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JI Túnez [a], HL Cappozzo [b], H Pavés [c], DA Albareda [d] & MH Cassini [a e]

[a] Grupo de Estudios en Ecología de Mamíferos, Departamento de Ciencias Básicas, Universidad Nacional de Luján and CONICET, Luján, Argentina

[b] Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN-CONICET), Buenos Aires, Argentina

[c] Instituto de Ciencias Marinas y Limnológicas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile

[d] Jardín Zoológico and Acuario de Buenos Aires, Buenos Aires, Argentina

[e] Laboratorio de Biología del Comportamiento, Instituto de Biología y Medicina Experimental (IBYME-CONICET), Buenos Aires, Argentina

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RESEARCH ARTICLE

The role of Pleistocene glaciations in shaping the genetic structure of South American fur seals (*Arctocephalus australis*)

JI Túnez[a]*, HL Cappozzo[b], H Pavés[c], DA Albareda[d] and MH Cassini[a,e]

[a]*Grupo de Estudios en Ecología de Mamíferos, Departamento de Ciencias Básicas, Universidad Nacional de Luján and CONICET, Luján, Argentina;* [b]*Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN-CONICET), Buenos Aires, Argentina;* [c]*Instituto de Ciencias Marinas y Limnológicas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile;* [d]*Jardín Zoológico and Acuario de Buenos Aires, Buenos Aires, Argentina;* [e]*Laboratorio de Biología del Comportamiento, Instituto de Biología y Medicina Experimental (IBYME-CONICET), Buenos Aires, Argentina*

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Analysing a 529 bp segment of the mitochondrial control region, we evaluated the role that Pleistocene glaciations may have had in shaping the genetic structure currently found in the two southernmost breeding areas of the South American fur seal, *Arctocephalus australis*. Additionally, we analysed if these two breeding areas correspond to different conservation units. We found 26 haplotypes in 54 individuals. Colonies from the Uruguayan breeding area did not show significant differences in haplotype frequencies, which suggest that they are remnants of a single ancient gene pool. The genealogical relationship between haplotypes revealed a pattern of phylogeographic structure with two main haplogroups corresponding to the different breeding areas. The analysis of molecular variance and the estimate of population divergence time also indicated significant genetic differences and a long period of isolation between Atlantic and Pacific colonies, suggesting that these breeding areas would correspond to different conservation units.

Keywords: *Arctocephalus australis*; population structure; Pleistocene glaciations; mtDNA control region; conservation units; southern South America

Introduction

Quaternary climatic fluctuations have been widely recognised as one of the main historical processes influencing the genetic diversity of natural populations in both the southern and northern hemispheres (Hewitt 2011; Sérsic et al. 2011). In Patagonia, a rich history of glaciations greatly altered the Patagonian landscape and influenced species' distributions (Rabassa et al. 2011). The Great Patagonian Glaciations (GPG) developed between 1.168 and 1.016 Ma (Early Pleistocene). After the GPG, 14–16 cold (glacial/stadial) geoclimatic events occurred intercalated with their corresponding warm (interglacial/interstadial) equivalents. Thirteen post-GPG moraines have been identified, some of the Early–Middle Pleistocene and others of the Last Glaciation (LG) which reached its maximum around 25,000 and ended nearly 16,000 years ago (Late Pleistocene) (Rabassa et al. 2005). All these climatic changes undoubtedly led to regional isolation of wildlife communities, novel associations between species, and local extirpations, which have shaped patterns of species and genetic diversity in temperate regions of South America (Ruzzante et al. 2006). The term 'glacial refugia' is used to describe the only suitable localities where

*Corresponding author. Email: nacho_tunez@yahoo.com.ar

temperate fauna and flora could have existed during full-glacial conditions. Based on geological and fossil evidence, coastal refugia existed in Malvinas (Falkland) Islands, the Atlantic coast of northern Patagonia, and in regions north of Isla Chiloé, Chile (Vuilleumier 1971).

