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Phylogeography and Demographic History of the Neotropical Otter (*Lontra longicaudis*)

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Abstract

The Neotropical otter (*Lontra longicaudis*) is a medium-sized semiaquatic carnivore with a broad distribution in the Neotropical region. Despite being apparently common in many areas, it is one of the least known otters, and genetic studies on this species are scarce. Here, we have investigated its genetic diversity, population structure, and demographic history across a large portion of its geographic range by analyzing 1471 base pairs (bp) of mitochondrial DNA from 52 individuals. Our results indicate that *L. longicaudis* presents high levels of genetic diversity and a consistent phylogeographic pattern, suggesting the existence of at least 4 distinct evolutionary lineages in South America. The observed phylogeographic partitions are partially congruent with the subspecies classification previously proposed for this species. Coalescence-based analyses indicate that Neotropical otter mitochondrial DNA lineages have shared a rather recent common ancestor, approximately 0.5 Ma, and have subsequently diversified into the observed phylogroups. A consistent scenario of recent population expansion was identified in Eastern South America based on several complementary analyses of historical demography. The results obtained here provide novel insights on the evolutionary history of this largely unknown Neotropical mustelid and should be useful to design conservation and management policies on behalf of this species and its habitats.

Key words: *Bayesian skyline plot, biogeography, conservation, divergence time, population structure, taxonomy*

The Neotropical otter (*Lontra longicaudis*) is a relatively common carnivore species with a broad distribution, ranging from Mexico to northern Argentina (Chehébar 1990). In the past, humans made heavy use of these otters for skins, as their pelts were very much in demand in the international market, especially during the first half of the 20th century (Chehébar 1990). Although some illegal hunting continues, this species has been relatively free of exploitation since the 1960s, when the hunting pressure declined significantly due to the growing concern about wildlife conservation and the enforcement of CITES designation for the Neotropical otter by Latin American countries (Chehébar 1990). However, this mustelid has been subjected to several other threats throughout its range, such as habitat loss and fragmentation, water pollution, road killing and direct persecution in retaliation for its supposed predation on fish stocks (Macdonald and Mason 1990). Like other otter species, it is among the first species to decline and disappear when the aquatic environment is degraded, as it plays a top predator role in local food chains (Foster-Turley et al. 1990). Therefore, it has been a particularly important focus for conservation efforts due to its potential role as an indicator of healthy aquatic environments. In spite of its relevance and conservation concern, very little is still known about this

species, which has led the IUCN (2011) to categorize it as "Data Deficient" (Waldemarin and Alvarez 2008). Although recent studies have addressed some ecological aspects of this otter, such as diet and habitat use (Gallo-Reynoso et al. 2008; Kasper et al. 2008; Chemes et al. 2010), other issues remain unexplored, such as population structure, current demography, and intraspecific evolutionary history.

Interestingly, traditional taxonomic studies have proposed that *L. longicaudis* might in fact be a species complex, based on variation in the rhinarium shape (Pohle 1920; Cabrera 1957; Harris 1968). Subsequent analyses suggested that those forms were conspecific but could be geographically subdivided into 3 subspecies: *L. longicaudis annenctes* (occurring in Mexico, Nicaragua, Costa Rica, Panama, Colombia, Venezuela, and Ecuador), *L. longicaudis enudris* (distributed through French Guiana, Suriname, Trinidad, and Peru), and *L. longicaudis longicaudis* (distributed through most of South America, including Brazil and Uruguay) (van Zyll de Jong 1972; Larivière 1999). However, such intraspecific taxonomic subdivision remains controversial, as no comprehensive revision of this species' morphological variation has so far been performed. Furthermore, recent observations have brought back into focus the hypothesis that this otter may in fact represent an assemblage of distinct species, which may or may not correspond to the proposed subspecies (Xth International Otter Colloquium, South Korea, 2007). Given this controversy and the currently scarce knowledge on variation across the species' geographic range, the use of any infraspecific classification has been discouraged until more detailed studies are conducted, which was deemed a high priority by the community of otter researchers (Xth International Otter Colloquium, South Korea, 2007).

In the context of defining species-level boundaries or intraspecific units, a useful approach is to investigate the phylogeographic patterns of molecular variation (Avise 2000). So far only one study has addressed the genetic diversity and geographic differentiation of *L. longicaudis* populations (Trinca et al. 2007). That study employed mitochondrial DNA (mtDNA) control region (CR) sequences to survey the variation present in otters from southern and southeastern Brazil and reported high levels of haplotype diversity, low nucleotide variation, and no signal of genetic structuring in those regions. However, given the focus of that study on a small portion of the otter's extensive geographic distribution, no comprehensive assessment of its overall phylogeographic structure and evolutionary history could be attempted.

In this study, we aimed to expand the investigation of *L. longicaudis* mitochondrial diversity by employing 3 different segments of the mtDNA to characterize a broad geographic sample spanning most of the species' range in South America. On the basis of this information, we investigated the phylogeographic structure and demographic history of Neotropical otter populations, including an evaluation of whether inferred historical partitions coincided with previously proposed subspecies. Because our focal species is a semiaquatic mammal, we also compared the observed phylogeographic patterns with some of those reported for terrestrial or aquatic taxa on a similar continental scale, aiming to test whether different life histories and dispersal capabilities have yielded contrasting or congruent subdivisions as reflected in current mtDNA diversity.

Materials and Methods

Sample Collection

Biological samples were collected from 52 Neotropical otters across a large portion of the species' range (Figure 1; Supplementary Table S1). Blood or faecal samples were obtained from captive individuals with known geographic origin; muscle and skin samples were collected opportunistically by collaborators from animals found dead (e.g., road killed), and additional faecal samples were also collected by field researchers working in different areas. Blood samples were preserved in a salt saturated solution (100 mM Tris, 100 mM ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate); tissue, faecal and hair samples were preserved in 96% ethanol. All samples were stored at −20 °C prior to DNA extraction. Samples of South American River Otter (*Lontra provocax*), Marine Otter (*L. felina*), North American River Otter (*L. canadensis*), Sea Otter (*Enhydra Lutris*), Asian Small-Clawed Otter (*Aonyx cinereus*), and Giant Otter (*Pteronura brasiliensis*) were also sequenced, so as to be used as outgroups in some of the analyses. For two of the mtDNA segments, we employed previously published sequences of *L. provocax* and *L. felina* (accession numbers GQ843782, GQ843800, GQ843819, and GQ843803).

Generation of Sequence Data

Genomic DNA was extracted from blood and tissue samples using a standard Proteinase-K digestion and phenol-chloroform-isoamyl alcohol protocol (Sambrook et al. 1989). DNA from faeces was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. The faecal DNA extractions were carried out in a separate laboratory, in a UV-sterilized laminar flow hood dedicated to the DNA analysis of non-invasive samples. DNA from hair and skin samples was extracted using the ChargeSwitch Forensic DNA Purification Kit (Invitrogen).

Three segments of the mtDNA genome were amplified by the Polymerase Chain Reaction (PCR) using primers developed or adapted for improved performance in carnivores: 1) ~550 bp of the 5' portion of the mtDNA CR, containing the first hypervariable segment, using primers MTLPRO2 and CCR-DR1 (Tchaicka et al. 2007); 2) a ~400 bp segment including the entire *ATP8* gene and part of the *ATP6* gene using primers ATP8-DF1 and ATP6-DR1 (Trigo et al. 2008); and 3) a ~750 bp segment of the *ND5* gene using primers ND5-DF1 and ND5-DR1 (Trigo et al. 2008).

