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# Diversity of mtDNA in Southern River Otter (*Lontra provocax*) from Argentinean Patagonia

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*Lontra provocax* is an endemic species from Patagonia that has been categorized as "endangered" by the International Union for Conservation of Nature. In this study, noninvasive molecular methods were used to investigate the genetic diversity and haplotype distribution of *L. provocax* in Argentinean Patagonia. We analyzed 150 scat samples collected from 1995 to 2006 and obtained 13 sequences of control region with 1 haplotype and 34 sequences of cytochrome *b* with 4 haplotypes. The population of the south of Patagonia (Tierra del Fuego and De los Estados Island) showed a relatively high haplotype diversity ($h$ = 0.71) and was statistically different to the population of the north (analysis of molecular variance, $F_{ST}$ = 0.15, $P$ = 0.018). We concluded that there are 2 different subpopulations of *L. provocax* that deserve conservation attention and that the southern population appears not to have suffered a human-induced population bottleneck of the sort typically experienced by various otter species around the world.

The huillín or Southern river otter, *Lontra provocax*, is an endemic species from Argentinean and Chilean Patagonia, categorized as "endangered" by the International Union for Conservation of Nature (Vogel 2004). In the past, the Southern river otter occupied almost the whole fringe of the Andean Patagonian forest of Argentina and several rivers that cross the steppe (Chehébar et al. 1986). However, the species was intensively hunted because of its valuable fur. This activity was prohibited by the middle of last century, and in the 1980s, several surveys showed that the Argentinean population had survived a critical period during which there had apparently been a high risk of extinction (Chehébar et al. 1986).

A recent, comprehensive survey shows that *L. provocax* is now found in 2 distinct areas in northern and southern Argentinean Patagonia, separated by more than 2400 km and a sea channel (Figure 1; Fasola et al. 2006). In the north, there are 2 subpopulations, the largest one occupying the Nahuel Huapi National Park and the other one restricted to a portion of the Limay River. In the south also, there are 2 subpopulations, 1 in Tierra del Fuego and another on De los Estados Island (Figure 1). We report the first investigation of mitochondrial DNA (mtDNA) sequence variation in Southern river otters in order to describe spatial patterns of genetic diversity between the Argentinean populations.

Low variability is characteristic of the control region (CR) of Lutrinae mtDNA (Larson et al. 2002; Ferrando et al. 2004), which is otherwise known as a highly polymorphic marker system in many mammals (Avise 1994). Other markers have been shown to possess more variability, such as cytochrome *b* (cyt b; Effenberger and Suchentrunk 1999) and microsatellites (i.e., Huant et al. 2005). In this regard, Ferrando et al. (2004) proposed that low variability is observed in the otter mtDNA CR because the highly variable "extended termination–associated sequences" domain is shorter than in other mammals.

Noninvasive molecular techniques, commonly using feces, are frequently the only available method for studying genetic characteristics of endangered mammal species. However, the efficiency of DNA extraction rapidly decreases with declining feces conditions (i.e., Hung et al. 2004). In this study, 2 mtDNA regions (CR and cyt b) from sample feces were evaluated as intrapopulation markers in

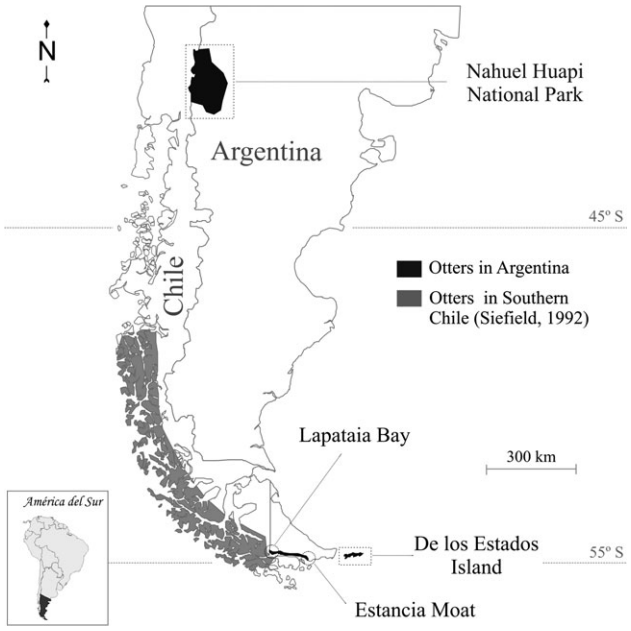


**Figure 1.** Distribution of Southern river otters in Argentina and southern Chile. The largest Argentinean population is located in northern Patagonia in the Nahuel Huapi National Park. The other population is located in southern Patagonia in Tiera del Fuego and De los Estados Island (Fasola et al. 2006). The population of southern Chile covers an extensive and isolated area (Sielfeld 1992).

order to describe spatial patterns of genetic diversity between the Argentinean populations.

