

# Phylogeography of the Marine Otter (*Lontra felina*): Historical and Contemporary Factors Determining Its Distribution

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## Abstract

The evolutionary history of a species can be revealed by phylogeographical analysis; nevertheless, not only historical but also contemporary processes can imprint on the distribution of genetic diversity. We report on the phylogeny of *Lontra* ssp. in South America, and the role of spatial heterogeneity in shaping the distribution and population structure of the endangered marine otter, *Lontra felina*. Analyzing a total of 2261 bp of mitochondrial DNA (mtDNA) revealed the recent divergence of *L. felina* from *L. provocax*. A strong population structure ( $\Phi_{st} = 0.83$ ,  $P < 0.0001$ ) and a significant pattern of isolation by distance were described for *L. felina* ( $n = 168$ ) across a wide geographical distribution (13°53' S to 43°36' S). *Lontra felina* mtDNA phylogeny is composed of 2 main clades: a clade from Peru and another composed of Chilean haplotypes. Northern populations show different divergent lineages and higher genetic diversity when compared with more recently colonized southern populations. Furthermore, long sandy beaches seem to act as barriers to dispersal, creating 2 evolutionary significant units in agreement with subspecies previous description, and at least 5 different management units (MUs). At a fine spatial scale, the size of rocky seashore patches, the distance between patches and anthropogenic factors also play important roles in species gene flow.

**Key words:** climatic change, conservation, fragmented habitat, phylogeny, phylogeography, otter

A total of 13 otter species are found in the world in 7 distinct genera, with *Lontra* the most representative genus in America. Most of these charismatic species are top predators in their respective communities and are vulnerable or at risk of extinction (IUCN 2010). Otters are widespread in aquatic environments from tropical to cold-temperate regions. Although otters are mostly dependent on freshwater habitats, all but 4 species have also been recorded along seacoasts (Kruuk 2006). Only 2 species, however, are adapted to a strictly marine existence: the sea otter (*Enhydra*

*lutris*) distributed along the North Pacific Ocean and the marine otter (*Lontra felina*) found in the southeast Pacific Ocean.

The evolution of the Lutrinae led to 3 major clades, based on nuclear and mitochondrial markers: a clade grouping the sea otter and river otters of Eurasia and Africa (*Aonyx*, *Enhydra*, *Hydricis*, *Lutra*, and *Lutrogale*); a second clade grouping the marine otter and river otters from North, Central, and South America (*Lontra*), and a basal lineage of *Pteronura brasiliensis* (Koeplfi and Wayne

1998; Koepfli, Deere, et al. 2008; Koepfli, Kanchanasaka, et al. 2008). From this phylogeny, South American *L. felina* and *L. longicaudis* appear to have diverged from the North American river otter (*L. canadensis*), approximately 2.8–3.4 million years ago (MA, Koepfli, Deere, et al. 2008), corresponding to the Panamanian land bridge formation. For many Mammalian taxa, including carnivores, a rapid radiation occurred soon after the colonization of South America (Marshall et al. 1979, 1982). For the genera *Lontra*, however, only 3 species are found, each with a markedly allopatric distribution. Among these species, *L. felina* is particularly interesting because it seems to be the most recently evolved species of mammal to have adapted to marine living conditions (Estes 1989).

The marine otter or “chungungo” as its known in Chile, lives on exposed rocky seashore (Castilla and Bahamondes 1979; Ostfeld et al. 1989; Medina-Vogel et al. 2006, 2007) along the Pacific coast from 9°12'S, near Chimbote (Perú; Apaza et al. 2003), to 56°S in Cape Horn and Isla de los Estados (Chile and Argentina; Olrog and Lucero 1981). The northern limit of the species distribution is determined by the end of the influence of the Humboldt Current System (HCS) and consequently the reduction in food availability. This distribution ranges from tropical to cold sub-Antarctic habitats, which contrasts with the apparently reduced capacity of *L. felina* to tolerate cold water temperatures. Indeed, due to its small size and reduced body fat, *L. felina* has to compensate for heat loss imposed by low water temperatures by increasing heat production and by spending limited time in the water (Medina-Vogel et al. 2007, 2008). Radio-tracking studies have shown that the marine otter spends more than 80% of the time on land, mostly resting inside dens, and going into the water mainly to feed (Medina-Vogel et al. 2007). Thus, there is an intriguing contrast between the potential to colonize a wide range of coastal habitat and a limited adaptation to the cool water environment.

Marine otter populations are distributed along a latitudinal range that experienced a series of historical and contemporary oceanographic and climatic events. For instance, the northern part of its range is recurrently affected by El Niño Southern Oscillations (ENSO). During ENSO events, the Chile–Peru cold marine waters are invaded by warm oceanic subtropical and equatorial waters, increasing superficial temperatures by 3–5 °C (Camus 1990). ENSO leads to shifts in primary productivity and plankton species composition, affecting trophic efficiency and interactions among higher level consumers (Thiel et al. 2007). Similarly, the southern limit of *L. felina* distribution likely experienced strong perturbations associated with glacial cycles. Throughout the Last Glacial Maximum (LGM, 23 000–17 000 years ago [ya]) an ice sheet covered southern Chile from 56 to 35°S in the Andes Mountains and to 41°S in the lowlands and at sea level (McCulloch et al. 2000). Glacial cycles generally result in species range contractions into lower latitudes followed by range expansions during interglacial periods (Hewitt 1996, 2000, 2004).

In contrast to the North Pacific sea otter (*E. lutris*), *L. felina* is solitary and highly dependent on the coastal marine

habitat. Dens are composed of a set of galleries under rocks with easy and close access to the water. Consequently, its presence in an area is associated with den availability and the short distance to food resources. Marine otters exhibit small home ranges, less than 4.1 km of lineal coast, and females exhibit intrasexual territoriality, which has not been detected for males or between genders (Medina-Vogel et al. 2007). It was recently demonstrated that *L. felina* incidence on the rocky seashore is also dependent on landscape spatial structure, such as the size of continuous rocky seashore patches and the distance between those patches (Medina-Vogel et al. 2008). Complete absence of *L. felina* in 2 of 8 study areas, of approximately 100 km long, was associated with the low percentage (<22%) of rocky seashore present around 35°58'S and 38°59'S, respectively (Medina-Vogel et al. 2008). At a broad scale, both the number and the extent of sandy beaches increase from north to central-south Chile, reaching a maximum between 34°S and 38°S (Thiel et al. 2007). Likewise, the southern coast of Peru is characterized by extended sandy beaches. *Lontra felina* dispersal ability is unknown, leading to the question of whether such habitat discontinuities can act as barriers to dispersal, thereby increasing isolation and genetic divergence.

*Lontra felina* is classified as an endangered species by the International Union for Conservation of Nature (IUCN 2010). The *L. felina* has been intensively killed for fur, being almost exterminated on the southern limit of its distribution (Chehébar 1990). Cases of otters predated by dogs has been increasing, adding to the threats of illegal hunting, habitat destruction, degradation, competition with humans for prey, and accidental death in crab pots (Medina-Vogel et al. 2004, 2006). Consequently, the rapid increment of human settlements along the coast has further increased population threats and isolation (Medina-Vogel et al. 2008).

