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RESEARCH ARTICLE

Morphological and genetic evidence for two evolutionarily significant units (ESUs) in the South American fur seal, *Arctocephalus australis*

Larissa Rosa de Oliveira · Joseph I. Hoffman · Erika Hingst-Zaher · Patricia Majluf · Mônica M. C. Muelbert · João Stenghel Morgante · William Amos

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Abstract The South American fur seal (*Arctocephalus australis*) is widely distributed, occurring along both the Atlantic and the Pacific coasts of South America. Previous work suggests there may be more than one subspecies, highlighting the need for further study. Here, we combine traditional and geometric morphometric analysis of skull shape and size with genetic data to compare two populations of South American fur seals, one from Uruguay and one from Peru. As a control group we used material from the closely related species *Arctocephalus gazella*. Both techniques of morphometric analysis reveal pronounced geographic variation in size and shape of the skull, with Peruvian specimens ($n = 102$) being larger than Uruguayan skulls ($n = 133$) and significant shape differences concentrated in the rostral region. Similarly, seven highly polymorphic microsatellite loci reveal highly significant differences in allele frequency. Moreover, Bayesian analysis implemented using the program STRUCTURE reveals two separate clusters corresponding perfectly to the two populations, with an assignment test correctly placing over 98% of specimens in their population of origin. This degree of differentiation for both genetic and morphological traits suggests complete and possibly prolonged isolation to the extent that we believe these populations should be considered distinct evolutionarily significant units.

Keywords South American fur seal · *Arctocephalus australis* · Skull morphometrics · Microsatellite · Evolutionarily significant units (ESUs)

Introduction

The partitioning of populations into smaller, isolated or semi-isolated units can have an important bearing on many demographic and evolutionary processes. Consequently,

L. R. de Oliveira · J. S. Morgante
Laboratório de Biologia Evolutiva e Conservação de Vertebrados (LABEC), Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, Cidade Universitária, Sao Paulo, SP 05508-090, Brazil

Present Address:
L. R. de Oliveira (✉)
Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul (GEMARS), Rua Felipe Néri 382/203, Porto Alegre, RS 90550-140, Brazil
e-mail: lari.minuano@gmail.com

L. R. de Oliveira
Centro de Estudos Costeiros, Limnológicos e Marinhos da Universidade Federal do Rio Grande do Sul (CECLIMAR/UFRGS), Av. Tramandaí, 976, Tramandai, RS 95625-000, Brazil

J. I. Hoffman · W. Amos
Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK

E. Hingst-Zaher
Laboratório de Mastozoologia, Museu de Zoologia da Universidade de São Paulo, Av. Nazaré, 481, Ipiranga, Sao Paulo, SP 04299-970, Brazil

P. Majluf
Unidad de Biología de la Conservación, Universidad Peruana Cayetano Heredia (UPCH), Armendáriz 445, Miraflores, Lima 18, Peru

M. M. C. Muelbert
Laboratório de Mamíferos Marinhos e Tartarugas Marinhas, Departamento de Oceanografia, Programa de Pós-Graduação em Oceanografia Biológica, Cx. Postal, 474, Rio Grande, RS 96201-900, Brazil

the identification of such units is required both to understand a species' biology and, where threatened, to formulate the most appropriate management and conservation strategies (Parsons et al. 2006). Marine mammals present a particularly potent challenge, on the one hand being capable of moving over huge distances (e.g., Martin et al. 1984; Fabiani et al. 2003) in an environment that generally lacks obvious physical population boundaries, yet on the often showing strong fidelity to breeding or feeding grounds (Wood 1998; Mate et al. 1999; Matthiopoulos et al. 2004). Indeed, several genetic studies have revealed patterns in which high levels of philopatry have created strong population sub-structure (e.g., Encalada et al. 1996; Goodman 1998; Tolley et al. 2001; Ovenden et al. 2004; McMillen-Jackson et al. 2005; Hoffman et al. 2006).

In conservation biology, the need to identify primary population subdivisions (Frasier and Bernatchez 2001) has led to the concept of 'evolutionarily significant units' (ESUs), objectively defined units below the level of species that should be prioritized for protection (Ryder 1986; Moritz 1994a; Chan et al. 2006; Hedrick et al. 2006; Robalo et al. 2007; Bottin et al. 2007) in the face of limited resources (Avise 1989). However, although the ESUs concept is embedded in the Endangered Species Act (Waples 1991, 1995), the Australian Endangered Species Protection Act (Moritz 1994a) and parallel legislation in other countries, a consensus as to how an ESU should be defined has proved hard to come by (e.g., see Moritz 1994b; Nielsen and Powers 1995; Karl and Bowen 1999; Crandall et al. 2000; Fraser and Bernatchez 2001). Like the species concepts, much of the debate concerns the level of emphasis placed on neutral versus selected variation, identifying the most relevant spatiotemporal scale (Fraser and Bernatchez 2001) and the problem of where to draw a line across what is often more or less a continuum (e.g., Moritz et al. 1995; Waples 1998).

The South American fur seal (*Arctocephalus australis*) is one of the most widely distributed South American otariid species. It occurs on the Atlantic coast southwards from Brazil, breeding at mainly island rookeries of Uruguay and Argentina, down to the Isla de los Estados and the Falklands Islands (Vieira 1955; Carvalho 1975; Vaz-Ferreira 1982), and there is a single record from South Georgia (56°0′ S; 33°0′ W) (Daneri et al. 1997). On the Pacific coast the species occurs from the Chiloé Island (42°–43° S) in Chile down to Cape Horn (55°10′ S; 67°40′ W). While there is no breeding colony or haul-out area between Chiloé and Mejillones (23°05′ S) in Northern Chile, further north the species occurs from North Chile to the Central Peruvian coast (Repenning et al. 1971; Guerra and Torres 1987). South American fur seals were hunted intensively for several centuries, with at least 750,000 animals being killed between 1873 and 1983 in Uruguay alone (Seal Conservation Society 2006), which in 1991 became the last country to prohibit hunting (Vaz-Ferreira and Bianco 1998). The species currently numbers are between approximately 300,000 and 450,000 and is stable, being listed in Appendix II under CITES.

Fur seals in general present considerable challenges to systematics. Whether due to their strongly philopatric population structure, allowing local adaptation, or conversely the ease with which they appear to be able to hybridise (e.g., Goldsworthy et al. 1999; Lancaster et al. 2006), there are a number of current debates about the position of species and/or populations (for a review see Rice 1998; Brunner 2004). The South American fur seal is no exception. Based on differences in skull length and width between animals from the Falkland Islands and the rest of the South American coast, King (1954) proposed three subspecies: *A. australis australis* on Falkland Islands, *A. australis galapagoensis* on the Galapagos Islands and *A. australis gracilis* on the remaining coast of South America. Repenning et al. (1971) later attributed species status to *A. galapagoensis* and emphasized the need for additional and more careful systematic studies on *A. australis*, while Oliveira et al. (2005) reported significant differences in the degree of cranial sexual dimorphism between Uruguayan and Peruvian populations, indicating a need for further investigation.

In the current paper we combine genetic and morphometric techniques to determine the level of differentiation between two contrasting South American fur seal populations, one from the Pacific Coast (Peruvian population) and one from the Atlantic Coast (Uruguayan population) of South America and discuss these results on the light of some ESU's concepts.

Materials and methods

Molecular data

Study sites and tissue sampling

For the genetic analysis, we collected South American fur seal tissue samples from two geographically distant populations: Punta San Juan in Peru and Rio Grande do Sul coast in southern Brazil (Fig. 1).