Because fecal samples tend to present degraded DNA, hampering the sequencing of long fragments, in such cases

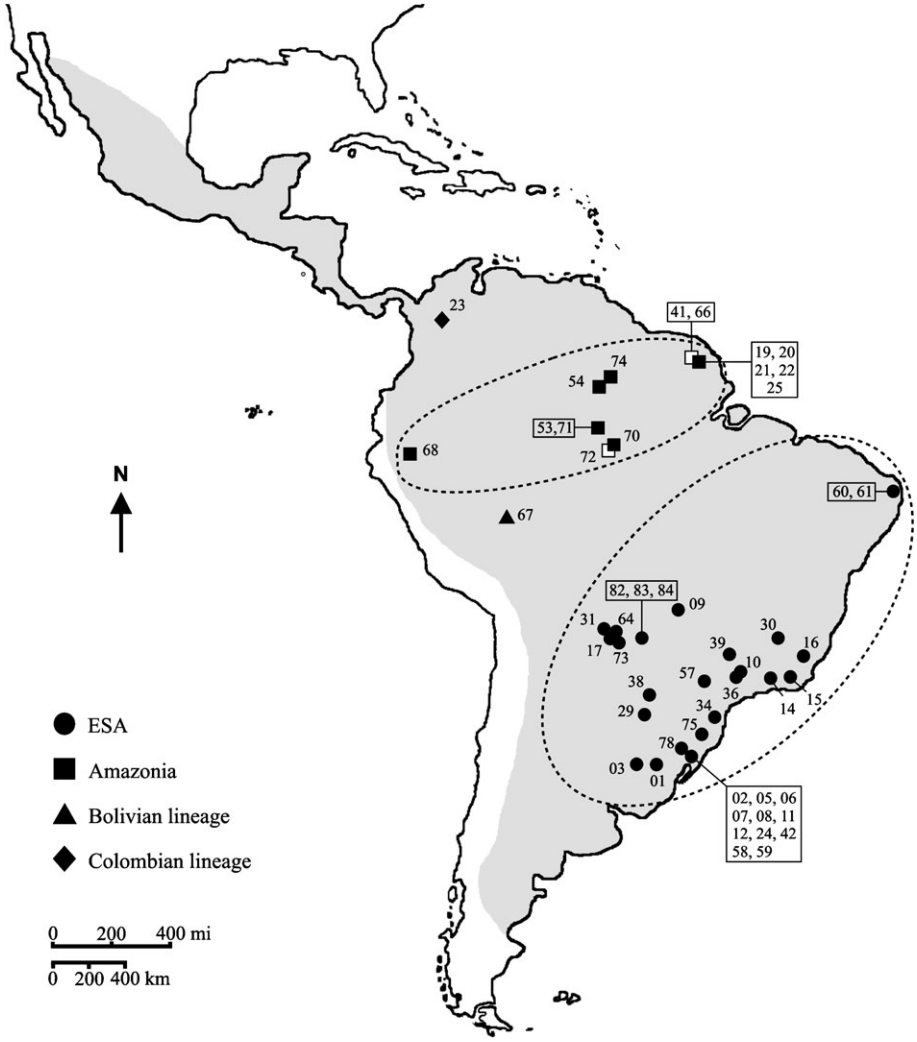

Figure 1. Map depicting the currently assumed geographic distribution (shaded area) of the Neotropical otter (modified from http://www.otterspecialistgroup.org/Species/Lontra_longicaudis.html), with sample collection sites. Numbers next to the collection sites are sample identification labels (number after "bLlo" in Supplementary Table S1) of *L. longicaudis* individuals in each area. Boxes indicate individuals from the same region. White squares are indicative of exchanged haplotypes between the 2 defined geographic groups (see Results). Dotted ellipses represent the geographically defined population groups (ESA and Amazonia).

we used the CR primers reported by Trinca et al. (2007), which divide our target segment into 3 shorter overlapping fragments (each one with approximately 250 bp). Each 20-µl PCR reaction contained 1–2 µl of empirically diluted template DNA, 1× PCR Buffer (Invitrogen), 2.0–2.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer and 0.5 unit of Platinum *Taq* DNA Polymerase (Invitrogen). The PCR conditions were the same for the 3 mitochondrial segments and began with one step of 94 °C for 3 min, 10 cycles ("Touchdown") of 94 °C for 45 s, 60–51 °C for 45 s (−1 °C per cycle), 72 °C for 1.5 min; this was followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1.5 min, and final extension of 72 °C for 3 min. The PCR conditions for amplification of DNA from faeces were similar as above, except for 5 cycles (Touchdown) of 94 °C for 45 s, 55–51 °C for 45 s, 72 °C for 1.5 min followed by 40 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1.5 min and final extension of 72 °C for 30 min. Products were visualized on a 1% agarose gel stained with GelRed 10× (Biothium) and purified either by precipitation methods with Polyethyleneglycol 8000 or amonium acetate, or by employing the enzymes Exonuclease I and Shrimp Alkaline Phosphatase. Purified PCR products were sequenced using the "DYEnamic ET Dye Terminator Sequencing Kit" (GE Healthcare), and analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare). Sequences were deposited in GenBank under accession numbers JQ038804–JQ038869.

Sequence Analyses

Sequences were visually checked and manually corrected using CHROMAS 2.0 (http://www.technelysium.com.au/chromas.html) or FinchTV 1.4.0 (www.geospiza.com/

Products/finchtv.shtml) and aligned with the CLUSTALW algorithm implemented in MEGA 4 (Tamura et al. 2007). The alignment of each mtDNA segment was checked and edited by eye separately. Statistics such as nucleotide (π) and haplotype (h) diversity were computed using MEGA, DnaSP v.5 (Librado and Rozas 2009) and ARLEQUIN 3.01 (Excoffier et al. 2005).

Phylogenetic analyses were performed separately for 2 mtDNA data sets: 1) full concatenation of all segments (CR + *ATP8/ATP6* + *ND5*) (Data Set 1, DS1) and 2) concatenation of the 3 coding fragments (*ATP8/ATP6* + *ND5*) (Data Set 2, DS2). The data sets were assessed for the best-fitting model of nucleotide substitution using the Akaike Information Criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall 1998). We inferred phylogenetic relationships among haplotypes using PAUP* 4.0b10 (Swofford 2002) for 3 different optimality criteria: 1) maximum likelihood (ML) employing the selected model and estimated parameters, with a heuristic search started from a neighbor joining (NJ) tree and using the tree-bisection-reconnection (TBR) branch-swapping method; 2) maximum parsimony using heuristic searches with 50 replicates of random taxon addition and TBR branch-swapping; and 3) distance based, using the NJ algorithm and ML genetic distances. Support of internal branches for all of the above methods was evaluated with 100 nonparametric bootstrap replicates. Additionally, we employed a Bayesian Inference approach with MrBayes 3.1 (Huelsenbeck and Ronquist 2001), using 2 independent Markov Chain Monte Carlo (MCMC) runs, each containing 4 Metropolis-coupled chains (1 cold and 3 heated) for 1 million generations. Trees were sampled every 100 generations, discarding the first 2500 trees as burn-in. Convergence was considered satisfactory when the average standard deviation of split frequencies between parallel runs was lower than 0.05. Finally, we also assessed the relationships among haplotypes using the relaxed phylogenetics approach implemented in BEAST 1.6.0 (Drummond and Rambaut 2007), in parallel with the divergence dating analyses. In all cases, trees were rooted using *Lontra provocax* and *L. felina* as outgroups.

