacella* (family Nacellidae; with subgenera *Nacella* and *Patinigera*) are distributed in Antarctic and Subantarctic waters, including the Magellanic Province at the southern tip of South America that extends along both the Atlantic coast of Argentina and Pacific coast of Chile (IBM-UNESCO, 1964).

Although several aspects on the biology and ecology of some species of this genus are well known (Morriconi, 1999; Fraser, Clarke & Peck, 2002; Smith & Simpson, 2002; Clarke *et al*., 2004; Astorga España, Rodríguez Rodríguez & Díaz Romero, 2005; Bazterrica *et al*., 2007; Malanga *et al*., 2007; Markowska & Kidawa, 2007; de Aranzamendi *et al*., 2008), taxonomic studies and distribution records are scarce in the Magellanic Province, especially on the Argentinean coast. The following species of the genus *Nacella* have been cited for southern South America and the neighbouring Antarctic region: *Nacella* (*Patinigera*) *magellanica* (Gmelin, 1791), *Nacella* (*P.*) *deaurata* (Gmelin, 1791), *Nacella* (*P.*) *delicatissima* (Strebel, 1907), *Nacella* (*P.*) *fuegiensis* (Reeve, 1855) and *Nacella* (*Nacella*) *mytilina* (Helbling, 1779) in Argentina and Chile; *Nacella* (*P.*) *venosa* (Reeve, 1855), *Nacella* (*P.*) *chiloensis* (Reeve, 1855), *Nacella* (*P.*) *clypeater* (Lesson, 1831) and *Nacella* (*P.*) *flammea* (Gmelin, 1791) in Chile and *Nacella* (*P.*) *concinna* (Strebel, 1908) in Antarctica.

This list does not reflect the real diversity of the genus *Nacella* in the southern region of South America, since species of this genus usually have large synonymies, reflecting both their high phenotypic intraspecific variability and the few morphological characters used in traditional malacology to identify species (shape, apex position, number and form of ribs, thickness and colour of the shell, radula sac, radula, colour of foot and mantle tentacles). Many doubts and contradictions have arisen in the taxonomy of the group since the nineteenth century. For example, Pilsbry (1891) rejected the validity of the species described by Gmelin (1791), Reeve (1855) and Rochebrune & Mabille (1885), considering several of them to be variations of the limpet originally described by Martyn (1784) as *Patella aenea*. At present, this species is considered as a *nomen dubium*, because its description combines characteristics of both *N. magellanica* and *N. deaurata* (Otaegui, 1974). Castellanos (1967) observed certain difficulties in the identification of *N. deaurata* and suggested that it would be necessary to use other diagnostic characters in order to define the limits in relation to related species. Dell

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(1971), based on samples collected by the Royal Society Expedition in southern Chile, discussed the validity of many of the species belonging to *Nacella*, recognizing only the species *N. magellanica* and *N. deaurata* as valid. However, Otaegui (1974) in his studies of limpets from Patagonia, revalidated *N. delicatissima* and did not report the presence of *N. fuegiensis* or *N. concinna*. Castellanos & Landoni (1988) in their catalogue of the 'Malacofauna Magallánica' mentioned the affinity between *N. fuegiensis* and *N. delicatissima*, forms showing overlapping geographical distribution, and doubted their validity as species. Valdovinos & Rüth (2005) considered *N. fuegiensis* to be a synonym of *N. magellanica*. They also accepted *N. delicatissima* as distinct, contradicting its treatment by Powell (1973) as a subspecies or variety of *N. deaurata*.

At present, molecular techniques are widely used to solve problems of taxonomic identification and phylogenetic relationships and to complement morphological studies. In the order Patellogastropoda, for example, Mauro, Arculeo & Parrinello (2003) used the partial sequence of the mitochondrial cytochrome oxidase I gene (COI) to differentiate three species of *Patella* with overlapping geographical distributions, which were difficult to identify on the basis of shell morphology. Using the same genetic marker, Simison & Lindberg (1999) analysed the genetic diversity of the limpet *Notoacmea fascicularis* and suggested that its morphological variability was not associated with the presence of different species, but represented a single species with a geographic effect. Espinosa & Ozawa (2006) found that two forms of *Patella ferruginea* correspond to ecotypes and not to different species. Using two mitochondrial genes (fragments of COI and 16S ribosomal RNA), Nakano & Ozawa (2005) distinguished four species of *Patelloida* that had been synonymized and treated as one species with two ecological forms.

In cases where protein-coding regions cannot resolve taxonomic problems and DNA sequences of the study species are not known, the use of inter simple sequence repeats (ISSRs) markers can be a successful alternative. In recent years, techniques based on PCR amplifications of ISSR have provided new nuclear genetic markers in non-coding DNA, which evolve at a much faster rate and are less constrained by selection than protein-coding regions. ISSR–PCR primers consist of repetitions in tandem of basic motifs of two to six nucleotides that amplify the fragments of DNA between inversely orientated microsatellite loci, with oligonucleotides anchored in microsatellites themselves. This technique does not require prior knowledge of DNA sequences, generates highly reproducible band patterns and reveals high levels of polymorphism (Zietkiewicz, Rafalski & Labuda, 1994; Bornet & Branchard, 2001), increasing the potential to detect genetic differentiation (Estoup *et al.*, 1998). Additionally, they allow for a rapid development and screening (unlike AFLPs) and show high reproducibility (unlike RAPDs). Although ISSRs have only recently been successfully employed in marine taxa (Casu *et al.*, 2005, 2006; Hou *et al.*, 2006; de Aranzamendi *et al.*, 2008; Lai, Ciruni-Galletti & Casu, 2008), they have been widely used in terrestrial taxa (Abbot, 2001; Bornet & Blanchard, 2001; Luque *et al.*, 2002; Hundsdoerfer, Kitching & Wink, 2005).

The purpose of this study was to evaluate the degree of genetic differentiation among the morphological forms and species of the genus *Nacella* of the Argentinian coast in the Magellanic Province of the southwestern Atlantic Ocean using different molecular markers, in order to establish if the morphological variability corresponds to the presence of different species, or if the forms belong to a small number of polymorphic species.