In order to clarify the evolutionary history of the marine otter, the objectives of this study were 3-fold: 1) to make inferences regarding the origin and colonization history of *L. felina* in the narrow coastal habitat of Peru and Chile; 2) to make inferences regarding the susceptibility of the endangered *L. felina* to climatic changes; and 3) to make inferences regarding the importance of spatial structure of its terrestrial habitat relative to effective population size and patch connectivity. Two scenarios can be addressed to explain marine otter speciation and colonization: 1) a northern origin resulting from the colonization of the west Andes mountains by *L. longicaudis* with adaptation to the coastal habitat, in a region characterized by very scarce rivers in the Atacama desert but a highly productive marine environment due the HCS or 2) a southern divergence from the southern river otter (*L. provocax*) distributed along the freshwater environment and the marine channels of Patagonia, with progressive adaptation to the coastal marine habitat. The phylogenetic relationships among the 3 South American *Lontra* species are described for the first time in this study to test these scenarios. The geographic distribution of intraspecific lineages was also analyzed to further investigate the evolutionary history of *L. felina*. Indeed, the genetic signature of the origin and colonization routes of the species

along the southeast Pacific coast could be altered by more recent processes, such as ENSO and glacial cycles, which likely affected the present day pattern of genetic diversity. Finally, we analyzed the correlation between the spatial structure of terrestrial habitat and the distribution of genetic diversity in order to investigate the potential role of terrestrial habitat availability on the species' evolution and on its potential susceptibility to local and global changes. We used mitochondrial DNA (mtDNA) control region (CR), NADH dehydrogenase subunit 5 (ND5), and cytochrome *b* gene (Cyt-*b*) sequences from tissue and feces samples of *L. longicaudis*, *L. provocax*, and *L. felina*, characterizing the phylogeographic pattern of marine otter across most of its range, with a more intensive sampling in 3 regions in order to investigate the role of the spatial partition of terrestrial habitat on its distribution and connectivity.

## Materials and Methods

### Sample Collection and DNA Extraction

A total of 419 samples were collected along the Peruvian and Chilean coasts, from 9°56'S to 43°36'S: 16 blood samples were extracted from captured individuals, 46 tissue samples from carcasses of natural mortality, and 357 scat (feces) samples were collected between the years 2004 and 2008. *Lontra felina* were captured for blood samples according to the protocol described by Soto-Azat et al. (2006, 2008). At a fine scale, 3 focal areas (1, 2, and 3) were selected for more intensive sampling, according to the degree of rocky seashore length, patchiness, and isolation by sandy beaches. All rocky seashore fragments from these 3 areas were entirely surveyed and all fresh feces ( $n = 233$ ) collected. Intensive prospecting in 2 other extensive areas evidenced a complete absence of the species from Algarrobo (33°22'S) to Peninsula Tumbes (36°36'S) and from Arauco Bay (37°10'S) to Queule (39°23'S). Two southern river otter (*L. provocax*) samples from different locations were used as out-group. Fresh feces were collected and preserved in 95% ethanol. For DNA extraction from feces, 2 ml ethanol from each samples was centrifugated at 13 000 rpm for 10 min, and the pellet was processed with DNeasy Tissue Kit (QIAGEN). Tissue and blood samples were directly extracted using the DNeasy Tissue Kit (QIAGEN) according to manufacturer's protocol.

### PCR Amplification

Based on *L. felina* CR mtDNA sequences obtained from primers L15926 (Kocher et al. 1989) and CCR-DR1 (5'-CTGTGACCATTGACTGAATAGC-3') (Tchaicka et al. 2007), we designed specific internal primers LfCR-F2 (5'-GCACCCAAAGCTGACATTCT-3') and LfCR-R2 (5'-GTTGTGCGATGCGGATAAAT-3') and the external primers LfCR-F1 (5'-CTCAAGGAAGAAGCGACAGC-3') and LfCR-R1 (5'-ACCTTATGGTTGTGCGATGC-3') to improve amplification from feces DNA. For NADH dehydrogenase subunit 5 (ND5) gen, we used primers ND5-

DF1 (5'-TTGGTGCAACTCCAAATAAAAAGT-3') and ND5-DR1 (5'-AGGAGTTGGGCCTTCTATGG-3') from Trigo et al. (2008) or ND5-DF1 and LfND5-R (5'-GGTTGGTCATGAAGATTTGTTG-3'), the latter designed from our *L. felina* sequences. Specific Cyt-*b* primers LfCYTB-1F (5'-ACCCACCCATTAGCCAAAAT-3') and LfCYTB-1R (5'-CCGACAGCTGGTATGAGGAT-3') were designed from a published *L. felina* sequence (AF057122; Koepfli and Wayne 1998). For some samples with lower DNA quality and amount, internal primers LfCYTB-2F (5'-TATCGGCCATCCATACATT-3') and LfCYTB-2R (5'-ATCGTGTTAGGGTGGCTTTG-3') were used. All primers were designed using Primer 3 (Rozen and Skaletsky 2000).

Polymerase chain reactions (PCRs) were carried out in a 50  $\mu$ l volume containing 4  $\mu$ l of DNA, 1 $\times$  reaction buffer, 1.5 mM of MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP, 0.4  $\mu$ M of each primer, and 1 unit of *Taq* DNA polymerase Platinum (Invitrogen). The PCR protocol was as follows: 10 min at 95°C, a touchdown of 95°C for 15 s, 60–50°C for 30 s, 72°C for 45 s, with 2 cycles at each annealing temperature, and 35 amplification cycles of 95°C for 15 s, 50°C for 30 s, 72°C for 45 s, followed by a final extension period of 30 min at 72°C. PCR product was checked with a 0.8% agarose gel electrophoresis, visualized with ethidium bromide, purified using QIAquick PCR purification Kit (QIAGEN), and sequenced by Macrogen Inc. (Seoul, South Korea). All samples were sequenced at least once in both directions. Most of the samples that did not amplified well at the first CR PCR were discarded. All *L. felina* CR, Cyt-*b*, and ND5 haplotype sequences have been deposited in the GenBank database under accession numbers GQ843765–GQ843802.

### Data Analysis

The sequences were aligned, and mutations were confirmed by eye, according to the chromatogram using Proseq v2.91 (Filatov 2002). All sequences were realigned using CLUSTALX v1.83 (Thompson et al. 1997).

For phylogenetic and phylogeographic analyses, we assessed the congruence of the evolutionary rates among CR, ND5, and Cyt-*b* by the partition homogeneity test (1000 permutation) using PAUP v4.0b8 (Swofford 2000), which identified congruence among the 3 data sets ( $P = 1$ ; CR, ND5, Cyt-*b*) and the 2 data sets ( $P = 1$ ; ND5, Cyt-*b*) allowing concatenating the different sequences. We used maximum parsimony (MP), maximum likelihood (ML), and Bayesian (BA) methods to reconstruct phylogenetic *L. felina* lineages relationship using CR, ND5, and Cyt-*b* and combined mtDNA sequences (CR+ND5+Cyt-*b*) with *L. provocax* and *Lu. lutra* as out-group. The most appropriate model of DNA evolution for the sequences was estimated with MODELTEST v3.06 (Posada and Crandall 1998), via the Akaike information criterion (AIC) for ML and BA reconstruction methods. The model selected for the 3 combined markers was HKY+I+G. MP and ML trees were constructed by PAUP v4.0b8 (Swofford 2000). ML was

performed with heuristic searches with 100 random sequence addition replicates, and nodal support was assessed using nonparametric bootstrap analysis with 1000 pseudoreplicates. BA was performed by MrBAYES v3.1.2 (Huelsenbeck and Ronquist 2001) using the general type of the best-fit model parameters defined for the data set, in which 4 independent analyses were run with 4 chains each, for 6 million generations and then sampled at intervals of 1000 generations. The first 25% of sampled trees were discarded to ensure stabilization and the remaining used to compute a consensus tree. The split frequency was below 0.004, confirming that sampling was from the posterior probability distribution.