The use of coasts for reproduction is a common feature of marine birds and pinnipeds. The availability of suitable coasts for reproduction was reduced during glaciations (Siegel-Causey 1997), producing strong detrimental effects on Patagonian populations of marine vertebrates. Several molecular studies have investigated patterns of haplotype differentiation in Patagonian pinnipeds and marine birds in relation to the dynamics of glaciations. Hoelzel et al. (1993) studied the genetic structure of the only continental colony of the South American elephant seal, *Mirounga leonina* (Linnaeus, 1758), located in Valdés Peninsula (Northern Patagonia, Argentina), finding that this colony suffered a drastic decrease in population numbers that derived in a population bottleneck that occurred 100,000 years ago. Túnez et al. (2007, 2010) obtained a similar result on South American sea lions, *Otaria flavescens* (Shaw, 1800). They analysed the pattern of haplotype differentiation and estimated that a possible bottleneck would have occurred approximately 64,000 to 110,000 yr BP, depending on the molecular marker used for the estimations. This bottleneck was followed by a demographic expansion of the southernmost colonies, whereas the northernmost ones would have acted as refugia during glacial periods. Thus, the historical population dynamics of *O. flavescens* in north-central Patagonia appears to be closely related with the dynamics of the Late Pleistocene glaciations. The genetic structure of rock shags, *Stictocarbo magellanicus* (Gmelin, 1789), in Patagonia was analysed by Siegel-Causey (1997), which suggests that the population subdivision found in this marine bird was probably caused by vicariant disjunction associated with the Llanquihue Glaciation (35,000–15,000 yr BP). The formerly continuous population was forced into refugia on the Pacific and Atlantic coasts, where they remained without contact for approximately 20,000 years. When the glacier retreated, Chubut and Falkland populations served as sources for the recolonisation, whereas the Fuegian population acted as a genetic sink.

Determining how species are divided into genetically distinguishable units is fundamental to the conservation of marine mammals. Because evolutionary processes act at the intraspecific level, genetic differences and locally adaptive characters will accumulate in these units over time. This reservoir of genetic and phenotypic diversity increases a species' ability to persist through environmental changes (Wang 2002). Thus, one of the main goals in conservation is to preserve the evolutionary potential of species by maintaining the diversity found in genetic units. There are two levels of genetic differentiation within a species, called evolutionary significant units (ESUs) and conservation units. An ESU is defined as a lineage demonstrating highly restricted gene flow from other such lineages within the higher organisational level of the species (Fraser & Bernatchez 2001), while a conservation unit is defined as any population that exchanges so few migrants with others as to be genetically distinct from them (Avise 2004). In practice, conservation units are identified by significant differences in allele frequencies at neutral marker loci. Mitochondrial haplotypes are especially powerful for identifying potential conservation units (Moritz 1994; Avise 1995) due to the special relevance of matrilines to population demography.

The South American fur seal, *Arctocephalus australis* (Zimmermann, 1783), is distributed along approximately 10,000 km of the southern coast of South America (King 1983). Breeding colonies are present in three main segments of coast: 1) Uruguay, with more than 80% of the total population of the species; 2) southern Chile–Isla de los Estados; and 3) northern Chile–central Peru (Fig. 1) (Repenning et al. 1971; Guerra & Torres 1987; Túnez et al. 2008).