MATERIAL AND METHODS

Sample collection

Nacella (*P.*) *magellanica*, *Nacella* (*P.*) *deaurata*, *Nacella* (*P.*) *delicatissima* and *Nacella* (*N.*) *mytilina* were collected from Río Negro, Chubut, Santa Cruz and Tierra del Fuego in Argentina (Fig. 1, Table 1). The identification of limpet specimens was based on morphological and morphometric characteristics of the shells following several authors (Powell, 1973; Otaegui, 1974; Castellanos & Landoni, 1988; Morriconi & Calvo, 1993; Forcelli, 2000; Valdovinos & Rüth, 2005). Most of the morphological range present in each of the species was included in the samples. Given that *N. magellanica* is the most abundant limpet of Patagonia and Tierra del Fuego and shows diverse morphotypes (Fig. 2), many more individuals of this species were sampled. *Nacella mytilina* is morphologically well distinguished from the other three species and inhabits a different environment (*Macrocystys pyrifera* kelp forests rather than rocky shores) and therefore only a few individuals were used. One sample of *N.* (*P.*) *concinna* from King George Island (Isla 25 de Mayo), South Shetlands, Antarctica (62°14′S, 58°38′W) was included for comparison. Samples were preserved in 80% ethanol.

DNA extraction, amplification and sequencing

Total genomic DNA was obtained from foot muscle tissue following phenol–chloroform extraction (Maniatis, Fritsh & Sambrook, 1982). DNA was stored in double-distilled water at 4°C until PCR amplification. A fragment of the mitochondrial COI and of the mitochondrial cytochrome *b* gene (Cyt *b*) was amplified using universal primer pairs: LCO1490 (5′-GGTC AACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TA AACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994), UCYTB151F (5′-TGTGGRGCNACYGTWATYACTA A-3′) and UCYTB272R (5′-GCRAANAGRAARTACCAY TC-3′) (Merritt *et al.*, 1998), respectively. For the amplification of the COI gene in *N. concinna*, the primers LCO1490 and COI-LEMR were used (Claudio A. González Wevar, personal communication). Double-stranded DNA was amplified in 50-μl reaction volumes containing 1 unit of Taq polymerase (Fermentas, Brazil), 5 μl of 10 × reaction buffer [75 mM Tris–HCl pH 8.8, 20 mM $(NH_4)SO_4$, 0.01% Tween-20], 2.5 mM $MgCl_2$, 200 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.2 μM of each primer and *c.* 15 ng of template DNA. PCR amplification was performed in an Eppendorf® Mastercycler programmed for 1 cycle of 3 min at 94°C, 35 (for COI) and 39 (for Cyt *b*) cycles of denaturation at 94°C for 1 min, annealing at 50°C (for COI) and 48°C (for Cyt *b*) for 1 min, and extension at 72°C for 90 s. A posttreatment of 7 min at 72°C and a final cooling at 4°C were performed. Double-stranded PCR products were purified with Qiaquik (Qiagen) and sequenced in both directions by Macrogen Inc. (USA). Sequencing results were analysed using the program CHROMAS version 2.23 (McCarthy, 1998) and manually edited. Sequences of COI and Cyt *b* were submitted to GenBank (accessing numbers EU870921-EU870924, EU870926-EU871007 for COI and EU870891-EU870920 for Cyt *b*).

Nuclear fingerprints

Four anchored and one nonanchored ISSR primers that showed a high number of clear, polymorphic bands were selected for the analyses: $(GTG)_3GC$, $(AC)_8C$, $(AG)_8Y$, $(CA)_6RG$ and $(CCA)_5$. ISSR–PCR amplification reactions consisted of 15 ng of template DNA, 0.6 units of Taq

