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PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

DIVERSIDADE GENÉTICA E ESTRUTURA POPULACIONAL DO LOBO-MARINHO SUL-AMERICANO (*ARCTOCEPHALUS AUSTRALIS,* MAMMALIA, CARNIVORA, OTARIIDE) AO LONGO DA COSTA ATLÂNTICA DA AMÉRICA DO SUL

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DISSERTAÇÃO DE MESTRADO

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SUMÁRIO

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RESUMO

O lobo-marinho sul-americano, *Arctocephalus australis*, está distribuído ao longo da costa do hemisfério sul com colônias reprodutivas localizadas desde o Peru até o Uruguai. Este trabalho foca na UES do Atlântico e cobre a maioria de suas colônias. No passado recente, várias colônias sofreram drásticas reduções populacionais com a caça e os eventos de El Niño. Muitos estudos focaram na análise da UES do Pacífico, no entanto, pouco se sabe sobre a UES do Atlântico. Neste estudo a estrutura populacional e a variabilidade genética destas populações foram avaliadas através da região controle do DNA mitocondrial e 11 *loci* de microssatélites. Os resultados encontraram alto nível de diversidade genética nesta região, sem sinal de gargalo genético recente, mas com sinais de uma expansão populacional iniciada entre 200.000 e 100.000 anos atrás. Um sinal de estruturação foi encontrado entre as colônias do Uruguai e Chubut quando avaliado a partir do DNA mitocondrial, provavelmente causado pela forte filopatria das fêmeas. No entanto, a análise de microssatélite não revelou a existência de estruturação, mesmo entre as diversas subpopulações mais distantes, sugerindo que o fluxo gênico seja mediado pelos machos. Para fins de conservação, estes resultados mostram que o lobo-marinho sul-americano da UES do Atlântico é uma única população, e por causa disso, medidas de segurança devem ser alinhadas entre os países de sua distribuição.

ABSTRACT

The South American fur Seal, *Arctocephalus australis*, is distributed along Southern Hemisphere coast with breeding colonies located since Peru until Uruguay. This work focuses on the Atlantic ESU and covers most of the colonies of the Atlantic coast. In recent past, several colonies underwent strong size reduction with hunting and El Niño events. Most studies have focused on investigate the Pacific ESU, little being known about the Atlantic populations. The population structure and genetic variability in this area were assayed with mitochondrial DNA control region and eleven microsatellite loci. The results found high levels of genetic diversity in the region, without evidence of recent genetic bottleneck but with evidence of a population expansion around 200-100 thousand years ago. A sign of genetic structure were found between colonies from Uruguay and Chubut when evaluated by the mtDNA. This is likely due to their strong female philopatry. However, microsatellite analysis did not revealed any existing structure, even between distant areas, supporting that most gene flow is mediated by males. For conservation purposes, these results shows that the South American fur seal Atlantic ESU is a single population and because of that, conservation measures should be aligned among the countries of its distribution.

APRESENTAÇÃO

O lobo-marinho sul-americano, *Arctocephalus australis*, é um dos otarídeos mais amplamente distribuídos ao longo do Hemisfério Sul, possuindo colônias reprodutivas tanto na costa Atlântica quanto Pacífica da América do Sul (Vaz-Ferreira, 1982). As principais colônias reprodutivas ocorrem no Uruguai (Cabo Polônio, 34°24 'S, 53°46'W; e Isla de Lobos, 35°00'S, 54°52'W), Argentina (Província do Chubut, entre os paralelos 42° e 46° de latitude sul) e ilhas vizinhas (Ilhas dos Estados, 54°50'S, 64°35'W; e Ilhas Falkland 51°41'S, 60°02'W), Peru (até Ilha Foca - 5°12´S, 81°12´W) e Chile. Neste último país a distribuição da espécie é interrompida entre a ilha de Chiloé (42°40'S, 73°59'W, litoral sul) e a região de Mejillones (23°06'S, 70°27'W, litoral norte) (Guerra & Portflitt,1991).

No Brasil (Ilha dos Lobos, Torres (29°20'S, 49°42'W)) bem como no norte da Argentina (*e.g.* Mar del Plata (38°06'S, 57°33'W)), não existem colônias reprodutivas da espécie, contudo, dezenas de exemplares de lobos-marinhos, possivelmente oriundos das colônias reprodutivas do Uruguai, chegam entre os meses de outono e primavera, favorecidos em seus deslocamentos pós-reprodutivos principalmente pela corrente fria das Malvinas (Pinedo *et al*., 1992; Simões-Lopes *et al*., 1995; Oliveira, 1999; Dassis et al., 2007).

Wynen *et al*. (2001) propôs que os otarídeos se originaram na região nordeste do Oceano Pacífico sob um clima temperado e que subsequentes dispersões resultaram na grande distribuição geográfica das diversas espécies atuais. Conforme Túnez *et al.* (2007), a distribuição das populações de *A. australis* não é contínua, podendo estar separadas por milhares de quilômetros uma da outra. Contudo, isto não quer dizer que estas populações estejam isoladas, pois os lobos-marinhos possuem uma grande capacidade de dispersão. No entanto, a descontinuidade na distribuição - mais de 2.000km na costa sul do Chile – implicou em diferenças evolutivas significativas entre as populações do Pacífico e Atlântico (Oliveira *et al.* 2008).

Dentre os estudos não-molecurares foram registradas diferenças morfológicas e comportamentais entre as populações. O primeiro estudo destacou a diferença do peso corporal médio das fêmeas adultas do Pacífico que é de 58,0 kg (Majluf, 1992), enquanto que o peso das do Atlântico é de 41,7 kg (Lima & Páez, 1995). O segundo demonstrou que os sistemas reprodutivos destas populações também diferiam.

Enquanto no Pacífico era observado o sistema de lekking (Majluf et al., 1996), onde os machos se encontram em pequenos territórios que são visitados por fêmeas receptivas (Boness, 1991); no Atlântico o sistema tradicional era o de harém, onde os machos defendem territórios fixos importantes para a termorregulação das fêmeas (Cappozzo et al., 1996). O terceiro estudo compara o tempo de permanência dos adultos nas colônias assim como do filhote com a mãe (Majluf, 1992). Nas colônias do Pacífico, os animais são encontrados nas praias durante todo o ano e os filhotes permanecem aproximadamente de 1-2 anos com a mãe. Já no Atlântico, os indivíduos deixam suas colônias após o período reprodutivo para forrageio, e os filhotes desmamam com menos de um ano (Majluf, 1992). Outros estudos utilizando dados osteológicos e/ou moleculares podem ser citados (Orr *et al.,* 1970; Brunner, 2002; Túnez *et al.,* 2007), em especial o de Oliveira et al. (2008), onde os autores sugeriram que dentre todas as populações de *A. australis* existentes na América do Sul, as populações do Pacífico e Atlântico apresentam diferenças significativas nas frequências alélicas e na morfologia craniana, o que indicaria que estas populações estariam isoladas geograficamente e seriam consideradas unidades evolutivamente significativas (UES) (do inglês ESUs - *evolutionarily significant units*). As UES identificam subdivisões populacionais que necessitam de priorização para proteção onde os recursos são limitados (Ryder 1986; Moritz 1994). O mesmo resultado foi observado em linhagens mitocondriais por Túnez et al. (2007) que encontraram monofilia recíproca entre as mesmas populações estudadas. Apesar de todas as informações existentes sobre as UES de *A. australis*, pouca ou nenhuma informação está disponível com relação a existência de estruturação dentro destas unidades.