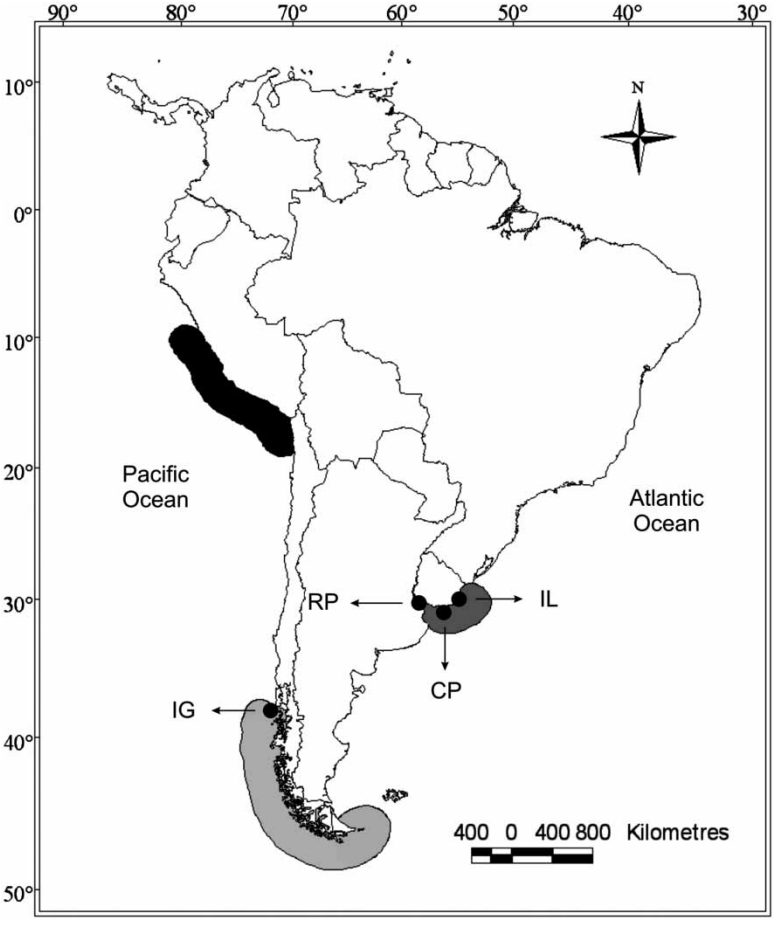

Figure 1 Distribution of South American fur seal (*Arctocephalus australis*) breeding activity and location of colonies sampled. Coloured areas correspond to the different breeding clumps: dark grey, Uruguay; light grey, southern Chile–Isla de los Estados; black, northern Chile–central Peru. CP (Cabo Polonio), IL (Isla de Lobos), RP (Rio de la Plata), IG (Isla Guafo).

These breeding areas are separated by 2200 and 2700 km, respectively. These large distances do not necessarily mean isolation between colonies because marine mammals show a large capacity of dispersal (Fabiani et al. 2003; Campagna et al. 2006, 2007). Two previous studies analysed the genetic diversity and population structure of the species. Using a 445 bp segment of the cytochrome *b* gene, Túnez et al. (2007) analysed the genetic diversity of the species in Cabo Polonio, Uruguay. *Arctocephalus australis* showed five haplotypes determined by 12 polymorphic sites. These haplotypes were compared with available sequences from the Peruvian colony of Punta San Juan (Wynen et al. 2001). None of them was shared between colonies. Oliveira et al. (2008) combined morphometric and genetic analyses to compare 48 individuals from Rio Grande do Sul, which were considered representative of the Uruguayan colonies, and 178 individuals from the same Peruvian colony. Seven polymorphic microsatellite loci reveal highly significant differences in allele frequencies between colonies and two separate clusters corresponding to them. Morphological traits also supported these genetic differences. Taken

together, all these results suggest complete isolation between oceans and that each population corresponds to a different ESU.

The study of genetic variability and population structure, especially from the mtDNA coding regions, is limited by the availability of a relatively small number of polymorphisms in the sequences (Torroni et al. 1996; Wallace et al. 1999). Alternatively, sequences from the first hypervariable (HVRI) segment of the rapidly evolving noncoding control region have been extensively used in pinnipeds in the last decade for the study of these topics (Burg et al. 1999; Wynen et al. 2000; Hoelzel et al. 2001; Mizuno et al. 2003; Trujillo et al. 2004; Weber et al. 2004, Hoffman et al. 2006; Matthee et al. 2006; Túnez et al. 2010). In this paper, we studied the population genetic structure of *A. australis* in Uruguay and southern Chile–Isla de los Estados, two of the breeding clumps previously described. We analyse the HVRI of the control region, and evaluate the role that Pleistocene glaciations may have had in shaping the genetic structure currently found. If Pleistocene glaciations were the main cause of the disjunction of Atlantic and Pacific populations, we expect a deep pattern of population structure between oceans and no genetic differentiation between the Uruguayan relictual colonies that survived through glacial episodes. Additionally, we used the genetic data obtained to determine if the different breeding areas of the species correspond to different conservation units.

Materials and methods

Sample collection

Samples were collected from dead animals found in beaches near the colonies of Cabo Polonio (34°24′S, 53°46′W) (CP, $n=18$) and Isla de Lobos (35°02′S, 52°55′W) (IL, $n=4$), Uruguay, and from live animals in Isla Guafo (43°36′S, 74°43′W) (IG, $n=17$), Chile (Fig. 1). Additional samples were obtained from live juveniles that, separated from their mothers, venture into the waters of the de la Plata River, go astray and are rescued and rehabilitated in the Buenos Aires aquarium (RP, $n=15$). These individuals were considered representative of the Uruguayan population for the analysis of population structure, as the second closest breeding colony is located at a distance more than 1300 km of the Rio de la Plata estuary. In the case of dead animals, tissue samples were taken and stored in preservation buffer containing 20% DMSO, EDTA 0.25N, pH 8.0 and saturated with NaCl. For live animals, tissue samples were taken following the methodology described in Cappozzo et al. (1991) and stored in ethanol 96%. Mitochondrial cytochrome *b* sequences from the same Cabo Polonio individuals used here were obtained in a previous work (Túnez et al. 2007; GenBank accession numbers AY712956 to AY712973).