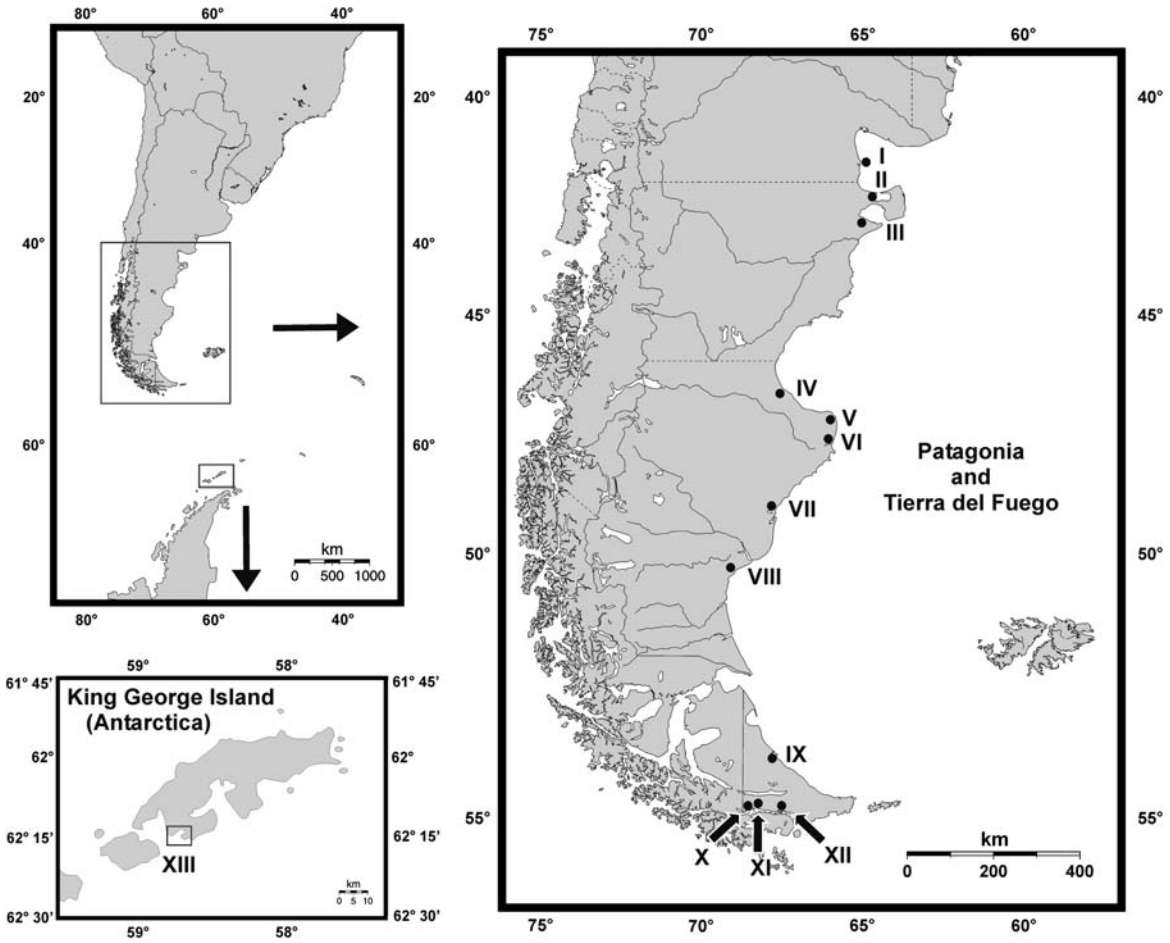

Figure 1. Sampling sites of *Nacella* species in Patagonia and Tierra del Fuego (Argentina) and King George Island (Antarctica). Key to localities is given in Table 1.

Table 1. Sampling localities and number of individuals analysed from each species of the genus *Nacella*.

Species	Localities	Sample size		
		COI	Cyt *b*	ISSR
Nacella magellanica	Islote de los Pájaros, Río Negro (I)	4	–	–
	Golfo San José, Chubut (II)	1	–	–
	Golfo Nuevo, Chubut (III)	–	–	2
	Intertidal reef between Comodoro Rivadavia and Caleta Olivia, Santa Cruz (IV)	12	3	14
	Cabo Blanco, Santa Cruz (V)	9	1	8
	Puerto Deseado, Santa Cruz (VI)	17	2	19
	San Julián, Santa Cruz (VII)	1	–	2
	Monte León, Santa Cruz (VIII)	7	1	10
	Cabo Peñas, Tierra del Fuego (IX)	1	–	2
	South of Ushuaia, Beagle Channel, Tierra del Fuego (X)	6	3	13
	Puerto Ushuaia, Tierra del Fuego (XI)	–	–	1
	Puerto Almanza, Beagle Channel, Tierra del Fuego (XII)	1	–	1
N. delicatissima	Puerto Deseado, Santa Cruz (VI)	2	2	4
	Monte León, Santa Cruz (VIII)	–	1	–
	South of Ushuaia, Beagle Channel, Tierra del Fuego (X)	6	4	3
N. deaurata	South of Ushuaia, Beagle Channel, Tierra del Fuego (X)	11	6	13
	Puerto Ushuaia, Tierra del Fuego (XI)	2	1	1
N. mytilina	Puerto Deseado, Santa Cruz (VI)	4	4	4
N. concinna	King George Island, South Shetland Islands (XIII)	1	1	–

The Roman numerals in brackets correspond to localities in Figure 1.

