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ORIGINAL PAPER

Mitochondrial and microsatellite assessment of population structure of South American sea lion (*Otaria flavescens*) in the Southwestern Atlantic Ocean

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Abstract Several major breeding areas have been defined for the South American sea lion (*Otaria flavescens*) along the Atlantic Ocean including the Uruguayan and Patagonian coasts. Together with a documented and severe reduction in population sizes caused by commercial hunting in the last century, these areas show opposite population trends. While Patagonian populations are recovering since hunting ceased, Uruguayan populations are declining. In this context, population genetic structure and genetic diversity were studied for the first time with both nuclear (microsatellites) and mitochondrial (control region) markers together. Alternative scenarios were found for both markers. While mitochondrial marker showed geographically structured populations, the nuclear loci showed a lack of geographical structure. These opposite patterns in genetic structure could be explained by female phylopatry and high male dispersion. The reduction in population size caused by commercial hunting did not leave a detectable footprint of bottleneck at the genetic level.

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Introduction

The South American sea lion *(Otaria flavescens*, Shaw 1800) is distributed along the Southwestern Atlantic Ocean from Torres, Brazil (29°20′S, 49°43′W) (Vaz-Ferreira 1976; Rosas et al. 1994) to Cape Horn in the extreme south of South America and from Cape Horn to Zorritos, Perú (3°40′S, 80°34′W) in the Southeastern Pacific Ocean (Riedmann 1990).

On the Atlantic distribution, breeding areas are concentrated in several regions. Two of those main areas with high concentration of breeding colonies are located along the Uruguayan and the northern Patagonian coasts. The former includes main rookeries at Isla de Lobos, Cabo Polonio and La Coronilla islands (Vaz-Ferreira 1982; Páez 2006). In northern Patagonia, more than sixty-five breeding rookeries and haul-out sites are found from 40 to 45 degrees south (Reyes et al. 1999; Dans et al. 2004; Reyes 2004; Grandi et al. 2008; Túnez et al. 2008).

As many other colonies along the distribution range, the Atlantic ones were severely exploited mainly during first half of twentieth century (Carrara 1952; Crespo and Pedraza 1991; Páez 2006). While direct exploitation for leather and oil is a matter of the past, in present days, threats on the sea lion vary along its distribution range and include effects from fisheries (Corcuera et al. 1994; Crespo et al. 1997, 2007; Szteren and Páez 2002) and tourism (Dans et al. 2004). Moreover, where rates of increase are known, the population trends are different or opposite. While in northern Patagonia, the population grows at a rate of 5.7% (Dans et al. 2004; Reyes 2004) in production of pups per year, in Uruguay it declines at rates of 4.5% per year (Páez 2006). Rates of increase are unknown in southern Patagonia and in the Falkland (Malvinas) Islands, the increase is positive but very low compared with

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Península Valdés (Thompson et al. 2005), 3.8% of increase in pup production per year.

Given this background, the continuity or isolation of putative stocks became a high-priority matter. Therefore, uncovering the potential connection between the breeding areas needs to be reviewed for conservation and management purposes.

There are four previous studies analysing the Atlantic population genetic structure of breeding colonies. The first one screened ten protein systems from two rookeries, Isla de Lobos (IL) (Uruguay) and Punta Norte (PN) (northern Patagonia (Szapkievich et al. 1999). Out of the 10 loci examined, only one (Transferrin) was polymorphic for both sea lion rookeries. The same alleles were found at PN and IL, and three different electrophoretic transferrin phenotypes were observed. The authors found no evidence of structure in nuclear protein-coding loci. More recently, Túnez et al. (2007) used 445 base pairs of the cytochrome b gene (cyt b) of the mitochondrial genome ($_{mt}$DNA) and proposed a structured scenario, where four haplotypes define genetic differences between Uruguayan and Patagonian areas, while no differences were found within each area. The other two works used control region and flanking tRNA sequences from $_{mt}$DNA (Freilich 2004; Túnez et al. 2010). Freilich (2004) analysed the north of Península Valdés and Malvinas (Falklands) colonies, whether Túnez et al. (2010) analysed only northern and central Patagonia colonies. Both works founded significant differences between colonies and signals of population expansion events related to late Pleistocene glaciations. A fifth work presented by Artico et al. (2010) analysed short fragments (287 bp) of the $_{mt}$DNA control region becoming from stranded animals from non-breeding sites of the species in the Brazilian cost near Uruguayan breeding colonies. The main contribution of this work was at the phylogenetic level, where monophyletic reciprocal groups were found each corresponding to Peruvian (Pacific Ocean distribution of the species) and Brazilian (Atlantic Ocean) samples.

However, some limitations have been stressed for each of the molecular markers chosen in previous studies; likely effect of stabilizing selection on protein-coding loci (e.g. Wlasiuk et al. 2003); allozyme technique limitation to detect synonyms changes at aminoacid level (Allendorf and Luikart 2007); and low variation and exclusive female contribution to population patterns in mitochondrial genes (cyt b and control region) (Avise 1995).