Punta San Juan population. Punta San Juan (15°22′ S, 75°12′ W) is an area in Peru that contains eighteen breeding colonies of fur seals and is protected from public access by a concrete wall (Majluf and Trillmich 1981). Tissue samples were collected in 1994 using piglet ear notch pliers (Majluf and Goebel 1992) from 178 pups born at a colony that has been extensively studied since 1984 (e.g., see Majluf 1987, 1992; Majluf and Trillmich 1981; Majluf et al. 1996, 2000; Arias-Schreiber and Rivas 1998;

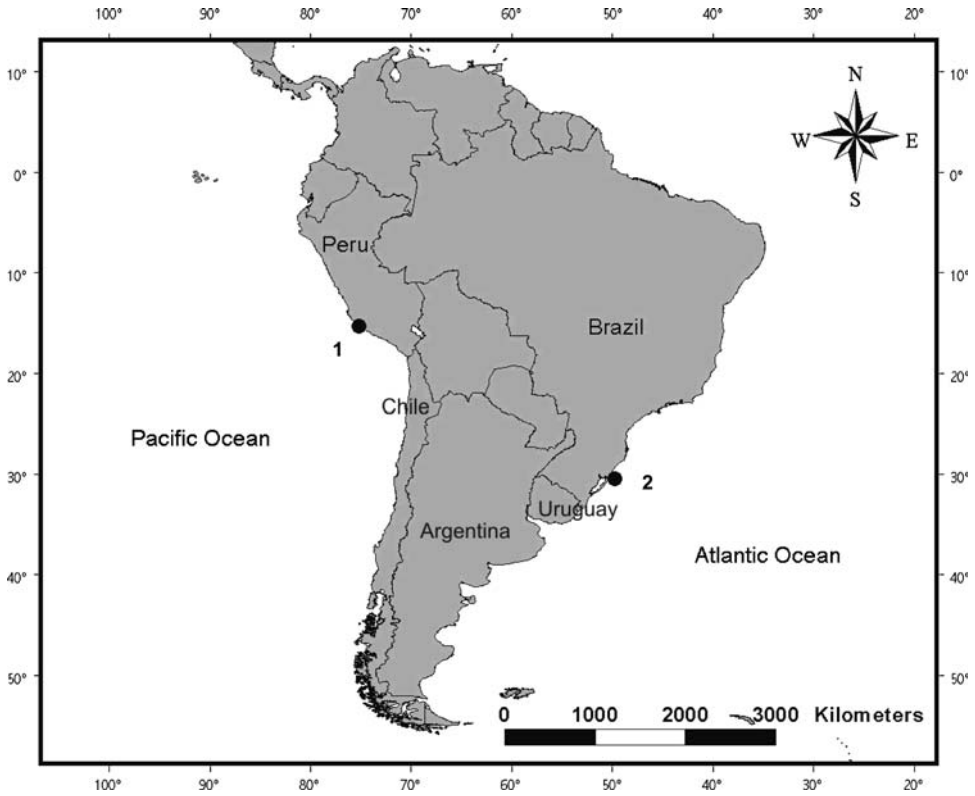

Fig. 1 Study area: 1. Punta San Juan, Southern Peruvian coast (15°22′ S) in the Pacific Ocean and 2. Southern Brazilian coast (29°20′ S) in the Atlantic Ocean, collected specimens belong to the Uruguayan population, according to Pinedo (1990) and Oliveira (2004), see text

Stevens and Bonness 2003; Oliveira et al. 2006). All sampling equipment was sterilized with ethanol between uses. Tissue samples were stored individually in the preservative buffer 20% dimethyl sulphoxide (DMSO) saturated with salt at −20°C (Amos and Hoelzel 1991).

Rio Grande do Sul population. Although there are no breeding colonies of pinnipeds along the Brazilian coast, every year many sea lions and fur seals are found there (Rosas et al. 1994; Simões-Lopes et al. 1995; Oliveira et al. 2001) especially during the austral autumn and spring months. These occur mainly along the coast of the Rio Grande do Sul state and are the result of the dispersal of individuals from their natal colonies after the breeding period. It has been suggested that these movements are influenced by the cold Falklands Current (Pinedo 1990). In addition, there is some tagging information as well as mtDNA cyt *b* and control region and morphology analysis (Oliveira unpublished data; Oliveira 2004; Tuñez et al. 2007) confirming that specimens found on the Brazilian coast are from Uruguay. In this sense, it is well accepted that sea lions and fur seals rest along the southern Brazilian coast during their northward foraging trips after their depart from breeding colonies in Uruguay where there are rookeries at Cabo Polonio (250 km south of the Eastern jetty of Lagoa dos Patos) and Isla de Lobos (Punta del Este, Uruguay) (450 km south of the Eastern jetty). The second closest colony is located at Chubut Province, Argentina, a distance more than 1,300 km of the Southern Brazilian coast. Consequently, in this study we collected 48 tissue samples in 1999 from an area comprising 270 km of sandy beaches between the Lagoa do Peixe National Park (31°15′ S, 50°54′ W) and the city of Torres (29°19′ S, 49°43′ W) in Southern Brazilian coast, and they were considered to be representative from the Uruguayan population.

DNA extraction and microsatellite amplification

Total genomic DNA was extracted using a modified Chelex protocol (Walsh et al. 1991) and genotyped using eight highly polymorphic microsatellite loci as described by Hoffman and Amos (2005): M11a from *Mirounga leonina* (Hoelzel et al. 1999), Hg6.3 and Hg8.10 from *Halichoerus grypus* (Allen et al. 1995), and PvcA, PvcE, Pv9, Pv11 and Pv17 from *Phoca vitulina* (Coltman et al. 1996; Goodman 1998). Any reactions that failed or yielded unclear banding patterns were repeated. To minimize the error rate, all genotypes were independently scored by two different observers and any discrepancies between the two sets of scores were corrected by reference to the original gels.

Data analyses

GENEPOP version 3.1 (Raymond and Rousset 1995) was used to calculate allele frequencies, expected (H_E) and observed (H_O) heterozygosities, to test for deviations from Hardy–

Weinberg equilibrium, homozygote excess and to test for linkage disequilibrium using a Markov chain method (10,000 dememorizations 1,000 batches, 50,000 iterations) following the algorithm of Guo and Thompson (1992). Null allele frequencies were calculated following Brookfield (1996) using the program MICRO-CHECKER (Van Oosterhout et al. 2004). To correct for multiple statistical tests being performed, Bonferroni adjustments (Hochberg 1988) with an α level of $P < 0.05$ were carried out on all tabulated results. A common problem with microsatellite genotyping is 'allelic dropout', in which one allele fails to amplify, leading to heterozygotes appearing as phenotypic homozygotes carrying only one allele (Walsh et al. 1992). Consequently, for loci that exhibited a significant excess of homozygotes, we re-amplified all homozygotes at three different template DNA concentrations. The resulting genotypes were highly concordant, suggesting that allelic dropout was not responsible for any observed deviations from Hardy–Weinberg Equilibrium (HWE).

All 226 individuals were analyzed for genetic variation, genetic differentiation, population structure and the assignment test. However, the number of studied loci after testing for Hardy–Weinberg equilibrium, homozygote excess and for linkage disequilibrium loci, was reduced from eight to seven, because locus Pv17 had to be omitted due to a high frequency of null alleles (see Results).

Using only unlinked loci that were in HWE, we tested the null hypothesis that allelic frequencies were identical across populations by conducting *G*-tests (Sokal and Rohlf 1981). Pairwise comparisons between populations were made for each locus and over all loci using GENEPOP version 3.1 (Raymond and Rousset 1995). We then estimated the extent of population subdivision using Wright's fixation index F_{st} (Wright 1965; Weir and Cokerham 1984), a measure of the reduction in heterozygosity of a subpopulation due to random genetic drift. For comparison, we also calculated R_{st}, an analogous measure designed for microsatellite data that incorporates a stepwise mutation model (Slatkin 1995).