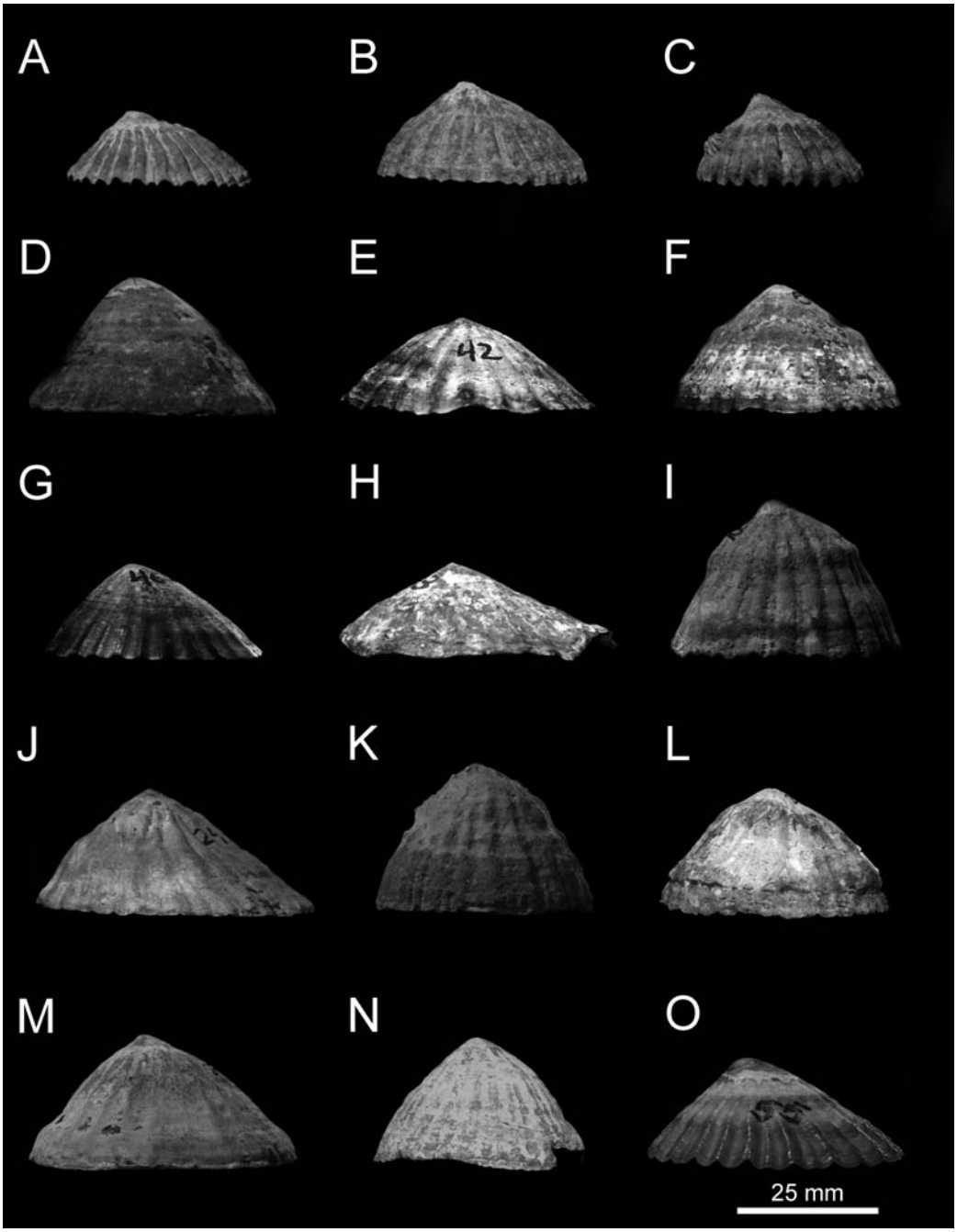

Figure 2. Variability in shell morphology of *Nacella magellanica* (lateral view) from Patagonia and Tierra del Fuego (Argentina). **A.**–**C.** Intertidal reef between Comodoro Rivadavia and Caleta Olivia (Santa Cruz). **D.**–**H.** Cabo Blanco (Santa Cruz). **I.**–**N.** Puerto Deseado (Santa Cruz). **O.** South of Ushuaia (Beagle Channel, Tierra del Fuego).

polymerase (Fermentas, Brazil), 1 μl of 10 × reaction buffer [75 mM Tris–HCl pH 8.8, 20 mM $(NH_4)SO_4$, 0.01% Tween-20], 2 mM $MgCl_2$, 62.5 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.2 μM of each primer and double-distilled water to a final volume of 25 μl. PCR amplification was performed in an Eppendorf® Mastercycler programmed for 1 cycle of 3 min at 94°C, 34–37 cycles of denaturation at 93°C for 1 min, annealing at 47–49°C for 1 min, and extension at 72°C for 90 s (Table 2). A posttreatment of 5 min at 72°C and a final cooling at 4°C were performed. Negative controls were included in each PCR. To verify the repeatability of ISSR results, replicates were included in each set of amplifications. PCR products were run in 1.5% agarose gels using 0.5 × TBE buffer. Five microlitres of 1 kb ladder (Invitrogen, Carlsbad, USA) were run for fragment size reference within each gel. Gels were run at 120 V for about 4 h and stained using a 0.005 μg ml^{-1} ethidium bromide solution. ISSR banding patterns were visualized using a UV transilluminator and recorded by digital photography.

Sequence analysis

Thirteen sequences obtained from GenBank database were included in the analysis for comparisons and as outgroups (for

COI: *N. magellanica*, *N. deaurata*, *N. mytilina*, *Cellana flava*, *C. nigrolineata*, *Patella vulgata*, *P. ferruginea*, *Helcion dunkeri*, *Cymbula compressa*, from Nakano & Ozawa, 2007, under accession numbers AB238545, AB238548, AB238567 - AB238569, AB238570, AB238575, AB238578, AB238580; for Cyt *b*: *Cellana flava*, *C. ornata*, *C. radians* from Goldstien, Schiel & Gemmell, 2006, DQ011463, DQ011496, DQ011500, and *Littorina saxatilis*, unpublished, EF114088). Sequences were aligned using the MUSCLE program (Edgar, 2004), with verification and minor manual adjustments. Once aligned, sequences were collapsed into haplotypes; the number of polymorphic sites and haplotypes were calculated using the program DNAsp 4.10 (Rozas & Rozas, 2003). Mean distances within and between species were calculated by Kimura's 2-parameter method (Kimura, 1980) using MEGA version 3.1 (Kumar, Tamura & Nei, 2004). Phylogenetic analyses were based on two approaches: the Maximum Parsimony (MP) method using the TNT program (Goloboff, Farris & Nixon, 2003, 2008) and Bayesian analysis with MrBayes v 3.1.2 (Ronquist & Huelsenbeck, 2003). For MP analyses, gaps were considered a fifth character. A heuristic search was performed with 200 random stepwise additions of taxa and tree bisection reconnection branch swapping. Bootstrap and jackknife analyses (based on 1,000 replications) were used to assess the stability of each node. For the Bayesian approach, the model of evolution that adjusted best with the haplotypes data set was selected using Modeltest 3.5 (Posada & Crandall, 1998). The average amount of sequence divergence within each species and among species was inferred with the selected HKY + γ evolution model (COI: A = 0.2241, C = 0.1933, G = 0.1934, T = 0.3893 and $\gamma = 0.2685$; Cyt *b*: A = 0.2607, C = 0.2249, G = 0.1500, T = 0.3643 and $\gamma = 0.9867$) (Hasegawa, Kishino & Yano, 1985) implemented in the program PAUP* 4.0b10 (Swofford, 2001). In the Bayesian analyses, four chains were used (the default parameters) and the starting tree was random. For Cyt *b* sequences, the analysis was run for 1,000,000 generations, with a sample frequency of 100. The first 2,500 trees were discarded as 'burn-in'. For COI sequences, due to the impossibility for the program to finish the analysis using the total number of specimens, the matrix was reduced to 40 representative individuals. The analysis was run for 2,000,000 generations, with a sample frequency of 100. The first 5,000 trees were discarded as 'burn-in'. The posterior probabilities supporting the nodes were calculated.

Table 2. Primer sequences used in the inter simple sequence repeats (ISSR) analyses, annealing temperature, number of cycles, number of polymorphic bands per primer and range of molecular weight in base pairs (bp) amplified by polymerase chain reaction ISSR for 97 individuals of *Nacella*.

Primer Sequences (5′–3′)	Annealing temperature (°C)	No. of cycles	No. of polymorphic bands	Size-range of polymorphic bands (bp)
$(GTG)_3GC$	48	37	16	480.5–1,293.8
$(AC)_8C$	49	37	11	630–1,317.9
$(AG)_8Y$	47	37	21	510.2–1,306.3
$(CA)_6RG$	49	38	9	662.3–1,066.5
$(CCA)_5$	48	35	11	714.3–1,155

ISSR–PCR analysis

The length of each fragment was estimated comparing with the molecular standard marker using the program DNAsize 1.0 (Raghava, 1994). Each variable, clear, high-intensity band of the ISSR–PCR fingerprints was scored as present (1) or absent (0) in a data matrix that was used for calculations. A matrix of genetic distances between pairs of individuals (1-% matching) was calculated from the matrix of presence/absence of bands with the program RAPDPLOT 3.0 (Black, 1995). A neighbour-joining tree (NJ) (Saitou & Nei, 1987) based on the genetic distances was generated using the programs NEIGHBOR and CONSENSUS in the PHYLIP package (Felsenstein, 2004) and bootstrapped 1,000 times. A principal components analysis (PCA) was performed to examine the genetic relationships among species using the PAST software (Hammer, Harper & Ryan, 2001). A minimal spanning tree (MST), which shows the shortest possible set of lines connecting all points in two dimensions based on Euclidean distance in the original space, was superimposed on the PCA plot to evaluate the multidimensional relationships among the individuals. Student's *t*-tests were used to assess if the principal components were significant among species using InfoStat software version 1.1 (InfoStat, 2002). Differences were considered significant at $P < 0.001$.