To reconstruct the *Lontra* genus phylogeny using the latter analysis and estimate divergence time, *L. felina* and *L. provocax* sequences were compared with *L. longicaudis*, *L. canadensis*, *Aonyx capensis* (Koepfli and Wayne 1998; Koepfli, Deere, et al. 2008; Koepfli, Kanchanasaka, et al. 2008), and *Lu. lutra* sequences (mitochondrion genome, NC\_011358). The more conserved genes ND5+Cyt-b were used for this phylogeny, and the model selected via the AIC was TIM+I. Divergence time among *Lontra* lineages (*L. canadensis*, *L. longicaudis*, *L. provocax*, and *L. felina* clades) was estimated based on the fossil record from the genus *Lontra* found in North America (3.6–1.8 MA; van Zyll de Jong 1972; Willemssen 1992). Rates were estimated using BA, as implemented by the program BEAST v1.4.8 (Drummond and Rambaut 2007). The molecular clock assumption was relaxed by allowing the rate to vary throughout the tree in an autocorrelated manner, with the rate in each branch being drawn from normal distribution whose mean was equal to the rate in its parent branch. Four independent runs of 10 000 000 steps were performed with parameters logged every 2000 steps, and burn-in of 1 000 000 trees. Parameters analysis was visualized in Tracer v1.4.1 (Drummond and Rambaut 2007).

Relationships between CR haplotypes within *L. felina* were examined by constructing median-joining network (MJN) by use of Network v4 (Bandelt et al. 1999). Molecular diversity indices, such as haplotype (*h*) and nucleotide ( $\pi$ ) diversity were calculated using ARLEQUIN v3.11 (Excoffier et al. 2005). We also analyzed the frequency distribution of pairwise differences between haplotypes. Sudden demographic expansion (Rogers and Harpending 1992) and spatial expansion (Excoffier 2004) models were fitted to the observed mismatch distribution using 1000 bootstrap. Tajima's *D* neutrality statistic (Tajima 1989) and  $F_s$  value (Fu 1997) were calculated to detect deviations from a neutral Wright–Fisher model of mutation-drift equilibrium.

A spatial analysis of molecular variance (SAMOVA) based on *F* statistics was performed with SAMOVA v1.0 (Dupanloup et al. 2002) to investigate the geographic partition of the genetic diversity. SAMOVA 1.0 implements a method to define groups of populations, geographically maximizing  $F_{CT}$  values (among group variance). SAMOVA was performed using data from 33 populations designated by each rocky seashore patch to identify groups of populations. We performed the analysis using 2–12 groups.

The significance of variance components and  $\Phi$  statistics was tested by 1000 random permutations. All estimations were performed using ARLEQUIN v3.01 (Excoffier et al. 2005). We also tested isolation by distance (IBD) by performing a Mantel test on  $F_{st}/(1 - F_{st})$  against geographic distances (km), with 10 000 permutations in ARLEQUIN v3.01. At the finer scale, isolation due to the extent of sandy beaches was investigated by estimating genetic substructure ( $\Phi_{st}$ ) in each of the 3 extensively sampled areas. In order to evaluate the effects of patch sizes and distances among them on the patch's genetic diversity and the genetic differentiation among patches, we compared *h*,  $\pi$ , and mean pairwise differences using a nonparametric Kruskal–Wallis test in SYSTAT v12 (Wilkinson et al. 1996). Patch size and distances between patches were estimated by satellite image in Google Earth. Patch sizes (1.64–33.81 km) were grouped into 4 categories, and pairwise distances between patches (2.3–61.4 km) were grouped into 5 categories.

To reduce the likelihood of replicate samples, we used the combination of mtDNA haplotype and 6 microsatellite loci (04OT17, 04OT05, 04OT22, 04OT07, Lut733, and Lut782; Dallas and Piertney 1998; Huang et al. 2005) for individual identification (Vianna JA, unpublished data). Each genotype was used to distinguished individuals in each rocky seashore patch or group of samples surrounding each location.

## Results

### Sequencing and Haplotype Identity

From the total of 419 samples, 168 were successfully sequenced for the mtDNA CR, including feces ( $n = 126$ ), blood ( $n = 16$ ), and tissue ( $n = 26$ ). Success rate for amplification and sequencing of feces samples was about 35% (124 of 357), reflecting the variable quality of these samples. Using the mtDNA haplotypes and microsatellites genotyping, we could identify a minimum of 89 individuals within the overall 126 feces samples. Moreover, no repeated multilocus genotypes were observed within a same sampled site or location (data not shown), suggesting that very few, if any, samples were replicates. Analysis of 570 bp CR sequences revealed 22 haplotypes and 18 polymorphic sites, including 4 indels (Table 1). ND5 sequences (684 bp) from a subset of 92 samples revealed 7 different haplotypes defined by 6 variable sites (Table 1). Nine Cyt-b haplotypes (1007 bp) were recovered from a subset of 38 samples. Based on the 3 linked markers, 19 haplotypes could be identified with 7 additional haplotypes only sequenced for CR and ND5, providing a total of 26 haplotypes (Tables 1 and 2; Figure 1). On the subsequent analyses, the more conserved mtDNA sequences, ND5+Cyt-b, were used to understand *Lontra* ssp. phylogenetic relationship, whereas the whole data set (CR+ND5+Cyt-b) were used for the understanding of *L. felina* phylogeography. Lastly, the faster evolving CR sequences were utilized for *L. felina* population structure and fine-scale analyses of focal areas.

**Table 1** The variable sites defining mtDNA haplotypes by CR, ND5, and Cyt-b sequences in *Lontra felina*

Haplotype	CR															ND5					Cyt-b																
	0	0	1	1	1	1	1	1	2	2	2	2	2	3	3	3	4	5	0	0	2	3	4	5	0	0	1	1	2	2	4	5	6	6	6	8	9
	6	8	2	3	3	3	4	4	1	1	2	6	9	4	6	6	5	5	2	8	9	5	0	2	2	9	0	4	3	7	9	5	4	5	6	7	5
A.I.A	T	T	—	C	—	—	C	A	C	C	A	A	A	T	C	A	A	C	C	C	C	A	G	C	G	A	A	T	G	C	A	A	T	G	A	G	A
B.I.B																																					G
C.II.C																																					A
C.II.D																																					A
D.II																																					N
E.III.D																																					N
F.II.D																																					N
G.II																																					N
H.III.D																																					N
I.III.D																																					N
I.IV																																					N
J.II																																					N
K.II.D																																					N
L.II.D																																					N
M.IV.E																																					N
N.IV.E																																					N
O.II.D																																					N
P.II.F																																					N
Q.II																																					N
R.V.G																																					N
S.IV																																					N
S.II.F																																					N
S.VI.H																																					N
T.VI.H																																					N
U.VI.I																																					N
V.VII																																					N

A total of 26 haplotypes are shown, 7 of which were not sequenced for Cyt-b and are represented by “N.” Dashed line represents indel on the alignment for CR sequences.