Next, we carried out assignment testing using the genotypes of all *A. australis* individuals plus the genotypes from 50 *A. gazella* pups (Hoffman et al. 2003), a closely related species (Deméré et al. 2003), as a control group of a full species not partitioned in more than one evolutionarily unit. We used the program Geneclass2 (Piry et al. 2004) to generate a three-dimension figure based on the log of likelihood of each genotype belonging to a different potential source population (Waser and Strobeck 1998).

Population structure was further investigated using a Bayesian model-based clustering algorithm implemented using the program STRUCTURE v.2 (Pritchard et al. 2000). This program clusters individuals into subpopulations and to reveal patterns of gene flow across the sampled area. STRUCTURE uses an iterative approach to cluster microsatellite genotypes into K populations regardless of the geographic locations of individuals. The approach is based on the assumptions of Hardy–Weinberg and linkage equilibrium within the resulting clusters, so that the likelihood of K is estimated from the genotype data alone. The highest likelihood value indicates the most likely number of populations in the sample. Individuals can be assigned to one or more populations, including the possibility of admixture. The first step of this analysis involved estimating the numbers of populations (K). Five independent runs for values of K ranging from 1 to 3 with a burn-in length of 10,000–500,000 iterations MCMC were performed, using no prior information and assuming uncorrelated allele frequencies and allowing admixture. In the second step of the analysis, individuals were assigned to each original geographic sample group (using $K = 2$; see Results). Finally to evaluate the STRUCTURE results in determining how indicative an individual's genotype was of the population from which it was sampled, we performed an assignment test (Paetkau et al. 1995). This approach simply calculates the likelihood of drawing a single multilocus genotype from different potential source populations based on the allele frequencies in those populations.

Morphological data

Skull collections

In order to assess morphological differences between the two studied populations we examined skulls of 235 adult specimens of *Arctocephalus australis* deposited in 19 institutions and museums between 1947 and 2004 (a list of all examined specimens is available in the appendix). Of these, 102 were from Peru and 133 were from Uruguay (including 110 specimens collected along the Southern Brazilian coast). As a control group we also examined five skulls from the closely related species Antarctic fur seal, *Arctocephalus gazella*. To avoid variability due to sexual dimorphism and growth, we selected only adult male skulls. Relative age categories were assigned on the basis of condylo-basal length and the degree of suture obliteration (Drehmer and Ferigolo 1997): specimens were considered adults when condylobasal length was >200 mm and the basioccipito-basisphenoid suture was totally fused and closed.

Data analyses

Geometric morphometrics. To analyze differences in size and shape between the Peruvian and Uruguayan populations we used geometric morphometric techniques (see Bookstein 1984, 1989, 1991; Marcus et al. 1993; Rohlf and

Marcus 1993; Monteiro-Filho et al. 2002). Geometric morphometric analysis (in two dimensions) requires digital photographs of whole, unbroken skulls on which a fixed series of landmarks can be identified. We collected 375 images, comprising 165 dorsal views (Uruguay = 103, Peru = 62) and 210 ventral views (Uruguay = 120, Peru = 90), this difference being due to damaged specimens in which one or more landmarks could not be plotted. Images were taken with a Pixera digital video camera connected to a portable computer with an 8–48 mm lens positioned parallel to the molar series. The standard resolution of all images was 800 × 600 pixels, and always included a scale. We also captured 10 images from five specimens of *Arctocephalus gazella* to analyze among-species differences. Thirty-eight anatomical landmarks (Fig. 2), each assumed to be morphologically and topologically equivalent across all of

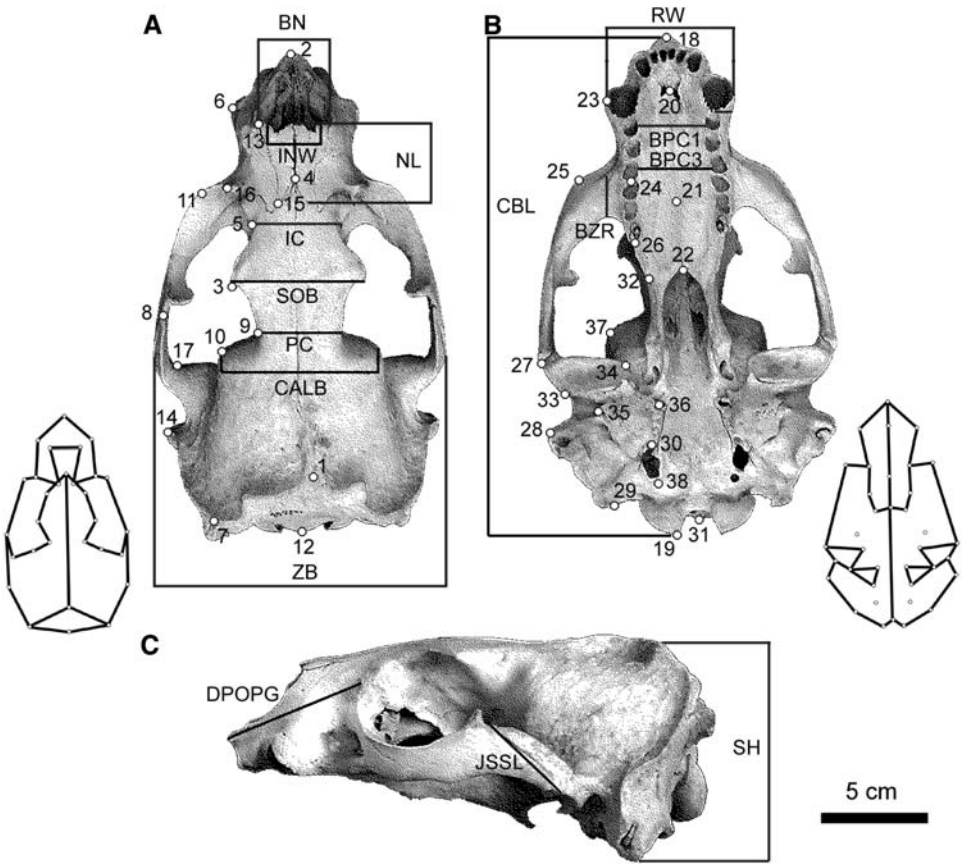

Fig. 2 Numbered landmarks and linear measurements for (**a**) dorsal, (**b**) ventral and (**c**) lateral views of the skull of *Arctocephalus australis*. CBL: Condylobasal length; ZB: Widest zygomatic breadth from posterior margin of squamosals; RW: Greatest rostral width; SH: Skull height, from the occipital crest on dorsal midline to the tympanic bulla; BPC1: Breadth of palate between first post canines; BPC3: Breadth of palate between third post canines; SOB: Supraorbital breadth; NL: Greatest length of nasals; IC: Interorbital constriction; PC: Postorbital constriction; BN: Breath of nasals; INW: Inner nasal width; CALB: Calvarium breath; BZR: Breadth of zygomatic root of maxilla; JSSL: Length of jugal-squamosal suture; DPOPG: Distance between the protuberance of orbit and protuberance of gnathion. Dorsal view landmarks (2A): (1) intersection between the posterior-most point on the sagittal crest and the sagittal extremity of the external nuchal crest; (2) rostral tip; (3) tip of the supraorbital process; (4) frontal–nasal suture; (5) interorbital constriction; (6) external-most point on the curve of the left side of the rostrum (canine alveolus); (7) left posterior-most point on the nuchal crest; (8) intersection between the jugal and squamosal bones; (9) post-orbital constriction; (10) external-most point on the curve of the left side of the calvaria; (11) external-most point of the jugal-maxillary suture; (12) lower-most point on the occipital crest (=occipital end); (13) anterior-most point on left nasal bone; (14) external-most point of the left mastoid process; (15) posterior-most point on left nasal bone; (16) pre-orbital process and (17) inner-most point on the internal squamosal curve. Ventral view landmarks (2B): (18) rostral tip; (19) posterior-most point on the curve of the occipital condyle; (20) point in the middle of incisive foramina; (21) maxilla-palatine suture; (22) rear-most point of palatines; (23) external-most point on the curve of upper right canine alveolus; (24) point between the third and fourth upper right alveoli; (25) point of maximum curvature of the right jugal; (26) posterior edge of the sixth upper right alveolus; (27) intersection between the posterior-most point of the squamosal zygomatic process and jugal; (28) anterior-most point of the mastoid; (29) posterior-most point of the mastoid (limit between the mastoid and exoccipital); (30) carotidal posterior canal; (31) anterior edge of foramen magnum; (32) inferior tip of the hamular process of the pterygoid; (33) external-most point on the curve of right glenoid fossa; (34) interior limit of the anterior part of right glenoid fossa; (35) auditory canal; (36) middle of anterior edge of the medium lacerated foramen (=carotidal internal foramen); (37) maximum curvature of the calvaria and (38) hypoglossal foramen

