

Non-invasive genetic analysis reveals high levels of mtDNA variability in the endangered South-American marine otter (*Lontra felina*)

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Abstract Scats from marine otters were collected from the entire Peruvian distribution range along the Pacific coast. Partial mtDNA control region sequences (265 bp) were successfully amplified and analysed in 37 out of 87 samples. Based on spatial distribution and home range information of marine otters we assumed our final data set to represent at least 24 different individuals, yielding surprisingly high genetic variability (11 haplotypes, $h = 0.86$, $\pi = 0.0117$). No unequivocal evidence of genetic substructuring, a bottleneck or isolation by distance could be detected. This study presents the first genetic data in this endangered species and highlights the significance of the Peruvian gene pool for the establishment of reserves, potential future expansion, recolonisation or translocations.

Keywords *Lontra felina* · Mitochondrial control region · Non-invasive genetic sampling · Peru

Introduction

The marine otter (*Lontra felina*) is the smallest marine mammal of the world and the only *Lontra* species confined solely to marine habitats. This monophyletic genus of otters comprises four species and is the sister to all remaining otters (*Hydrictis*, *Enhydra*, *Lutra*, *Lutrogale* and *Aonyx*) except for the most basal species, the giant otter (*Pteronura brasiliensis*) (Koepli et al. 2008). The historical distribution of the marine otter has been reduced in the

last decades due to anthropogenic factors such as habitat destruction, pollution and poaching, and in the southern part of their distribution range (Cape Horn and Southern Tierra del Fuego) marine otters are on the brink of extinction (Red List of Threatened Species Version 2009.1; Parera 1996; Apaza et al. 2004). It is likely that the population will decrease by at least 50% in the next 30 years (Red List of Threatened Species Version 2009.1). Therefore, the species is listed as Endangered by the IUCN, in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), in Appendix I of the Convention on Migratory Species (CMS), and it is protected by Peruvian law.

Marine otters occur along the Pacific coast of South America from 6° S (today more probably 8° S, personal observation) to 56° S in Peru, Chile and Argentina, but because they depend upon habitats with rocky outcroppings and caves above the water at high tide, the distribution is disjunct (Larivière 1998; Red List of Threatened Species Version 2009.1). Chile holds the largest number of animals; studies on spatial distribution (Medina-Vogel et al. 2008) showed that strong predictors for marine otter presence are rocky seashore patches, and studies on spacing behaviour (Medina-Vogel et al. 2007) showed home ranges of <5 km along coastlines and suggested availability of land refuges and food as decisive factors in the species' distribution.

Research on the status of *L. felina* in Peru has so far been limited to some basic census surveys with divergent results: While earlier studies described numbers of about 200–300 (Castilla and Bahamondes 1979), a more recent survey yielded an estimate of c. 700 animals for the Peruvian coast (Apaza et al. 2004). In light of the bleak prospect for the coming decades, the species is in urgent need of help but as yet, no genetic analyses on marine

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otters have been carried out, although knowledge on genetic variability and structure is of great relevance to conservation measures. The present study from the Peruvian part of the species' distribution range, therefore, aims at closing this gap by providing the first molecular data for this endangered mustelid. We particularly want to quantify the amount of genetic diversity left in marine otters and also analyse its spatial distribution to find out if there are already clear signs of disrupted gene flow among the remnant populations along the coast.

Materials and methods

Scat samples were collected along the Peruvian Pacific coast from Vesique (9°12'S, 78°29'W) to the port of Vila (18°07'S, 70°43'W), covering more than 1400 km of coastline and the entire distribution range of marine otters in Peru (IUCN-OSG 1998), in August and September 2008. Samples came from four main regions: Norte, Lima, Ica and Sur (Fig. 1) which were separated by 340, 347 and 370 km of unsampled coastline. Altogether 25 localities were visited, and a total of 87 samples were taken at 20 of them. Faeces were primarily collected at latrines used for scent-marking. Fresh scats were preferred as it has been shown that success rates of DNA amplification drop substantially after a few days (Murphy et al. 2007). Samples were taken using surgical gloves, stored in 96% ethanol, sealed with Teflon tape and transferred to a refrigerator as quickly as possible. DNA was extracted from the samples after they had been transferred to Germany (about 2 months after collection) using the QIAMP DNA stool mini kit. A blank extraction control was included to monitor potential contamination. A portion of the mitochondrial control region was amplified with the primers DLH (5'-CCTGAAGTAAGAACCAGATG-3') and ProL (5'-CACCACCAACACCCAAAGCT-3') at an annealing temperature of 57°C and 35 amplification cycles. Amplicons were then sequenced with an automated MegaBACE sequencer.