Mitochondrial DNA extraction and PCR amplification

Tissue samples were incubated overnight at 37 °C in extraction buffer containing 10 µl of proteinase K, 10 mg/ml; 5 µl of RNase, 20 mg/ml and 10% SDS. DNA was isolated from the mixture by phenol-chloroform extraction and alcohol precipitation, dried at room temperature, resuspended in buffer TE, pH 8.0 and stored at −20 °C.

Aliquots of total DNA were used as templates in polymerase chain reaction (PCR) to amplify a double-stranded DNA product of 529 bp from the 3′ end of the mitochondrial tRNA-Pro gene and the adjacent 5′ end of the HVRI. Each PCR had a reaction volume of 100 µl and contained 20 µl of 5 ng/µl DNA, 20 µl of 5X Green GoTaq Reaction Buffer (7.5 mM $MgCl_2$, pH 8.5), 0.5 µl of 20 mM premixed deoxynucleotide triphosphates, 5 µl of 10 mg/ml bovine serum albumin, 1.25 units of GoTaq polymerase (Promega, Madison, USA), 4 µl of 5 mM oligonucleotide primers and water to reach the final volume reaction. The primer pairs used were L16274, 5′-TACACTGGTCTTGTAAACC-3′ and H34, 5′-CCAAATGCATGACACCA-CAG-3′ (Lamont et al. 1996). Amplification protocol consisted in 35 cycles of PCR, each one

involving denaturation at 94 °C for 1 min, annealing at 46 °C for 30 s and extension at 72 °C for 1 min, and was carried out in a MyCycler thermal cycler (Bio-Rad, California, USA). PCR products were resolved in 1% agarose gel electrophoresis, visualised and photographed under UV light, purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and sent to an external laboratory (Macrogen Inc., Seoul, Korea) where sequencing was performed with the same oligonucleotide primers used in PCR reactions.

Data analyses

Genetic structure

Sequences were aligned and analysed for polymorphic sites using ClustalX (2.0). Absolute and relative frequencies of haplotypes within colonies, haplotype and nucleotide divergence and pairwise comparisons of percent sequence divergence were computed using ARLEQUIN software, version 3.11 (Excoffier et al. 2005). The same program was used to perform an analyses of molecular variance (AMOVA; Excoffier et al. 1992) in order to estimate the partitioning of genetic variation. Genetic differences were calculated using the method of pairwise differences. Population pairwise F_{ST} values were calculated for every pair of colonies. Sequential Bonferroni corrections were applied to adjust the statistical significance levels for multiple simultaneous comparisons (Rice 1989). F_{ST} estimates were then used to test for isolation by distance using the Mantel procedure (Manly 1986). Correlation was examined between $F_{ST}/1 - F_{ST}$ and the logarithm of swimming distance, through ocean currents, between colonies, following the method proposed by Rousset (1997). These distances were measured in kilometres over a digital map of the study area using ArcGIS 9.1 software.

The median-joining network method (Bandelt et al. 1999) implemented in the Network 4.5 program (Fluxus Technology Inc., Suffolk, UK) was applied to our dataset in order to estimate the genealogical relationship between haplotypes. This method, using a parsimony criterion, combines the minimum-spanning trees (MSTs) with a single network, allowing more detailed population information than strictly bifurcating trees (Posada & Crandall 2001).

The substitution rate at the HVRI was calculated following the method of Alter and Palumbi (2009). In brief, we used a previously published 445 bp segment of the cytochrome *b* gene obtained from the same individuals used here (haplotype A; Túnez et al. 2007) and calculate control region substitution rate as:

$$\mu_{CR} = x \times \mu_{cytb} \times n_{cytb}/0.5.n_{CR}$$

where x was mean control region pairwise distance for individuals identical at cytochrome *b* haplotype (7.65 ± 0.48 substitutions), μ_{cytb} was substitution rate for synonymous changes in cytochrome *b* (3.26% per Myr in pinnipeds, from Phillips et al. 2009), n_{cytb} was the number of four-fold degenerate sites in cytochrome *b* (61) plus 1/3 the number of twofold degenerate sites (93), and n_{CR} was the number of nucleotides in our control region fragment (529). This method suggested a substitution rate of $8.66 \pm 0.54\%$ per Myr, that was used to convert mutational time (τ) into real time according to equation: $\tau = 2\mu t$, where t is the time in years. The calculated rate is similar to other HVRI rates obtained in different pinnipeds, including 5%–10% per Myr estimated from fossil calibrations for the southern elephant seal, *M. leonina* (Slade et al. 1998), and 10.3% for the northern fur seal, *Callorhinus ursinus* (Linnaeus, 1758; Pinsky et al. 2010).