the specimens, were selected to describe the variation in skull shape and were digitized using the software TpsDig 1.32 (Rohlf 2003).

To avoid inflation of degrees of freedom related to the two bilaterally symmetrical views (dorsal and ventral), landmarks were digitized in one half of each skull and analyses were conducted using this configuration (symmetrical skulls presents exactly the same structures in both sides of the skull, in this sense using half skull analyses we will avoid that the same landmarks be positioned twice and the introduction of extra erroneous degrees of freedom). For graphical representation, skull coordinates were duplicated along the sagittal line using the software GRFND (Slice 1994), following the steps described in Hingst-Zaher et al. (2000). The coordinates produced by TpsDig (Rohlf 2003) were converted into millimeters using the scale included in the image.

Landmark configurations were aligned by General Procrustes Alignment (GPA) using the software TpsRelW 1.25 (Rohlf 2002) with the options $\alpha = 0$, projection orthogonal and include uniform component. The GPA method computes a consensus configuration (least-squares Procrustes average) based on the landmark coordinates of all specimens (see Bookstein 1991, for methodological details). Then, deviations of each individual specimen from the consensus were used to compute a matrix of partial warp scores with the α parameter set to zero to give equal weight to partial warps regardless of scale (Rohlf 1993). Relative warp (RW) scores were computed over the covariance matrix of the partial warp scores (Bookstein 1991), these being equivalent to principal components (PC) of a distribution of shapes in a space tangent to Kendall's shape space. RW scores describe the axes of greatest variation of shape across all specimens. Each relative warp, expressed as a direction of shape change about the mean form, can be interpreted in terms of a transformation that can often be summarized as a thin-plate spline diagram.

As a measure of size that is largely independent of variation in shape, we used centroid size, the square root of the sum of squared distances of a set of landmarks from their centroid (Bookstein 1991). Calculations were performed using the software TPS Regr (Rohlf 2000). Centroid sizes obtained for *A. australis* and *A. gazella* were compared using analysis of variance (ANOVA) followed by a Tukey *post hoc* test.

The scores of the specimens on the two first RW axes were examined to explore the extent to which the skulls' shapes reveal natural groupings. To assess the degree of shape difference between the three groups defined at sampling, we used a Canonical Discriminant Analysis (CDA) (Zelditch et al. 2004) over the partial warp scores (including uniform component). Finally, for a graphical representation we generated thin-plate spline diagrams of skull shape changes of each population, through the regression of shape coordinates over the canonical scores using the software TPS Regr v.1.25 (Rohlf 2000).

Traditional morphometrics. Since geometric morphometrics techniques are relatively new, we also used traditional (linear) morphometrics (Marcus 1990) based on 16 measurements from 235 skulls to provide a comparison with previous studies such as King (1954) and Brunner (2000). Measurements were taken using a 300 mm digital caliper connected to a portable computer and were based on those taken previously for pinnipeds (Reppenning et al. 1971; Kerley and Robinson 1987; Drehmer and Ferigolo 1997; Oliveira et al. 1999) (see Fig. 2). We examined differences among populations and species for each measurement using ANOVA. To detect any *a priori* groups we did a PCA over the covariance matrix of the log-transformed measurements, including five skulls of *A. gazella*, a closely related species (Deméré et al. 2003) to show any species-level differences. The groups thus identified were used in a canonical discriminant analysis (CDA) in order to optimize the differences between populations and minimized within populations (Neff and Marcus 1980).

All statistical analyses were performed using SAS 8.02 (SAS Institute 2003), SPSS 8.0 (SPSS for Windows, Chicago, IL) and Systat 10 (Systat Software Inc., Point Richmond, CA).

Results

Molecular data

A total of 226 individuals from the Peruvian and Uruguayan populations were genotyped at eight highly polymorphic microsatellite loci for genetic diversity analyses (see Table 1 for summary statistics). All of the loci except for Pv17 were in Hardy–Weinberg equilibrium in both populations (Table 1). Because locus Pv17 also exhibited significant linkage disequilibrium with Pvc11 in the Peruvian population, this locus was removed from subsequent analyses. Consequently, all genetic analyses were performed using only seven loci. These were all highly polymorphic, yielding at least six alleles in any population. Allelic richness was somewhat higher in the Uruguayan population (7.83) compared with the Peruvian population (6.65).

Genetic differentiation

The Peruvian and Uruguayan populations differ significantly in their allele frequency distributions (G-test, df = 16, $P < 0.001$, Fig. 3), a result supported by both F_{st} (0.076) and R_{st} (0.136) values, both of which are significant at $P < 0.05$.

Table 1 Measures of genetic diversity of Uruguayan and Peruvian populations of South American fur seal, *Arctocephalus australis*

	Uruguay								
	N^a	NEA[b]	AR[c]	SR (bp)[d]	Heq[e]	Ho[f]	HWE[g] (*P*)	FNA[h]	HD[i]
M11 A	8	2	7.704	146–168	0.812	0.794	ns[l]	0.011	ns
Hg 6.3	10	2	9.669	224–242	0.876	0.923	ns	−0.026	ns
Pvc A	6	0	5.713	149–165	0.738	0.700	ns	0.026	ns
PvcE	10	2	9.006	120–138	0.833	0.756	ns	0.048	*[m]
Pv11	11	4	9.462	156–180	0.817	0.750	ns	0.043	ns
Hg 8.10	9	1	8.135	176–190	0.777	0.643	ns	0.094	ns
Pv 9	7	1	6.911	166–178	0.685	0.739	ns	−0.037	ns
Pv 17	6	2	6.000	159–173	0.733	0.318	***[n]	0.395	***
Mean	8.38	1.75	7.825	–					
	Peru								
	N	NEA	AR	SR (bp)	Heq	Ho	HWE (*P*)	FNA[h]	HD[i]	F_{st}[j]	R_{st}[k]
M11 A	10	3	7.877	146–168	0.816	0.785	ns	0.019	ns	0.0308	−0.005
Hg 6.3	9	1	7.344	224–244	0.805	0.776	ns	0.018	ns	0.0324	0.100
Pvc A	9	3	8.122	149–165	0.805	0.794	ns	0.007	ns	0.1261	0.247
PvcE	9	1	7.014	118–136	0.785	0.837	ns	−0.032	ns	0.0680	−0.009
Pv11	8	1	6.518	164–180	0.780	0.839	ns	−0.036	ns	0.0535	0.161
Hg 8.10	8	2	6.284	178–192	0.744	0.699	ns	0.031	*	0.0749	0.324
Pv 9	8	2	5.534	168–196	0.721	0.654	ns	0.049	ns	0.1416	0.183
Pv 17	6	2	4.475	159–173	0.518	0.223	***	0.398	***	0.1138	−0.006
Mean	8.38	1.88	6.646	–			–	–	–	0.0763*	0.1361*