Given the evidence for saturation in the CR, divergence times for inferred phylogeographic partitions were estimated for DS2 only. Moreover, to further minimize the effects of saturation, we limited this analysis to genus *Lontra*, thus excluding any more distant outgroups. Divergence dates were estimated with BEAST, applying molecular calibrations reported in a previous phylogenetic study of the Mustelidae (Koepfli et al. 2008) for 2 different divergence points in the subfamily Lutrinae: 1) base of the genus *Lontra*; 2) *Lontra longicaudis* versus *L. felina* + *L. provocax*. The age of these nodes (corresponding to nodes 14 and 15 in Koepfli et al. 2008, respectively) was assumed to lie within the credibility intervals reported in that study: 1) 1.6–5.2 million years ago (Ma) and 2) 0.5–3.2 Ma, respectively. To apply these molecular calibrations in a conservative fashion, we used these minimum and maximum ages as boundaries in a uniform prior distribution for the node age. We employed 2 distinct strategies to model nucleotide substitution in our data set: 1) estimating the best-fit model for each segment using Modeltest and 2) assuming a segment-specific SRD06 model (Shapiro et al. 2006), in which the 1st and 2nd codon positions were grouped, and the 3rd codon position was placed in a separate category. In every case, we assumed an uncorrelated lognormal relaxed molecular clock and a tree prior based on a Yule process. The MCMC procedure was run for 10–100 million generations, with samples taken every 1000–10 000 steps. Results were analyzed with the program Tracer v. 1.5 (Rambaut and Drummond 2007) removing the initial 1–10 million steps (10% of each run) as burn-in.

In addition to the phylogeny-based approaches, haplotype networks were constructed using the median-joining approach (Bandelt et al. 1999) implemented in Network 4.5.1.0 (http://www.fluxus-engineering.com) to depict phylogenetic, geographic, and potential ancestor-descendent relationships among the sequences. Population structure analyses were performed assuming broad geographic units based on the observed phylogeographic pattern. The testing of additional alternative scenarios of geographic subdivision could not be fully performed with this approach due to limitations of sample size for some of the included areas which are represented by only one to few individuals. As measures of differentiation among populations, we estimated fixation indices (F_{ST}) (Wright 1965), using an Analysis of Molecular Variance (AMOVA) approach (Excoffier et al. 1992) implemented in ARLEQUIN. Finally, we tested the null hypothesis of no correlation between geographic and genetic distances using a Mantel test (Mantel 1967) as implemented in AIS 1.0 (Miller 2005), with statistical significance assessed from 1000 random permutations.

To investigate the historical demography of *L. longicaudis*, we used DnaSP and ARLEQUIN to perform neutrality tests, namely Tajima's D, Fu and Li's F* and D*, and Fu's Fs (Tajima 1989; Fu and Li 1993; Fu 1997), and also to conduct Mismatch Distribution Analyses (Rogers and Harpending 1992). In addition, we used BEAST to generate Bayesian Skyline plots (BSP), which allow an assessment of historical patterns of change in N_e over time. For this intraspecific analysis, we assumed a strict molecular clock and a piecewise-constant Bayesian skyline tree prior. The substitution model and evolutionary rate for each segment were derived from the initial BEAST run that provided the best fit to the data set (see Results). We employed a segment-specific clock rate, which was entered as a range of values reflecting the 95% highest posterior density (HPD) interval resulting from the best-fitting initial BEAST run.

Results

A 516-bp fragment of the CR was sequenced for 51 *L. longicaudis* individuals. Due to ambiguous alignment in one hypervariable segment, 25 sites of the CR were excluded from all further analyses, yielding a final data set of 491 bp for this fragment. Sequences of the *ATP8/ATP6* fragment

Table 1 mtDNA diversity estimates for the Neotropical river otter and related species

Segment[a]	Length (base pairs)[b]	*N*	Number of haplotypes	*S*[c]	PI[d]	π[e]	d_{xy} (*Lontra felina*)[f]	d_{xy} (*Lontra provocax*)[f]	d_{xy} (*Lontra canadensis*)[f]	d_{xy} (*Pteronura brasiliensis*)[f]
CR	491 (438)	51	29	29/37	24/32	0.01149 ± 0.00270	0.03484 ± 0.00702	0.03763 ± 0.00776	0.05296 ± 0.00950	0.08611 ± 0.01124
ATP8/ATP6	329 (295)	52	15	20/38	6/24	0.00432 ± 0.00142	0.06572 ± 0.01344	0.06263 ± 0.01292	0.17632 ± 0.01968	0.20043 ± 0.02052
ND5	651 (651)	52	19	37/64	14/38	0.00741 ± 0.00177	0.05217 ± 0.00765	0.05122 ± 0.00778	0.12242 ± 0.01142	0.17166 ± 0.01410
ATP8/ATP6 + *ND5*	977 (925)	52	24	57/102	20/62	0.00639 ± 0.00129	0.05666 ± 0.00723	0.05500 ± 0.00677	0.14026 ± 0.01081	0.18117 ± 0.01170
CR + *ATP8/ATP6* + *ND5*	1471 (1337)	51	37	86/139	44/94	0.00814 ± 0.00131	0.04942 ± 0.00548	0.04922 ± 0.00522	0.11125 ± 0.00743	0.14967 ± 0.00963

[a] CR = mtDNA control region; *ATP8/ATP6*: ATPase subunit 8/ATPase subunit 6; *ND5*: nicotinamide adenine dinucleotide dehydrogenase subunit 5.

[b] Values in parentheses are segment lengths after exclusion of all sites containing gaps or missing information.

[c] *S* = Segregating (polymorphic) sites; values are given for the *L. longicaudis* data set/data set, including *L. longicaudis* + *L. felina* + *L. provocax*.

[d] PI = Parsimony-informative sites; values are given for the *L. longicaudis* data set/data set, including *L. longicaudis* + *L. felina* + *L. provocax*.

[e] π = Nucleotide diversity per site.

[f] d_{xy} = mean nucleotide divergence (*p*-distance) between *L. longicaudis* sequences and selected outgroups.

(329 bp, including164 bp of *ATP8* and 165 of *ATP6*, with an overlap of 40 bp) and the *ND5* fragment (651 bp) were obtained for 52 Neotropical otters each. Outgroups were sequenced for these mtDNA regions yielding the same sequence length, except for *Aonyx cinereus*, whose CR segment was 1 bp longer than the remaining individuals. All segments were concatenated for use in various analyses, leading to a combined alignment containing 1472 bp (1471 bp when excluding the *Aonyx* sequence).

For all 3 segments, haplotype (gene) diversity was moderate to high, while nucleotide diversity was low to moderate (Tables 1 and 2). The CR was the most variable segment but showed evidence of saturation when other otter species were included in the comparisons (see Table 1 and Figure 2). This led us to conduct separate analyses with and without this fragment. Alignments containing concatenations of the full data set (DS1; $n = 51$) or the 2 coding fragments (DS2; $n = 52$) led to the observation of 37 and 24 unique haplotypes, respectively (Supplementary Table S2). The TIM + I + G and the TrN + G nucleotide substitution models were found to provide the best fit to DS1 (−lnL = 4888.1147) and DS2 (−lnL = 1986.4604), respectively and were applied in all subsequent model-based phylogenetic analyses.

Phylogenetic trees produced with all different methods were consistent with respect to major topological features, with mostly subtle differences in nodal support. Support for major groups was strong with all methods, indicating robust resolution of the main clades (Figure 3). A clear phylogeographic pattern could be discerned, with 2 major clades retrieved by all analyses (Figure 3 and Supplementary Figure S1) and supported by robust bootstrap values (>75% with DS1 and >88% with DS2) and Bayesian posterior probabilities of 1.0 for both DS1 and DS2. Clade 1 contained almost all sequences sampled in Brazil, along with 2 sequences from French Guiana (bLlo41 and bLlo66). Clade 2 contained all other samples from French Guiana, along with most individuals from the Brazilian Amazon and one sample from Peru.