**Phylogeny and Phylogeography**

Among the South American otters, MP, ML, and BA analyses for ND5+Cyt-b sequences (Figure 2) revealed that *L. felina* and *L. provocax* are sister species and monophyletic with *L. longicaudis*. *Lontra felina* and *L. provocax* are closely related species with 1.4% of sequences divergence when compared with 5.4% divergence between *L. longicaudis* and *L. provocax* + *L. felina*. The divergence time between *L. longicaudis* and *L. provocax* + *L. felina*, based on a *Lontra* spp. radiation, was estimated at 1.57 MA. (95% highest posterior density (HPD): 0.56–2.61 MA) for concatenated ND5+Cyt-b, whereas the sister species *L. provocax* and *L. felina* diverged about 883 000 ya (95% HPD: 0.16–1.89 MA). Two major divergent haplogroups were observed for *L. felina*, one composed with Peruvian haplotypes and the other including all Chilean haplotypes. Divergence among Peruvian and Chilean haplogroups was estimated at 490 500 ya (95% HPD: 0.05–1.24 MA). The diversification of Chilean *L. felina* haplogroup was estimated at 308 300 ya (95% HPD: 0.04–0.90). The most recent divergence occurred about 35 100 ya (95% HPD: 0.0001–0.21 MA) between the 2 southernmost haplotypes.

Phylogenetic reconstruction (MP, ML, and BA) of CR+ND5+Cyt-b for *L. felina* haplotypes confirmed the

high divergence and reciprocal monophyly of Chilean and Peruvian haplogroups (Figure 3). Sequence divergence between the 2 clades was 0.5%, a higher value when compared with the within Chilean clade divergence of 0.2%. Separate and combined phylogenetic reconstruction for mitochondrial sequences also showed the same Peruvian and Chilean divergence. However, single markers lacked phylogenetic resolution within Chilean clades. Concatenating the 3 markers (CR+ND5+Cyt-b) showed 6 clusters within the Chilean haplogroup in the Bayesian analysis, according to their geographic locations: clade 2 composed by haplotypes from Norte Grande and Norte Chico; clade 3 and 4 from Norte Chico; clade 5 and 6 from Central Chile, and clade 7 from Central-South and South. Consequently, higher divergence characterized the northern Chilean haplotypes (Norte Grande, Norte Chico) compared with other areas. Central-South haplotypes were the most basal, whereas southern Chilean haplotypes showed the most recent divergence within clade 7.

As in the phylogenetic reconstruction, the MJN for CR and CR+ND5+Cyt-b haplotypes showed differentiated clusters according to the geographical locations (Supplementary Material 1A,B). The most widespread CR haplotype (S) was

**Table 2** Distribution of *Lontra felina* CR, ND5, and Cyt-b haplotypes by sampled geographic location. Gray shading represent the haplotype presence in the location. Boxes around locations are the 3 focal study areas (1, 2, and 3). Horizontal black bar represents the discontinuity areas without *L. felina* populations

Localities	Latitude (S)	N	mtDNA		Haplotypes																					
			Cytb	CR	A	B	C-D	D	D	D	D	D	D	D	D	E	E	E	F	F	G	H	H	I		
			ND5	CR	I	I	VII	II	II	III	II	II	II-IV	IV	II	II	II	IV	IV	II	II	II	V	VI	VI	VI
Lagunillas	13°53'	1																								
La libertad	15°29'	1																								
Matarani	17°00'	2																								
Punta Coles	17°42'	1																								
Burros	18°03'	1																								
Vila Vila	18°10'	3																								
Chanavaya	20°53'	4																								
Guanillos	21°13'	4																								
Tocopilla	22°05'	1																								
Caleta Fierro	22°38'	1																								
Punta Lobos	23°03'	1																								
Mejillones	23°28'	1																								
Punta Coloso	23°47'	3																								
Caleta El Cobre	24°20'	2																								
Pan Azucar	26°08'	3																								
Soldado	26°10'	11																								
C. Castillo	26°12'	4																								
C. Chañaral	26°16'	2																								
Punta Choros	29°15'	4																								
Dama Island	29°15'	5																								
Arrayan	29°43'	15																								
Panul	30°03'	10																								
Lagunillas	30°05'	1																								
Guanaqueros	30°11'	5																								
Tongoy	30°15'	1																								
Punta L. Vaca	30°17'	2																								
Talcaruca	30°28'	1																								
Limarí	30°47'	1																								
C. Morrito	31°06'	1																								
C. Sierra	31°07'	1																								
Norte L. Vilos	31°49'	1																								
Palo Colorado	32°01'	10																								
Puquen	32°11'	8																								
La Ballena	32°18'	1																								
Papudo	32°26'	16																								
El Pangue	32°31'	2																								
Cachagua Island	32°35'	3																								
Quintero	32°46'	3																								
Quintay	33°11'	12																								
33°21'-36°36'			[Horizontal black bar]																							
Colcura	37°07'	7																								
37°10'-39°23'			[Horizontal black bar]																							
Mehuín	39°25'	3																								
Pilolcura	39°40'	2																								
Puñuil-Chiloé Island	41°55'	4																								
Ahuenco-Chiloé Island	42°06'	1																								
Guafo Island	43°36'	2																								

a central haplotype on the MJN (Supplementary Material 1A). However, the CR haplotype S was differentiated by the other 2 sequences (ND5 and Cyt-b), according to each geographical location (north, central, and south; Tables 1 and 2).

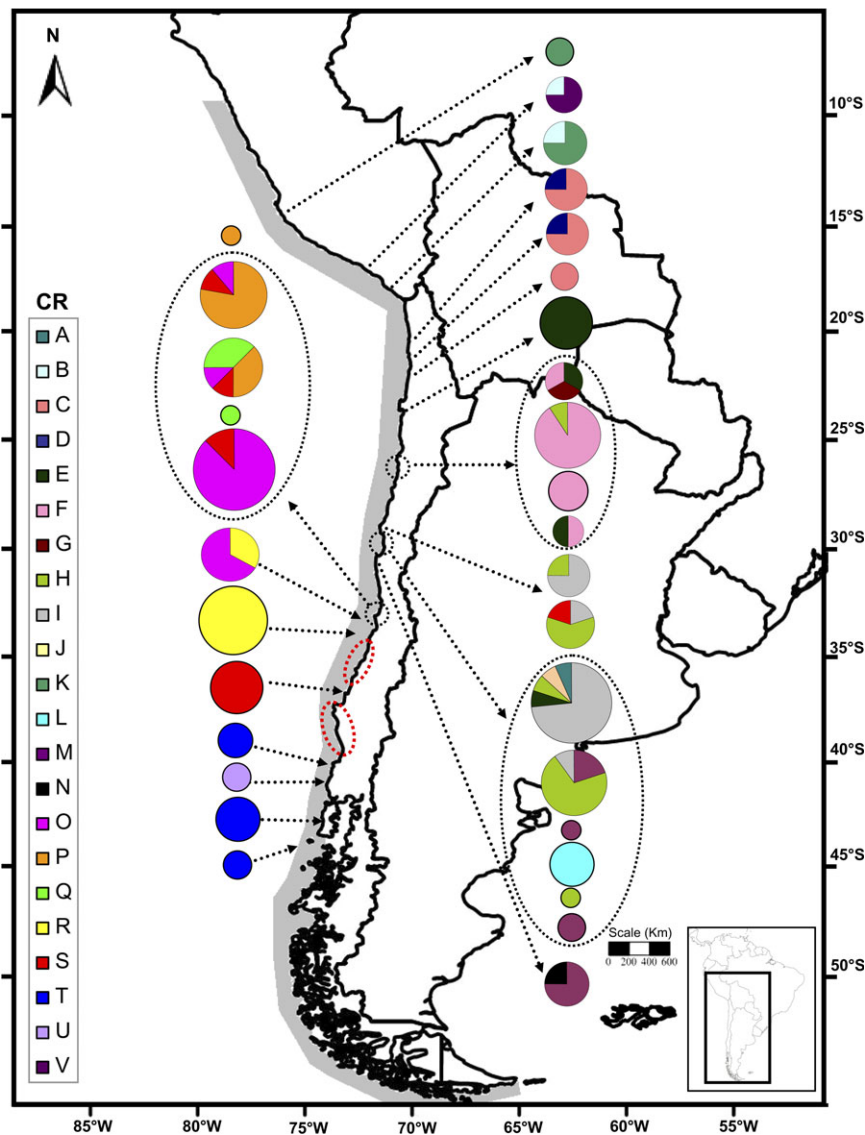
***Lontra felina* Population Structure**