Given all this background, the objective of this work was to study the population structure and population demographic history through the analysis of two molecular markers, the sequence of the $_{mt}$DNA hiper-variable control region and thirteen polymorphic microsatellite loci covering most of the Atlantic Ocean distribution of the species. Combined molecular markers studies of both mitochondrial and nuclear genomes have become of relevant interest for better resolution of population genetic structure in wild populations (Bos et al. 2008; Lukoschek et al. 2008) including pinniped (Bickham et al. 1998; Hoffman et al. 2006; Coltman et al. 2007; Wolf et al. 2007; González-Suárez et al. 2009).

Materials and methods

Tissue sampling, localities of collection and DNA extraction

Tissue samples were collected throughout the two major breeding areas of the Southwestern Atlantic and some breeding colonies from the Central-South Patagonia distribution (Fig. 1). Samples were obtained from individuals stranded or biopsied during tagging or experimental activities from 1990 to 2008 in Patagonia and from 2006 to 2008 in Uruguay. Most of the samples were taken from rear flipper punches of living pups, or muscle, liver or skin from recently stranded animals. The samples were preserved in 20% dimethyl sulphoxide (DMSO) saturated with salt (Amos and Hoelzel 1991) for Patagonian samples and in alcohol 95% for Uruguayan ones. Total DNA was extracted following Miller et al. (1988).

Mitochondrial DNA control region sequencing

Twenty-eight samples from Uruguayan and Patagonian distribution were chosen for $_{mt}$DNA control region amplification. Pinniped-specific primers were designed aligning and extracting consensus sequences from sequences of mitochondrial proline and phenylalanine tRNA (Genbank accession numbers: NC_004030, NC_008415, NC_008420, NC_008419, NC_008418, NC_008416). Designed primers, $tRNA_{pro}$ (5′-GCAACAGCCCCACCAYCAACACCC-3′) and $tRNA_{phe}$ (5′-CATCTAGGCATTTTCAGTGCCTTG-3′), succeeded to amplify the complete control region with the following PCR profile: one cycle of 240 s at 94°C; thirty-five cycles at 94°C, 56°C, 72°C of 30 s each step; and finally elongation for 180 s at 72°. All PCRs (25 µl) contained 2.5 mM Tris–Cl (pH 8.7), 5 mM KCl(NH4) 2SO4, 200 mM each dNTPs, 10 mM primers and 0.5 units Taq polymerase with varying MgCl2 (between 1.5 and 3.0 mM). PCR products were purified using ExoSAP-IT (Affymetrix), sequenced in ABI3730XL, edited using PROSEQ3.0 (Filatov 2002) and aligned in CLUSTAL X (Thompson et al. 1997) and verified by eye. Thirty-nine (427 bp) previously available sequences from Freilich (2004) and forty-nine (508 bp) sequences from Túnez et al. (2010) were added for population analysis (Table 1). Therefore, a total of 115 $_{mt}$DNA control