Neste sentido, o presente estudo tem como objetivo avaliar a variabilidade genética e estruturação geográfica das populações de *A. australis* localizadas ao longo da costa Atlântica da América do Sul. Estudos desta natureza são fundamentais para a orientação das políticas de manejo e conservação dos países que compartilham a mesma população genética de lobos-marinhos, alinhando suas medidas de proteção, já que seus espécimes não respeitam fronteiras geopolíticas. Por outro lado, entre os países em que as populações forem geneticamente diferentes, suas estratégias de conservação devem ser condizentes com suas distintas histórias evolutivas, bem como adequadas às suas respectivas ameaças locais à conservação.

Para a realização deste estudo foram feitas análises de sequências da região controladora do DNA mitocondrial e de 11 *loci* de microssatélites, ambos marcadores altamente variáveis, seletivamente neutros e amplamente utilizados em estudos populacionais (Avise, 1994; Schlötterer, 2004; Oliveira, et al. 2006). É importante salientar também que este é um dos primeiros estudos realizados abrangendo uma ampla distribuição da espécie na Bacia do Oceano Atlântico além de analisar dois marcadores altamente informativos.

Este trabalho é apresentado no formatado de artigo científico de acordo com as normas de submissão da Journal of Biogeography. Contudo, a fim de facilitar a leitura do documento as figuras e tabelas foram colocadas ao longo do texto.

Genetic diversity and population structure of the South American fur Seal, *Arctocephalus australis*, along the South American Atlantic coast

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Abstract

The South American fur Seal, *Arctocephalus australis*, is distributed along Southern Hemisphere coast with breeding colonies located since Peru until Uruguay. This work focuses on the Atlantic ESU and covers most of the colonies of the Atlantic coast. In recent past, several colonies underwent strong size reduction with hunting and El Niño events. Most studies have focused on investigate the Pacific ESU, little being known about the Atlantic populations. The population structure and genetic variability in this area were assayed with mitochondrial DNA control region and eleven microsatellite loci. The results found high levels of genetic diversity in the region, without evidence of recent genetic bottleneck but with evidence of a population expansion around 200-100 thousand years ago. A sign of genetic structure were found between colonies from Uruguay and Chubut when evaluated by the mtDNA. This is likely due to their strong female philopatry. However, microsatellite analysis did not revealed any existing structure, even between distant areas, supporting that most gene flow is mediated by males. For conservation purposes, these results shows that the South American fur seal Atlantic ESU is a single population and because of that, conservation measures should be aligned among the countries of its distribution.

Introduction

The South American fur seal, *Arctocephalus australis*, is an otariid seal with a geographic distribution that comprises much of the Atlantic and Pacific coasts. Throughout its distribution (Figure 1) there are breeding colonies on the coast of Peru, Chile, Falkland Islands, Estados Island, Argentina and Uruguay, with the larger one, Isla de Lobos, located in the latter country, with approximately 150,000 individuals (Vaz-Ferreira, 1982; Ximenez & Langguth, 2002). However, this distribution is not continuous. In the Pacific, there is a gap of about 2,000km long between Mejillones and Chiloé Island in Chile, subdividing the species in two geographically isolated populations: one at the northern Pacific and the other comprising the southern Pacific and the Atlantic region (Figure 1). Molecular and craniometrical studies (Oliveira et al., 2008) suggested that the Pacific and Atlantic populations have significant differences and that they were geographically isolated,

so that these populations should be considered Evolutionarily Significant Units (ESU).

For the Northern South Pacific population, which is the most studied, recently, Oliveira et al. (2009) demonstrated that this ESU suffered with the El Niño Southern Oscillation (ENSO) phenomenon occurred between 1997 and 1998. Due to limited food availability, the population declined from 23,481 to 8,223 individuals and lost genetic variability linked to the reduction of its effective population size. Majluf (1992) also reported that this same population previously suffered a bottleneck in the ENSO of 1982-1983. The importance of these phenomena and their relationship to the fur seal population viability lies in the fact that, according to meteorological models, the ENSOs will become stronger and more frequent due to global warming. Interestingly, Oliveira et al. (2009) found no genetic bottleneck in the Atlantic population studied, reflecting different demographic histories between these two units. However, they analyzed a single Atlantic population, representing the Uruguayan breeding colony.

Hunting was also an important factor in the reduction of the species population size. Records indicate that the beginning of South American fur seal exploitation was about 6,000 years ago along the Atlantic coast by the aborigines (Schiavini, 1987). Commercial exploitation in the Uruguayan coast begun in 1515 and was banned only in 1991 (Ximenez & Langguth, 2002). The same happened on the Pacific coast, where the population was almost exterminated between 1900 and 1946 (Majluf, 1987b; Bonavia, 1982). Although hunting of South American fur seals is no longer allowed in all its distribution, its impact on the genetic diversity of the species was evaluated only for seven nuclear microsatellites and for a single population in the Atlantic coast (Oliveira et al., 2009), having no information for mitochondrial DNA (mtDNA).

In this species, as well as for other pinnipeds, females are known to be philopatric to their birth site, with males being responsible for gene flow among breeding colonies (Riedman, 1990; Fabiani et al., 2003). Therefore, studying both mtDNA and bi-parental nuclear markers such as microsatellites DNA is fundamental in achieving a comprehensive inference about the evolutionary processes acting on the species genome as a whole (e.g. genetic drift and its relationship with possible fluctuations and demographic subdivisions or gene flow between populations).

This study analyzed the genetic variability, population structure, gene flow and the demographic history of the South American fur seal populations along the Atlantic coast through the use of mtDNA and 11 microsatellite loci.

Materials and Methods

Samples

Biological samples were obtained from 96 South American fur Seals collected along approximately 2,200 km between the southern coast of Brazil (city of Torres - 29°20'S, 49°42'W) and the Chubut Province (between parallels 42° e 46° south latitude). Samples were collected from six areas: Rio Grande do Sul, southern Brazil; Cabo Polonio (34°24 'S, 53°46'W) and Isla de Lobos (35°00'S, 54°52'W), Uruguay; and Mar del Plata (38°06'S, 57°33'W), Northern Chubut and Southern Chubut, Argentina (Figure 1, Table S1, Supporting information). Since there is no breeding site in the Brazilian coast, the individuals sampled are mainly from the Uruguayan breeding colonies and stay in Brazil only because of their winter post reproductive displacement (Pinedo et al., 1992; Dassis et al., 2007; Oliveira et al., 2008). All samples were stored individually and kept in 70% ethanol or DMSO, according to the protocol established by Amos and Hoezel (1991).

Most genetic diversity analysis were performed merging near collecting sites (see Fig. 1) in four populations, as follow: Uruguay (A - C); Mar del Plata (D); Northern Chubut (E - I) and Southern Chubut (J - M).