The CR sequences of *L. felina* shared a pattern of high haplotype diversity ( $0.9315 \pm 0.0055$ ) but low nucleotide

diversity ( $0.004749 \pm 0.002810$ , Table 3). Each geographical location showed characteristic haplotypes (Table 2, Figure 1).

The SAMOVA of CR reported increased  $F_{CT}$  values ( $F_{CT} = 0.45-0.75$ ,  $P < 0.0001$ ) related to an increase in number of groups ( $K = 2-10$ ). High population structure was found for all group combinations ( $\Phi_{st} = 0.86-0.77$ ,  $P < 0.0001$ ). During most of the SAMOVA simulations,

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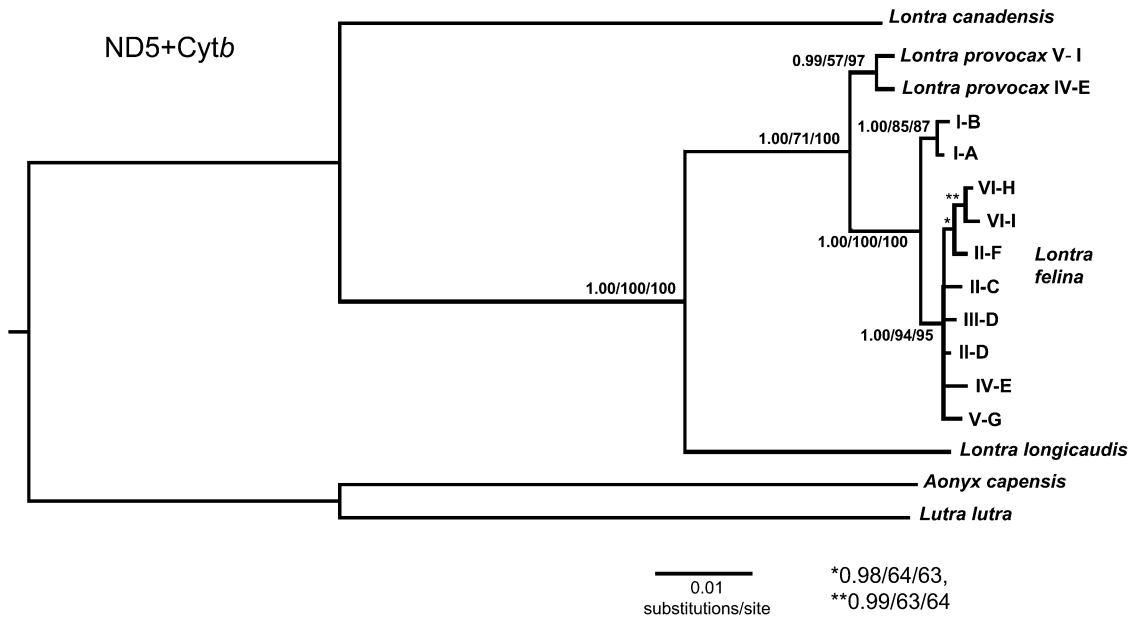


**Figure 1.** The distribution of marine otter (gray shading), and the regional frequencies of mtDNA CR haplotypes (colored piecharts) in Chile and southern Peru represented. The circle area of piecharts reflects the sample size, and disk sectors are proportional to haplotype frequencies depicted as distinct colors. A total of 26 concatenated haplotypes are represented, however, haplotypes C.II.C and C.II.D are joined in one column, as well the haplotypes I.II.D and I.IV.D. Black dotted circles around piecharts are the 3 study focal areas (Area 1, Area 2, and Area 3), and red dotted circles are areas with no reports of the species.

Peru was separated from all Chilean samples. However, no partition was spatially coherent to the lineal *L. felina* distribution allocated by SAMOVA. Thus, we also performed a SAMOVA for the CR+ND5 concatenated sequences which also showed an increase in  $F_{CT}$  values ( $F_{CT} = 0.38-0.73$ ,  $P < 0.0001$ ) and  $\Phi_{st}$  values ( $\Phi_{st} = 0.86-0.81$ ,  $P < 0.0001$ ) related to an increase in K. In this case, the SAMOVA partition spatially coherent to the lineal *L. felina* distribution was 5 groups for the concatenated CR+ND5, with  $\Phi_{st} = 0.82$  and  $P < 0.0001$ . Although all southern samples were grouped together because of their common ND5 haplotype, we separated these samples a posteriori into 2 different groups (Central-South and

South) based on their geographic isolation by extensive sandy beaches. In addition, this partition did not significantly modify the overall genetic structure ( $\Phi_{st} = 0.83$ ,  $P < 0.0001$ ). Consequently, 6 groups were finally defined as the best partition of mtDNA structure: Peru ( $13^{\circ}$  to  $18^{\circ}$ S), Norte Grande ( $20^{\circ}$  to  $24^{\circ}$ S), Norte Chico ( $26^{\circ}$  to  $31^{\circ}$ S), Central ( $31^{\circ}$  to  $33^{\circ}$ S), Central-South ( $37^{\circ}08'S$ ), and South ( $39^{\circ}$  to  $43^{\circ}$ S).

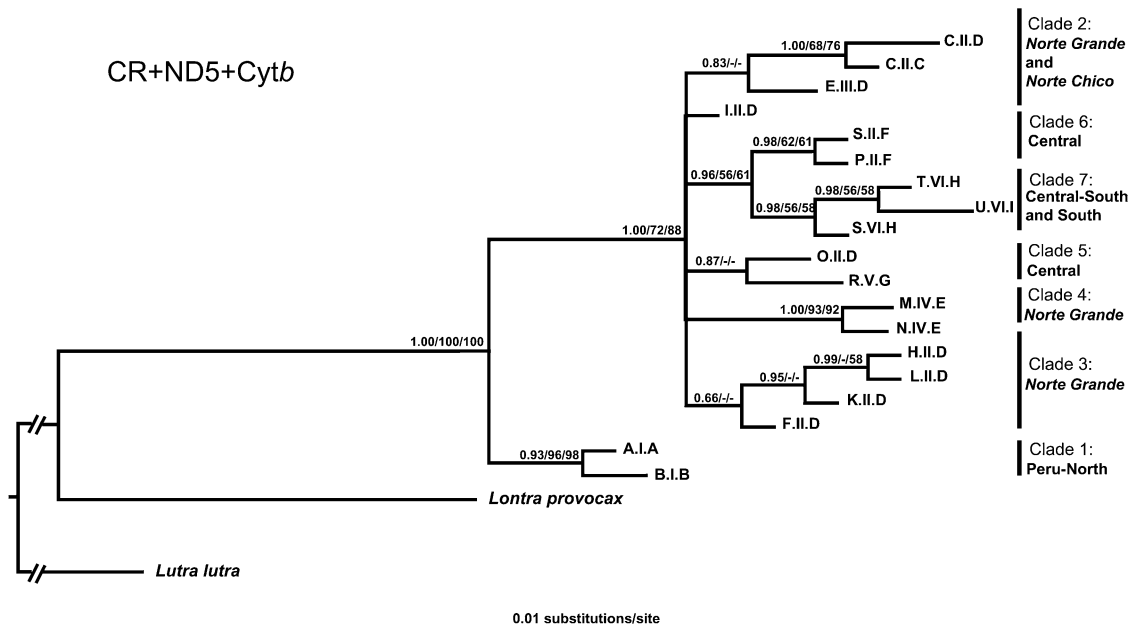
Higher CR genetic diversity was found for Peru ( $b = 0.67$ ), northern ( $b = 0.63$  and  $0.83$ ), and Central ( $b = 0.74$ ) Chilean groups compared with South ( $b = 0.39$ ) and Central-South ( $b = 0$ ; Table 3). The number of haplotypes was higher in Norte Chico (Hap = 11) followed by Central