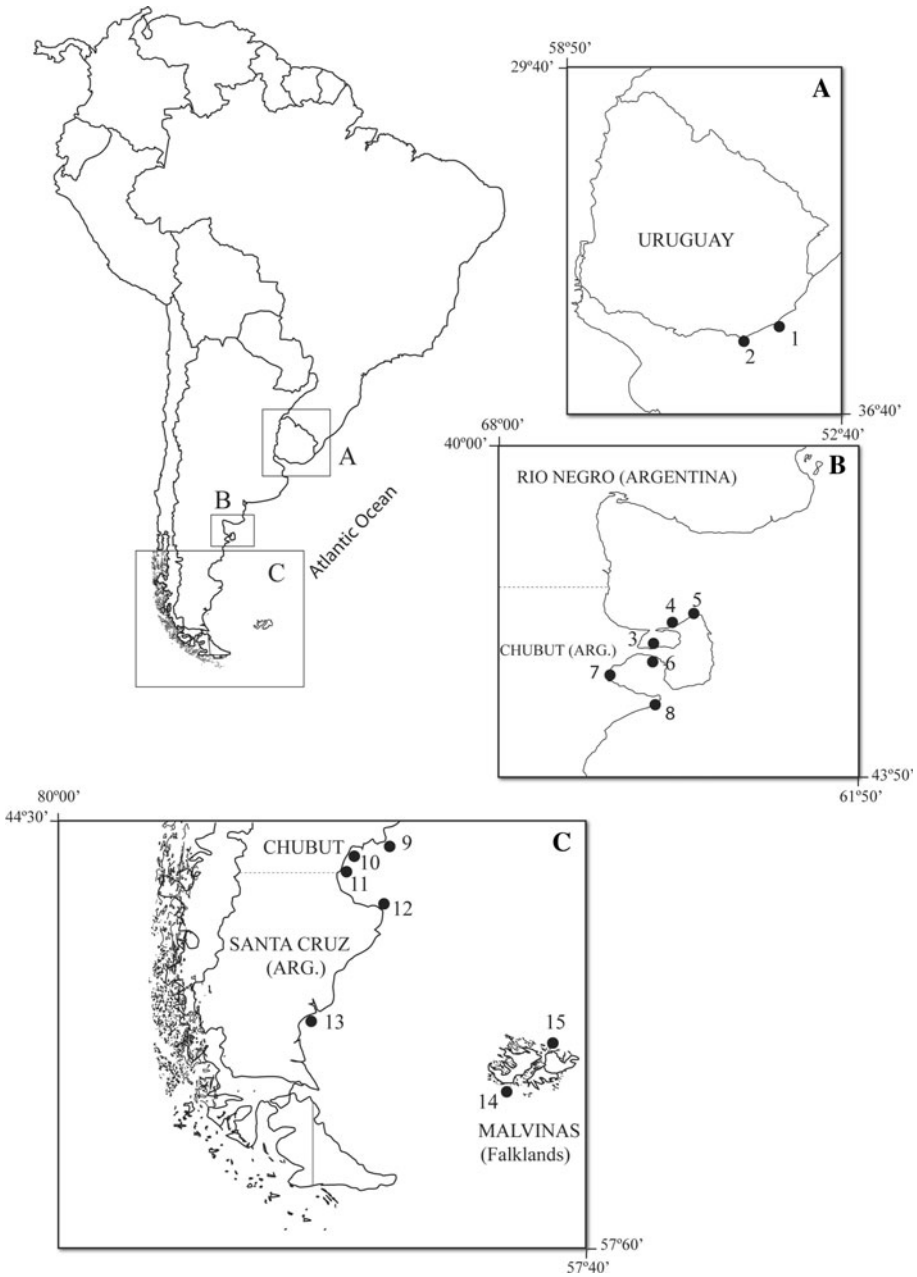

Fig. 1 Sample localities. **a** Rocha (*1*), Isla de Lobos (*2*). **b** Playa Larralde (*3*), Pta. Norte (*4*), Faro Bs. As. (*5*). Puerto Pirámide (*6*), Puerto Madryn (*7*), Pta. León (*8*). **c** Isla Arce (*9*), Isla Vernacci Oeste (*10*), North Golfo San Jorge (*11*), Monte Loayza (*12*), Cerro Bayo (*13*), Stick in the mud Islet (*14*) and Seal bay (*15*)

region sequences were used for population structure and demographic analysis. Also for phylogeny analysis, five additional sequences from Perú were available at Genbank (GI:14389105/6/7/8/9 Wynen et al. 2001), but were only used for this analysis because they were only 289 bp in length.

Microsatellite genotyping

We selected twenty-one microsatellite loci previously studied on Pinnipeds (Allen et al. 1995; Gemmell et al. 1997; Goodman 1997; Hoffman et al. 2007) (Online resource 1), thirteen of them also cross-amplified in *O. flavescens*. Initial screening of polymorphic loci and amplification optimization were done over a panel of ten individuals. Afterwards, eight loci were discarded for absence of polymorphism or failed amplification. For extant thirteen loci, each forward primer were fluorescent labelled (Online resource 1) and amplified for sixty samples using polymerase chain reaction (PCR) with the following profile: one cycle of 240 s at 94°C; five cycles at 94°C, 56°C, 72°C of 30 s each step; five cycles of 94°C, 54°C, 72°C of 30 s each step; thirty cycles of 30 s at 94°C, 50°C, 72°C of 30 s each step; and finally elongation for 300 s at 72°. Reaction conditions were taken from Kretzmann et al. (2001). PCR products were run in

Table 1 Number of samples used for analyses, assigned region for each locality and reference work or accession numbers

Region	Locality	Number of samples (control region/microsatellite)	Genbank accession no or reference article
		Total: 115–60	
Uruguay		Sub-total: 13–17	
Uruguay (Uru)	1 Isla de Lobos	4–7	HM467619-22
	2 Rocha	9–10	HM467623-31
		Sub-total: 102–43	
Patagonia			
North Península Valdés (NPV)	3 Playa Larralde	1–4	HM467633
	4 Pta. Norte	24–1	HM467635; Freilich (2004), Túnez et al. (2010)
South Península Valdés (SPV)	5 Pta. Buenos Aires	8–0	Freilich (2004)
	6 Puerto Pirámide	7–0	Túnez et al. (2010)
	7 Puerto Madryn	4–20	HM467634, 38, 39, 41
	8 Pta. León-Rawson	5–2	HM467632, 36, 37, 40, 42
South Chubut-Santa Cruz (SCh-SC)	9 Isla Arce	10–0	Túnez et al. (2010)
	10 Isla Vernacci Oeste	10–0	Túnez et al. (2010)
	11 North Golfo San Jorge	0–5	–
	12 Monte Loayza	10–0	Túnez et al. (2010)
	13 Cerro Bayo	4–9	HM467643-46
Falklands (Malvinas) Islands	14 Stick in the mud Islet	14–0	Freilich (2004)
	15 Seal Bay	5–0	Freilich (2004)
Other seq.			
Perú	Perú	5–0	GI:14389105-9 from Wynen et al. (2001)

ABI3730XL and genotyped using PEAKSCANNER V1.0 software (from http://marketing.appliedbiosystems.com/mk/get/PS1login).