## Methods

### Study Area and Fecal Sampling

Patagonia occupies all of southern Argentina, approximately between latitudes S 36° and S 55°, and from the Andean mountain chain to the Atlantic Ocean (Figure 1). It is dominated by 2 main ecosystem types: the steppe, which occupies most of the region, and the temperate forest, which forms a narrow fringe between the steppe and the mountains. Precipitation shows an abrupt longitudinal gradient from west to east (from 2000 to 700 mm within a few kilometers), which explains the rapid transition between forest and an arid environment.

There were 3 sources of Southern river otter scat samples. In 1995, 16 scats were collected from Nahuel Huapi Lake and 25 scats from De los Estados Island. The main scat sample set was collected in a large survey conducted between January and May 2005 (described in detail in Fasola et al. 2006). Three teams surveyed most of the Patagonian forest strip, covering a total length of 1800 km, from 38°52′01″S to 54°52′22″S. Four hundred and forty seven sites were visited, located along the coastlines of 67 lakes and 62 rivers and streams. At each site, we surveyed 600 m along the coast (Mason and Macdonald 1983). We collected 109 scats for genetic studies in this survey, but the quality of scats was not equal for all samples. Unfortunately, fresh fecal samples were difficult to find due to the low population density, typical of Southern river otters, and the remoteness of the sites where they live. We considered that only 25 fecal samples could be classified as "fresh," and these were preserved in ethanol at 4 °C. The rest of the scats, already powder dry at the time of collection, were preserved dry at room temperature.

### Genetic Analysis

"Control" samples were obtained from 2 dead South American river otters, 1 found at the end of Castor River in Tierra del Fuego and 1 on the coast of the Nahuel Huapi Lake (samples kindly provided by the National Park Administration). Both samples were successfully amplified, sequenced, and manually edited using Chromas, version 2.23. Specific primers for cyt b (reverse: 5′-CATCTCAA-CATGATGAAACTTC-3′ and forward: 5′-GTACCATT-CAGGCTTGAT-3′) and the CR (reverse: 5′-CTATAGGT ATTTGTATACTTTTG-3′ and forward: 5′-GACGTG-TACCTCTTCTCG-3′) were developed, based on sequences of closely related species, published in GenBank. A 671-bp cyt b sequence and a 516-bp CR sequence were obtained. These sequences were published in GenBank with access numbers DQ341273 and DQ368686.

Each fecal sample (250 mg of otter scat) was mixed in a vial containing 1 ml of undiluted ethanol and 2 ml of CTAB (hexadecyltrimethylammonium bromide) and then incubated for 1 h at 70 °C. CTAB buffer was prepared according to Parsons et al. (1999), using 100 mM Tris–HCl (pH = 8.0), 1.4 M NaCl, 20 mM EDTA, and 2% CTAB. DNA was extracted using phenol–chloroform and alcohol precipitation. Precipitated DNA samples were resuspended in 10 mM Tris and 1 mM EDTA, pH = 8.0, and stored at −20 °C. DNA was purified with the Dneasy Tissue Kit (Qiagen). The amplification reaction consisted of 35 cycles of 1 min at 94 °C, 30 s at 50 °C, and 1 min at 72 °C. Polymerase chain reaction (PCR) volume was 100 µl, containing 10 µl of sample, 20 µl of 5 ng/µl DNA, 5 µl of 5 mM $MgCl_2$, 5 µl of 10× reaction buffer, 0.5 µl of 20 mM premixed deoxynucleotide triphosphates (Invitrogen, Life technologies), 5 µl of 10 mg/ml bovine serum albumin, 1.25 UTaq polymerase (Invitrogen, Life technologies), 4 µl of 5 µM oligonucleotide primers, and water to achieve the final reaction volume. Twenty microliters of the PCR products were electrophoresed in 1.5% agarose gel electrophoresis, inspected, and photographed under ultraviolet light. The rest of the amplification products (80 µl) were purified using a Wizard SV Gel and PCR Clean-Up System (Promega). Purified DNA was sent to an external laboratory (Macrogen) for sequencing in both directions with the same oligonucleotide primers used in PCR, using an ABI 377 Automated DNA Prism Sequencer (Applied Biosystems, Inc).

**Table 1.** Haplotypes (I, II, III, and IV) of cyt b (671 bp) of 34 sequences of *Lontra provocax* for the subpopulations

| | Sample size | | | | Location of base-pair mutations | | |
|---|---|---|---|---|---|---|---|
| Haplotype | LB | EI | EM | NH | 44–45 | 448 | 637 |
| I | 1 | | | | ACAC**GG**CCACA | GGT**A**ATC | CAT**T**CCCG |
| II | 3 | 6 | | | ACAC**AA**CCACA | GGT**G**ATC | CAT**T**CCCG |
| III | 3 | | 2 | 3 | ACAC**AA**CCACA | GGT**A**ATC | CAT**T**CCCG |
| IV | 8 | | | 8 | ACAC**AA**CCACA | GGT**G**ATC | CAT**C**CCCG |

LB, Lapataia Bay; EI, De los Estados Island; EM, Estancia Moat; NH, Nahuel Huapi National Park. The first 3 populations were from southern Patagonia, and NH was from northern Patagonia.