**Figure 2.** Bayesian consensus phylogram of *Lontra felina* and other otters (*L. provocax*, *L. longicaudis*, *L. canadensis*, and *Aonyx capensis*, and *Lu. lutra*) constructed using 1691 bp of mtDNA ND5 and Cyt-b sequences. Nodes are numbered with posterior probabilities (BA) and bootstrap (ML and MP).

Chile (Hap = 5) and declining toward the northern and southern extremes of the distribution (Hap = 1 to 3). A significant pattern of IBD was detected analyzing CR marker when comparing all locations ( $R^2 = 0.44$ ,  $P < 0.0001$ ) or the 6 previously mentioned groups ( $R^2 = 0.39$ ,  $P = 0.08$ ).

*Lontra felina* showed significant deviation from neutrality for Fu's test ( $F_s = -6.83$ ,  $P = 0.03$ ) but not for Tajima's  $D$  test ( $D = 0.06$ ,  $P = 0.63$ ). Mismatch distribution was slightly bimodal for all *L. felina* samples. Sudden demographic expansion and spatial expansion models showed low bootstrap support for the observed mismatch



**Figure 3.** Bayesian consensus phylogram of *Lontra felina* haplotypes, *Lontra provocax* and *Lu. lutra* as out-group, constructed using 2261 bp of mtDNA CR, ND5, and Cyt-b sequences. Nodes are numbered with posterior probabilities (BA) and bootstrap (ML and MP).



**Table 3** Sample size (*N*), polymorphic sites (*S*), and number of haplotypes (Hap) for each of the mtDNA sequences (CR, ND5, and Cyt-b), and CR haplotype (*b*) and nucleotide diversity ( $\pi$ ) for 6 groups defined by geographical areas for *Lontra felina*

Geographic areas	Geographic location (latitude)	CR-570 bp			Diversity		ND5-684 bp			Diversity		Cyt-b-1007 bp		
		N	S	Hap	<i>h</i>	$\pi$	N	S	Hap	<i>h</i>	$\pi$	N	S	Hap
Peru-North	13°53'–18°09'	9	8	3	0.67	0.0034	7	1	2	0.48	0.00070	2	1	2
Norte Grande	20°53'–24°20'	17	3	3	0.63	0.0022	10	1	2	0.56	0.00081	4	2	2
Norte Chico	26°08'–31°08'	67	7	11	0.83	0.0034	35	1	2	0.36	0.00053	10	2	2
Central	31°49'–33°11'	56	2	5	0.74	0.0017	28	1	2	0.52	0.00075	12	3	3
Central-South	37°08'	7	0	1	0	0	7	0	1	0	0	3	0	1
South	39°25'–43°36'	12	1	2	0.39	0.00069	5	0	1	0	0	3	1	2
Total	13°53'–43°36'	168	18	22	0.93	0.0047	92	6	7	0.85	0.00135	38	13	9

distribution (0.51 and 0.57, respectively), and similar values were found for Chilean haplotypes only (0.49 and 0.51, respectively).

### Fine-Scale Analyses of Focal Areas

Similarly to the *L. felina* phylogeography results of regional scale, the fine-scale analysis shows the significant influence of the habitat spatial pattern over the distribution of genetic diversity. All 3 areas showed population structure for CR sequences when considering each rocky seashore patch as a population (Figure 4, Table 4). Area 1 showed the lowest mtDNA diversity and population structure ( $\Phi_{st} = 0.33$ ,  $P = 0.023$ ) and a predominant haplotype (F). Higher population structure was found for Areas 2 and 3,  $\Phi_{st} = 0.56$  and  $0.66$ , respectively ( $P < 0.0001$ ). Area 2 is characterized by patches of different sizes and a larger patch separated by 2 large cities of around 300 000 people, La Serena and Coquimbo. Area 3 patches are of equivalent sizes and distances. Although patches shared haplotypes, each one had a characteristic haplotype in high frequency. Mantel tests showed that IBD was not significant within each of the 3 areas:  $R^2 = -0.44$ ,  $P = 0.86$  for Area 1;  $R^2 = 0.09$ ,  $P = 0.38$  for Area 2; and  $R^2 = 0.30$ ,  $P = 0.45$  for Area 3. The nonparametric Kruskal–Wallis test showed that both rocky seashore distance and size affects the genetic diversity and divergence between patches (Table 5). However, patch size was not significant for  $\Phi_{st}$  between pairwise patches. Distance between rocky seashore patches was significant for all parameters except nucleotide diversity.

**Table 4** Characteristics of all 3 study areas including the number of rocky seashore patches (*N*), average size, distance between patches and its standard deviation, percentage of rocky seashore habitat, degree of landscape division (*D*) followed by each area haplotype and nucleotide diversity, and  $\Phi_{st}$ 

Study area	Geographic location	Total length (Km)	Rocky Seashore Patch			Percentage of rocky seashore (%)	Degree of landscape division ( <i>D</i> )	Diversity		
			<i>N</i>	Size	Distance			haplotype	nucleotide	$\Phi_{st}$
Area 1 (Pan Azucar)	26°08'–26°18'	33	4	4.66 ± 3.51	3.54 ± 1.62	57	0.88	0.36	0.0013	0.33*
Area 2 (La Serena)	29°37'–30°17'	150	11	8.9 ± 10.9	5.18 ± 6.26	65	0.91	0.78	0.0037	0.56*
Area 3 (Palo Colorado)	32°01'–32°30'	79	6	11.1 ± 5.89	3.85 ± 2.29	71	0.88	0.69	0.0018	0.66*