RESULTS

COI gene sequences

The COI sequences revealed 32 haplotypes in 84 specimens from Patagonia and the Argentinean sector of Tierra del Fuego. Thirty-seven sites of the 573 bp sequenced exhibited variation and 15 of them were phylogenetically informative. No deletions, insertions or inversions were detected. *Nacella mytilina* presented exclusive haplotypes differing by two to nine mutations from *N. magellanica*. *Nacella delicatissima* shared haplotypes with *N. magellanica* or *N. deaurata*. The most frequent haplotype (h 31) was shared between *N. magellanica*, *N. deaurata* and *N. delicatissima*.

The intraspecific distances (0.003–0.007; not shown) were similar to interspecific ones (0.004–0.01) among the Southwestern Atlantic limpets. Genetic distances between *N. mytilina* and each of the other three Magellanic limpets are slightly higher than the other interspecific distance values. The

Table 3. Mean molecular distances for interspecific pairwise comparison.

	N. magellanica	*N. delicatissima*	*N. deaurata*	*N. mytilina*	*N. concinna*
N. magellanica	–	0.004 ± 0.002	0.004 ± 0.002	0.011 ± 0.005	0.102 ± 0.017
N. delicatissima	0.004 ± 0.001	–	0.003 ± 0.001	0.009 ± 0.004	0.099 ± 0.017
N. deaurata	0.004 ± 0.001	0.004 ± 0.001	–	0.009 ± 0.004	0.101 ± 0.017
N. mytilina	0.01 ± 0.003	0.01 ± 0.003	0.009 ± 0.003	–	0.108 ± 0.018
N. concinna	0.093 ± 0.013	0.093 ± 0.013	0.093 ± 0.013	0.098 ± 0.013	–

Distances values ± standard errors are shown. Above diagonal: Cyt *b* values. Below diagonal: COI values.
N., *Nacella*.

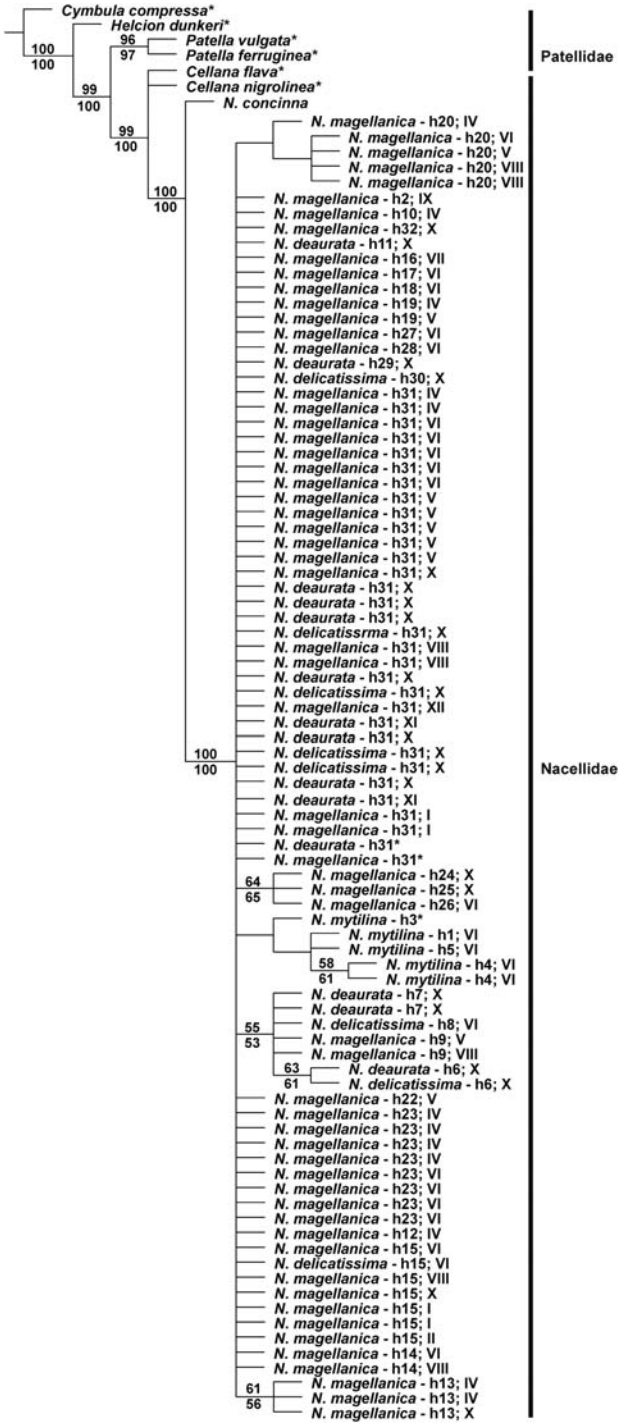

Figure 3. Maximum parsimony strict consensus tree of partial COI gene analysis. Bootstrap values are shown above branches and jackknife values are below (both based on 1,000 replications). Only values above 50% are indicated (16 equally parsimony trees, 728 length; consistency index CI = 0.72, retention index RI = 0.809). The haplotype number and locality of origin are indicated next to each individual. Abbreviations: *N.*, *Nacella*; h, haplotype. Asterisks indicate sequences from GenBank.

genetic distances between *N. concinna* and each of the other species of *Nacella* were high (Table 3).