[a] Number of alleles

[b] Number of exclusive alleles

[c] Allelic richness (mean number of allele per locus)

[d] Size range

[e] Expected heterozygosities

[f] Observed heterozygosities

[g] Hardy–Weinberg Equilibrium

[h] Frequency of null alleles

[i] Heterozygous deficiency after Bonferroni adjustments

[j] Fixation index by Wright (1965)

[k] Fixation index by Goodman (1997)

[l] Non significant to * $P < 0.05$; *** $P < 0.001$

[m] Significant to $P < 0.05$

[n] Significant to $P < 0.001$

Next, we conducted assignment tests using the program Geneclass2, including the genotypes from 50 *A. gazella* pups as a control group of a full species not partitioned in more than one evolutionarily unit. Overall, 93.4% of the specimens were assigned correctly to their own species or original population, the breakdown being 100% of the *A. gazella* individuals, 89.65% of the Uruguayan fur seals and 97.04% of the Peruvian fur seals. This suggests that while *A. australis* genotypes are highly representative of their original colony, greater levels of gene flow exist within *A. australis* relative to between *A. australis* and *A. gazella* (Fig. 4).

Next we used Bayesian analysis within the program STRUCTURE to evaluate the most likely population subdivision scenario for *A. australis* without using the known geographic origin of each individual. The mean likelihood value for five independent runs was greatest at $k = 2$, showing that the two collection sites do indeed reflect two strongly differentiated populations ($k = 2$; ln = −5478.96). Subsequently these two populations' designations were used in an assignment test (see Fig. 5). The results for STRUCTURE for 226 individuals (just using the two populations of *A. australis*) reveal that 98.5% of the

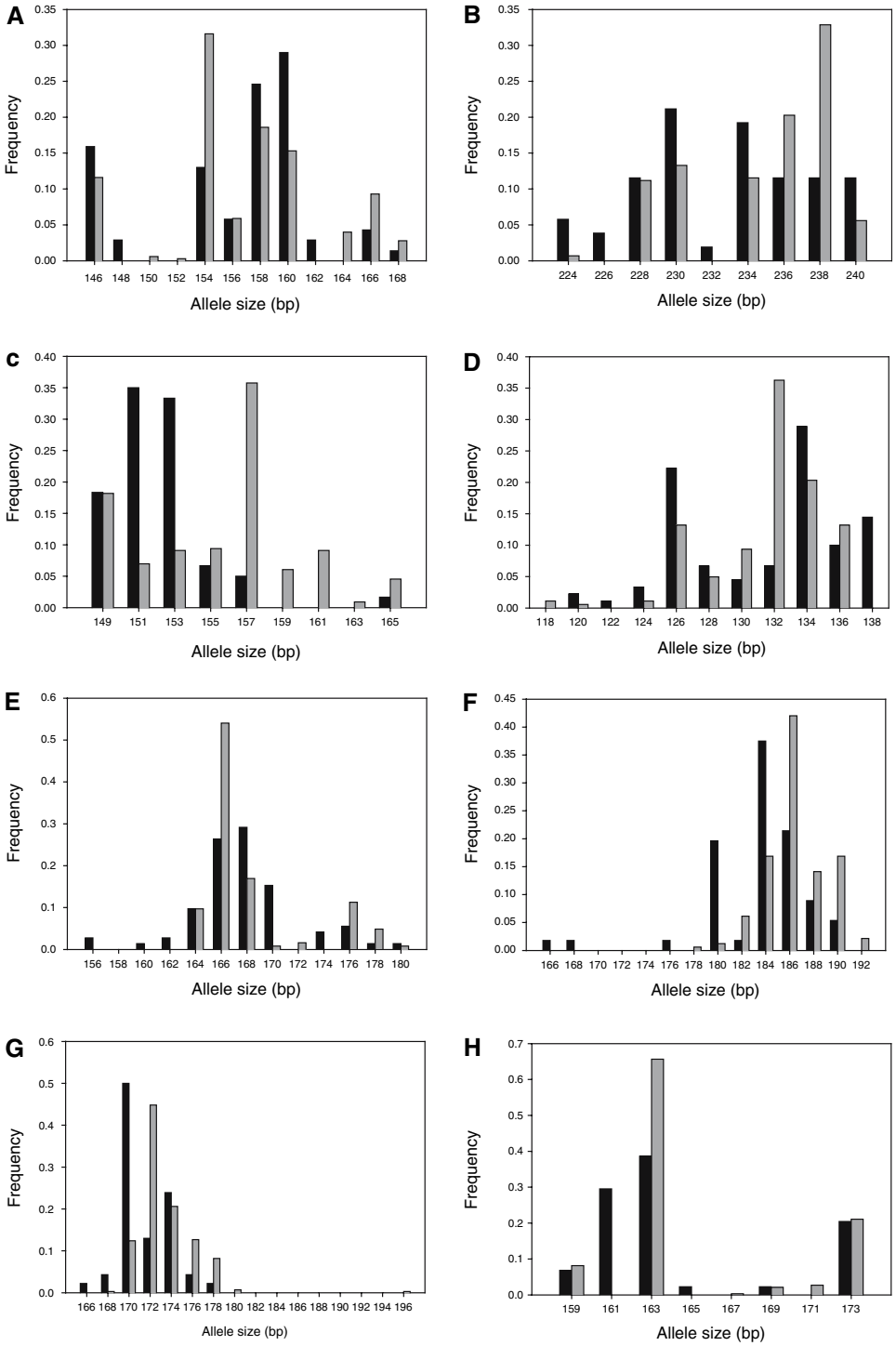

Fig. 3 Allele frequencies to Uruguayan (black) and Peruvian (grey) populations of South American fur seal, *Arctocephalus australis* of eight microsatellite loci. (**a**) Locus M11A; (**b**) Locus Hg6.3; (**c**) Locus PvcA; (**d**) Locus PvcE; (**e**) Locus Pv11; (**f**) Locus Hg 8.10; (**g**) Locus Pv9 and (**h**) Locus Pv17

Uruguayan and 98.8% Peruvian specimens were correctly attributed to their original colony and that no cases of mixed ancestry were inferred (i.e., individuals with membership allocated to both groups of populations and with mean values of the percentage of membership higher than 0.8). Overall, these results indicate a considerable degree of genetic isolation, with gene flow having been low or absent for many generations.

Morphological data

Geometric morphometric analysis

Size. Centroid size (CS) differs significantly between Uruguayan and Peruvian populations (ANOVA: dorsal, df = 169, $F = 4.91$, $P = 0.009$ and ventral, df = 214, $F = 15.29$, $P < 0.0001$) and in both views Peruvian skulls

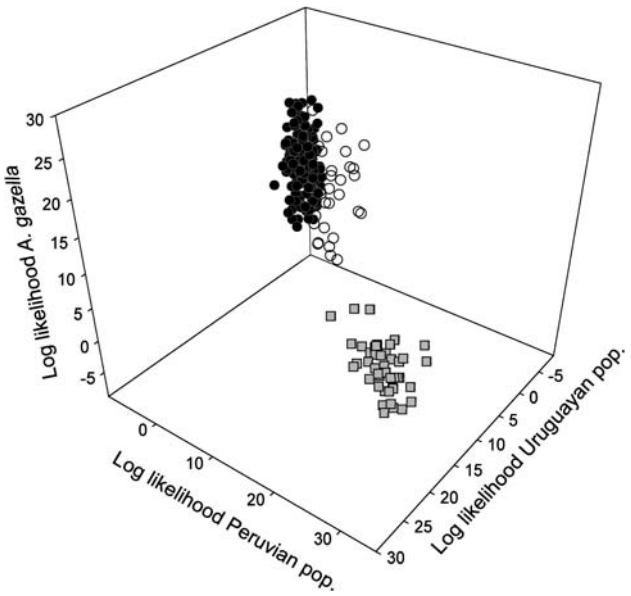

Fig. 4 Assigned genotypes from Antarctic fur seal, *Arctocephalus gazella* (grey squares), Peruvian (black circles) and Uruguayan (white circles) populations of South American fur seal, *Arctocephalus gazella* using log likelihoods calculated from a minimum from four to seven loci (locus Pv 17 was excluded)

(dorsal mean: 268.64; ventral mean: 317.42) were larger than Uruguayan ones (dorsal mean: 261.81; ventral mean: 305.33). Neither *A. australis* populations differed in size from *A. gazella*, probably due to the very small sample size of *A. gazella* ($n = 5$) (dorsal mean: 272.78; ventral mean: 311.24).