Mitochondrial DNA control region analyses

Number of haplotypes, number of polymorphic sites, nucleotide diversity π (Nei 1987) and haplotypic diversity (h) were estimated for each subpopulation and the total population using ARLEQUIN V3.1 (Excoffier et al. 2005). Population subdivision and structure were estimated using an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) that computes Wright's hierarchical *F*-statistics analogues, Φ-statistics, which incorporate evolutionary distance between haplotypes in addition to frequency data. For the population structure analysis, sampling localities were assigned to five regions in a latitudinal array of the study area. The first region corresponds to the Uruguayan coast (named as Uru), three to Patagonia (NPV, for Northen Península Valdés; SPV, for Southern Península Valdés; and SCh-SC, for South Chubut-Santa Cruz region) and the last one to the Falklands (Malvinas) Islands (Table 1). Definition of these regions does not respond to geographic barriers delimiting the Atlantic distribution, instead, published demographic information based on annual direct counts and census was used. For this purpose, samples from adjacent zones to focal colonies defined by Grandi et al. (2008) and samples from different important breeding areas for the species presented by Túnez et al. (2008) were defined as separated units for analyses.

To estimate gene flow dynamics, pairwise population fixation indices between regions were also calculated using pairwise differences for computations. The significance of the fixation indices was tested using a null distribution obtained from ten thousand permutations, in a non-parametric approach described in Excoffier et al. (1992). In order to investigate the existence of higher level of structure above region level, geographic groups were defined base on the breeding areas of importance for the species and also analysed with the AMOVA. For this purpose, three higher grouping were tested defining two, three or four geographic units, respectively: (A) Uruguay (Uruguayan region), Patagonia (all Argentinean regions plus Falklands (Malvinas) Islands one); (B) Uruguay, Patagonia and Falklands (Malvinas) Islands; (C) Uruguay, North Patagonia (Northern and Southern Península Valdés regions), Central-South Patagonia (South Chubut-Santa Cruz region) and Falklands (Malvinas) Islands. As a complement to this, the program SAMOVA (Dupanloup et al. 2002) was used to define partitions of sampling sites

that are maximally differentiated from each other but without any a priori assumption about geographic structure (giving the possibility to test from 2 to 4 geographic units randomly).

Tajima's D (Tajima 1989), Fu's F_S (Fu 1997) and mismatch distribution (Rogers and Harpending 1992; Rogers 1995; Excoffier 2004) were used to investigate historical demography in ARLEQUIN. For mismatch analysis using ARLEQUIN, we employed the parametric bootstrapping to test the goodness of fit of the observed mismatch distribution to that expected under the sudden expansion model using the sum of squared deviations (SSD) statistic. A population expansion event in the recent past may result in a unimodal mismatch distribution, while a multimodal distribution is encountered in populations at demographic equilibrium. If evidence of population expansion was found, the parameter tau (τ, in mutational units) was computed (Schneider and Excoffier 1999) and analysed. This parameter enables us to estimates the time since expansion using the equation, $t = \tau/2\mu$, where μ is the mutation rate for the locus used and t is the time since expansion. The mutation rate used was 27.45% per million years as published for the Steller sea lion control region (*Eumetopias jubatus*) (Phillips et al. 2009). Expansion parameters and confidence interval were computed using a bootstrap approach with $\alpha = 0.05$ (Schneider and Excoffier 1999). Unrooted phylogenetic reconstruction of Atlantic and Peruvian haplotypes relationships was done with Paup 4b10, using Neighbour-Joining (Saitou and Nei 1987) method, Kimura two parameters (Kimura 1980) and 10 thousands of bootstrap replicates.

A Mantel test (1967) was used to asses a correlation between the log(migrants) and log(distance) between regions as proposed originally by Slatkin and Maddison (1990) using XLSTAT (http://www.xlstat.com/, Addinisoft 2009). Geographical distance was calculated as shortest sea-lion-travel distance between regions defined and measured in kilometres.

Microsatellite data analyses

GENEPOP (Raymond and Rousset 1995, web version: http://genepop.curtin.edu.au/index.html) was used to detect significant departures from Hardy–Weinberg equilibrium (H–W), using a procedure described in Guo and Thompson (1992), analogous to exact test of H–W (Fisher 1922; Haldane 1954), and to estimate observed (Ho) and expected (He) heterozygosity and allele numbers for each locus by population and globally. To minimize type I error in case of multiple comparisons, we performed sequential Bonferroni corrections (Holm 1979; Rice 1989) on P values for each region. Linkage disequilibrium (LD) was tested for each pair of loci in ARLEQUIN V3.1 (Excoffier et al. 2005).