In addition to these 2 main clades, 2 other distinct haplotypes were observed in the phylogenetic analysis, each of them represented by a single sample in this study. One of them was the haplotype found in Bolivia (Ll-ANC33 and Ll-AN18 in DS1 and DS2, respectively), whose exact placement was not identical with the 2 data sets: in DS1, it was the sister group of Clade 2, whereas in DS2, it was positioned in a trichotomy with respect to Clades 1 and 2 (Figure 3 and Supplementary Figure S1). This suggests that Bolivia may contain a third phylogeographic lineage separate from Clades 1 and 2, whose exact relationships should be further investigated with additional sampling. Furthermore, the individual from Colombia (bLlo23) contained a very distinct haplotype (Ll-ANC35 and Ll-AN13 in DS1 and DS2, respectively) presenting at least 17 mutation steps relative to any other haplotype and being positioned as the most basal lineage of all *L. longicaudis*.

An analysis of phylogenetic and geographic structure within Clade 1 revealed some further patterns. The samples

Table 2 Nucleotide and gene diversity observed in the *Lontra longicaudis* mtDNA segments (specified separately for the two different geographic groups: ESA and Amazonia)

Segment	Geographic group	Nucleotide diversity (SE)[a]	Gene diversity (SE)[a]
CR	ESA	0.00620 ± 0.00180	0.8187 ± 0.00849
	Amazonia	0.01210 ± 0.00258	0.9487 ± 0.00592
	Total	0.01149 ± 0.00270	0.9043 ± 0.00477
ATP8/ATP6 + ND5	ESA	0.00153 ± 0.00040	0.7873 ± 0.00896
	Amazonia	0.00586 ± 0.00124	0.8791 ± 0.01093
	Total	0.00639 ± 0.00129	0.8906 ± 0.00486
CR + *ATP8/ATP6 + ND5*	ESA	0.00310 ± 0.00070	0.9361 ± 0.00408
	Amazonia	0.00822 ± 0.00133	0.9890 ± 0.00439
	Total	0.00814 ± 0.00131	0.9694 ± 0.00210

[a] Calculated using *p*-distances.

from French Guiana (individuals bLlo41 and bLlo66—for haplotype numbers, see Supplementary Table S2) were consistently placed in basal positions. With DS1, they grouped with one haplotype from northeastern Brazil (Ll-ANC17), while with DS2, they were the sister group to all other samples (see Supplementary Figure S1). In the latter case, the haplotype from northeastern Brazil (Ll-AN16) was the next one to diverge and thus remained at a relatively basal position. All other haplotypes (representing a broad sample of individuals from across eastern, central, and southern Brazil) clustered in a single internal clade, which was robustly supported by most analyses. Its internal phylogeny exhibited very short branches, little structure and no evidence of geographic substructure, suggestive of a recent population expansion. On the basis of these results, we defined a geographic group named Eastern South America (ESA), containing most samples from Clade 1 and no haploytpe from Clade 2 (see Figure 1). A second group named "Amazonia" was defined as the geographically delimited sample set containing all sequences from Clade 2 plus 3 haplotypes from Clade 1 (see Figure 1 and Supplementary Table S2).

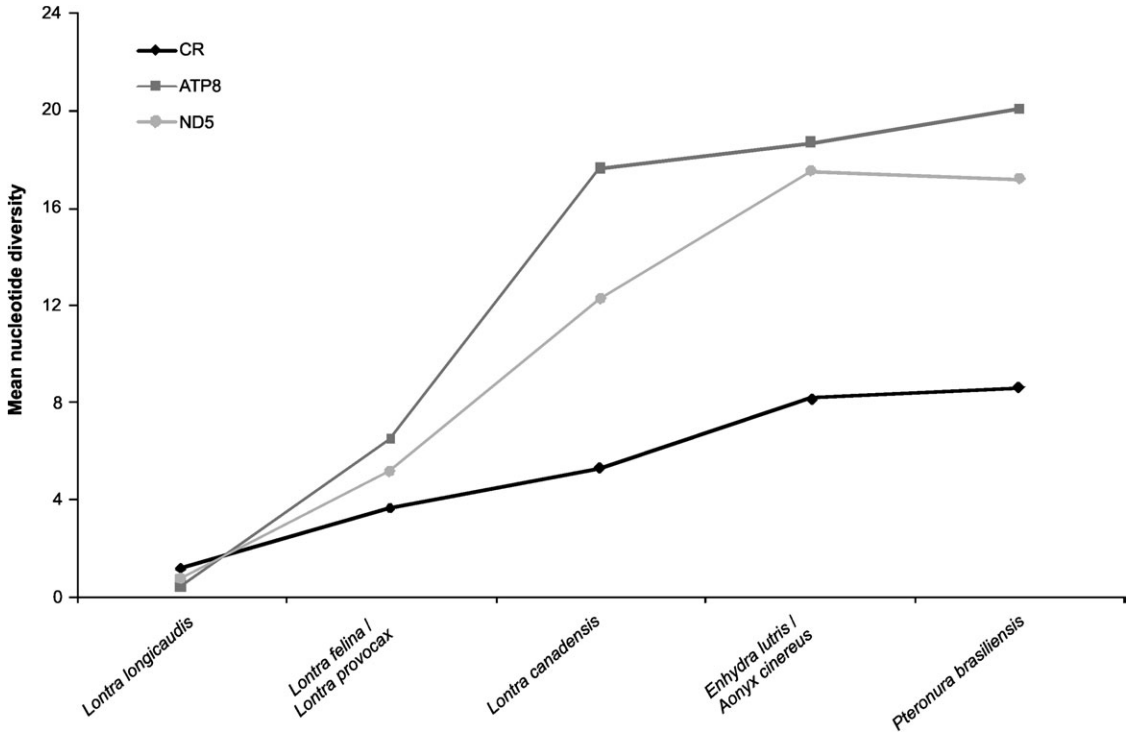

Figure 2. Graph depicting an analysis of saturation at the 3 mtDNA segments employed in the present study. Values in the *y* axis represent nucleotide diversity (π) for *L. longicaudis* or mean *p*-distance (D_{xy}) for all pairwise comparisons relating Neotropical otter sequences with those of each of several related otter species. The sequence of species placement on the *x* axis reflects their evolutionary divergence relative to *L. longicaudis* but is not strictly positioned on a timescale (for divergence date estimates among these species, see Koepfli et al. 2008).