Sequences were aligned using BioEdit (Hall 1999) and collapsed into haplotypes using the collapse function implemented in the FaBox package available at <http://www.birc.au.dk/fabox/> (Villesen 2007). Because we used faecal material, we used the following approach to validate our results: each sample was sequenced in both directions, and all haplotypes were confirmed by at least two independent PCR and sequencing runs, i.e. singletons were re-analysed, and in case of haplotypes occurring more than once, at least one sample was re-analysed. No differences were detected within any pair of runs for the same individual which we interpreted as evidence of reliable sequences.



Fig. 1 Geographic location of the sample sites of marine otter faeces along the Peruvian coast within four sampling groups

Marine otters are mostly solitary, and radio-tracking of animals in Chile revealed that home ranges comprised no more than 4134 m of coastline (Larivière 1998; Larivière and Jennings 2009; Medina-Vogel et al. 2007). Therefore, identical haplotypes were only treated as different individuals if the sample locations were at least 5 km apart.

Haplotype and nucleotide diversity were calculated with DnaSP (Rozas and Rozas 1999). Differentiation among the four sample regions was tested for with Arlequin (Excoffier et al. 2005) by means of an analysis of molecular variance (AMOVA) and pairwise Φ_{ST} -values under the Tamura and Nei model of nucleotide substitution (this model was closest to the GTR model inferred for our data set with Findmodel (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>) which is not implemented in Arlequin). To test for a pattern of isolation by distance we applied a Mantel test on matrices of the number of pairwise nucleotide differences and geographical distances between individuals.

Haplotype relationships were depicted by a median-joining network with the Network software (Bandelt et al. 1999). Networks are more appropriate for intraspecific phylogenies than tree algorithms because they explicitly allow for the co-existence of ancestral and descendant alleles in a sample, whereas trees treat all sequences as terminal taxa (see Posada and Crandall 2001 for a review). Demographic history was inferred by a mismatch analysis and calculation of Fu's F_s (Fu 1997) with the Arlequin software. Mismatch analyses depict the frequency distribution of pairwise differences between the sequences found in a sample. This distribution is dependent upon demographic

processes. Unimodal distributions are typical of populations that experienced a recent expansion, while ragged and multimodal distributions are found in populations at demographic equilibrium (Rogers and Harpending 1992) and bimodal distributions often result from admixture of two previously separate lineages. Bottlenecked populations typically produce L-shaped distributions with a single peak at low pairwise differences. Arlequin tests for a mismatch distribution fitting the sudden expansion expectations. Fu's Fs statistic is a neutrality test but can also be used to infer demographic histories with large and statistically significant positive values of Fs being indicative of a deficit of rare haplotypes compared to expectations for a stable population and thus being a sign of a bottleneck, while large and significant negative values suggest an excess of recent mutations (many rare haplotypes), which is typical of recent expansions.

Results and discussion

Forty-one out of the 87 collected scat samples were amplified for a fragment of the mtDNA control region. Four samples yielded shorter sequences and were not included in subsequent analyses, leaving 37 successfully sequenced samples. The final alignment comprised 265 bp. The success rate of amplification was 43%, a value similar to those from other scat-based analyses (e.g. Centrón et al. 2008; Sharma et al. 2009). GenBank comparisons ruled out the possibility of contamination from other species by showing highest sequence similarity of our data with the North-American *Lontra canadensis* (no marine otter sequences are available). Thirteen polymorphic sites with 16 mutations (nine transitions, two transversions and five indels) defined 11 haplotypes (accession numbers GU982296-GU982306) one of which (LF08) was present in all four sampling groups

Table 1 Distribution and frequencies of the 11 haplotypes found in the present study with geographic coordinates within four sampling groups