Hierarchic genetic structure of *O. flavescens* in the Southwestern Atlantic Ocean distribution using microsatellite loci was also studied in ARLEQUIN using the locus-by-locus AMOVA with 10100 data permutations (Excoffier et al. 1992) recommended when missing data could exist (Weir and Cockerham 1984). Pairwise Rho_{st} and F_{st} between regions were also estimated as done for mitochondrial control region and using ARLEQUIN, but the geographic population structure at a higher level was done only assuming two or three breeding areas in the Atlantic Ocean (samples for microsatellite genotyping were not available for Falklands (Malvinas) Islands region, disabling to test four breeding areas).

A Bayesian clustering analysis was also done using STRUCTURE V2.3.1 (Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009) to evaluate population structure. This analysis assumes K (unknown, in this case unknown breeding areas in the Atlantic Ocean) populations, which have different allele frequencies at a set of independent loci. The K with the highest estimated likelihood value indicates the most likely K number of population in the sample. Three independent runs of 2 millions iterations each with a burn-in period of 150 thousand iterations were set for the analysis of K values ranging from 1 to 5. We assumed correlated allele frequencies among putative subpopulations and an admixture model.

Two single-sample methods for detecting recent bottlenecks were used, Wilcoxon sign-rank test (Luikart 1997) of heterozygosity excess (Cornuet and Luikart 1997) and mode-shift indicator of allele frequency distribution (Luikart et al. 1998) implemented in BOTTLENECK V1.2.02. (Cornuet and Luikart 1997). The infinite allele model (IAM), stepwise mutation model (SMM) and the two-phase model of mutation (TPM, with 90% of SMM) were used.

As with the mitochondrial DNA, we used the test of Mantel (1967) to investigate the correlation between the log(migrants) and log(distance) between regions using XLSTAT (http://www.xlstat.com/, Addinisoft 2009).

Results

Mitochondrial DNA control region

A product of 750 base pairs was correctly sequenced and edited for 28 new individuals. When sequences reported by Freilich (2004) and Túnez et al. (2010) were included, 340 bp were correctly aligned. For the total of 115 sequences used for population analyses, 45 polymorphic sites were found, defining 53 distinct haplotypes: Uru 12; NPV 17; SPV 12; SCh-SC 12; and Falkland (Malvinas) Islands 8 haplotypes. Between regions, NPV and SPV 4 haplotypes were shared; NPV and SCh-SC shared 5

haplotypes; SPV and SCh-SC shared 2 haplotypes. No other haplotypes were shared between regions.

Haplotype diversity ranged from 0.841 in SCh-SC to 0.987 in Uru. Nucleotide diversity values ranged from 0.008 at Falkland (Malvinas) Islands to 0.021 at Uru, with mean values of 0.014 for the rest of the regions.

Demographic analyses showed non-departure from neutrality using Tajima's D test; however, Fu' Fs which is considered a more powerful test to detect population expansion (Ramos-Onsins and Rozas 2002) was negative and significant ($P < 0.05$) for Uru, NPV and SPV. Also, multimodal mismatch distributions for Uru, SVP, SCh-SC and Malvinas regions (Online Resources 2) and unimodal distribution for NVP region (Fig. 2) were found. As evidence of population expansion event was founded for NVP, timing of expansion was estimated to be, $\tau = 5.0$ mutational units ($CI_{95\%} = 2.05$–9.58).

If a unique Atlantic population is considered for analyses, Tajimas'D was negative but still not significant different from zero, Fu' Fs was negative (−24.00) and significant, and a unimodal mismatch distribution was observed. With $\tau = 4.283$ mutational units ($CI_{95\%} = 1.28$–20.13).

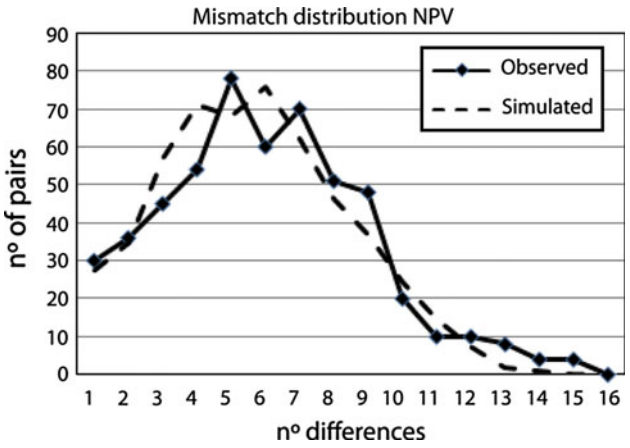

Fig. 2 Mismatch distribution graphic for NPV region (SSD = 0.0015; *P*(SSD) = 0.95). Observed data distribution (*solid rhombus and full line*); Simulated data distribution (*broken line*)

A well-supported clade (96 bootstrap values) was recovered by phylogenetic analyses (Fig. 3) separating Peruvian haplotypes and two haplotypes from the most austral Atlantic locality (Cerro Bayo, region of SCh-SC) from the remaining Atlantic haplotypes. Two other clades with moderate (78) and high (99) bootstrap value were found. If the geographic distribution is considered, none of these clades were monophyletic and presented a mix of haplotypes from two or three different regions.