Shape. For the dorsal view, the first relative warp explains 25.88% of the shape variation, while the second explains 13.81%. There was a discreet separation between Uruguayan and Peruvian populations of *A. australis*, and *A. gazella* groups with the first one. For the ventral view, first relative warp explains 17.25%, and the second explains 12.53% of the shape variability. Both relative warps are clearly delineating the three groups, showing shape differences among the studied populations and species. For the partial warps analysis, the first canonical axis of the canonical discriminant analysis explains 80% of the observed variation for dorsal view and 81.24% for ventral view. Dorsal and ventral shape differences are summarized in Fig. 6a and b respectively. In both views, *A. australis* populations revealed a clear separation on the first axis while separation between *A. australis* and *A. gazella* is along the second axis. More specifically, for the dorsal view (Fig. 6a), the Peruvian and Uruguayan specimens show a separation along the first axis, with Uruguayan skulls presenting the rostral region, supra-orbital process and post-orbital constriction broader than Peruvian skulls and also a longer brain case. The Peruvian specimens have in general a compact and compressed braincase and narrower nasal bones compared to Uruguayan specimens. For the ventral view (Fig. 6b), skull shapes range from a square to triangular braincase, have an accentuated jugal angle and a broader zygomatic arch in the Uruguayan than in the Peruvian population (Fig. 6b). *A. australis* and *A. gazella* specimens were separated along the second canonical axis in both views (Fig. 6). In the dorsal view *A. gazella* skulls have a highly compressed rostral region when compared with the Uruguayan and Peruvian populations and for the ventral view *A. gazella* specimens are more compressed in the middle part of the skull, (e.g., see grid lines in Fig. 6b).

Mahalanobis distances are significant for the two populations of *A. australis* and *A. gazella* (dorsal: Wilks' lambda = 0.1574, df = 60/276, $P < 0.0001$; and ventral: Wilks' lambda = 0.0841, df = 76/350, $P < 0.0001$). In general, the Uruguayan and Peruvian specimens are closer to one another (dorsal: D2 = 11.59, $F = 12.35$; ventral: D2 = 18.84, $F = 21.05$) than to *A. gazella* (dorsal: D2 *A. gazella*—Peru = 36.83, $F = 4.69$; D2 *A. gazella*—

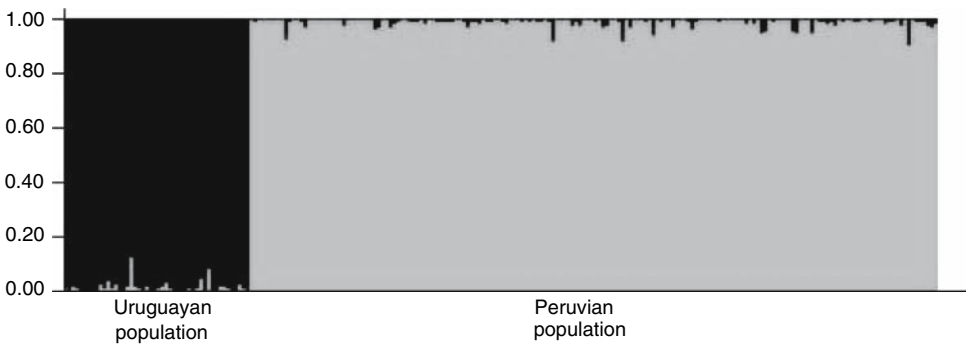

Fig. 5 Best population clustering result ($k = 2$ clusters) in a Bayesian analysis of seven microsatellite loci data (locus Pv 17 was excluded). Assigned individuals were grouped by sampling area: Uruguayan in dark grey and Peruvian in light grey. The bars represent the proportion of ancestry attributed to each population of population of South American fur seal, *Arctocephalus australis*. Plot of STRUCTURE population assignment results coinciding with initial analyses that designated samples from two sampling localities as originating from two groups

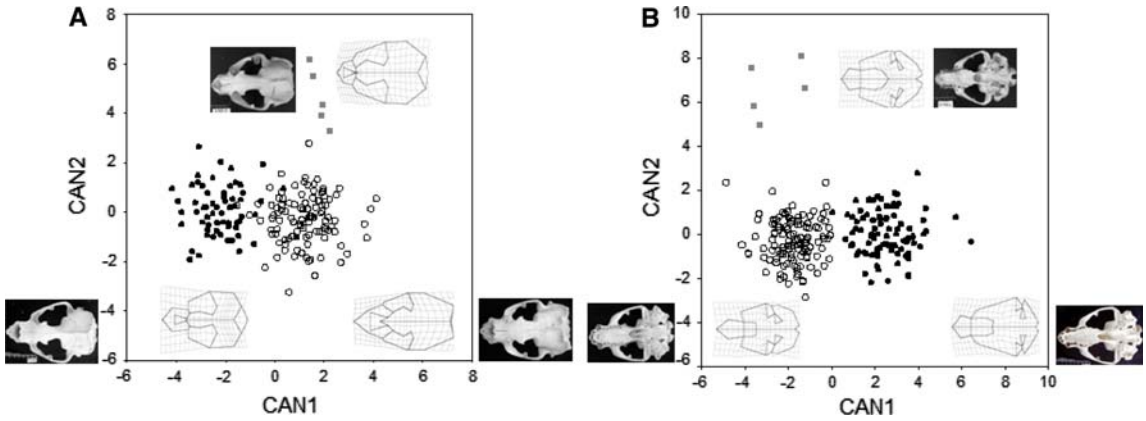

Fig. 6 Scores of the specimens on the first axis of the canonical variates analysis for (**a**) dorsal and (**b**) ventral views. Diagrams are representing extreme skull shapes resulting from regression of shape coordinates over canonical scores (effect intensified 3×). Black circles, *A. australis* from Peru; open circles: *A. australis* from Uruguay and grey squares, *A. gazella*. Pictures from specimens representing each studied population (Peru: PSJ 241; Uruguay: G. 173; *A. gazella*: K.7321)