Haplotype	Sampling groups				Total
	Norte	Lima	Ica	Sur	
LF01	–	–	–	3 17°00'S 72°06'W 17°01'S 71°02'W 17°17'S 71°28'W	3
LF02	–	1 12°28'S 76°47'W	–	–	1
LF03	–	1 12°28'S 76°47'W	–	–	1
LF04	1 9°43'S 78°17'W	–	–	–	1
LF05	–	1 13°01'W 76°29'W	–	–	1
LF06	–	–	–	1 17°17'S 71°28'W	1
LF07	–	1 13°01'S 76°29'W	1 15°26'S 75°04'W	2 18°00'S 70°53'W 18°01'S 70°50'W	4
LF08	3 9°13'S 78°29'W 10°05'S 78°10'W 9°56'S 78°13'W	1 12°57'S 76°30'W	1 15°26'S 75°04'W	3 18°00'S 70°63'W 18°01'S 70°50'W 18°07'S 70°43'W	8
LF09	–	–	–	1 17°59'S 70°53'W	1
LF10	–	–	–	1 18°07'S 70°43'W	1
LF11	–	1 12°28'S 76°47'W	–	1 18°00'S 70°53'W	2
Total	4	6	2	12	24

(Table 1). Thirteen samples of the 37 were excluded from further analyses because they showed haplotypes identical to others within a distance of 5 km (possible pseudoreplication). Therefore, calculations were based on a final data set comprising 24 sequences assumed to be from different individuals (see Table 1). This sample size is identical or similar to previous genetic analyses of other *Lontra* species in South America (Centrón et al. 2008; Trinca et al. 2007).

Overall haplotype and nucleotide diversity was 0.86 (± 0.053 SD) and 0.0117 (± 0.002 SD), respectively. None of the calculated overall or pairwise Φ_{ST} -values was significantly different from zero (all $P > 0.18$). A significant substructuring could, therefore, not be detected with our data. However, in spite of the ubiquitous distribution of LF08 this may be due largely to the small sample sizes within the four groups. In the absence of further data these groups should be considered as sample regions rather than genetically defined units. Further sampling and high-resolution markers such as microsatellites may elucidate differentiation along the Peruvian coast. We found no significant correlation of pairwise genetic and geographical distances between individuals (Mantel test, $r = -0.024$, $P = 0.795$).

Network analysis (Fig. 2) revealed two frequent central haplotypes, but also a number of relatively distant satellite haplotypes of low frequency. On average, two sequences differed by 3.094 mutations from one another, and the mismatch analysis did not reject the null hypothesis of a sudden demographic expansion (sum of squared deviations

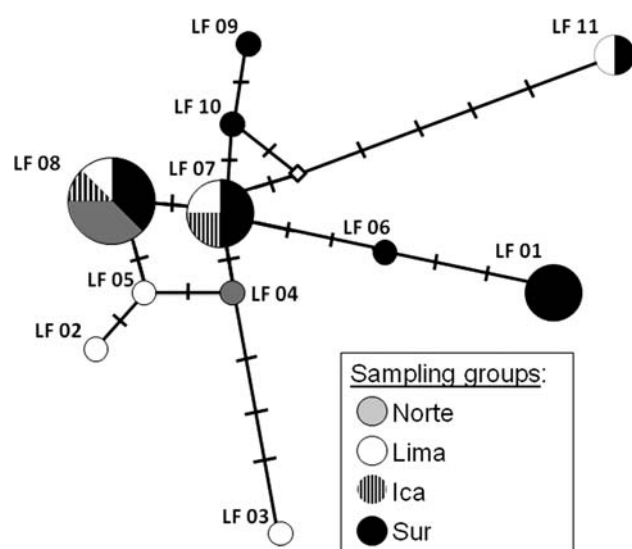


Fig. 2 Median-joining network of the eleven marine otter mtDNA control region haplotypes found in this study. Circles are proportional to haplotype frequencies and show haplotype origin. The white diamond denotes a median vector; black dashes refer to mutational events

between observed and expected mismatch distribution: 0.01, $P = 0.68$; Harpending's raggedness index: 0.03, $P = 0.84$). Fu's F_s statistic was negative (-2.94) and just above the significance threshold ($P = 0.063$) when transitions, transversions and indels were all weighted equally, but a significant ($P = 0.007$) and slightly negative (-0.785) value was yielded when mutations were weighted according to their intrinsic ratio (1:1.8:4.5 for transitions, indels and transversions, respectively). These results may tentatively be interpreted as hinting at an expansion event (in line with the mismatch analysis).