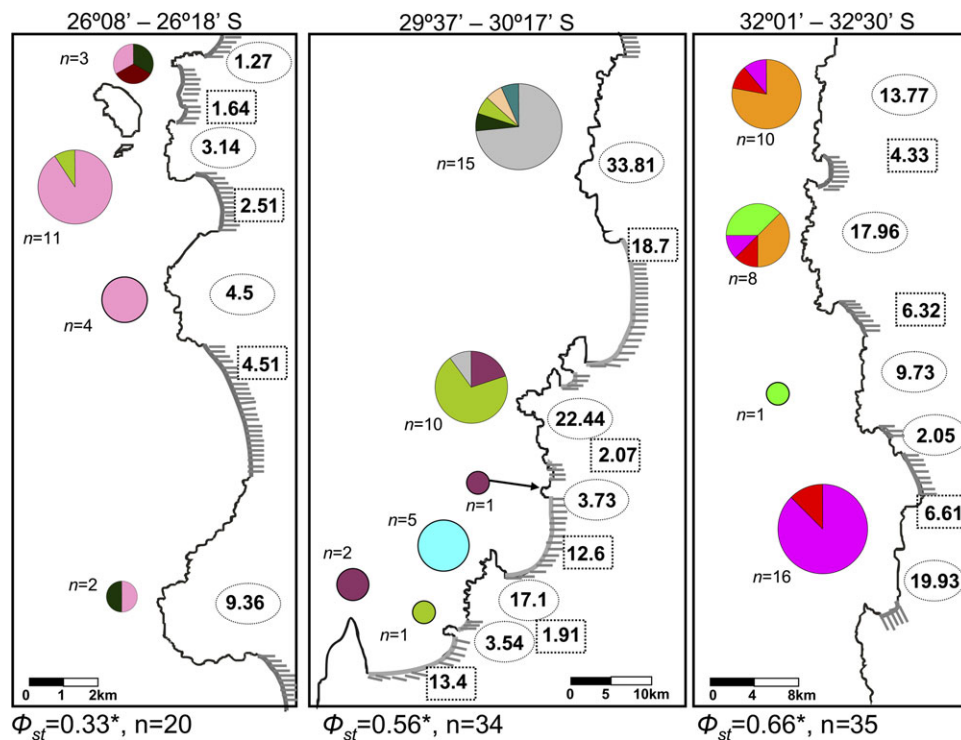
$P < 0.001$  for  $\Phi_{st}$  is represented by \*.

## Discussion

### *Lontra* in South America

The phylogenetic relationships among all *Lontra* species confirmed that *L. felina* and *L. provocax* are sister species and monophyletic with *L. longicaudis*. Divergence between *L. felina* + *L. provocax* and *L. longicaudis* occurred around 1.57 MA, consistent with the date described for the *L. felina* and *L. longicaudis* split using 8 fossil calibration points along the Mustelidae phylogeny (Koepfli, Deere, et al. 2008). Our date is also consistent with the oldest record of *L. longicaudis* in South America, found in Argentina (Rusconi 1932; Berta and Marshall 1978), in levels that could be dated between 1.8 and 0.98 MA (Soibelzon et al. 2005). *Lontra felina* and *L. provocax* are isolated from *L. longicaudis* in most of its distribution by the Andes Mountains in the east, and the Atacama Desert in the north, suggesting that divergence from *L. longicaudis* likely occurred as an allopatric process. Phylogenies of different groups of carnivores usually exhibit a similar pattern of lineages distribution within South America, with basal species distributed mainly in the north as opposed to more recently evolved southern species (Marshall et al. 1982). Similarly, the Andes played an important role in carnivore speciation, such as for the Neotropical Felidae of the Ocelot lineage *Leopardus* sp. that recently radiated in South America. The most recent speciation occurred in the southernmost regions between *L. geoffroyi* and *L. guigna* (0.74 MA), associated to an isolation by the Andes (Johnson et al. 2006).

*Lontra felina* and *L. provocax* are closely related species (1.4% of divergence) that diverged in the recent Pleistocene



**Figure 4.** The local frequencies of mtDNA CR haplotypes in of 3 study areas (1, 2, and 3 from left to right) of coastal seashore at different scales. The circle area of piecharts reflects the sample size, and disk sectors are proportional to haplotype frequencies depicted as distinct colors. Black lines represent rocky seashore and gray lines with horizontal lines sandy beaches. Left of each line is the Pacific Ocean. The extent of each seashore rocky fragment (km) is presented in the dotted circles and the extent of sandy beaches inside the dotted squares. Below each figure is the  $\Phi_{st}$  values and sample size ( $n$ ).

(883 000 ya). Our data support the hypothesis that speciation of *L. felina* occurred from the *L. provocax* and not from *L. longicaudis*. This result supports the hypothesis that speciation may have occurred along the marine channels of Patagonia, after an adaptation to the marine habitat. Although coastal environments have abundant food resources that otters can exploit, species have to adapt in various ways to cope with the problems of salt water (Kruuk 2006). Except for the 2 marine species (*E. lutris* and *L. felina*), otters distributed along the seacoast need access to freshwater for drinking and washing their dense fur to remove accumulating salt and allow for thermoinsulation (Kruuk 2006). Marine otter occur in areas without rainfall and in absence of freshwater and its insulation dilemma is

partly solved by spending less time in the water (Medina-Vogel et al. 2007).

It has been demonstrated that recent ecological processes as well as historical factors contribute to the structure of modern day communities (Vitt and Pianka 2005). Divergence in ecological traits and competition varies according to the degree of phylogenetic relatedness among species (Vitt and Pianka 2005). Consequently, as seen in other mustelid species, divergence in ecological traits should be greatest between sister species occupying the same area (Koepfli, Deere, et al. 2008). Although both *L. provocax* and *L. felina* co-occur in southern Chile, they are found in different habitat (Sielfeld 1990). Interestingly, the ecological differences between sheltered environments of fjords and

**Table 5** Kruskal-Wallis test results of effects of seashore patch size and distance on the genetic diversity ( $h$  and  $\pi$ ) and divergence between patches (pairwise differences and  $\Phi_{st}$ ) of all 3 study areas

Rocky seashore patch	Diversity		Genetic distance	
	Haplotype ( $h$ )	Nucleotide ( $\pi$ )	Pairwise difference	$\Phi_{st}$
Size	$P < 0.001$ $H = 32.9$ ; $df = 3$	$P < 0.001$ $H = 56$ ; $df = 3$	$P < 0.03$ $H = 9.1$ ; $df = 3$	NS $H = 4.7$ ; $df = 3$
Distance	$P < 0.01$ $H = 13.6$ ; $df = 4$	NS $H = 7.8$ ; $df = 4$	$P = 0.001$ $H = 17.9$ ; $df = 4$	$P < 0.001$ $H = 24.5$ ; $df = 4$

df, degrees of freedom; NS, not significant.

channels strongly influenced by freshwater and the wave exposed coast of the easternmost islands are found from 42°S to 56°S, offering a wide area in which parapatric speciation could have occurred. Likewise, competition likely contributed to the reduction in gene flow and to the evolution of reproductive isolation. Indeed, Ebensperger and Botto-Mahan (1997) suggested that both species segregate habitats to avoid interspecific competition for food. Thus, there is a positive feedback between ecological processes and evolutionary forces favoring *L. felina* speciation. This scenario holds even though the recent speciation and low divergence of *L. felina* and *L. provocax* suggests that hybridization could occur in nature, as reported for other neotropical carnivores (Johnson et al. 1999; Trigo et al. 2008).

### *Lontra felina* Evolutionary and Demographic History

Marine otter exhibit high haplotypic diversity but low levels of nucleotide diversity ( $b = 0.9315$ ,  $\pi = 0.0047$ ) of the mtDNA CR. Similar diversity levels were described for other otter species from South America such as *L. longicaudis* in south and southeast Brazil ( $b = 0.819$ ,  $\pi = 0.0039$ – $0.0067$ , Trinca et al. 2007) and *P. brasiliensis* across a wide distribution in Brazil ( $b = 0.867$ ,  $\pi = 0.006$ , Garcia et al. 2007). In the case of *L. lutra* in Europe, low mtDNA CR diversity was attributed to a single Pleistocene refugia, although, a conservative pattern of molecular evolution of the mtDNA CR in otter was also suggested (Mucci et al. 1999; Cassens et al. 2000). Moreover, the North Pacific sea otter (*E. lutris*) illustrate reduced genetic variation on remnant and translocated populations (Lidicker and McCollum 1997; Bodkin et al. 1999; Larson, Jameson, Bodkin, et al. 2002) attributed to bottleneck related to fur trade (Larson, Jameson, Etnier, et al. 2002).