Significant genetic differentiation was found between regions. The global AMOVA indicated that 37% of variation ($\Phi st = 0.37$, $P < 0.05$) was explained as differences between regions. All pairwise populations AMOVAs were also high and significant (Table 2). For higher levels of geographic units' structure, no significant values were obtained, neither for the three possible grouping proposed nor the SAMOVA's recovered groups (Table 3a).

An isolation by distance pattern was not supported by a Mantel test ($r = 0.80$, $P > 0.05$) assessing the correlation between log Nm and log Distance matrixes (Online Resource 3).

Microsatellites loci

Allelic diversity found for each region per locus was similar and moderate compared with those observed for other pinnipeds (Gemmell et al. 1997). It ranged from 2 alleles in locus Aa4 in SCh-SC to 11 in locus ZcwG04 in Uru. No deviation from H–W equilibrium or linkage disequilibrium was found in the data set after sequential Bonferroni corrections for multiple tests (Online Resource 4).

Global AMOVA using microsatellite loci was low and not significant ($F_{st} = 0.019$, $P = 0.3$; $Rho_{st} = 0.1$, $P = 0.09$). Neither of the pairwise comparisons between regions (Table 2) nor the higher levels of geographic structure tested were significant. (Table 3b).

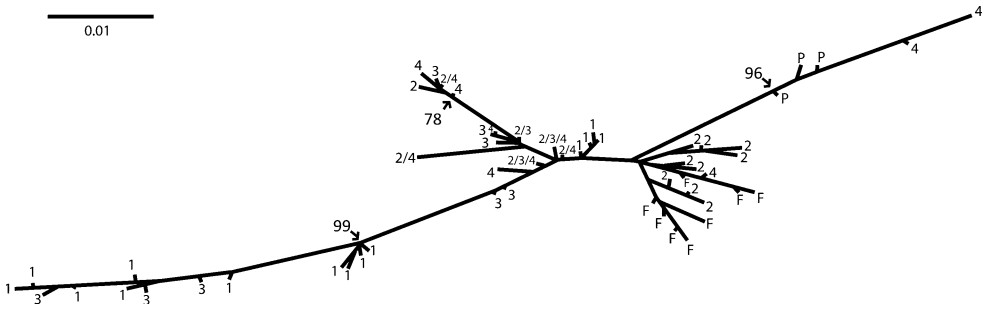

Fig. 3 Unrooted phylogram of haplotypes (56, 53 Atlantic haplotypes plus 3 from Perú), using PAUP4.0 with NJ method and K2P evolution model. Bootstrap values (after 10 thousands replicates) over 50 are shown. Uru (*1*); NPV (*2*); SPV (*3*); SCh-SC (*4*); Falkland (Malvinas) Islands (*F*); Perú (*P*)

Table 2 Pairwise Fst comparisons for control region and microsatellites, values marked with (–) were not able to be computed

	Control region	Microsatellites	
	ϕ_{st}	F_{st}	R_{st}
$AMOVA_{global}$ =	0.374 ($P = 0.001$)	0.019 ($P = 0.342$)	0.116 ($P = 0.09$)
Uru versus NVP	0.521 ($P = 0.001$)	0.005 ($P = 0.783$)	0.164 ($P = 0.122$)
Uru versus SVP	0.330 ($P = 0.001$)	−0.009 ($P = 0.837$)	0.062 ($P = 0.166$)
Uru versus SCh-SC	0.525 ($P = 0.001$)	0.007 ($P = 0.396$)	0.058 ($P = 0.085$)
Uru versus Malvinas	0.616 ($P = 0.001$)	–	–
NVP versus SVP	0.196 ($P = 0.001$)	−0.006 ($P = 0.891$)	0.191 ($P = 0.080$)
NVP versus SCh-SC	0.148 ($P = 0.001$)	−0.003 ($P = 0.765$)	0.287 ($P = 0.078$)
NVP versus Malvinas	0.322 ($P = 0.001$)	–	–
SVP versus SCh-SC	0.135 ($P = 0.001$)	0.024 ($P = 0.216$)	0.114 ($P = 0.068$)
SVP versus Malvinas	0.517 ($P = 0.001$)	–	–
SCh-SC versus Malvinas	0.487 ($P = 0.001$)	–	–

Table 3 Geographic structure tested above region level for Atlantic samples. A, control region AMOVA and SAMOVA results; B, presents AMOVA and STRUCTURE results for microsatellite loci. (*), for $K = 4$ using SAMOVA, the geographic structure clustering recovered was Uruguay versus SPV versus (NPV plus SCh-SC) versus Malvinas