Although widespread and endangered, relatively little is known about marine otters in spite of a recent conservation focus on this species (e.g. Medina-Vogel et al. 2007, 2008). Perhaps the most important of our results is the unexpectedly high genetic variability. The 24 marine otters of the present study yielded more haplotypes at a short fragment of the control region than did the entire European population of *Lutra lutra* at a longer one. Overall haplotype diversity and mean nucleotide diversity in Eurasian otters were reported as 0.16 and 0.0006, respectively (Ferrando et al. 2004 and references therein), but recent studies have revealed higher diversity for Eurasian otters in the UK (0.73 and 0.003, Stanton et al. 2008) and Ireland (haplotype diversity of 0.75, Finnegan and Néill in press). Still, marine otter haplotype diversity and nucleotide diversity were considerably higher, the latter at least by an order of magnitude, although population sizes of *L. lutra* are doubtless much larger. This genetic depletion is even more pronounced than that of the sea otter (*Enhydra lutris*), which suffered a loss of an estimated 99% of its population due to persecution but still yielded mean haplotype diversities of 0.41 (Larson et al. 2002). Comparable data for other *Lontra* species are rare, but a recent study of Southern river otters (*Lontra provocax*), also classified as Endangered by the IUCN, from two disjunct regions in Argentinean Patagonia yielded only a single mtDNA control region haplotype in 13 individuals, although four different cytochrome *b* haplotypes were found in 34 animals analysed (Centrón et al. 2008). In an analysis of 491 bp of the mtDNA control region the third South-American *Lontra* species, the Neotropical otter (*Lontra longicaudis*), was found to exhibit a haplotype diversity similar to ours (0.82), but a more than twofold lower nucleotide diversity of 0.0049 (Trinca et al. 2007). Neotropical otters are also considered to be threatened, but, due to lack of information, their present IUCN status is Data Deficient. As it seems, the marine otter is genetically the most diverse South-American *Lontra* species. Both the network with its various rare and divergent haplotypes and the demographic tests (mismatch analysis and Fu's F_s) did not show obvious signs of a bottleneck but rather some weak indication of a population expansion.

It is also noteworthy that we did not find significant substructuring in the mitochondrial genome, suggesting that either there is gene flow among the sample localities or that the recent disruption of the meta-population has not yet resulted in significant differentiation through genetic drift. The fact that we did not find an isolation-by-distance pattern has to be viewed with caution given our approach: because we excluded identical sequences within the same 5 km, we actually might have considered whole maternal lineages as a single individual, which may bias correlations between geographical proximity and genetic similarity. In any case, disjunct occurrence is not only the result of human-caused habitat fragmentation; the specific habitat requirements of marine otters result in a naturally disjunct distribution pattern, and adaptations to dispersal may facilitate gene flow despite human pressure and mitigate the negative effects of fragmentation (long-distance swimming has been observed by one of us, J.V., in the field). Future analyses making use of high-resolution markers (microsatellites) are needed to further evaluate if there is differentiation along the Peruvian coast or not.

The bleak prospect of future population reductions of 50% or more within the next 30 years will certainly lead to a substantial loss of gene pool diversity. Our results are a first step towards a genetic underpinning for future conservation which should include information on the partitioning and distribution of genetic diversity. They are also interesting with respect to possible reserve areas. Some twenty islands and ten mainland sites along the Peruvian coast are managed by the government as guano harvesting areas. Based on the law Decreto Supremo N° 024-2009 MINAM, these areas will be transformed into a system of reserves (*Reserva Nacional Islas y Puntas Guaneras*) comprising a total area of more than 100000 ha to protect threatened populations of seabirds (Humboldt penguin, Peruvian diving petrel, inka tern, Peruvian booby), marine mammals (South American fur seal, South American sea lion, marine otter) and reptiles (sea turtles). Although this measure will help maintain meta-populations of marine otters and facilitate gene flow, areas with high levels of genetic diversity along the southern Peruvian coast are not included. The genetic data presented here suggest that protection in the south should be implemented in the conservation program and also provide information to select appropriate individuals should translocations and re-stocking of abandoned areas become necessary. Further genetic studies based on larger sample sizes, nuclear loci and, above all, covering also the Chilean and Argentinean part of the distribution range are what is now most urgently needed.

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