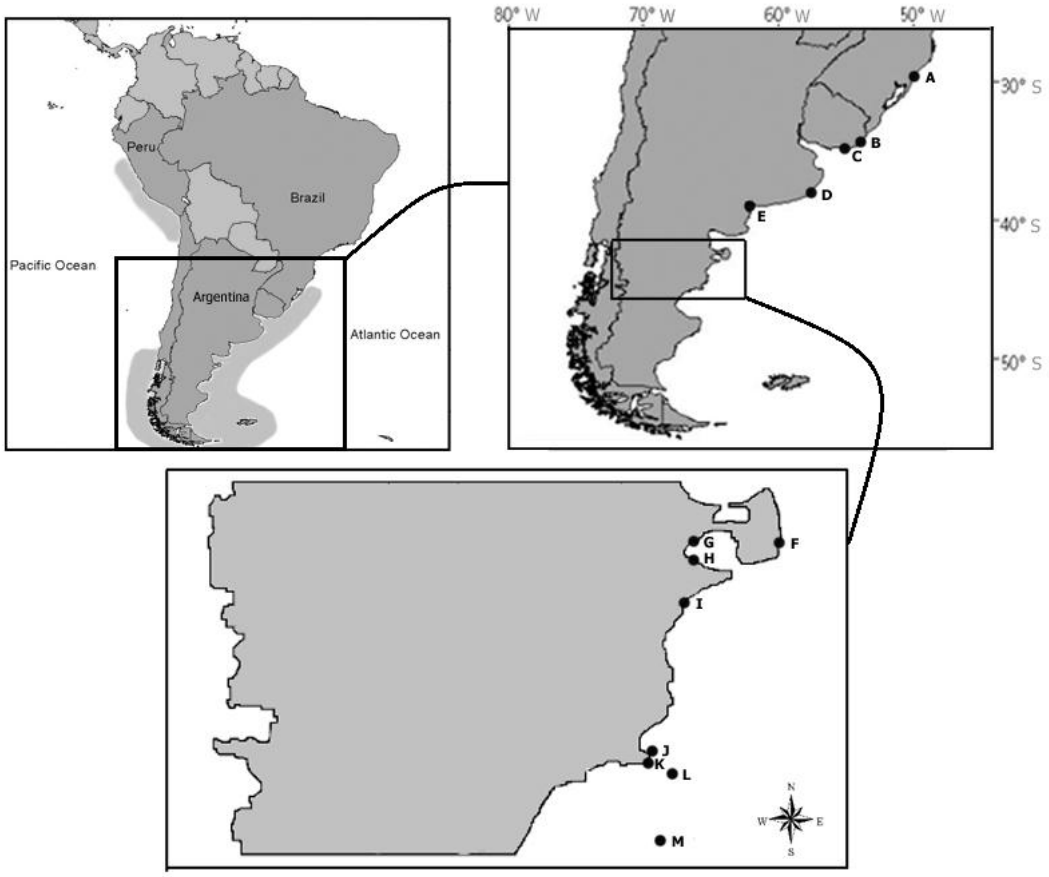

Figure 1: Map of the species distribution (in light gray) and collecting areas along the Atlantic coast: A: Rio Grande do Sul (Brazil; N=31); B: Cabo Polonio (Uruguay; N=3); C: Isla de Lobos (Uruguay; N=5); D: Mar del Plata (Buenos Aires; N=10); E: Las Grutas (Rio Negro; N=1); F: Punta Delgada (Chubut; N=1); G: El Doradillo (Chubut; N=1); H: Muelle Almirante Storni (Chubut; N=1); I: Rawson (Chubut; N= 1); J: Islote Moreno (Chubut; N=2); K: Isla Rasa (Chubut; N=50); L: Isla Arce (Chubut; N=3); M: 46° 00´S; 64° 00´W (Chubut; N=1).

DNA extraction and amplification

Genomic DNA extractions were carried out following standard phenol-chloroform protocol (Sambrook et al., 2001) or by the use of the kit DNeasy Tissue Kit (Quiagen). Extraction verification was performed in 1% agarose gel and quantified by comparison with the molecular weight marker “DNA low mass” (Invitrogen). For the mtDNA control region PCR amplification, the following primers were used: R3 (L15926) THR 5´- TCA AAG CTT ACA CCA GTC TTG TAA ACC - 3´ (Kocher et al., 1989); TDKD (H16498) 5´- CCT GAA GTA GGA ACC AGA TG - 3´(Meyer et al., 1990). The final volume of each amplification reaction was 20 μl consisted of: ≈ 10 ng of genomic DNA, 3,5 mM of MgCl2, 1X PCR buffer, 0,2 mM of all dNTPs, 0,2 μM of each the reverse and the forward primers and 0,05 U of Taq DNA polymerase enzyme. The amplification conditions used were established by

Wynen et al. (2000) with modifications, consisting of an initial denaturing period of 90 s at 94°C, 39 cicles of 30 s at 94°C, 45 s at 56°C and 70 s at 72°C, followed by a final extension period of 10 min at 72°C and cooling at 4°C. Amplified products were purified by incubation with alkaline phosphatase and exonuclease I enzymes.

For microsatellite amplification, previously developed loci for pinnipeds were used: Hg8.10, Hg6.3 and Hg4.2, described for *Halichoerus grypus*; PvcE, Pv9 and Pv11 for *Phoca vitulina*; M11a for *Mirounga sp*.; and ZcwE12, ZcwF07, ZcwA12, ZcwG04 e ZcwB07 for *Zalophus californianus* (Allen et al. 1995; Coltman et al. 1996; Gemmel et al. 1997; Hoffman et al. 2007). The final volume for each amplification reaction was 10 μl consisted of: ≈ 10 ng of genomic DNA, 1,5 mM of MgCl2, 1X PCR buffer, 0,1 mM of all dNTPs, 0,05 U of Taq DNA polymerase enzyme, 0,016 μM of Forward primer marked with M13 tail, 0,25 μM of Reverse primer and 0,2mM of M13 fluorescent-labeled primer (Boutin-Ganache et al. 2001). The amplification conditions were the same for all primers, varying only the annealing temperature (Hg8.10,Hg4.2, ZcwE04 and ZcwA12 = 56°C; Hg6.3, Pv11 and ZcwB07 = 58°C; M11a, ZcwG04 and ZcwE12 = 52°C; Pv9, ZcwF07 and Hg1.3 = 54°C). The general amplification conditions consisted of an initial denaturing period of 3 min at 94°C, 29 cycles of 45 s at 94°C, 45 s at <temperature of the primer being amplified>, 90 s at 72°C, followed by a final extension period of 3 min at 72°C and cooling at 4°C. The sequences of the mtDNA control region were generated and the eleven microsatellite loci were genotyped in the automated DNA sequencer MegaBACE 1000.

Mithocondrial DNA analysis

The chromatograms were analyzed and assembled by the Phred-Phrap-Consed package (Ewing et al. 1998, Gordon et al. 1998). The resulting consensus sequences were aligned automatically using ClustalX (Thompson et al., 1997) and manually edited in BioEdit (Hall, 1999).

A haplotype network was generated by median-joining method (Bandelt et al., 1999) implemented in Network 4.6 (http://www.fluxus-engineering.com) in order to verify the haplotypes relationship, distribution and frequency identified across the sampling area. *Arctocephalus australis* sequences were aligned with sequences from other species of the same genus (obtained in Genbank) to estimate haplotype

divergence times and the time to most recent common ancestor (TMRCA) of the species using BEAST 1.6 (Drummond and Rambaut, 2007). BEAST was run with 50 million iterations sampling each 5,000 chains and the first 10% iterations were discarded as burn-in. The runs were visually inspected using TRACER 1.5, summarized in TreeAnnotator 1.6.1, and the inferred tree visualized in FigTree 1.3.1 (http://beast.bio.ed.ac.uk/Tracer).

Measures of genetic variability like haplotype diversity, nucleotide diversity and mean number of differences among haplotypes, as well as Fu's Fs (Fu, 1997) and Tajima's D (Tajima, 1989) neutrality tests were performed using Arlequin 3.11 (Excoffier et al., 2005). Levels of genetic differentiation among the studied populations were evaluated in Arlequin by pairwise fixation index (F_{ST}) and the Analysis of Molecular Variance (AMOVA), the latter using the haplotype frequency (F_{ST}) and the proportional distance (Φ_{ST}) approaches. Two different scenarios of population subdivisions were tested on the Atlantic coast. In the first scenario four populations were considered, one composed of specimens collected in Rio Grande do Sul (Brazil), Cabo Polonio and Isla de Lobos (Uruguay), and the other three remaining populations in Argentina: Mar del Plata, Northern Chubut and Southern Chubut. The second scenario considered two populations, one composed of specimens collected in Rio Grande do Sul, Cabo Polonio, Isla de Lobos and Mar del Plata (Atlantic I), and another represented by specimens from Northern Chubut and Southern Chubut (Atlantic II).