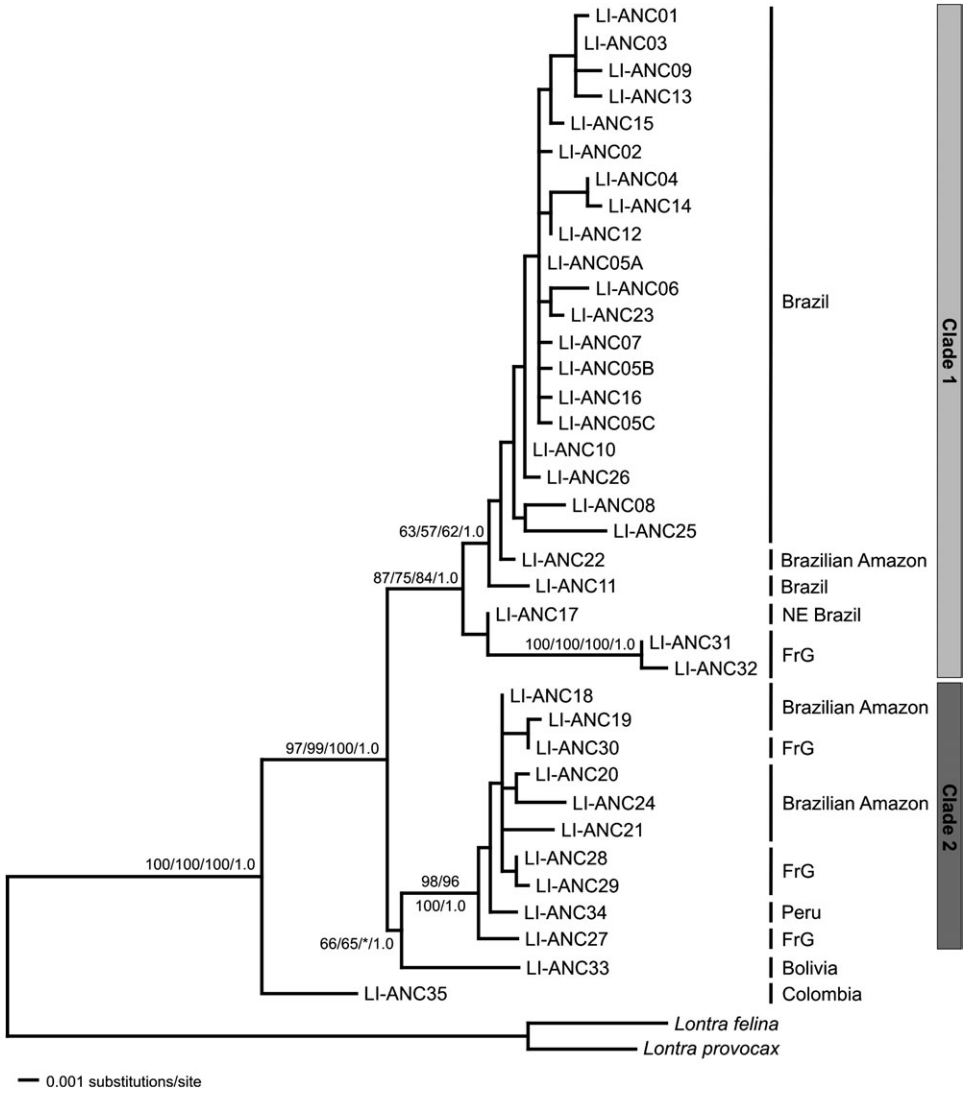

Figure 3. ML tree of *L. longicaudis* mtDNA haplotypes identified in this study, based on 1471 bp of concatenated CR + *ATP8/ATP6* + *ND5* sequences (DS1). Labels are haplotype identification numbers (see Supplementary Table S2). Values in the branches indicate support for the adjacent node based on ML/MP/NJ/BI. The asterisk represents nodal support <60%.

The median-joining network produced with the full concatenation (DS1) was not entirely efficient at resolving the relationships among haplotypes (see Supplementary Figure S2), probably due to saturated mutation sites in the CR, which may have led to reticulation and a high number of median vectors. Nevertheless, a star-shaped pattern could be observed in the set of ESA haplotypes. In contrast, the haplotype network produced with DS2 depicted a clear phylogeographic pattern, with at least 7 mutational steps separating the samples belonging to Clades 1 and 2 (Figure 4). The ESA haplotypes, contained within Clade 1, exhibited a star-shaped pattern with several localized lineages connected by short branches to a more common, widespread sequence. Again, individuals bLlo41 and bLlo66 (for haplotype identification, see Supplementary Table S2) were positioned near this group instead of being associated with other samples from French Guiana or the Brazilian Amazon. This analysis also showed Clade 2 as a separate group, which comprised most haplotypes from French Guiana, one sample from Peru and almost all animals from the Brazilian Amazon (see Figure 4). Interestingly, one Amazonian haplotype (Ll-AN21) deviated from this pattern by nesting within Clade 1, directly connected to its central sequence.

Given these results and the availability of denser sampling on a regional scale, we focused subsequent geography-based analyses on the 2 broad phylogeographic units defined above (ESA vs. Amazonia), which were assessed in terms of their genetic diversity and differentiation. The AMOVA results indicated that 62–72% (DS1 and DS2, respectively) of the observed genetic variability

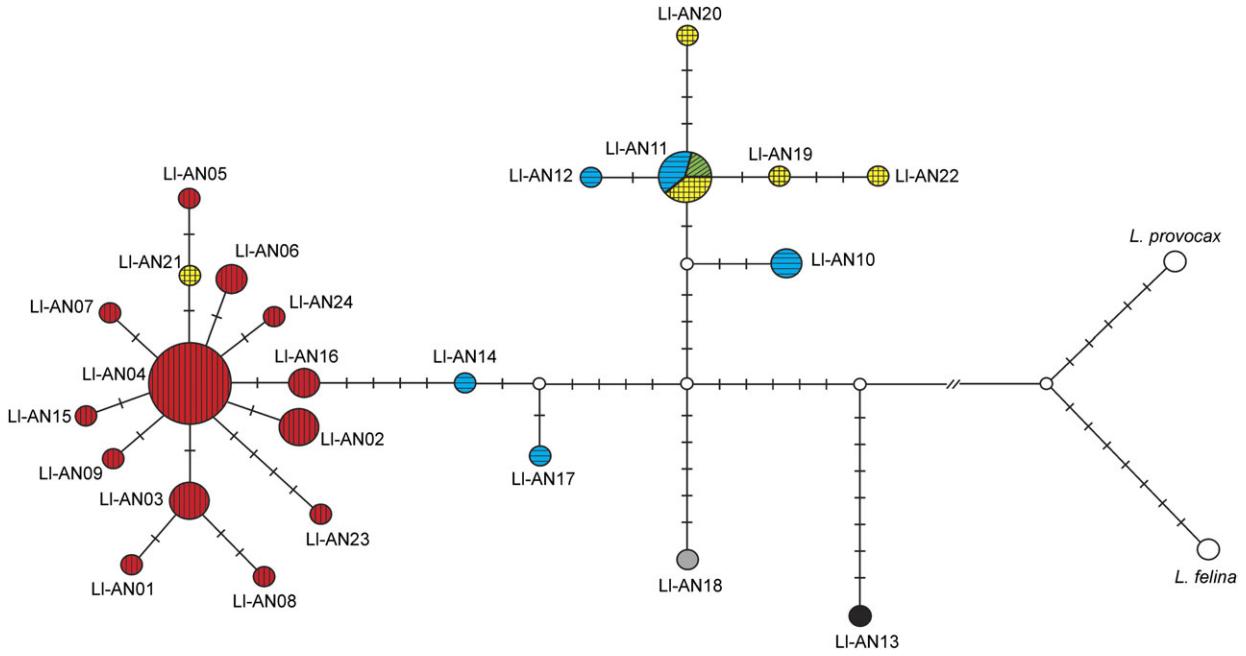

Figure 4. Median-joining network of *L. longicaudis* mtDNA haplotypes based on concatenated *ATP8/ATP6* + *ND5* sequences (DS2; 681 bp were used; all sites containing indels or missing information were excluded). Cross marks are nucleotide substitutions inferred to have occurred on each branch. Circles correspond to haplotypes, whose frequency in our sample is indicated by the circle size. Geographic origins of haplotypes are as follows: red (vertical lines in the print version): ESA; blue (horizontal lines in the print version): French Guiana; yellow (cross hatched in the print version): Brazilian Amazon; light green (diagonally hatched in the print version): Peru; light gray: Bolivia; black: Colombia; and white: outgroups.

(excluding the samples from Bolivia and Colombia) corresponded to differences between these populations. The F_{ST} between these 2 geographic groups was very high (0.63, $P = 0.000$ and 0.72, $P = 0.000$, for DS1 and DS2, respectively), as expected given their almost perfect reciprocal monophyly. Finally, the Mantel test did not reveal any significant relationship between genetic and geographic distances when the entire sample was compared ($r = 0.19$, $P = 0.98$), or when Clade 1 ($r = 0.28$, $P = 0.96$) or Clade 2 ($r = -0.12$, $P = 0.31$) were analyzed separately.