MDIV (Nielsen & Wakeley 2001) was used to estimate divergence time between Atlantic and Pacific populations. This program uses a Bayesian approach to estimate this parameter between pairs of populations that are assumed to have diverged from a common ancestral population. MDIV was run multiple times with different random seeds and lengths of the Markov chain to assess the stability of the results. Final results were obtained using the

following parameters: finite sites (HKY) model (Hasegawa et al. 1985); Markov chain simulation for 5,000,000 steps, where the first 500,000 were discarded as burn-in; and a uniform prior distribution between 0 and 10 for T (divergence time/2 N_e). MDIV measures divergence time in units of effective population time (N_e) that can be calibrated into generations and hence years when a specific mutation rate and generation time are assumed. The generation time used for the calculations was 8 years, based on data obtained in other two Arctocephalinae species, the Antarctic and sub-Antarctic fur seals, *A. gazella* (Peters, 1875) and *A. tropicalis* (Gray, 1872) (Lunn et al. 1994; Dabin et al. 2004). The modes of the posterior distribution for both population divergence time and θ (where $\theta = 2 N_e\mu$, and μ is the mutation rate per sequence per generation) were used to estimate divergence times between populations, and to explore the probability that the signatures of population segregation were congruent with Pleistocene timescale. For this purpose, Atlantic colonies were analysed as one population; taking into account the results of the AMOVA analysis (see below). A confidence interval for the divergence time between populations was estimated using the mutation rate of HVRI at the upper and lower estimates of $8.66 \pm 0.54\%$ per million years. This method is considered appropriate in this instance since it is not possible to estimate an upper confidence limit for the unbounded distribution of T.

Historical population dynamics

The demographic history of Atlantic (CP, IL and RP analysed separately or altogether) and Pacific populations was examined using a mismatch distribution analysis. This method, based on an assumed stepwise growth model (Rogers & Harpending 1992), was used to evaluate: 1) whether there was signature of population expansion; and 2) the timing of demographic expansion measured in units of mutational time. Typically, a population with a constant size in the past has a multimodal and ragged mismatch distribution, while a population that has undergone expansion usually shows a unimodal and smooth distribution (Rogers & Harpending 1992; Harpending 1994; Rogers 1995). Approximate confidence intervals for growth parameters are obtained by a parametric bootstrap approach (1000 replicates). The validity of the estimated stepwise expansion model is tested using the same parametric bootstrap approach by a goodness-of-fit test between the observed distribution of the pairwise differences pattern and the simulated one based on the estimated model parameters. To quantify the smoothness of the observed haplotype frequency distribution, Harpending's (1994) raggedness index was applied. A non-significant index would indicate a good fit of the data to a population expansion model. All these computations were also performed in ARLEQUIN 3.11 (Excoffier et al. 2005). Mutational time was converted into real time as described above.

Results

From the 54 samples analysed, we found 26 haplotypes with a length of 529 bp and 67 polymorphic sites. Haplotypes 01 and 02 were the most frequent, each one being found in five of the 54 individuals analysed (9.26%). None of the 26 haplotypes was present in all sampling sites. Results for the sampling sites located in the Atlantic Ocean indicated that: 1) Cabo Polonio shared five of its 10 haplotypes with Rio de la Plata; 2) Isla de Lobos shared two of its four haplotypes with Rio de la Plata; 3) Rio de la Plata showed three other haplotypes; and 4) Cabo Polonio and Isla de Lobos did not share any haplotype. Moreover, none of the eight haplotypes from Isla Guafo was shared with the Atlantic Ocean sampling sites.