Population genetic parameters such as population growth rate and migration rates were estimated using Lamarc 2.0 (Kuhner, 2006) considering a single population. To that end, a chain of 500,000 genealogies was performed and the genealogies were recorded at an interval range of 100 items and burn-in of 50,000 samples discarded. The substitution rate used was $3.68E^{-8}$ site/year (μ) (Tchaika et al., 2007). Fluctuations in population size over time were also evaluated by BEAST with a Bayesian Skyline plot tree prior using the parameters described above.

Microsatellite analysis

The obtained genotypes were checked against stutters, allele drop-out and null alleles with Micro-Checker (Van Oosterhout et al. 2004). Genetic diversity was estimated by the number of alleles (allele richness) and by the expect heterozygosity

(He) and observed heterozygosity (Ho) under Hardy-Weinberg equilibrium (HWE) established using Genepop 3.1 (Rousset & Raymond, 1995). Linkage equilibrium among loci, HWE deviation and estimates of fixation index F_{ST} and R_{ST} were also performed by Genepop and Arlequin. Pairwise F_{ST} and AMOVA did not include Northern Chubut population due to the reduced number of individuals genotyped. Genetic structure was also evaluated by the approach implemented in Structure 2.2 (Pritchard et al. 2000) with 1.000.000 iterations and burn-in of 500.000 discarded samples and assuming the admixture and the correlated allele frequencies models. Independent runs were performed with K raging from 1 to 8, each value of K repeated 10 times to evaluate the consistency of the data.

Evidence for recent genetic bottleneck on these populations were evaluated by Bottleneck 1.2.02 (Cornuet and Luikart 1996) using two methods: Stepwise Mutation Model (SMM) and Infinite Allele Model (IAM). Population genetic parameters were estimated similarly to that used above with mtDNA. In this case, the microsatellite mutation rate used was 10^{-4} and 10^{-5} per generation (Hongyan, Chakraborty and Fu, 2005).

Results

Mitochondrial DNA

The 96 mtDNA sequences (267bp) obtained were grouped in 43 distinct haplotypes that present 39 polimorphic sites. The haplotipic (h) and nucleotide (π) diversities (%) observed for the species as a whole were h=91.69±2 and π=1.29±0.73. Basic sequence statistics are shown in Table 1.

Table 1: *Arctocephalus australis* mtDNA basic statistics.

Population	N	# of variable sites	H	h±SE (%)	π±SE (%)
Atlantic I	41	22	20	84.51±4.91	1.00±0.61
Uruguay	32	17	18	88.71±4.57	1.06±0.64
Mar del Plata	9	8	4	69.44±14.7	1.00±0.67
Atlantic II	55	32	31	95.49±1.57	1.48±0.84
Northern Chubut	5	7	4	90.00±16.1	1.52±1.06
Southern Chubut	50	30	30	95.84±1.57	1.45±0.82

Uruguay = Rio Grande do Sul + Cabo Polonio + Isla de Lobos.
Atlantic I (Grande do Sul + Cabo Polonio + Isla de Lobos + Mar del Plata), Atlantic II (Northern Chubut + Southern Chubut).
N = number of individuals analyzed, H = number of haplotypes, h = haplotype diversity, π = nucleotide diversity

The haplotype network (Figure 2) show that the central haplotypes are the most frequent and shared between sampling sites, the two most frequent are shared by all six populations sampled. On the contrary, the tip (external) haplotypes present low frequency and are restricted to a single population. Also there is no clear geographic structure in the relationship between the haplotypes. Most haplotypes are very similar, with few mutations between them, some parts of the network show a clear ‘’star” pattern, suggesting an event of population expansion in the evolutionary history of the species. In addition to the results presented by the network, the mtDNA phylogenetic tree (Figure 3) suggests the first divergence within the present day Atlantic *A. australis* haplotypes occurred around 600 thousand years ago (kya) by three haplotypes from Southern Chubut (Hap_37, Hap_36 and Hap_43), but most of the genetic differentiation occurred between 200 and 100 kya.

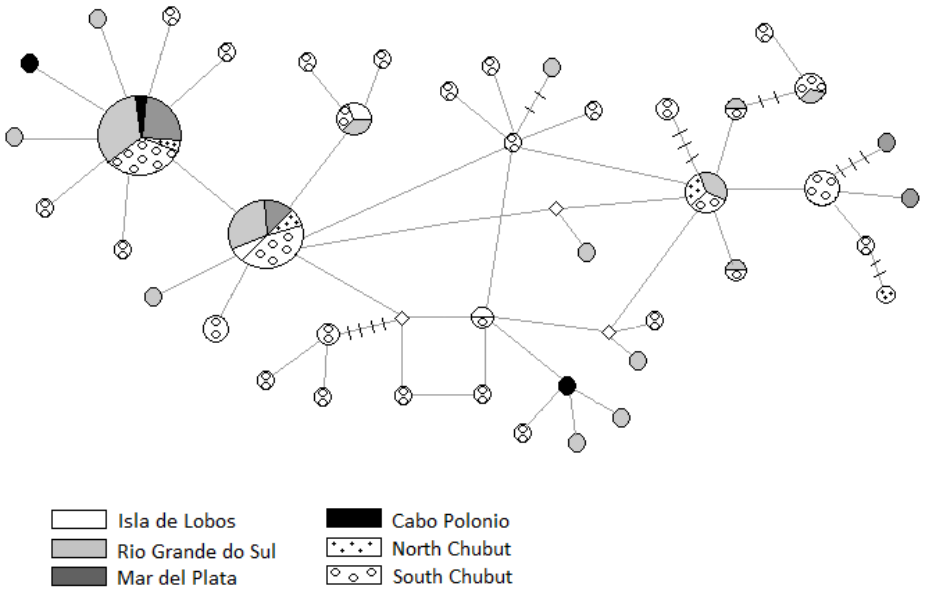

Figure 2: Median Joining network. Circle size is proportional to the frequency. The connector represents one mutational step. Each trace represents one mutational step. Diamond represents potential intermediate haplotypes that were not sampled.