Initial rounds of divergence dating analyses with BEAST were focused on establishing the best-fit model of nucleotide substitution for each segment (in this case, we treated *ATP8* and *ATP6* as distinct segments, including the overlapping 40 bp only in the latter; additionally, the initial 3 bp of the *ATP8* fragment were excluded so as to exactly match the coding region of this genetic locus). The AIC assessment with Model test indicated that HKY + G provided the best fit to *ATP8*, HKY + I to *ATP6*, and GTR + I to *ND5*. Although the analysis employing such models provided a good fit to the data, we observed that a considerable improvement (i.e., significantly higher posterior probability) could be attained with the use of the codon-based SDR06 model. We therefore employed this approach in all subsequent analyses with BEAST, including divergence dating and BSP. The mean divergence time between *L. longicaudis* and *L. felina* + *L. provocax* was estimated to be 1.15 Ma (95% HPD: 0.53–1.99 Ma), while the mean coalescence of all *L. longicaudis* haplotypes (Time to the Most Recent Common Ancestor) was estimated at 0.58 Ma (95% HPD: 0.22–1.07 Ma). Furthermore, the age of haplotype coalescence within each of the 2 main *L. longicaudis* internal clades (Clades 1 and 2) was estimated at 0.28 Ma (95% HPD: 0.097–0.52 Ma) and 0.19 Ma (95% HPD: 0.056–0.38 Ma), respectively. The divergence time between *L. felina* and *L. provocax* was estimated to have occurred approximately 0.28 Ma (95% HPD: 0.076–0.59 Ma).

To test the hypothesis of a recent population expansion in *L. longicaudis*, we performed mismatch distribution analyses and neutrality tests for each of the mtDNA haplogroups. Mismatch distribution analyses revealed some cases of smooth unimodal patterns, indicative of a population expansion following a genetic bottleneck (Figure 5). This was especially the case for Clade 1 (and even more so its ESA subgroup) when assessed with DS2. Clade 2 did not show such a clear pattern but did exhibit a unimodal distribution when assessed with DS1 (Figure 5). These results were congruent with those obtained with the neutrality tests (Table 3), which yielded negative values for all the assessed groups, consistent with the inference of historical population expansion. Significantly negative values were observed in all tests for the full sample of *L. longicaudis* when assessed with DS2 (but only with Fu's Fs when using DS1). The strongest signal for population expansion

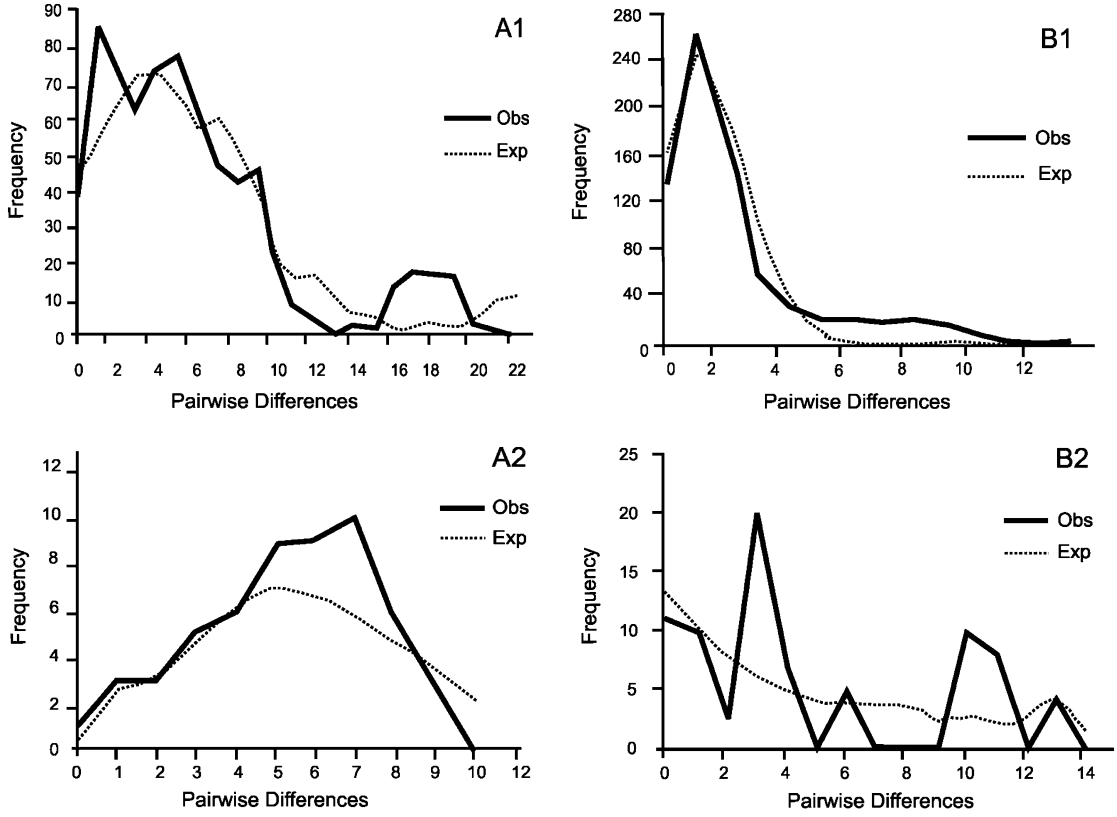

Figure 5. Mismatch distribution analysis of the 2 data sets employed in this study (DS1 [**A**] and DS2 [**B**]) for Clade 1 (A1, B1) and Clade 2 (A2, B2). The continuous line indicates the observed frequency of pairwise differences among haplotypes, while the dotted line depicts the expected frequency under a sudden population expansion model.

(significantly negative values across most or all tests) was observed for Clade 1 and ESA when assessed with DS2, with a trend for stronger departure from the neutral model when the analysis was restricted to the latter, geographically defined, set of sequences.

The BSP were consistent with a scenario of population growth in Clade 1 (Figure 6A), with a clear signal of demographic expansion starting approximately 0.025 Ma. In contrast, there was only a modest trend suggesting gradual population increase in Clade 2 but no marked signal of recent demographic expansion.

Discussion

Genetic Diversity

As observed in a previous study based on a smaller data set (Trinca et al. 2007), the present results support the view that

Table 3 Neutrality test results for the two data sets used in this study (DS1 and DS2), as well as different phylogenetic or geographic subgroups (see text and Figure 1)

		Fu's *Fs*	Fu and Li's *F*	Fu and Li's *D*	Tajima's *D*
DS1	All samples	**−12.869 (*P* = 0.000)**	−2.310 ($P > 0.05$)	−2.337 ($P > 0.05$)	−1.254 ($P > 0.10$)
	Clade 1[a]	**−10.155 (*P* = 0.000)**	−1.741 ($P > 0.10$)	−1.401 ($P > 0.10$)	−1.596 ($P > 0.05$)
	ESA[b]	**−10.916 (*P* = 0.000)**	−2.306 ($P > 0.05$)	−2.149 ($P > 0.05$)	−1.552 ($P > 0.10$)
	Clade 2	**−4.288 (*P* = 0.012)**	−0.726 ($P > 0.10$)	−0.514 ($P > 0.10$)	−1.008 ($P > 0.10$)
DS2	All samples	**−7.003 (*P* = 0.001)**	**−3.523 ($P < 0.02$)**	**−3.694 ($P < 0.02$)**	**−2.013 ($P < 0.05$)**
	Clade 1[a]	**−9.654 (*P* = 0.000)**	**−2.751 ($P < 0.05$)**	−2.410 ($P > 0.05$)	**−2.156 ($P < 0.05$)**
	ESA[b]	**−9.981 (*P* = 0.000)**	**−3.189 ($P < 0.05$)**	**−2.987 ($P < 0.05$)**	**−2.124 ($P < 0.05$)**
	Clade 2	−1.088 ($P = 0.162$)	−1.089 ($P > 0.10$)	−0.862 ($P > 0.10$)	−1.264 ($P > 0.10$)

Numbers highlighted in bold are significant for $\alpha < 0.05$.