Table 2 Mean standard deviation (SD) of the 16 skull measurements (mm) from adult male specimens of *Arctocephalus australis* from the Uruguayan and Peruvian populations, and also of *Arctocephalus gazella.* See Fig. 2 for a description of the measurements

	t-test										Anova						
	Uruguay					Peru					*A. gazella*						
Measurements	*n* [a]	Mean		SD [b]	*n*	Mean		SD	df [c]	*P* (*t*-test) [d]	*n*	mean		SD	*F*	df	*P* (Anova) [d]
CBL	131	230.99	+/−	9.67	101	235.33	+/	9.04	230	0.001	5	236.67	+/−	(4.67)	6.55	2	0.002
ZB	129	134.89	+/	7.52	101	140.26	+/	7.97	228	0.0001	5	145.41	+/	(6.22)	52.20	2	0.0001
RW	118	49.91	+/	4.07	85	51.70	+/	4.28	201	0.003	5	53.85	+/	(2.71)	6.08	2	0.003
SH	134	95.49	+/	5.22	101	98.30	+/	5.44	233	0.0001	5	98.41	+/	(4.52)	8.34	2	0.0001
BPC1	130	25.36	+/	2.69	100	25.47	+/	2.76	228	[e]ns 0.773	5	28.10	+/	(1.83)	2.46	2	ns 0.088
BPC3	131	28.48	+/	2.82	101	29.47	+/	2.82	230	0.009	5	31.58	+/	(1.56)	5.77	2	0.004
SOB	134	52.20	+/	2.77	100	52.03	+/	2.81	232	ns 0.636	5	52.74	+/	(0.81)	0.23	2	ns 0.79
NL	107	36.34	+/	3.03	67	35.33	+/	3.59	172	0.048	5	37.13	+/	(4.66)	42.40	2	0.0001
IC	133	33.86	+/	3.42	100	33.68	+/	2.86	231	ns 0.666	5	35.19	+/	(3.96)	0.56	2	ns 0.57
PC	133	30.14	+/	3.19	100	27.31	+/	2.88	231	0.0001	5	34.58	+/	(3.63)	32.49	2	0.0001
BN	99	31.45	+/	2.97	66	27.63	+/	2.20	163	0.000	5	32.26	+/	(1.42)	42.40	2	0.0001
INW	129	32.21	+/	2.79	97	31.37	+/	2.58	224	0.019	5	35.92	+/	(1.06)	8.51	2	0.0001
CALB	132	107.29	+/	5.79	101	110.11	+/	6.33	231	0.0001	5	116.33	+/	(5.73)	10.39	2	0.0001
BZR	132	16.13	+/	1.85	100	17.91	+/	1.96	230	0.0001	5	20.07	+/	(2.02)	31.82	2	0.0001
JSSL	128	36.34	+/	4.01	95	40.81	+/	4.30	221	0.0001	5	30.93	+/	(2.78)	39.87	2	0.0001
POPG	129	70.77	+/	3.95	94	71.03	+/	3.79	221	ns 0.618	5	71.27	+/−	(2.39)	0.15	2	ns 0.859

[a] Number of analyzed specimens

[b] Standard deviation

[c] Degrees of freedom

[d] Significance level for *t* test and Anova

[e] All *P*-values are significant to $P < 0.05$ with except the values with ns = non significant

Uruguay = 24.40, $F = 3.21$; ventral: D2 *A. gazella*—Peru = 69.04, $F = 7.10$; D2 *A. gazella*—Uruguay = 48.48, $F = 5.05$). Nevertheless, the distances between these populations of *A. australis* are highly significant ($P < 0.0001$).

Traditional morphometrics

Descriptive statistics for the linear measurements taken from all three groups are shown in Table 2. Skulls from the

Peruvian population were generally larger than those from Uruguay, with *A. gazella* skulls being the largest of all. Results of the one-way ANOVA, comparing the two populations and also *A. gazella*, indicate that 12 measurements were statistically different between populations (see Table 2) and also between species, suggesting the existence of geographic variation among the Uruguayan and Peruvian populations. For the multivariate analysis, the first principal component (PC1) explained 42.29% and the second (PC2) 14.04% of the total observed variability. The measurements with highest loadings on PC1 were the breadth of the palate between first post canines, the zygomatic root of maxilla and the interorbital constriction. Those on PC2 were the postorbital constriction and the nasals. Most of these measurements are related to skull width, particularly in the rostral region. All loadings for the eigenvectors of the first principal component are positive, indicating that this component mainly reflects differences in size. In contrast, half of the eigenvectors of the second principal component are negative, indicating an important role of skull shape in the separation represented by this axis.

The CVA (Fig. 7) revealed significant differences between the two populations of *A. australis* and also when then compared with *A. gazella* specimens (Wilks' $\lambda = 0.1415$; $F = 12.75$; df = 32/246; $P < 0.0001$). Differences between the two *A. australis* populations were mainly along the first canonical axis (Fig. 7) showing again a strong evidence of geographical variation in skull size of *A. australis.* In addition, the *A. australis* and *A. gazella* specimens are separated along the second canonical axis, probably related to differences in skull shape.

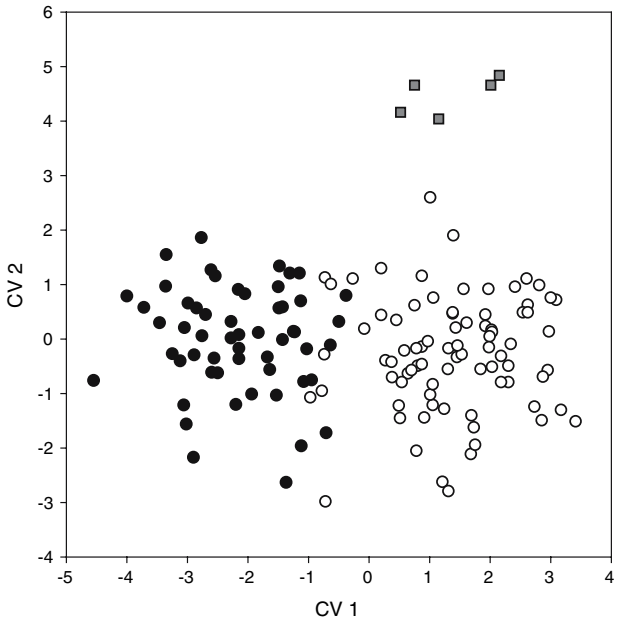

Fig. 7 Axis projection of canonical discriminant analysis for 16 skull measurements in *Arctocephalus australis* belonging to Uruguayan (open circles) and Peruvian (black circles) populations of South American fur seal, *Arctocephalus australis* and Antarctic fur seal specimens, *Arctocephalus gazella* (grey squares). CV1: canonical variant 1; CV2: canonical variant 2

Discussion and conclusions

Here we explored levels of genetic and morphometric differentiation between two geographically isolated populations of the South American fur seal, finding clear differences in both cases. Ours is one of very few studies to combine morphometrics and genetic data to evaluate geographic variation in marine mammals (e.g., Hoelzel et al. 2000; Wang et al. 1999, 2000; Wada et al. 2003).

Lying on opposite sides of the South American continent, it is perhaps not surprising to find genetic differences between the Peruvian and Uruguayan populations, though whether this reflects complete isolation for a short period of time or a potentially much longer period of partial isolation, with limited gene flow occurring via more southern populations remains unclear. Our results support a preliminary analysis of mitochondrial DNA (Túnez et al. 2007), where sequences from Uruguay differed from published sequences from Peruvian seals (Wynen et al. 2001). Neutral genetic differentiation might be hastened by periodic bottlenecks due to El Niño Southern Oscillation (ENSO) events which can drastically reduce food availability and cause major population reductions (Glantz 1996; Majluf 1998). However, in recent times, even strong ENSO events have not pushed the effective population size below approximately 2000, suggesting that in reality the effect of these events may be slight (Oliveira et al. 2006).

In fact, the ENSO events may impact genetic diversity in the Peruvian population rather little because long-lived species with overlapping generations can exhibit a "storage effect", whereby adults ride out tough seasons and usually make it to at least some "good years" when they can transmit their 'stored' variability (Warner and Chesson 1985). Although this effect was originally defined in demographic terms, later studies (Ellner and Hairston 1994, Gaggiotti and Vetter 1999) showed that it is also applicable to the genetic structure. The larger the generation overlap, the smaller the impact of environmental fluctuations on the level of genetic variability maintained by a population (Gaggiotti and Vetter 1999). Indeed, the large environmental fluctuations and intense commercial hunting (Seal Conservation Society 2006; Stevens and Bonness 2003) appears not to have caused any large loss of genetic variability.