The genealogical relationship between haplotypes is illustrated in Fig. 2. It shows a pattern of phylogeographic structure with two main groups, one containing haplotypes from Capo Polonio, Isla de Lobos and Rio de la Plata (01–18) and the other containing haplotypes from Isla Guafo (19–26). However, within each

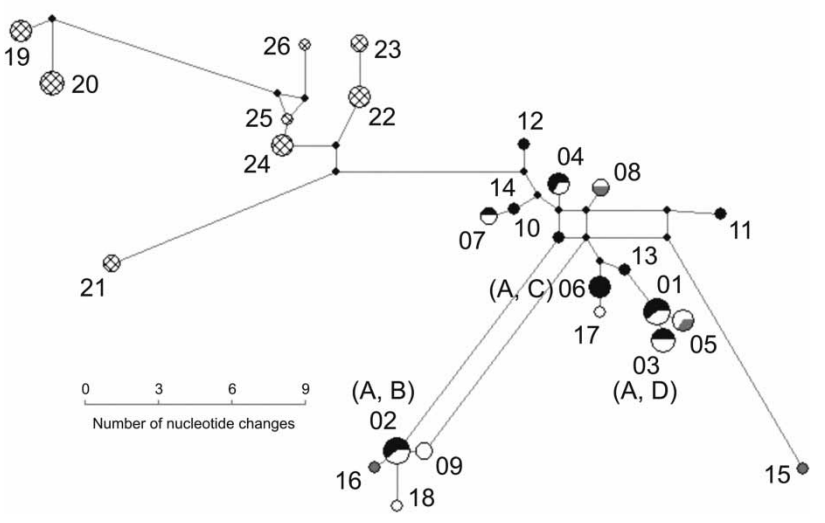

Figure 2 Genealogical relationships between South American fur seals (*Arctocephalus australis*) haplotypes. Circle areas are proportional to haplotype frequencies and length of the branches to the number of changes from one haplotype to the following. Black, Cabo Polonio; grey, Isla de Lobos; white, Rio de la Plata; cross-hatched, Isla Guafo. Cytochrome *b* haplotypes previously found for control region haplotypes 02, 03 and 06 between brackets.

main group, some haplotypes (02, 09, 15–16 and 18 in the first group and 19–21 in the second) show a level of genetic differentiation from the other haplotypes in the group, comparable with the level of differentiation between groups. The relationship between previously published cytochrome *b* haplotypes and HVRI ones is also shown in Fig. 2. Individuals with HVRI haplotypes 02, 03 and 06 carried cytochrome *b* haplotypes A and B, A and D, or A and C, respectively, which suggests some level of homoplasy at the HVRI.

The results of the AMOVA analysis showed significant differences between sampling sites ($F_{ST}=0.38$, $P=0.0001$). Pairwise comparisons of F_{ST} values indicate significant differences between Isla Guafo and the sampling sites on the Atlantic Ocean ($F_{ST}=0.46$, 0.36 and 0.48 for CP, IL and RP, respectively, $P<0.0001$). Differences were not significant between sampling sites on the Atlantic Ocean ($P>0.34$). Significant differences were confirmed after sequential Bonferroni correction for multiple simultaneous tests ($P<0.0083$). Correlation between genetic and geographical distances was not significant (Mantel test, $P>0.12$).

The posterior distribution for population divergence time between Atlantic and Pacific populations is shown in Fig. 3. The population divergence time, calculated from $T_{max}=1.160$, $\theta_{max}=9.686$ and a substitution rate for the HVRI of $8.66\pm0.54\%$ per Myr, was estimated at between 0.92–1.05 Ma suggesting that the divergence between these two populations occurred at Early Pleistocene, in a time interval between the developing and the end of the GPGs.

Overall values of haplotype and nucleotide divergence were high and similar in all the four colonies, ranging from 0.90 to 1 and 0.014 to 0.027, respectively, and suggesting no recent population expansions. The observed distribution of pairwise differences did not differ significantly from the simulated pattern based on a sudden expansion model for the Atlantic colonies analysed separately or altogether