[a] See Figure 3 for definition of Clade 1.

[b] See Figure 1 for definition of ESA.

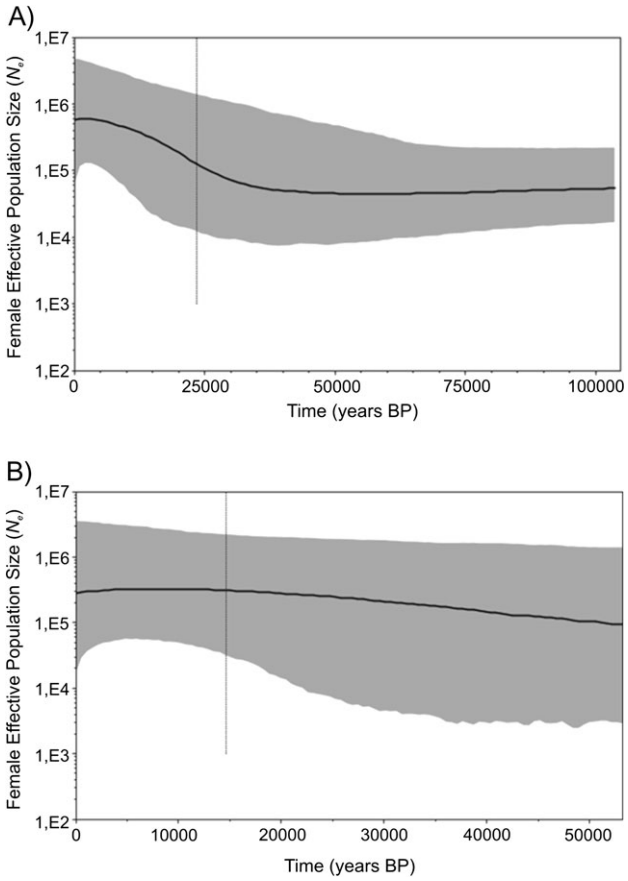

Figure 6. BSPs generated with BEAST 1.6.0 based on Data Set 2 (*ATP8*/ATP6 + *ND5* segments). Separate analyses are shown for Clades 1 (**A**) and 2 (**B**). The analysis employed segment-specific substitution rates that were previously estimated with BEAST in parallel to divergence time calculations (for details, see text).

the Neotropical otter presents moderate genetic diversity in its mtDNA CR. Levels of polymorphism in this segment were similar to those reported for other otters, such as *Lontra felina* and *Pteronura brasiliensis* (Garcia et al. 2007; Valqui et al. 2010; Vianna et al. 2010) and also comparable with those observed in other carnivores, such as jaguars (Eizirik et al. 2001) and crab-eating foxes (Tchaicka et al. 2007). However, diversity in *L. longicaudis* tended to be higher than that found for *Lontra provocax* in the Argentinean Patagonia (Centrón et al. 2008) and for *Lutra lutra* in Europe (Effenberger and Suchentrunk 1999; Mucci et al. 1999; Cassens et al. 2000; Ferrando et al. 2004; Pérez-Haro et al. 2005; Stanton et al. 2009). In contrast, Larson et al. (2002) reported an about 8-fold higher estimate of nucleotide diversity for the sea otter *Enhydra lutris*.

In addition to the CR results, our data sets allowed an assessment of diversity levels in the mitochondrial genes *ATP8/ATP6* and *ND5* (Tables 1 and 2). The observed variability in the *ATP8/ATP6* and *ND5* segments could not be directly compared with other otters, due to the absence of polymorphism data from additional species. Nevertheless, data from 2 small Neotropical felids (*Leopardus tigrinus* and *L. geoffroyi*) reveal a somewhat lower diversity in both segments (Trigo et al. 2008; Trigo T, personal communication) relative to *L. longicaudis*, suggesting that the latter species harbors considerable variability in these fragments, albeit lower than that observed in the CR.

In spite of its high diversity relative to other mtDNA segments, our analyses suggest that the CR may not be the best mitochondrial marker for phylogeographic studies in *L. longicaudis* and perhaps other related species. Variable sites in this segment seem to be saturated even at the intraspecific level, leading to a lower signal-to-noise ratio than observed for the other 2 fragments (Figure 2). Conversely, the *ND5* segment used here seems to be very informative and less prone to saturation at recent levels, which has also been observed for other carnivores studied by our group (Matte EM, Fontoura-Rodrigues ML, Bornholdt R, Figueiró HV, unpublished data).

Phylogeographic Structure

The most evident pattern provided by the mtDNA data was the deep partition between 2 broad groups of populations, separated in a northwest–southeast fashion, in addition to divergent lineages in Bolivia and Colombia (see Figures 1, 3, and 4). The 2 main phylogeographic groups (ESA and Amazonia) were strongly supported by all analyses, including phylogenies, haplotype networks, and AMOVA. Overall, our results indicate that *L. longicaudis* is composed of at least 4 geographically structured phylogroups: 1) Colombia, 2) Bolivia, 3) Amazonia, encompassing all samples from the Amazon basin (except Bolivia) and French Guiana, and 4) ESA, containing several other drainages. It may be noted that Colombia and Bolivia were represented by a single sample each (whose genetic divergence from the other individuals indicates deep phylogeographic partitions involving these areas), so that further sampling is needed to ascertain their evolutionary uniqueness.

Interestingly, the separation of the Colombian haplotype from any other *L. longicaudis* implies at least 17 mutational steps, which is considerably deeper than the divergence estimated between *Lontra felina* and *L. provocax* (12 mutational steps). This substantial differentiation is also reflected in the estimates of divergence times, with the separation between the Colombian sample and the remaining *L. longicaudis* inferred to have been 0.575 Ma, while that between *L. felina* and *L. provocax* would have occurred approximately 0.28 Ma (for a different but overlapping estimate of the divergence age for this node, see Vianna et al. 2010). This Colombian sample originated from the Magdalena river valley, which is separated from other regions on either side by the Cordillera Central and the Cordillera Oriental, respectively. The deep genetic divergence observed in this region relative to the remaining samples could be due to fact that the Magdalena river flows northward into the Caribbean Sea (Eisenberg and Redford 1999), possibly hampering the genetic connectivity between the 2 sides of the Cordillera Oriental.

In addition to the interest in unraveling the biogeographic processes shaping this evolutionary distinction, such a deep divergence could imply that a taxonomic revision in this group may indeed be warranted.

The almost complete reciprocal monophyly of the 2 better-sampled phylogroups (Amazonia and ESA) is remarkable, with only 3 individuals (bLlo41, bLlo66, and bLlo72) found in a geographic region inconsistent with their phylogenetic placement (French Guiana and Brazilian Amazon). The position of these 3 samples in Clade 1 is intriguing and raises 2 alternative hypotheses, namely relictual sister-group relationship due to ancestral colonization versus secondary gene flow between the 2 regions (Avise 2000). The observed phylogenetic pattern supports the former hypothesis for bLlo41 and bLlo66 (which are solidly placed at the base of Clade 1) and the latter for bLlo72 (which is clearly positioned within Clade 1). We may thus postulate that the ESA group (almost completely bearing Clade 1 haplotypes) derives from a recent episode of colonization from the north of South America, so that northern populations are paraphyletic with respect to more southerly ones. Another observation that is consistent with this inference is the position of the single haplotype (sampled in 2 different individuals) from the Brazilian northeast, which is the most basal of all Brazilian lineages within Clade 1 (e.g., Ll-ANC19 in Figure 3; see also DS2 tree in Supplementary Figure S1). This hypothesis should be further tested with additional sampling in northern Brazil and adjacent areas.