The MP and Bayesian trees showed a polytomy comprising most of the analysed individuals of the genus *Nacella* (MP tree in Fig. 3; Bayesian tree not shown). Although *N. mytilina* belongs to a different subgenus (*Nacella*) than the other species included in this study (*Patinigera*), it is included in the same unresolved clade. Individuals of the morphologically recognized species did not group together.

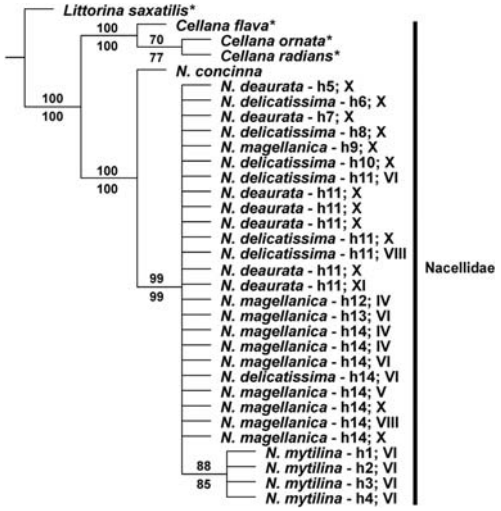

Figure 4. Maximum parsimony strict consensus tree of partial Cyt *b* gene analysis. The values above the branches indicate bootstrap percentages and below the branches are jackknife values (both based on 1,000 replications). Only values above 50% are indicated (14 equally parsimony trees, 592 length; CI = 0.731, RI = 0.696). The haplotype number and locality of origin are indicated next to each individual. Abbreviations: *N.*, *Nacella*; h, haplotype. Asterisks indicate sequences from GenBank.

Cyt b *gene sequences*

Partial mitochondrial Cyt *b* gene sequences of 399 bp were obtained from 29 specimens of *Nacella*. A total of 14 haplotypes were detected, with 16 variable sites, four of which were parsimoniously informative. Given that this initial screening with Cyt *b* gene sequences revealed a very low number of variable and informative sites, no more individuals were sequenced for the analysis. *Nacella magellanica*, *N. deaurata* and *N. mytilina* did not share any haplotype, but they only differed in two to four mutations. Some individuals of *N. delicatissima* showed the same haplotypes as *N. magellanica* or *N. deaurata*.

Mean pairwise intraspecific distances (0.002–0.004; not shown) were similar to the interspecific (0.003–0.004) ones among *N. magellanica*, *N. deaurata* and *N. delicatissima*. Genetic distances between *N. mytilina* and each of the other three Magellanic limpets are slightly higher than the interspecific distances among the other species studied. On the other hand, mean distance values among Magellanic species and *N. concinna* were considerably higher (Table 3).

In the MP and Bayesian trees, the clade of Magellanic limpets was well supported (bootstrap and jackknife values 100%). Individuals of *N. mytilina* formed a separate well-supported clade, but all specimens of the other three species formed a large polytomy (MP tree in Fig. 4; Bayesian tree not shown).

Inter simple sequence repeat–polymerase chain reaction

ISSR amplification patterns were highly repeatable. Primers $(GTG)_3GC$, $(AC)_8C$, $(AG)_8Y$, $(CA)_6RG$ and $(CCA)_5$ produced 68 polymorphic bands, in the range of 480–1,318 bp, that could be accurately scored (Table 2).