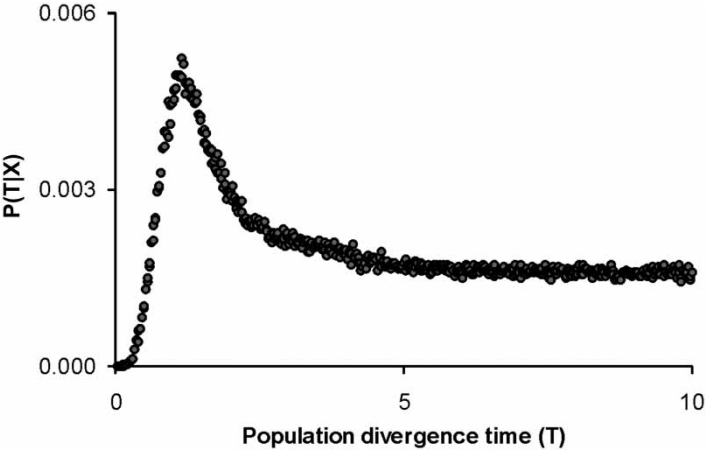

Figure 3 MDIV results for the posterior distribution of T (population divergence time) between Atlantic and Pacific populations of South American fur seals (*Arctocephalus australis*).

($P \geq 0.40$), supporting the demographic expansion hypothesis. Harpending's raggedness index also provided statistical support for these results. Table 1 shows the parameters estimated under the model. The tau value (τ), which reflects the location of the mismatch distribution crest, provides a rough estimate of the time when rapid population expansion started (Rogers & Harpending 1992; Rogers 1995). The tau value for the Atlantic population was 13.479, which corresponds to an estimated expansion time of 293,673 years BP, assuming a divergence rate of 8.66% per million years (Table 1). For the Pacific population the observed distribution of pairwise differences differ from the simulated pattern ($P < 0.001$) indicating no evidence of population expansion.

Discussion

Arctocephalus australis shows a patchy distribution of breeding activity with breeding colonies separated in three different clumps: 1) Uruguay; 2) southern Chile–Isla de los Estados; and 3) northern Chile–central Peru. In the present study, we analysed the genetic differentiation between colonies from the first two clumps. In spite of extremely high philopatry (a

Table 1 Parameters estimated under the Stepwise expansion model for Atlantic and Pacific populations of South American fur seals (*Arctocephalus australis*).

	Parameters	Atlantic	Pacific
Stepwise model[1]	$\theta_0 = 2uN_0$	0.000 [0.000–3.945]	0.000 [0.000–16.636]
	$\theta_1 = 2uN_1$	15.148 [8.507–78.312]	103.552 [57.809–99 999]
	$\tau = 2ut$	13.479 [3.729–19.930]	35.246 [21.465–41.557]
	P (obs vs. sim)	0.677	0.001
Raggedness index	r	0.012	0.047
	P	0.787	0.194
Expansion time (BP)	t	293 673	767 920

Note: [1]Parameters are given as estimates (95% confidence limits).

well-known phenomenon in female pinnipeds), which can accelerate the process of genetic differentiation between colonies (Trujillo et al. 2004; Matthiopoulos et al. 2005; Hoffman et al. 2006; Campbell et al. 2008; Wolf et al. 2008), fur seals from Uruguay did not show significant differences in haplotype frequencies. The low genetic divergence among Uruguayan colonies is consistent with the idea that these colonies are remnants of a single ancient gene pool belonging to a relictual population that survived through the several glacial episodes that characterise Patagonian geological history during the last million years. Low levels of genetic differentiation could also be caused by an homogenising effect of gene flow at small scale (Wolf et al. 2008) or by homoplasy at hotspots for substitutions in the mitochondrial control region, a process that has been described in several species of mammals (e.g. Tamura & Nei 1993; Fernando et al. 2000; Herrnstadt et al. 2002; Galtier et al. 2006). However, a low level of homoplasy was detected in our control region data. *Otaria flavescens*, the other otariid species that inhabits our study area, shows in north-central Patagonia an historical population dynamics that appears to be closely related with the dynamics of the Late Pleistocene glaciations (Túnez et al. 2010). Genetic analyses based on control region mitochondrial sequences suggest that the sea lion population in Patagonia suffered a demographic contraction during the glacial period, followed by a population expansion when the glaciers retracted. Northern colonies could have acted as refugia during glaciations while the southern ones were recolonised after the glaciers retracted. The results obtained here suggest a similar process in *A. australis* and *O. flavescens* in a different timescale. Habitat contractions caused by Pleistocene glacial cycles across temperate regions have been proposed as a major process in reducing genetic diversity and shaping the current genetic structure in populations from the northern hemisphere (Rising & Avise 1993; Hewitt 1996; Merilä et al. 1997; Zink 1997; Milá et al. 2000). Other evidence came from studies of different species from the southern hemisphere, which include several species of plants (Allnutt et al. 1999, 2003; Premoli et al. 2002; Muellner et al. 2005) and fishes (Zattara & Premoli 2005; Ruzzante et al. 2006); the rock shag, *S. magellanicus* (Siegel-Causey 1997) and the saxicolous mouse, *Phyllotis xanthopygus* (Waterhouse, 1837) (Kim et al. 1998). Historical harvesting has also been proposed as a process that shaped the current patterns of genetic differentiation found in several species of fur seal (e.g. Wynen et al. 2000; Weber et al. 2004). It may have contributed to shape the genetic population structure currently found in *A. australis* by means of local extirpations and posterior recolonisation from refuge populations. However, all the available information to date suggest that no local population extirpations occurred: 1) Túnez et al. (2008) showed that the Atlantic distribution of breeding and non-breeding colonies of *A. australis* was maintained during the second half of the 20th century, even with a ten-fold population increase; 2) the scarce archaeological evidence in the region suggests that the distribution of the species along the Atlantic coast did not vary significantly over the past 5000 years (Schiavini 1993; Castro et al. 2004); and 3) all available records of exploitation from the 16th century in our study area came from colonies in which fur seals are now present (Cabrera & Yepes 1940; Vaz-Ferreira 1982; Bastida & Rodríguez 1994).