Within Clades 1 and 2, there was little evidence of geographic structure, with no regional clustering of haplotypes or clear associations to river basins (Figures 3 and 4). The apparent lack of genetic structure within Clade 1 and Clade 2 could derive from extensive gene flow among populations in those areas and/or from a recent origin of each set of populations from a common ancestral gene pool. Our divergence date estimates indicate that both clades have a considerably recent coalescence (0.28 Ma for Clade 1 and 0.19 Ma for Clade 2), suggesting that this may be an important component underlying the observed lack of internal geographic structure.

The phylogeographic patterns observed here are largely congruent with the results reported by Hubert and Renno (2006) for South American characid fishes, which indicated that different drainages such as the Amazon and Paraná comprised distinct species pools. In the case of river dolphins, molecular analyses allowed the recognition of 2 distinct mtDNA lineages from the Bolivian Amazon and the Colombian Orinoco/Colombian Amazon, supporting the recognition of 2 Evolutionarily Significant Units (ESUs) in those areas (Banguera-Hinestroza et al. 2002). Also, a phylogeographic pattern similar to those observed here was reported by Camargo et al. (2006) for the Neotropical frog *Leptodactylus fuscus*, a species that occupies open habitats and river edges. In that species, 3 well-supported clades were observed, one from central and northern South America (including most of the Amazon basin), another from Bolivia, and the third comprising almost the entire territory of the Brazilian shield and Argentina. Those clades did not seem to overlap geographically, supporting the hypothesis of *L. fuscus* being a "species complex" (Camargo et al. 2006). Such congruent patterns in different taxa are suggestive of common underlying processes leading to regional population isolation and differentiation, likely influenced by the history of river basins and its impact on associated species.

Finally, some aspects of the *L. longicaudis* phylogeographic structure are comparable to those previously reported for a related and largely sympatric species, the giant river otter *Pteronura brasiliensis* (Garcia et al. 2007). In both cases, there is evidence of significant genetic structure on a broad geographic scale. On the other hand, both species show evidence of long-range historical connectivity among regions leading to some haplotype sharing between different biomes (in the case of *Pteronura*) or relatedness among haplotypes sampled in very distant areas (in *L. longicaudis*). These observations (still restricted to matrilineal markers and possibly even more visible when male lineages are surveyed) are likely a result of these species' semiaquatic habits and consequent dispersal capabilities within and among drainages.

Demographic History

When intraspecific levels of molecular variation were assessed for *L. longicaudis*, we observed that the ESA group presented lower nucleotide and haplotype diversity than the Amazonian group, a pattern that was consistent across all data partitions (Table 2). Given the larger sample size available for ESA relative to Amazonia, this observation may in fact be a reflection of contrasting demographic histories between these 2 regions. Such pattern prompted us to investigate in more detail the population history of each region, based on multiple complementary approaches.

The network analysis placed haplotype Ll-AN04 at a central position in the ESA portion of Clade 1, with its high frequency also suggesting an ancestral status within this lineage. The pattern observed with the mtDNA phylogenies and haplotype networks is consistent with a recent population expansion in ESA, with most haplotypes from this region differing from each other by only 1 or 2 mutational steps, connected in a star-shaped fashion (e.g., Figure 4). This inference is also supported by the mismatch distribution analyses (Figure 5) and the neutrality tests (Table 3) performed for Clade 1 (which contains all ESA lineages). The mismatch distribution results for this clade based on DS2 (less noise in terms of phylogenetic signal, with less homoplasy than inferred for the CR) fit particularly well, the expected pattern under a sudden demographic expansion (Rogers and Harpending 1992), with a single smooth prominent peak. Although there was also some suggestion of recent demographic expansion in Clade 2 (especially when DS1 was analyzed—see Figure 5), it was not as conclusive as that observed for Clade 1. The same conclusion emerges from an inspection of the neutrality test results (Table 3), which showed consistent support for

a demographic expansion in Clade 1 (and ESA within it), and only modest support for an equivalent process in Clade 2 (significant only for Fu's Fs estimated with DS1).

A congruent pattern was also observed in the Bayesian skyline plot analyses (Figure 6), with Clade 1 exhibiting a much clearer signal of recent population expansion than Clade 2. An interesting inference derived from this approach was the estimation of the time frame for this demographic expansion affecting Clade 1. Our results suggest that Clade 1 lineages remained rather stable in effective population size until approximately 25,000 years ago, when they underwent a substantial (approximately 10-fold) demographic increase.

Taxonomy

The taxonomy of *L. longicaudis* has been the target of recent discussion, since morphological variation in the rhinarium (which is often argued to underlie species or subspecies differentiation in otters—Larivière 1999) has been reported by researchers from different parts of the species' distribution (Xth International Otter Colloquium, South Korea, 2007). Although the variation in rhinarium shape has not yet been thoroughly characterized across the species' range, preventing a more complete assessment of the putative subspecies, our phylogeographic results demonstrate considerable agreement with the proposed geographic range of these infraspecific partitions. The ESA phylogroup largely agrees with the proposed range of *L. longicaudis longicaudis*, presumed to occur in most of South America. The Amazonian phylogroup may represent the subspecies *L. longicaudis enudris*, believed to occur in French Guiana, Suriname, Trinidad, and Peru. Finally, the divergent Colombian lineage could be considered to be suggestive of historical differentiation of the subpecies *L. longicaudis annenctes*, which would be restricted to Central America and northwestern South America (Larivière 1999). A possible discrepancy between the proposed subspecific scheme and our phylogeographic results is the indication that Bolivia contains a fourth major mtDNA lineage in *L. longicaudis*, suggesting that further sampling may reveal additional evolutionary partitions in this otter species.

However, any intraspecific taxonomic decision on the Neotropical otter is premature at this point, given the still modest geographic sampling across the species' range and the breadth of the present data set. Although our phylogeographic results largely match the currently proposed subspecies, additional analyses should be conducted employing both molecular and morphological approaches, so as to further ascertain and refine the observed geographic partitions. Molecular analyses should include nuclear markers, which would complement the matrilineal mtDNA sequences analyzed here. Likewise, more in-depth morphological assessments should include a broad suite of characters (e.g., cranial measurements) in order to complement and test the perceived differentiation based on rhinarium shape.

Implications for Conservation

The results presented here have important implications for the conservation and management of this species in the wild and in captivity as well as its habitats. In spite of being represented in this study by a single sample each, the divergent lineages observed in Colombia and Bolivia suggest that these areas may be sufficiently differentiated to indicate their provisional recognition as distinct evolutionary entities. Given the limited sampling, we do not propose that they be treated as fully recognized ESUs but rather point out that they should be a priority for further study in this regard.

The main phylogeographic groups identified here (ESA and Amazonia) indicate that these regions have likely been historically isolated, at least with respect to female lineages. However, some historical female connectivity between these areas may also be inferred from our data, as illustrated by sample bLlo72, which was collected in the Brazilian Amazon but harbors an mtDNA haplotype contained in Clade 1. Nevertheless, the genetic differentiation detected between these 2 regions was substantial, indicating little gene flow, and consequently they may be viewed as distinct ESUs or at least Management Units (Moritz 1994). Each of these groups is widespread and associated with broad watersheds and diverse ecoregional units (Olson et al. 1998), likely justifying specific efforts in terms of conservation and management actions. In parallel, to further assess the demographic distinctiveness of these units, additional analyses should be performed including independently evolving markers such as nuclear loci with biparental inheritance as well as Y-chromosome loci, so as to investigate the existence of male-based gene flow in this species. Furthermore, additional demographic units may exist in the Neotropical otter and will potentially emerge as more detailed studies are performed on the basis of expanded geographic sampling. Nevertheless, the results from this study are compelling in the context of demonstrating that this species presents substantial phylogeographic structure, opening up new research avenues targeting its demographic history and dispersal patterns at multiple spatial scales.

Supplementary Material

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

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