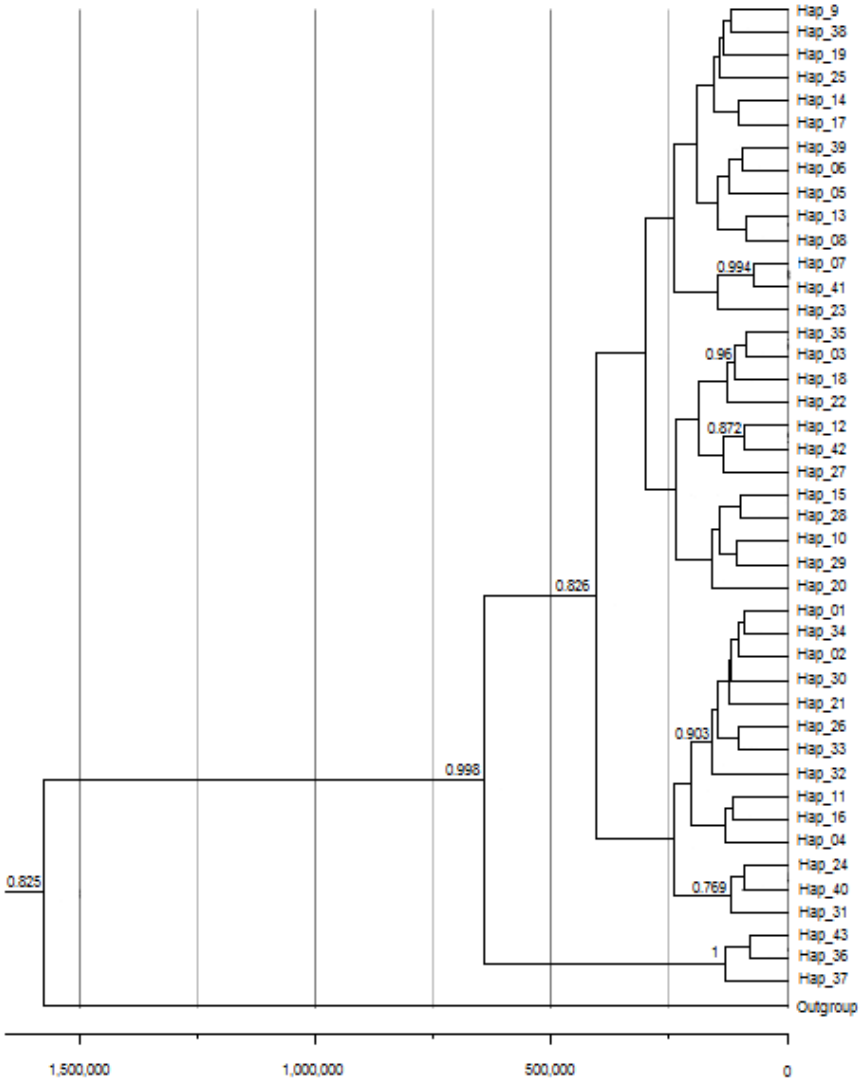

Figure 3: Bayesian haplotype tree with divergence time estimates in years. Branch numbers are posterior probabilities shown only for values above 0.7.

The mtDNA AMOVA analysis showed that more than 95% of the variation occurs within the populations in the two subdivision scenarios, suggesting the existence of a single population along the study area (Table 2). However, some pairwise F_{ST} and Φ_{ST} indices were significant (Table 3), suggesting some restriction to gene flow among some of these populations. Significant values were found in the first scenario between Atlantic I and Atlantic II populations (Φ_{ST}) and in the second between Mar del Plata and Southern Chubut (F_{ST}) and between Uruguay and Northern Chubut (Φ_{ST}). The Bayesian analysis performed by Lamarc showed relatively high values of gene flow among all populations considered in both scenarios evaluated (Table 4), the mean number of migrants per generation being

sufficient to genetically homogenize different subpopulations (Lacy 1987; Mills & Allendorf, 1996).

Table 2 - AMOVA analysis based on F_{ST} and Φ_{ST} for the mtDNA control region for different scenarios of population subdivision of *A. australis*.

Scenario		Percentage of variation		
		Within populations	Among populations	Fixation Index
4 populations[a]	F_{ST}	98.58	1.42	0.01420
	Φst	97.16	2.84	0.02837*
2 populations[b]	F_{ST}	98.10	1.9	0.01903*
	Φst	98.72	1.28	0.01279

*p<0.05
[a] = Uruguay(Rio Grande do Sul + Cabo Polonio + Isla de Lobos), Mar del Plata, Northern Chubut, Southern Chubut.
[b] = Atlantic I (Grande do Sul + Cabo Polonio + Isla de Lobos + Mar del Plata), Atlantic II (Northern Chubut + Southern Chubut).

Table 3 – Pairwise values for F_{ST} (above diagonal) and Φ_{ST} (below diagonal) between populations for each scenario. Figures based on p-distance method for the mtDNA control region.

Scenario					Scenario		
4 populations	Uy	MDP	NC	SC	2 populations	AI	AII
Uy		-0.008	0.008	0.006	AI		0.019*
MDP	-0.002		0.069	0.053*			
NC	0.190*	0.138		0.005	AII	0.012	
SC	0.003	0.012	0.099				

*p<0.05
Uy (N=32) = Uruguay (Rio Grande do Sul + Cabo Polonio + Isla de Lobos); MDP (N=9) = Mar Del Plata; NC (N=5) = Northern Chubut; SC (N=50) = Southern Chubut. AI (N=41) = Atlantic I (Grande do Sul + Cabo Polonio + Isla de Lobos + Mar del Plata); AII (N=55) = Atlantic II (Northern Chubut + Southern Chubut). N = number of samples

The neutrality indices for the whole data (Fu's Fs: -26.22, P<0.05; Tajima's D: -1.67, P<0.05) suggest evidence for a past population expansion, in agreement with the growth rate calculated from the g parameter estimated by Lamarc (g = 524.86 [260.56; 952.67]). Finally, the Bayesian Skyline plot analysis (Figure 4) also suggests the occurrence of population expansion beginning between around 200-100 kya.

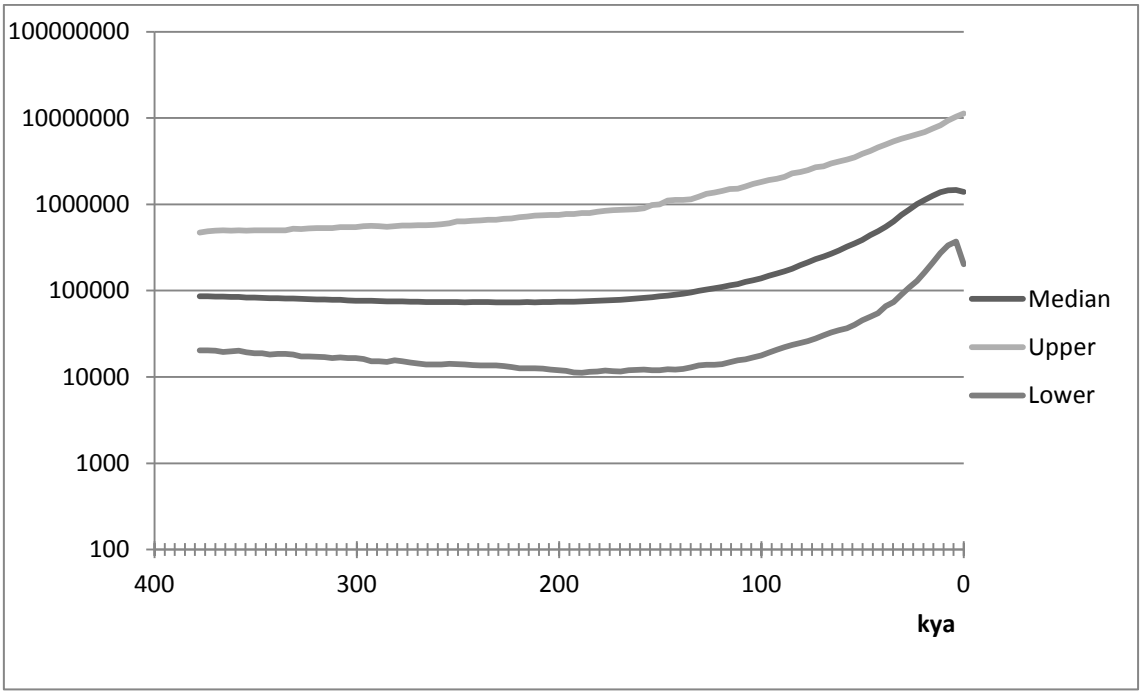

Figure 4: Bayesian Skyline plot. Y axis: female effective population size.