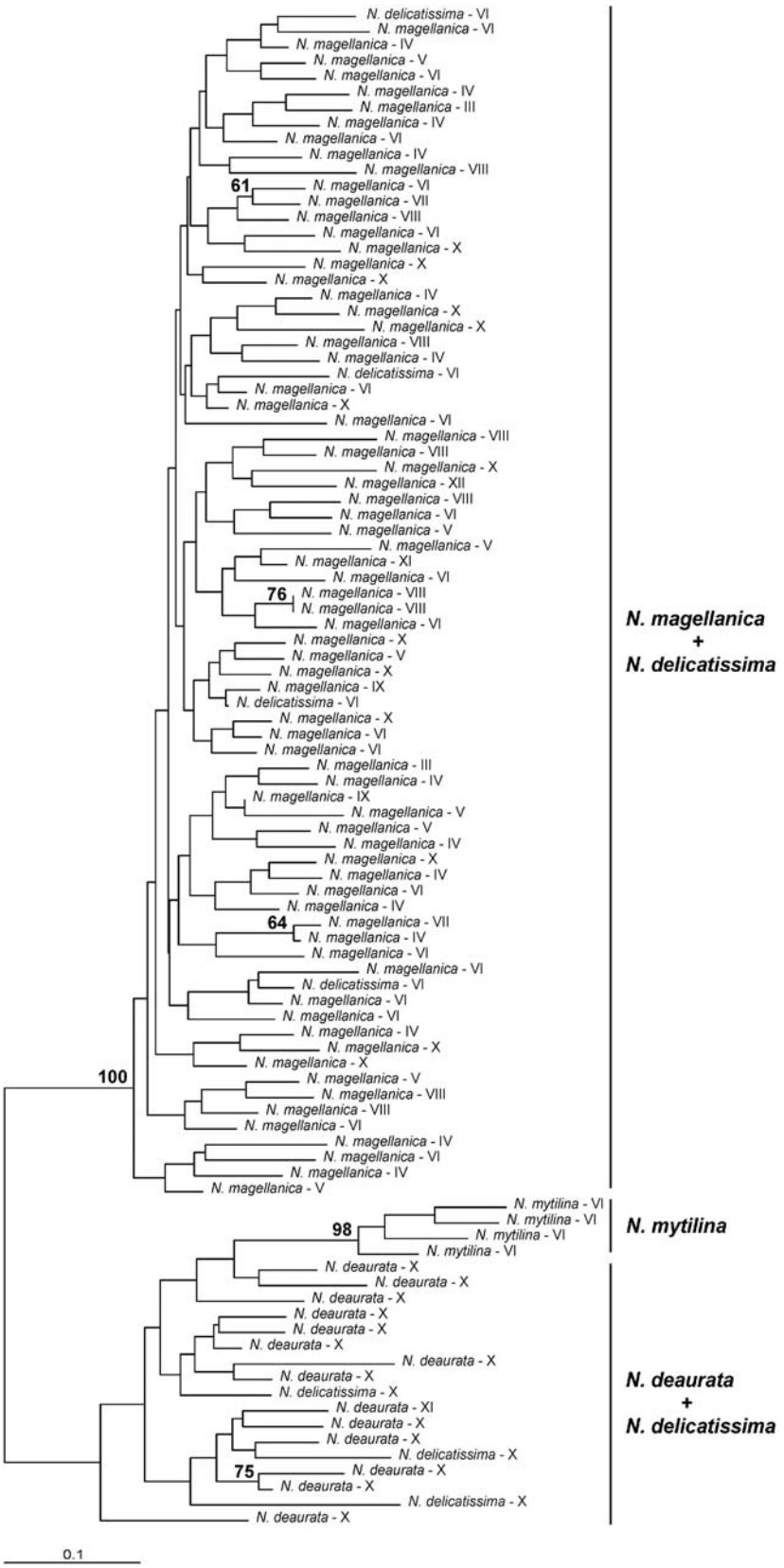

Figure 5. Neighbour-joining unrooted tree of ISSR fingerprints in *Nacella* samples based on 68 scored fragments. Roman numerals indicate sampling localities. The values above the branches are bootstrap percentages (based on 1,000 replications). Only values above 50% are shown.

Four private bands were detected in *N. magellanica* and five in *N. mytilina;* 13 bands were shared between *N. magellanica* and *N. delicatissima*, and four between *N. deaurata* and *N. delicatissima*. In addition, some of the bands were absent in only one of the species: four in *N. magellanica*, three in *N. deaurata* and nine in *N. mytilina*.

The NJ tree based on 97 individuals differentiated, with high bootstrap support, three of the morphological species: *N. magellanica*, *N. deaurata* and *N. mytilina*. The specimens of *N. delicatissima* were intermixed with the individuals of *N. magellanica* or *N. deaurata* (Fig. 5). Individuals from the same sample sites did not form discrete clusters in the analysis, indicating the absence of geographical patterns. The plot of the first and second principal components, which accounted for 31.7 and 5.1% of the total variation, respectively, supported the results given by the NJ analysis (Fig. 6). The MST confirmed that the relationships between samples in the PCA plot are preserved in multidimensional space (not shown). Student's *t*-tests for principal component 1 indicated a significant differentiation ($P < 0.001$) between *N. magellanica* and *N. deaurata*, *N. magellanica* and *N. mytilina*, *N. deaurata* and *N. mytilina*. Principal component 2 showed significant differences between *N. mytilina* and the other species (Table 4).

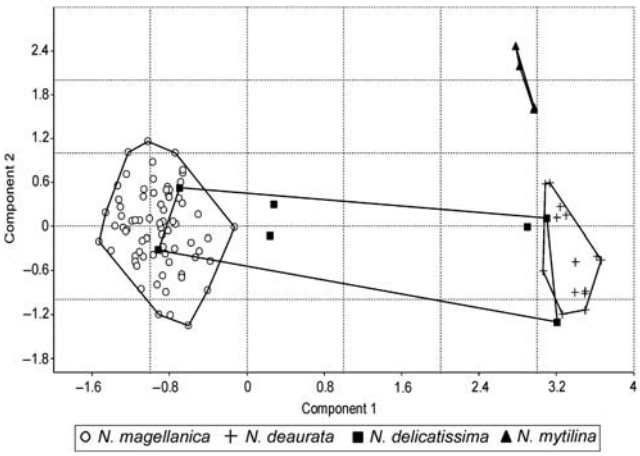

Figure 6. Principal components analysis based on ISSR fingerprints in *Nacella* samples. The plot shows the first and second components, which explain 31.7 and 5.1% of the total variation, respectively. Each convex polygon groups all individuals of each species.

DISCUSSION

Our results represent the first attempt to clarify the complex taxonomy of the genus *Nacella* in the Magellanic Province using molecular markers. Although mitochondrial DNA sequences were not useful for identification of species, the analysis of arbitrarily primed nuclear markers clearly grouped specimens of each of *Nacella magellanica*, *Nacella deaurata* and *Nacella mytilina*, the most frequent forms of the genus observed on the coast of Patagonia and Tierra del Fuego in Argentina.

The two genes analysed exhibit very low levels of sequence variation when comparing samples of the four nominal Magellanic species sampled (COI: 6.2% variable sites with only 2.5% of them being informative; Cyt *b*: 3.16% variable sites with 0.79% informative). These results are quite different from the values obtained in several other studies of closely related species of molluscs. For example, Baker *et al.* (2003) demonstrated the presence of cryptic species in freshwater mussels (Hyriidae) using partial sequence of COI, isozymes and morphological data. The authors found, for the COI gene, 137 variable sites (29%) in a segment of 473 bp, with 106 sites (22.4%) parsimony informative. Terranova, Lo Brutto & Mitton (2007), using partial mitochondrial sequences of 16S and COI genes, detected three cryptic species in *Brachidontes variabilis* (Mytilidae). The COI sequences of 618 bp had 187 variable sites (30.3%), of which 168 (27.2%) were parsimony informative.

Intraspecific (COI: 0.003–0.007; Cyt *b*: 0.002–0.004) and interspecific (COI: 0.004–0.01; Cyt *b*: 0.003–0.011) genetic pairwise distances among the four Magellanic *Nacella* species were very low and similar. Knowing that *N. mytilina* belongs to a different subgenus and that it is easy to differentiate from the other *Nacella* species using morphological characters, it was unexpected that the genetic distances between *N. mytilina* and the other three limpets (COI: 0.009–0.01; Cyt *b*: 0.009–0.011) were only slightly higher than the interspecific distance values among the three species of the subgenus *Patinigera* (COI: 0.004; Cyt *b*: 0.003–0.004). These genetic distance values are much lower than those reported for other closely related species of gastropods. Nakano & Osawa (2005), using two mitochondrial genes (16S and COI), obtained interspecific genetic distance values of one order of magnitude higher than the intraspecific ones in four species of *Patelloida* (Lottidae) that had been treated as a single species. Mauro *et al.* (2003), utilizing a COI gene fragment, demonstrated much higher interspecific genetic distance among three closely related *Patella* species (0.122–0.129) than those reported here for Magellanic *Nacella* (0.004–0.01).