We started working both with CR and cyt b markers. However, the amount of DNA was insufficient for PCR of both markers for all fecal samples. Therefore, the objective was to obtain a first set of sequences and compare the haplotype diversities calculated for this subsample with both markers. The first 13 sequences of CR were identical, whereas the same number of sequences of cyt b offered 3 haplotypes (Table 1). We therefore opted to use cyt b as the marker whereby the diversity and population structure would be estimated for the remaining samples. Genetic data were statistically analyzed using Arlequin ver 2.000 software.

## Results

All 150 scat samples were processed for DNA extraction, but only 40 showed detectable mtDNA PCR product. In addition, the amount of extracted DNA from the collected samples was usually sufficient for only one mtDNA PCR reaction and insufficient for analysis of nuclear DNA. Therefore, the success rate for DNA extraction was 26.6% for all fecal samples. However, for feces of good quality ($n = 25$), success rate of DNA extraction and amplification was 80% (20/25).

Despite these limitations, we were able to obtain 13 sequences of 516 bp of CR and 34 sequences of 671 bp of cyt b. These sequences showed 1 haplotype of CR and 4 haplotypes of cyt b (Table 1). There were 4 polymorphic sites in the cyt b, corresponding to 4 transitions: a G–A at positions 44 and 45, a A–G at position 448, and a T–C at position 637. Neither deletions nor insertions were found.

In Argentina, there are 2 populations of *L. provocax* in northern and southern Patagonia (Fasola et al. 2006). The northern population is divided into 2 subpopulations (Nahuel Huapi National Park and Limay River), and the southern population also occurs as 2 subpopulations (Tierra del Fuego and De los Estados Island). The northern samples sequenced were obtained from the Hermoso Lakes and Limay River in Nahuel Huapi National Park, an area of approximately 3800 km$^2$ (Figure 1). The northern population had 2 haplotypes, a haplotype diversity ($h$) of 0.44, and a nucleotide diversity ($\pi$) of 0.13%. The southern samples were collected in 3 different sites, 2 located on the Tierra del Fuego coast (Lapataia Bay and Estancia Moat) and the third from several points around the coast of De los Estados Island (Figure 1). This southern population exhibited all 4 haplotypes, with $h = 0.71$ and $\pi = 0.16\%$. The northern and southern populations were compared with an analysis of molecular variance that indicated significant differences between them ($F_{ST} = 0.15$, $P = 0.018$).

## Discussion

The 516 bp of CR sequenced from 13 Southern river otter fecal samples exhibited only 1 haplotype. On the other hand, cyt b diversity was different in northern and southern Patagonia. In the north, it reached similar values of mtDNA diversity as reported for species of otter in other parts of the world (Ferrando et al. 2004). However, the southern population showed relatively high haplotype diversity ($h = 0.71$). Indeed, in Lapataia Bay, we found all 4 haplotypes along only 31 km of coast. Lapataia Bay is the southeast extreme of the distribution of the presumed largest population of *L. provocax* of the world, which occupies the marine coast of southern Chile (Sielfeld 1992). This Chilean population is located in an extremely isolated area, which had remained almost undisturbed during the period when the other populations of Southern river otters suffered strong human impacts (Sielfeld 1992). Therefore, it is probable that the southern Patagonia population has avoided a population bottleneck that affected the population in northern Patagonia and, indeed, is typical in many populations of otters around the world (Effenberger and Suchentrunk 1999; Larson et al. 2002).

Historical records indicate that the northern and southern populations of *L. provocax* have been geographically separated throughout recorded history, although their distributions were formerly more extensive (Chehébar et al. 1986). These hundreds of years of population isolation could explain the genetic difference between the north and the south, which also suggests the occurrence of 2 distinct stocks of *L. provocax* in Argentina. This genetic distinctiveness should be taken into account when planning the conservation of Southern river otters. The northern subpopulation is the largest one in Argentina, with an extent of occurrence of 12 140 km$^2$, and it is within protected areas in most of this range (Fasola et al. 2006). The southern subpopulation is restricted to the marine

coasts of the Beagle Channel and De los Estados Island, which have a total coastline of 571 km, most of it without public protection. A more comprehensive genetic comparison between the Chilean and the Argentinean populations of southern Patagonia is required to identify potential genetic divisions and design a common conservation strategy.

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