A

Control region

	AMOVA	SAMOVA	
(A) Uruguay versus Patagonia	$\phi_{ST} = 0.371$ ($P = 0.199$)	$K = 2$	$\phi_{ST} = 0.371$ ($P = 0.192$)
(B) Uruguay versus Patagonia versus Malvinas	$\phi_{ST} = 0.379$ ($P = 0.109$)	$K = 3$	$\phi_{ST} = 0.379$ ($P = 0.098$)
(C) Uruguay versus North Patagonia versus Central-South Patagonia versus Malvinas	$\phi_{ST} = 0.239$ ($P = 0.302$)	$K = 4$*	$\phi_{ST} = 0.319$ ($P = 0.088$)

B

Microsatellite

	AMOVA	STRUCTURE	
		$K = 1$	ln $P(D) = -1{,}753$ $P \approx 1$
(A) Uruguay versus Patagonia	$F_{ST} = 0.009$ (P = 0.188)	$K = 2$	ln $P(D) = -1{,}864$ $P \approx 0$
(B) Uruguay versus North Patagonia versus Central-South Patagonia	$F_{ST} = 0.005$ ($P = 0.356$)	$K = 3$	ln $P(D) = -1{,}902$ $P \approx 0$
		$K = 4$	ln $P(D) = -1{,}886$ $P \approx 0$
		$K = 5$	ln $P(D) = -1{,}914$ $P \approx 0$

Bayesian analysis of genetic structure also indicates $K = 1$ as the most likely value ($P_{k=1} \approx 1$) suggesting a unique population in the Southwestern Atlantic Ocean.

Analysis of historical population demography using BOTTLENECK resulted in significant heterozygosity excess, suggesting a decline for the Southwestern Atlantic population with AIM (0.01) and TPM (0.03) mutational models, but not for SMM (0.90) or the mode-shift indicator of allele frequency distribution.

A Mantel test ($r = 0.41$, $P > 0.05$) again did not support a correlation between matrices of migration and geographic distances (Online Resource 3).

Discussion

In the present work, gene flow, population structure and changes in gene diversity after the intense reductions in population sizes caused by commercial hunting have been studied with mitochondrial and nuclear DNA markers. Specifically, control region sequences and allelic diversity from thirteen microsatellites loci were analysed from individuals sampled from five regions covering most of the distribution of the species in the Southwestern Atlantic Ocean.

The history of the different Atlantic putative stocks of South American sea lions analysed in this paper is

relatively dissimilar. Sea lions from Argentine coast were taken mainly for leather and oil. The heaviest extraction lasted from the twenties to the fifties (Carrara 1952; Godoy 1963). Northern Patagonian colonies were reduced from an estimated population size of at least 160,000 individuals in the early twentieth century to less than 10,000 in the sixties and started to increase in the eighties. (Crespo and Pedraza 1991; Koen-Alonso and Yodzis 2005). In contrast, the Uruguayan stock continued to be exploited until 1991 when the extraction of individuals ceased. The population trend in Uruguay in the twentieth century is not documented, but in the present decade, it was estimated that the population declines a rate of 1–4% annually (Páez 2006).

A preliminary study of the potential connection between breeding areas was published by Szapkievich et al. (1999), who found no polymorphic protein locus with the exception of the transferrin. The authors also mentioned migrating tagged individuals between areas. Years later, the studies by Freilich (2004), Túnez et al. (2008, 2010) indicated some degree of population structure based on $_{mt}$DNA.

For the understanding of population demography and structure through the analysis of molecular markers, some properties are worth reviewing, whereas alternative evolutionary scenarios in population history may yield similar results in the analysis.

The first to consider is natural selection, where selective sweeps over a specific locus may overlap population changes or subdivision, erasing earlier historic population demography recorded on that locus (e.g. Wakeley and Hey 1997; Kuhner et al. 2000; Avise 2004). Secondly, deviation from gene flow and genetic drift equilibrium or violation of Wright's Island model (Wright 1951) could lead to imprecise results and to gene flow estimations reflecting historical, rather than current connections. Finally, other differences between markers as effective population size (Fay and Wu 1999), mutation rate (Hoelzel et al. 1993; Primmer et al. 1996), inheritance and sex-dispersal bias need to be taken into account.

The most plausible scenario proposed by the conjunction of both sets of molecular markers, nuclear and mitochondrial, is a contrast between female philopatry and high rates of male dispersion throughout the Atlantic Ocean. This is supported by the lack of population genetic structure found with microsatellite loci and highly structured mitochondrial variation. Moreover, this study is concordant with previous studies using allozymes, cyt b (Szapkievich et al. 1999; Túnez et al. 2007) and D-loop control region (Túnez et al. 2010) for the species and provides a more detailed picture of the genetic interactions between Patagonia and Uruguay breeding areas.