Table 4. Student's *t*-test results for the principal components analysis, using ISSR–PCR data.

	N. magellanica	*N. deaurata*	*N. delicatissima*	*N. mytilina*
Principal component 1				
N. magellanica	–	−54.2	−2.99	−26.82
N. deaurata	<0.0001*	–	3.12	4.54
N. delicatissima	0.0244	0.0205	–	−2.46
N. mytilina	<0.0001*	0.0003*	0.0488	–
Principal component 2				
N. magellanica	–	2.19	0.45	−7.23
N. deaurata	0.0312	–	−0.91	−7.07
N. delicatissima	0.6544	0.3732	–	−6.13
N. mytilina	<0.0001*	<0.0001*	0.0002*	–

Above diagonal: *t* values. Below diagonal: *P* values.

N., *Nacella*.

*$P < 0.001$.

Both the mitochondrial genes, COI and Cyt *b*, produced phylogenetic trees of similar topology. The COI trees obtained by MP and Bayesian methods failed to distinguish the four species of *Nacella*; individuals were clustered neither according to the nominal species nor according to the sample sites. The Cyt *b* gene trees showed a well-supported clade only for *N. mytilina*. The trees obtained show that all Magellanic *Nacella* included in this study belong to one clade, and suggest a recent speciation event. The fossils records of *N. deaurata* and *N. magellanica* in Argentinean Patagonia are from the late Pleistocene and mid-Holocene and in Tierra del Fuego (Beagle Channel, Argentina), from the mid- and late Holocene (*c.* 8,000–4,000 BP); the general conditions of the littoral environment in these regions during those periods were similar to those prevailing at present (Gordillo, 1999; Aguirre, Negro Sirch & Richiano, 2005). These stable environmental characteristics could have led to low levels of genetic differentiation between these new species. However, the Antarctic species *N. concinna* is clearly separated from the clade grouping all Magellanic specimens (>45 mutations for COI genes; >34 mutations for Cyt *b*, data not shown), which suggest an older separation between them. Individuals could have spread from Antarctica to South America, or in the opposite direction, along the Scotia arc after the opening of the Drake Passage (25–30 Ma BP) and the present oceanographic conditions could have led to the isolation of the marine fauna of the Antarctic Ocean (Clarke & Crame, 1992).

In contrast to the mitochondrial DNA results, the ISSR fingerprint analyses presented here confirm the division of *N. magellanica*, *N. deaurata* and *N. mytilina*. Individuals of these nominal species are clearly separated into three groups using both NJ and PCA analyses. Specimens from the same sample sites are not grouped together in the analyses, indicating that the clustering results are not explained by their geographic origin.

The individuals of *N. delicatissima* do not group together in the NJ or in the PCA analyses; rather, these specimens are scattered in the graphics. Some of the individuals are grouped with specimens of *N. magellanica* and others with *N. deaurata*. Therefore, the ISSR markers do not support *N. delicatissima* as a distinct genetic entity, which is in line with its overlapping morphological characteristics with *N. magellanica* and *N. deaurata*.

ISSR molecular markers were more useful in revealing genetic differences among closely related species of the genus *Nacella* than mitochondrial DNA sequences. This could be explained by the high potential for detecting differences using arbitrary primers that amplify nuclear noncoding DNA sequences that evolve at a much faster rate and are less constrained by selection than mitochondrial genes in relatively recently formed species. It is noticeable that the presence of private bands in two species, and absence of particular bands in only one of the species analysed, supports the utility of these genetic markers for identifying them.

Greater morphological than molecular evolution rates and incongruence between nuclear and mitochondrial data have been suggested in other gastropod taxa (Dillon & Frankis, 2004; Glaubrecht & Köhler, 2004; Köhler *et al*., 2004; Von Rintelen *et al*., 2004; Köhler & Glaubrecht, 2006; Lee *et al*., 2007). Even when some relationships are well supported in analyses using single-locus markers, the trees obtained are only gene trees, which might differ from the species tree as a result of incomplete lineage sorting of ancestral polymorphisms during speciation (Avise, 2004). Therefore, in a group of species that might have undergone a recent speciation process such as the Southwestern Atlantic *Nacella* species, it may be very difficult to resolve the species phylogeny, even if several DNA segments with different substitution rate are assayed.

In spite of its low genetic differentiation from the other Southwestern Atlantic species, *N. magellanica* is highly variable in shell morphology and colour patterns (Fig. 2). This variability was also observed by us (R.B., unpubl.) in Holocene (10,000 BP) and Late Pleistocene (40,000 BP) fossiliferous strata at Rada Tilly (Chubut, Argentina) and Caleta Olivia (Santa Cruz, Argentina). In some cases, phenotypic plasticity has been argued as responsible for the morphological variation of limpets, suggesting that morphological differences in shell shape could be environmentally determined (Corte-Real, Hawkins & Thorpe, 1996; Mauro *et al*., 2003). Some of this morphological variation could be explained by environmental factors such as erosion by suspended sediments, mechanical action of waves and desiccation according to tidal level. Nevertheless, the individuals also vary in kind and number of ribs, shell height and, in extreme cases, by the presence of undulations of the margin of very flat shells; it is not known whether such variation is connected with phenotypic plasticity or other causes (Fig. 2). Although *N. deaurata* has been reported all along the coasts of the southern tip of South America, in this study it was only found in the Beagle Channel (Tierra del Fuego). Individuals of this species reported in Patagonia could represent part of the morphological variability found in *N. magellanica*. Like *N. deaurata*, some specimens of *N. magellanica* show the apex towards the anterior margin of the shell, which could mislead the correct classification of these two species. *Nacella mytilina* is a species that is easily recognized by the structure and coloration of the shell, and in addition it inhabits a different environment, the sublittoral community of *Macrocystis pyrifera* kelp. *Nacella delicatissima* was found in very low frequency during the sampling. According to our results, *N. delicatissima* may not be a separate species but an infrequent morphotype of both *N. magellanica* and *N. deaurata*, characterized by a thin and low shell, with a frequent incidence of epizoic calcareous algae (*Lithothamnia* type).

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