Table 4: Estimates of *A. australis* migration produced by Lamarc mtDNA analysis

From	To			
	Uruguay	Mar del Plata	Northern Chubut	Southern Chubut
4 populations				
Uruguay	-	0.00008 (**2.94**) 4.22	0.0001 (**4.24**) 6.53	6.42 (**17.6**) 41.1
Mar del Plata	0.0004 (**2.24**) 16.41	-	0.0001 (**2.36**) 5.8	0.0007 (**2.79**) 14.68
Northern Chubut	0.0003 (**2.5**) 9.68	0.00004 (**2.47**) 4.03	-	0.0007 (**2.81**) 9.59
Southern Chubut	5.84 (**19.06**) 19.91	0.00007 (**2.63**) 4.34	0.0001 (**4.11**) 6.32	-
From	To			
	Atlantic I		Atlantic II	
2 populations				
Atlantic I	-		8.28 (**19.71**) 40.97	
Atlantic II	5.87 (**19.03**) 19.91		-	

Most probable estimates (MPE) of Nm (=number of migrant individuals per generation) are shown in bold flanked by upper and lower 95% confidence values in regular text.

Microsatellite loci

Samples from populations of Rio Grande do Sul (N=12), Isla de lobos (N=5), Cabo Polonio (n=1), Mar del Plata (N=4) and Southern Chubut (N=44) were

genotyped for eleven autosomal microsatellite loci. All of them were polymorphic with high levels of genetic variability (Table 5, Figure 5). The entire sample had an average of 11.7 allele per locus and an observed (Ho) and expected (He) mean heterozygosity of 0.78 and 0.8, respectively (Table 5). The analysis performed by Micro-Checker revealed that M11a locus presents homozygote excess, possibly caused by the existence of null alleles. However, no deviation of HWE was detected for the entire population after Bonferroni correction. Only one evidence of significant linkage disequilibrium was found between Hg6.3 and ZcwG04 loci (significance level adjusted with a Bonferroni correction for 55 comparisions).

Organizing the samples in three populations, Uruguay, Mar del Plata and Chubut, departures from HWE were detected for the Uruguayan population at M11a locus and for Chubut at M11a and Pv9 loci. The three populations present similar statistics, Chubut with the highest mean number of alleles (10.8) but Mar del Plata presenting the highest mean Ho (0.87) and He (0.86).

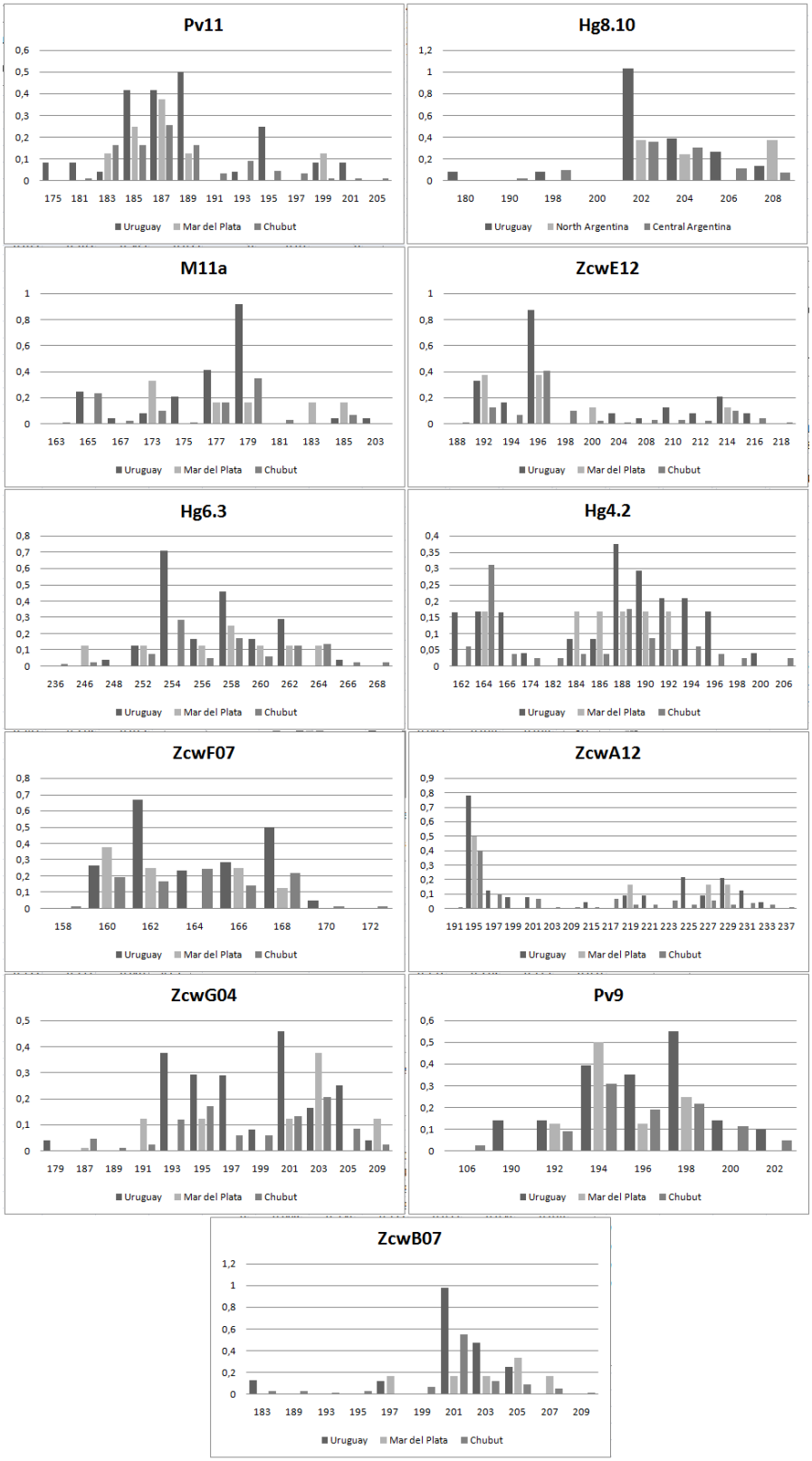

Figure 5: Allelic frequence for each *locus* per population.

Table 5 – Genetic diversity of each locus for each one of the three populations considered and for the entire sample. A = number of alleles, E = number of exclusive alleles,Ho = observed heterozygozity, He = expected heterozygozity. * Loci that deviated from H-W equilibrium after Bonferroni correction.