Using each of the 3 mtDNA sequences independently or combined, phylogenetic trees clearly point to a divergence of the *L. felina* Peruvian clade from the Chilean clade. The mtDNA uncovered a significant differentiation and a pattern of IBD among *L. felina* populations spanning the 3500 km of coastline. Among the 6 regional groups of haplotypes inferred from the geographical distribution, Norte Chico and Central Chile had the highest genetic diversity, declining to the 2 extremes of the distribution (North-Peru, Norte Grande, Central-South, and South). No clear evidence of a recent demographic expansion was detected from the genetic diversity, even when excluding the highly divergent haplotypes from the northernmost region. However, the network of haplotypes suggests that most of the present day diversity evolved from ancestral haplotypes that are no longer present but to which *L. provocax* seems connected.

Two main conclusions can emerge from these analyses. The low diversity and divergence but high isolation pattern in the southernmost part of the sampled distribution strongly supports a hypothesis of recent colonization toward the south. Whether or not this southward colonization is a postglacial process remains undetermined because the divergence of this haplotype from the Central-South one

was dated approximately 35 000 ya. This event predate the LGM, which is dated around 23 000 to 17 000 ya. Additional sampling south of 43°36' is necessary to further support the hypothesis of southern population extinction and postglacial colonization followed ice sheet covered retreat. On the contrary, the high divergence observed in the northern lineages (haplotype V, A, and B) fits the hypothesis of an ancient distribution in this region. The reduced diversity observed there, compared with central Chile, is an expected pattern for this region where periodic events of ENSO can reduce effective population size and thus increase genetic drift. Both scenarios suggest that *L. felina* is susceptible to climatic changes such as ENSO or glaciations which likely caused strong demographic fluctuations.

The geographic pattern of population differentiation also reveals possible consequences of historical physical barriers associated with current restrictions to dispersal and distribution of suitable habitat. The southern area of the Peruvian coast and northern Chile (17° to 19°S latitude) is mainly composed of long sandy beaches, up to 56 km long. Those areas constitute permanent physical barriers to *L. felina*, restricting gene flow (see Discussion below on the fine scale), thereby favoring the genetic isolation of peripheral Peruvian populations. Peruvian populations are the northern limit of the *L. felina* range, and the species' 1D distribution following the coast restricts gene flow exclusively from southern populations. Reduced genetic diversity and increased population structure is often recorded in peripheral populations compared the core of the species distribution, as described for the mustelid *Martes pennanti* (Wisely et al. 2004). Moreover, isolation of peripheral populations is a known mechanism of peripatric speciation (Mayr 1982), which can be exemplified by the high divergence of Peruvian haplotypes being external from the Chilean monophyletic clade. Differences between *L. felina* specimens from Peru and Chile were previous distinguished. Gervais (1841) described the species *L. peruviansis*, which it was then described as subspecies, *L. felina peruviansis* (Osgood 1943). Subsequently, *L. felina* was again characterized into 2 different subspecies: the typical *L. felina felina* and the Northern marine otter (“chungungo del norte”) = *L. felina peruviansis* characterized by its shorter and lighter color fur (Housse 1953; Sielfeld 1983).

### Coastal Habitat and Population Structure

A small number of *L. felina* haplotypes are shared between neighboring regions following a pattern of IBD and confirming its low dispersal ability. No concatenated haplotype was shared between North-Peru, Norte (Grande and Chico), Central, Central-South, and South. Apparently, 2 geographic barriers seem to prevent the animal's gene flow: 1) from Algarrobo (33°22'S) south toward Peninsula Tumbes (36°36'S) and 2) south from Arauco Bay (37°10') to Queule (39°23'). Both areas correspond to the longest sandy beaches present in Chile with few small patches of rocky seashore (Thiel et al. 2007) where marine otter is

absent. This is in agreement with the susceptibility of *L. felina* to large disruptions of its habitat reported by Medina-Vogel et al. (2008).

High population structure was observed at a large but also at a more local scale in all 3 areas. At the fine scale, the population structure was dependent on the patchy structure of rocky seashore, mainly the distance between them. Area 1 showed low population structure compared with Areas 2 and 3 and is characterized by short distances (i.e., 1.64–4.51 km) between extended rocky seashore patches, equivalent to the species home range of 3.2–4.2 km of lineal coast (Medina-Vogel et al. 2007). Our results showed a high dependence on large rocky seashore patches to maintain genetic diversity. In most mammal species, including otters, females are more philopatric than males which are more likely to disperse from their natal area (Greenwood 1980). Evidence of male-based dispersal has been noted for coastal North America river otter, *L. canadensis* (Blundell et al. 2002). Males had a greater potential for dispersal among close populations (16–30 km), whereas some females were able to disperse 60–90 km. In the case of the southern river otter, dispersal of up to 46 km was registered by radio-tracking (Sepúlveda et al. 2007). Our molecular data based on maternal lineage of mtDNA markers indicate female philopatry. However, it is necessary to further investigate male dispersal patterns using biparental or paternal markers to better understand dispersal and the effective role of habitat structure.

#### Integrating Historical and Contemporary Factors: Implications for Conservation

The spatial structure of terrestrial habitat is responsible for population connections and isolation in this marine otter and it is particularly relevant to *L. felina* evolutionary history and speciation. Higher mtDNA diversity is found along Central and Norte Chico, likely due to long-term demographic stability for *L. felina* and a favorable distribution of rocky seashore and den availability. However, this area also holds the highest human density in Chile and the coast is highly impacted by anthropogenic activities, which raise concern about the conservation of this endangered species. According to the geographic structure of the genetic diversity, 2 evolutionary significant units (ESUs) can be defined: the Peruvian clade and the Chilean clade. By definition, an ESU is monophyletic for mtDNA and is a primary source of species historical genetic diversity (Moritz 1994). Consequently, defining ESU is necessary to ensure that the evolutionary heritage is recognized and protected by maintaining the capability of different ESUs to evolve (Moritz 1994). Furthermore, our data agree with the previous suggested *L. felina* subspecies division (Gervais 1841; Osgood 1943; Housse 1953; Sielfeld 1983). Similarly, management units (MUs) is a term used to differentiate a population that exchanges few migrants with others and is thus genetically differentiated (Moritz et al. 1995). The SAMOVA allowed the identification of 5 genetically differentiated groups of populations in Chile, suggesting similar number of MUs. Although contemporary gene flow

may not be accurately estimated by phylogeographic markers, our results clearly demonstrated the susceptibility of *L. felina* to habitat disruptions, suggesting that relatively small scale MU's should be defined for conservation purposes. In particular in the case of local extirpation, natural reintroduction might be unlikely during an ecological time scale relevant for management interests (Avice 1995). Furthermore, given the 1D distribution of *L. felina* following the lineal coastline, population extirpation can increase north–south isolation and further decrease probabilities of natural recolonization. *Lontra felina* susceptibility to historical climatic changes and anthropogenic impact associated with low dispersal ability and a possible reduced population size, therefore, impose a high risk of extinctions along large areas.

#### Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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