The finding of corresponding morphological differences among the populations was perhaps more surprising. The

magnitude of the morphological differences found using traditional morphometric approaches was considerable, with PC1 (size, Neff and Marcus 1980) explaining 42.29% and PC2 (size and shape) explaining a further 14.04% of the total variation. Similarly, geometric morphometric techniques detected significant differences in both centroid size and shape in the dorsal and ventral views of the skull belonging to the two populations of *A. australis*. Shape variation detected using geometric morphometrics was particularly impressive (Fig. 6).

The strong morphological variation between the two populations presented here supports conclusions from previous non-molecular studies. Differences between Peruvian and Uruguayan populations were first observed in female body weight, with Peruvian animals (58 kg, Majluf 1992) being heavier than those in Uruguay (41.7 kg, Lima and Paez 1995). One possibility is that this reflects selection (Lima and Paez 1995; Peters 1983). The Peruvian population of South American fur seal is the second most tropical fur seal population in the world, behind the Galápagos fur seal (*Arctocephalus galapagoensis*), and, as mentioned, faces unpredictable fluctuations in food supply due to El Niño (Cane 1983; Limberger et al. 1983; Majluf 1987, 1991). Such periodic stress may lead to selection for flexible patterns of behavior (Majluf 1991) and perhaps even for a larger body size to provide some level of buffering against lean years. Other differences in the shapes and sizes of the skulls may reflect different life history strategies (Oliveira et al. 2005). For example, breeding behavior appears to be based on leks in Peru but harem holding in Uruguay. The latter implies more intense physical confrontations, potentially selecting for greater robustness in male skulls (Oliveira et al. 2005).

Within the genus *Arctocephalus*, skull morphology can be used for species identification (Repenning et al. 1971). However, this is hampered by the high levels of variability in *A. australis* (King 1954) where the diversity of shape has led to the proposal of three subspecies: *A. australis australis* on the Falkland Islands, *A. australis galapagoensis* on the Galapagos Islands and *A. australis gracilis* on the remaining coast of South America. Subsequently, while Repenning et al. (1971) attributed species status to *A. galapagoensis*, Brunner (2004) reported that males from the Falkland Islands and Punta del Diablo overlapped. In this context, how big and significant are the differences we have found in *A. australis*? Should the two populations be considered evolutionarily significant units (ESUs)? The debate over what unit should be used in conservation biology has been long and convoluted (Cracraft 1983; Ryder 1986; Avise and Ball 1990; Wayne 1992) and one outcome is the concept of the ESUs (Vogler and DeSalle 1994; Moritz 1994a; Waples 1995). ESUs are now widely applied and, according to Karl and Bowen (1999), often correspond to species or subspecies boundaries, but their definition varies from author to author. For example, Moritz (1994a) proposed a definition based on genetic criteria: "ESUs should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies al nuclear loci". Data from mtDNA presented by Túnez et al. (2007) associated with our findings in microsatellites loci support Moritz ESUs concept for the studied populations of South American fur seal.

According to a more general definition provided by Waples (1991): "An ESU is a population (or group of populations) that (1) is substantially reproductively isolated from other conspecific populations, and (2) represents an important component in the evolutionary legacy of the species". In our study, the combination of genetic and morphological differences indicated that Peruvian and Uruguayan populations are reproductively isolated and it is easy to argue that both populations represent an important evolutionary legacy. In particular, their habitats differ considerably. The Peruvian population, despite living in cold waters, is the second most tropical fur seal population in the world, and this may well have led to considerable adaptation, perhaps reflected in differences in breeding systems (Cappozzo et al. 1996; Majluf et al. 1996) and female weight (Majluf 1992) when compared to Uruguayan population Lima and Paez (1995).

In conclusion, we have found significant differences both genetically and morphologically between two populations of *A. australis* that breed on both sides of South America. Although smaller in magnitude than those found between *A. australis* and *A. gazella*, these differences strongly suggest reproductive isolation to the extent that these populations could be considered ESUs. Consequently, we recommend they be managed separately, according to their own life histories and particular conservation problems.

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Appendix

Specimens examined in the skull morphometrics study. The 240 adult specimens (235 *Arctocephalus australis* and 5 *A. gazella*) used in this study were obtained from the following collections:

Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul, Brazil (GEMARS: 0173; 0176; 0185; 0208; 0218; 0256; 0259; 0263; 0278; 0280; 0293; 0297; 0298; 0302; 0308; 0316; 0321; 0338; 0359; 0361; 0364; 0368; 0425; 0429; 0436; 0439; 0445; 0450; 0537; 0542; 0544; 0558; 0561; 0578; 0581; 0582; 0584; 0586; 0589; 0655; 0661; 0681; 0694; 0706; 0721; 0739; 0801), Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul, Brazil (MCN-FZB: 2630; 2637; 2688; 2706; 2886), Laboratório de Mamíferos Aquáticos da Universidade Federal de Santa Catarina, Brazil (LAMAQ-UFSC: 1057; 1063; 135; 1142; 1143; 1149; 1153; 1154; 1156; 1157; 1158; 1159; 1160; 1163; 1166; 1167; 1169; 1170; 1228; 1274), Laboratório de Mamíferos Aquáticos e Tartarugas Marinhas da Fundação Universidade do Rio Grande, Brazil (LMM-FURG: s/no.7; 0101; 0608; 0609; 0663; 0684; 0726; 0731; 0732; 0750; 0754; 0840; 0863; 0890; 1258; 1282; 1336; 1338; 1340; 1341; 1342; 1346; 1431; 1435; 1437; 1438; 1442; 1444; 1464; 1535; 1549; 1554; 1657; 1690; 1738; 1742; 1748; 1781; 1808; 1813; 1815; 1824; 1859; 1898; 1903; 1985; 2045; 2084; 2121; 2267), Centro Nacional Patagónico, Argentina (CENPAT: Aa16), American Museum of Natural History, USA (AMNH: 205916; 205917; 205918; 254562; 254563; 254564; 254565; 254569), Facultad de Ciencias Naturales, Uruguay (FCN: 1522; 1580), National Museum of Natural History – Smithsonian Institution, USA (NMNH: 239140; 504895), British Museum of Natural History, UK (BMNH: 1947.7.16.4; 1984.911; 1984.912; 1984.918; 1984.920; 1984.921; 1984.923; 1984.924; 1984.926; 1984.927; 1984.928; 1984.930; 1984.931; 1984.932; 1984.933; 1984.934; 1984.935; 1984.939; 1984.942a; 1984.947; 1984.948; 1984.949; 1984.969; 1984.972; 1984.973; 1984.975; 1984.978), Proyecto Punta San Juan, Peru (PSJ: 0005; 0008; 0009; 0078; 0143; 0168; 0178; 0180; 0209; 0210; 0216; 0217; 0220; 0221; 0222; 0234; 0236; 0237; 0238; 0239; 0240; 0241; 0242; 0261; 0262; 0263; 0264; 0265; 0266; 0267; 0268; 0287; 0295; 0297; 0298; 0300; 0302; 0304; 0306; 0307; 0319; 0320; 0321; 0322; 0323; 0324; 0325; 0326; 0327; 0328; 0329; 0330; 0331; 0367; 0368; 0369; 0370; 0371; 0372; 0373; 0374; 0375; 0376; 0377; 0378; 0379; 0417; 0418; 0447; 0448; 0450; 0460; 0461; 0462) and Museum of Zoology, University of Cambridge, UK (K.7321K; K.7321L; K.7321M; K.7321N; K.7321O). Total sample examined: *Arctocephalus australis* from Uruguay ($n = 133$) and *Arctocephalus australis* from Peru ($n = 102$); *Arctocephalus gazella* ($n = 5$).

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