Locus	Allele size	Uruguay				Mar del Plata				Chubut				Overall		
	Interval (bp)	A	E	Ho	He	A	E	Ho	He	A	E	Ho	He	A	Ho	He
Pv11	175-205	7	0	0.88	0.84	5	0	1.0	0.85	12	3	0.9	0.85	13	0.9	0.85
Hg8.10	180-208	4	0	0.76	0.73	3	0	1.0	0.75	7	2	0.86	0.75	8	0.84	0.74
M11a	163-203	4	0	0.55*	0.76	5	1	1.0	0.93	9	2	0.48*	0.78	11	0.53	0.79
E12	188-218	7	0	0.94	0.76	4	0	0.75	0.78	13	3	0.81	0.79	13	0.84	0.78
Hg6.3	236-268	6	0	0.72	0.8	7	0	1.0	0.96	11	2	0.75	0.84	12	0.75	0.84
Hg4.2	162-206	8	0	0.94	0.9	6	0	1.0	1.0	14	3	0.9	0.85	15	0.91	0.88
F07	158-172	5	0	0.81	0.8	4	0	0.75	0.82	8	2	0.82	0.81	8	0.81	0.81
A12	191-237	7	1	0.7	0.83	4	0	0.66	0.8	17	6	0.83	0.81	18	0.78	0.81
G04	179-209	6	0	0.88	0.87	6	0	0.75	0.89	11	1	0.92	0.87	12	0.9	0.87
Pv9	106-202	7	0	0.82	0.79	4	0	0.75	0.75	7	1	0.76*	0.8	8	0.78	0.79
B07	183-209	5	0	0.42	0.64	5	0	1.0	0.93	10	5	0.62	0.66	11	0.59	0.68
Mean		6		0.76	0.79	4.8		0.87	0.86	10.8		0.78	0.8	11.7	0.78	0.8

Regarding the genetic structure of the Atlantic ESU, Structure analysis conducted to assess the number of genetically distinct populations (k) contained in our total sample showed the highest mean probability of the data for K = 1, indicating the existence of a single population (Figure 6). Both AMOVA (Tables 6) and pairwise F_{ST} and R_{ST} values (Table 7) obtained in the two scenarios tested were all non-significant and extremely low, also indicating the absence of geographic differentiation in the bi-parental markers.

With respect to the demographic history of this ESU (given the results above, the following analyses considered a single population), the genetic bottleneck analysis showed no evidence of recent population decline, with allele frequency presenting a normal L-shaped distribution (as expected under mutation drift equilibrium). The growth rate, calculated from g parameter estimated by Lamarc (g = 0.06 [0.05;0.23]), also indicated a growing population, but recently in a lower rate, in agreement with the Bayesian Skyline plot for mtDNA.

Table 6 – AMOVA analysis based on F_{ST} and R_{ST} for microsatellites for different scenarios of population subdivision of *Arctocephalus australis*.

Scenario		Percentage of variation		
		Within populations	Among populations	Fixation Index
3 populations[a]	F_{ST}	100.19	-0.19	-0.00187
	R_{ST}	101.04	-1.04	-0.01042
2 populations[b]	F_{ST}	100.32	-0.32	0.00316
	R_{ST}	100.17	-0.17	-0.00168

None of the values obtained were significant.
[a] = Uruguay(Rio Grande do Sul + Cabo Polonio + Isla de Lobos), Mar Del Plata, Chubut.
[b] = Atlantic I (Grande do Sul + Cabo Polonio + Isla de Lobos + Mar del Plata), Atlantic II (Northern Chubut + Southern Chubut).

Table 7 – Pairwise values for F_{ST} (above diagonal) and R_{ST} (below diagonal) between populations for each scenario for microsatellites.

Scenario				Scenario		
3 populations	Uy	MDP	C	2 populations	AI	AII
Uy		0.00474	-0.00043	AI		-0.00316
MDP	-0.04818		-0.01123			
C	-0.00367	-0.02581		AII	-0.00168	

None of the values obtained were significant.
Uy (N=18) = Uruguay (Rio Grande do Sul + Cabo Polonio + Isla de Lobos); MDP (N=4) = Mar Del Plata;
C (N=44) = Chubut. AI (N=22) = Atlantic I (Grande do Sul + Cabo Polonio + Isla de Lobos + Mar del Plata);
AII (N=44) = Atlantic II (Chubut). N = number of samples.

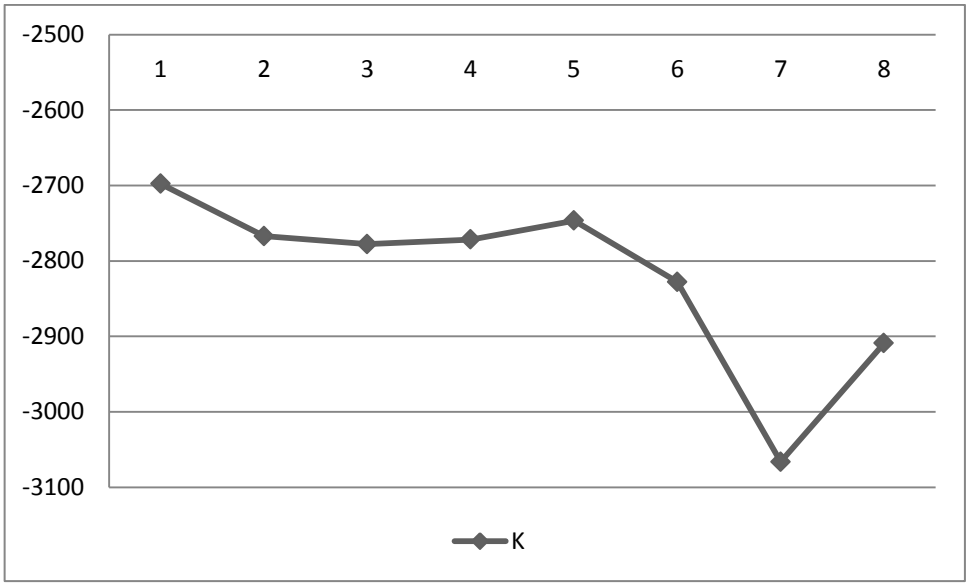

Figure 6 – Results of the Bayesian analysis conducted in program Structure to assess the number of genetically distinct populations (K) contained in our total sample. Y axis = Estimated Ln probability of data.

Discussion

The matrilineal and bi-parental markers evaluated here presented relatively high levels of genetic diversity for the Atlantic ESU as for other marine mammals (González-Suárez et al., 2009; Quéroil et al., 2007), as found for the Brazilian sample by Oliveira et al. (2009) for seven microsatellite loci. Besides, no evidence of recent genetic bottleneck was found in our more comprehensive study of the Atlantic ESU, in accordance with Oliveira et al. (2009). These suggest that although its history shows a strong exploitation suffered in recent centuries and only recently being banned, hunting does not seem to have significantly affected this population genetic diversity.

The large distribution of the Atlantic ESU does not seem to prevent a high gene flow between some populations in the area. According to microsatellite data, the F_{ST} and R_{ST} indices and the Bayesian analysis performed by Structure indicated high levels of gene flow between all populations sampled with no suggestion of an expressive geographic structure. Wright (1969) estimated that a single migrant per generation would be sufficient to prevent the complete differentiation. Recent studies suggest that this number should vary from 5 to more than 10 migrants to compensate population size fluctuations (Lacy 1987; Mills & Allendorf, 1996; Vucetich & Waite,

2000). In this study, when the existence of two populations is considered, the migration rate for the bi-parental markers is sufficiently high (mean of 19.37 migrants per generation) to maintain the genetic equilibrium among the populations.

Considering mtDNA results, the migration analysis performed in Lamarc had also found high levels of gene flow among all populations in agreement with microsatellite results. However, pairwise F_{ST} and Φ_{ST} values evidenced same level of structure between some pairs of populations. Significant and relatively high levels of these differentiation indexes were found between Mar del Plata vs Southern Chubut and Uruguay vs Northern Chubut populations. A possible explanation for the conflicting signals between the matrilineal mtDNA and the bi-parental microsatellites is the strong female philopatry in the species (Burg et al. 1999; Hoffman et al. 2006). This difference in gene flow estimates between mtDNA and microsatellite data is found in several species and indicates a dispersal pattern and migration mediated by males (Petit & Mayer 1999; Ruedi & Castella 2003; Weyandt et al. 2005). However, we cannot discard that some bias could have occurred due to sampling size difference among some localities and there are important colonies not studied here. Future research should explore the Argentinean colonies that were not sampled in this work like Ushuaia, Falkland Islands and Estados Island (54° 49' S, 64° 29' W) and the southern coast of Chile in order to test if the results of this study is valid to its entire distribution.