Values of haplotype and nucleotide divergence do not show any signature of recent population expansion. On the other hand, mismatch distribution analysis supports the demographic expansion hypothesis for the Atlantic population, while the population size in Isla Guafo appears to have remained constant. The Atlantic population showed a τ value of 13.479, which corresponds to an estimated expansion time of approximately 300,000 yr BP (Middle Pleistocene). As previously stated, several glacial events intercalated with their corresponding warm equivalents have been identified in Patagonia during this geological

stage (Rabassa et al. 2005). All these glaciations greatly altered the Patagonian landscape and influenced species distributions. In this context, it is possible that the population expansion in Uruguay is associated with the dynamics of Pleistocene glaciations, though the reasons remain unclear. Another member of the subfamily Arctocephalinae, *A. pusillus pusillus* (Schreber, 1775), experienced an historical population expansion probably between c. 37,000–18,000 yr BP (Matthee et al. 2006). This date coincides with the height of the last glacial maximum in the area. The authors suggested that the population expansion could be attributable to the high ocean productivity that characterises maxima glacial periods which made resources abundant in the South Atlantic.

AMOVA analysis indicated significant genetic differences between Uruguay and southern Chile–Isla de los Estados. These two breeding areas are separated by 4800 km of coast where breeding activity is almost negligible (Túnez et al. 2008). However, correlation between genetic and geographical distances was not significant, which suggests that other environmental factors, such as geographic barriers or environmental restrictions to gene flow, may affect the observed genetic structure. These genetic differences between Atlantic and Pacific breeding areas were also supported by: 1) the genealogical relationship between haplotypes, which showed two main groups of haplotypes that correspond to these breeding areas; and 2) the MDIV results, which suggest a long period of isolation between areas that would have started 1 million years before the present, at Early Pleistocene, in a time interval between the developing and the end of the GPGs. However, this divergence time estimation needs to be regarded with extreme caution, as it was calculated using data from a short sequence of the mitochondrial genome. Even the most sophisticated coalescence approaches show discrepancies in divergence time even though full genome sequences are available (Hobolth et al. 2007; Locke et al. 2011).

Previous studies using different molecular markers and morphological traits showed complete isolation between oceans and suggests the existence of different ESUs (Túnez et al. 2007; Oliveira et al. 2008). Several requisites are needed for the definition of ESUs. From the genetic perspective, ESUs should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci (Moritz 1994). *Arctocephalus australis* fulfils these genetic requisites when Uruguayan and Peruvian colonies are compared (Túnez et al. 2007; Oliveira et al. 2008). Here, we found evidence of a high level of genetic differentiation between oceans at a smaller geographical scale. Colonies from Uruguay and southern Chile–Isla de los Estados showed reciprocal monophyly and significant differences in haplotype frequencies, which suggests that these two breeding areas would correspond to different conservation units. Recent studies in pinnipeds that compared male and female rates of migration based on the comparison between microsatellites and mtDNA data, showed that dispersion is biased towards males (Burg et al. 1999; Trujillo et al. 2004), as in most mammals (Chepko-Sade & Halpin 1987). A more comprehensive analysis, using both mitochondrial and nuclear markers, is required to better understand the current conservation status of the species.

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