Female sea lions are expected to disperse to breeding sites as close to their natal site as possible. Grandi et al. (2008) found that in northern Patagonia, the process of formation of new colonies and population expansion was a consequence of a complex dynamics involving philopatry dispersal to available and suitable habitat and reproductive success in different social-structure contexts. On the other hand, large distance movements in males have been recently demonstrated by Giardino et al. (2009), where sea lions males tagged on Mar del Plata and Puerto Quequén (Buenos Aires province) were re-sighted during the breeding season in Uruguayan and Península Valdés rookeries, approximately 500 and 800 km, respectively, from tagging localities. This male-biased pattern of dispersal has also been described for other pinniped species (e.g. Burg et al. 1999; Hoelzel et al. 2001; Hoffman et al. 2006; González-Suárez et al. 2009).

Regions distributed throughout the Southwestern Atlantic Ocean and used for the analyses showed no structure with microsatellites and no support for female evolutionary significant units (ESUs, defined in Moritz 1994), contrary to Artico et al. (2010) results, with $_{mt}$DNA. However, mitochondrial management units (MUs, also defined in Moritz 1994) could certainly be defined (supported by AMOVA results). These units represent a matrilineal structure but also aggregate nearby colonies and where migration of both males and females (upper intensity in males) is expected. Thus, these units intended to provide a conservative clustering for management purposes, which reflects overall spatial dynamics and not genetic isolated lineages.

Using τ and assuming the mutational rate for mitochondrial control region proposed by Phillips et al. (2009) of 27.45% per million years, the estimated date for detected expansion event on NPV region is 27 thousand years ($CI_{95\%}$ = 11–50 thousand years) before present. This was also found, but with a bigger $CI_{95\%}$, assuming a unique Atlantic population for analyses (23 thousand years, ($CI_{95\%}$ = 7–107 thousand years). Similar results for estimated date were also found for the same species (Túnez et al. 2010). These findings have been associated with environmental changes during the Pleistocene (Mercer 1968), specifically to those in the last glacial maximum period, 21 thousand years ago (e.g. Rabassa 2008; Lessa et al. 2010). Environmental changes affecting the accession to shoreline habitats for breeding activities (Hoelzel et al. 1993; Túnez et al. 2007, 2010) or the abundance of oceanic resources (Matthee et al. 2006) have been the main explanation to historical population changes in the South Atlantic Ocean. Túnez et al. (2010) suggested a population expansion after glaciers retracted from central Patagonia (NPV and SPV regions in this study) to southernmost coasts. Our results could not support this scenario because no evidence of population expansion was found for southernmost region (SCh-SC), on the contrary, population

expansion evidence for central Patagonia region of NPV was found. Until the moment, mitochondrial genes bring evidence supporting different demographic events in some of the structured mitochondrial regions. For the cyt b and control region, these events were dated approximately on 110 and 27 thousand years before present, respectively. As our results suggest, North Península Valdés showed a population expansion also suggested by Túnez et al. (2010) for the central Patagonia area. Given all this, strong evidence exist supporting a population expansion events, but, there is still no enough evidence to determine the existence nor the location of plausible glacial refuges and the direction of re-colonization for *O. flavescens*.

In this context, the complete history and details of population structure and demography of the species will not be completely understood until South Patagonia from the Pacific and Atlantic Oceans (e.g. Tierra del Fuego colonies) could be correctly sampled and included into the analysis with a diversity of molecular markers, like those already used for the species and new ones such as single nucleotide polymorphisms (SNPs).

Analyses of microsatellite data suggest a low signal of bottleneck at the genetic level, despite the severe reduction in sea lion population size registered during commercial exploitation of the last hundred years. Moreover, this signal was not recovered through all the mutational models used for microsatellite bottleneck analyses. Similar results were obtained by Oliveira et al. (2009) for the Atlantic population of the South American fur seal, *Arctocephalus australis*. While a severe population reduction was registered, the population did not show significant deviations from equilibrium expectations, thus, there is no suggestion of a population genetic bottleneck. In this context, two factors should be mentioned to understand the limited impact on genetic neutral diversity registered. First, the high mobility of males for reproduction should facilitate a rapid re-distribution of genetic variants among colonies. Secondly, Gaggiotti and Vetter (1999) showed that the larger the generation overlap, the smaller the impact of environmental fluctuations on the level of genetic variability maintained by a population. Genetic variability may be only weakly affected by population size as a result of a storage effect of genotypes which helps maintain genetic variability even at small population sizes. This effect occurs in species with long and overlapping generations, such as sea lions that have an approximate generation time of 12 years and a lifespan of more than 20 years (Hernández-Camacho et al. 2008).

If population dynamics of breeding areas is considered, Uruguay and Patagonia show opposite population trends. The latter is positive, while the former is negative. The causes of this contrast are far from being understood and may not rely only on genetic but on ecological factors. The hypothesis of genetically isolated populations coupled with inbreeding depression of the Uruguayan genetic pool appears to be easily rejected by our results. Therefore, ecological, more likely, than genetic factors should be used in order to model these tendencies. Anthropogenic factors like incidental or direct mortality or other species interactions like competition for food resources with *A. australis* may be of extreme importance and should be incorporated into future research on the biology and conservation of the species.

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