The TMRCA indicated that much of the current genetic diversity of the Atlantic ESU emerged relatively recent, around 200 kya, and both the tree and the haplotype network showed a suggestive pattern of past population expansion in the history of this population. Past population expansion is corroborated by results of the neutrality indices for mtDNA data and the growth rate estimated by Lamarc for both mitochondrial and microsatellite data. According the Bayesian Skyline analysis this population expansion started around 200 - 100 kya.

Additionally, the detailed analysis of mtDNA haplotype network showed that all shared haplotypes are the more frequent and central (older), and the tip (more recent) haplotypes are of low frequency and occur in single populations. This pattern is indicative of the occurrence of an ancient gene flow (or of the existence of a smaller ancient population) that expanded to colonize present day breeding areas

and that actual maternal gene flow between these colonies is much reduced. This recent isolation might be mainly due to an increment in the geographic distance between new populations in new colonized areas, affecting mainly the genetic structure of the females which exhibit lower dispersion rates and higher rates of philopatry. This extensive ancient haplotype sharing may also explain the weak signals of geographic structure detected in our results based on mtDNA data in spite of this well known philopatry.

Conservation implications

Based on the results described above and taking in consideration the prohibition of hunting along all the distribution of the species and the El Niño events, it is possible to conclude that this population has great viability, because its high genetic variability provided adaptation to these strong selective pressures. The maintenance of this genetic diversity and habitat protection must be the focus of conservation efforts.

This work shows that the Atlantic ESU of South American fur seal maintain high gene flow by males along all its distribution, has high genetic diversity and large population size, and then, it is a single population in the Atlantic. However, the genetic corroboration that the females are highly philopatric means that any breeding colony that goes extinct is unlikely to be naturally recovered since males alone could not establish new breeding colonies. At the same time, the conservation measures for this population as a whole should be aligned among the countries of the distribution regardless of cultural, political and geopolitical borders differences, since any regional threat can impact the population as a whole.

The establishment of conservation policies is of great importance because knowing how pinnipeds are distributed would probably help to understand why some species are at extinction risk while others are not. Such knowledge would help concentrate efforts for the conservation of species currently at risk instead of those which may be predisposed to an eventual danger (Ferguson & Higdon, 2006). The identification of a particular subpopulation leads to a correct definition of areas for the establishment of parks and reserves; the proper management of endanger

populations; and the correct strategy planning for captive breeding (O'brien & Mayr, 1991).

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Supplementary Material

Table S1. Samples analyzed.

Locality (site)	Samples	Institution/contact
46° 00´S; 64° 00´W (Chubut Province)	Aa05*	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de la Patagonia - Enrique Alberto Crespo
Muelle Alte. Storni, Pto. Madryn (Província do Chubut)	Aa25*	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de la Patagonia - Enrique Alberto Crespo
Islote Moreno (Chubut Province)	Aa29*, Aa125*	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de la Patagonia - Enrique Alberto Crespo
Rawson (Chubut Province)	Aa34*	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de la Patagonia - Enrique Alberto Crespo
P. Delgada (Chubut Province)	Aa38*	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de la Patagonia - Enrique Alberto Crespo
Playa El Doradillo (Chubut Province)	Aa47*	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de La Patagonia - Enrique Alberto Crespo
Las Grutas (Chubut Province)	Aa130*	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de La Patagonia - Enrique Alberto Crespo
Isla Rasa (Chubut Province)	Aa101*, Aa102*, Aa106*†, Aa107*†, Aa108*†, Aa109*†, Aa117*†, Aa118*,	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de

	Aa124*, Aa160*†, Aa170*†, Aa181*†, Aa183*†, Aa188*†, Aa189*†, Aa190†, Aa191*†, Aa192†, Aa193*†, Aa194*, Aa195*†, Aa197*†, Aa199*†, Aa200*†, Aa2101*†, Aa2106*†, Aa2107*†, Aa1_22*, Aa2_22*†, Aa3_22*†, Aa5_22*, Aa6_22†, Aa7_22*†, Aa9_22†, Aa10_22*†, Aa11_22*†, Aa12_22*†, Aa13_22*†, Aa14_22*†, Aa15_22*†, Aa16_22*†, Aa17_22*†, Aa18_22*†, Aa19_22*†, Aa20_22*†, Aa21_22*†, Aa22_22*†, Aa23_22†*, cria1†, cria2†	la Patagonia - Enrique Alberto Crespo
Isla Arce (Chubut Province)	Aa103*, Aa104*†, Aa105*	Laboratório de Mamíferos Marinos, Centro Nacional Patagónico (CONICET) e Universidad Nacional de la Patagonia - Enrique Alberto Crespo
North Argentina (Mar del Plata)	24-99*, 86-00*, AF01-2001*, BV*†, 71-00*†, 78-00*†, 85-00*, 87-00*, 110-00*, 69-00†	Departamento de Ciencias Marinas, Facultad de Ciencias Exactas y Naturales, Universidad de Mar del Plata - Diego Rodríguez
Uruguay (Cabo Polonio)	CP1*†, CP2*, CP3*	Sección Zoología Vertebrados, Facultad de Ciencias - Diana Szteren
Uruguay (Isla de Lobos)	1uy†, 2uy*†, 3uy†, 4uy*†, 5uy*†	*Proyecto Pinnípedos Cetáceos Uruguay, Facultad de Ciencias, Uruguay* - Valentina Franco Trecu
Brazil (Rio Grande do Sul)	556*, 586*, 591*, 656*†, 657*†, 660*, 662*†, 664*, 665†, 668*, 671*, 676*†, 683*, 687*, 689*, 691*†, 692†, 693†, 694*, 696*, 705*, 708*, 712*†, 714*, 722*, 728*, 729*, 730*†, 731†, 959†, Parna*	Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul - Larissa Rosa de Oliveira

*samples analyzed for mtDNA control region; † samples analyzed for microsatellites.

CONCLUSÃO

Os resultados do presente trabalho mostram que a UES do Atlântico é uma única população genética, com grande distribuição e alto fluxo gênico de machos entre as diversas colônias. A rede de haplótipos mostra um compartilhamento antigo de haplótipos, além de indicar um sinal de expansão. Os índices de fixação apontaram uma provável estruturação entre as colônias do Uruguai e do Chubut em relação ao DNA mitocondrial. Já os dados de microssatélite não mostraram essa tendência. O comportamento filopátrico por parte das fêmeas já foi identificado como uma característica da espécie, sendo os machos, então, os mediadores do fluxo gênico.

Uma forte ameaça sofrida por esta espécie foi a caça, que apenas em 1990 foi proibida no Uruguai. Esta exploração vem acontecendo há cerca de 6.000 anos, primeiros pelos aborígenes, depois fortemente pela caça comercial. Apesar disso, nenhum sinal de gargalo genético foi detectado a partir dos dados. Os eventos de El Niño recentes também parecem não ter influenciado na diversidade genética da espécie. Tanto os dados do DNA mitocondrial quanto os de microssatélite apontaram expansão populacional que começou entre 200.000 e 100.000 anos atrás.

A continuidade deste trabalho é muito importante, pois nem toda a distribuição foi amostrada. As populações do extremo sul da Argentina e da costa sudoeste do Chile também precisam ser avaliadas para verificar se os mesmos resultados ainda se mantêm. Para a conservação, os resultados deste estudo são importantes, pois as políticas devem considerar a existência de uma única população no Atlântico além de terem de ser alinhadas entre todos os países onde a espécie se encontra distribuída.

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