Eurasian Otters, *Lutra lutra*, Have a Dominant mtDNA Haplotype From the Iberian Peninsula to Scandinavia

Ainhoa Ferrando, Montserrat Ponsà, Josep Marmi, and Xavier Domingo-Roura

From the Departament de Biologia Cellular, de Fisiologia i d’Immunologia, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain (Ferrando and Ponsà), and Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Dr. Aiguader 80, 08003 Barcelona, Spain (Ferrando, Marmi, and Domingo-Roura).

Address correspondence to A. Ferrando at the first address above, or e-mail: ainhoa.ferrando@irta.es.

Abstract

The Eurasian otter, *Lutra lutra*, has a Palaearctic distribution and has suffered a severe decline throughout Europe during the last century. Previous studies in this and other mustelids have shown reduced levels of variability in mitochondrial DNA, although otter phylogeographic studies were restricted to central-western Europe. In this work we have sequenced 361 bp of the mtDNA control region in 73 individuals from eight countries and added our results to eight sequences available from GenBank and the literature. The range of distribution has been expanded in relation to previous works north towards Scandinavia, east to Russia and Belarus, and south to the Iberian Peninsula. We found a single dominant haplotype in 91.78% of the samples, and six more haplotypes deviating a maximum of two mutations from the dominant haplotype restricted to a single country. Variability was extremely low in western Europe but higher in eastern countries. This, together with the lack of phylogeographical structuring, supports the postglacial recolonization of Europe from a single refugium. The Eurasian otter mtDNA control region has a 220-bp variable minisatellite in Domain III that we sequenced in 29 otters. We found a total of 19 minisatellite haplotypes, but they showed no phylogenetic information.

The Eurasian otter (*Lutra lutra*, L. 1758) has suffered an important decline throughout much of the Western Palearctic in the last century, a decline which has led to local extinctions in many regions of Europe (Conroy and Chanin 2000; Macdonald and Mason 1994; Ruiz-Olmo and Delibes 1998). The species is protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and is the focus of important conservation efforts, including programs to reintroduce it into its former range (Saavedra and Sargatal 1997). It is becoming clear that there is an urgent need to determine the genetic relationships of otters across their distribution range to support reintroductions and management programs.

The mitochondrial (mt) DNA control region is widely used as a genetic marker in phylogeography (Avise 1998), and is divided into three domains: a conserved central domain (CCD), and two hypervariable flanking regions that can have repetitive regions known as the extended termination associated sequences (ETAS) domain and the conserved sequence blocks (CSB) domain. Genetic studies on Eurasian otter mtDNA have been restricted to central-western Europe and have detected extremely low levels of variability (Cassens et al. 2000; Effenberger and Suchentrunk 1999; Mucci et al. 1999). These results may suggest that otters spread across Europe from a single refugium, though its location is unknown. The main models suggested for the postglacial recolonization of Europe are based on the expansion of taxa from one or more southern refugia during the Pleistocene, although more eastern refugia also may have been involved (Hewitt 1999; Taberlet et al. 1998; but see Stewart and Lister 2001). The importance of the Iberian Peninsula as a glacial refugium is demonstrated by the high levels of genetic variability and numerous endemisms expressed as unique mtDNA haplotypes in the fauna (Bilton et al. 1998; Hewitt 1999).

The aims of this study are (1) to determine the genetic variability and principal genetic relationships among European otter populations, exploring new areas not well studied previously, including otter samples from the Iberian Peninsula, Scandinavia, and Eastern Europe; (2) to evaluate
the variability and phylogenetic information of the minisatellite found in the 3' end of the control region; and (3) to explore the possible existence of otter colonization routes, and glacial refugia in particular, in the Iberian Peninsula.

Materials and Methods

Sampling and DNA Extraction

Samples of 73 Eurasian otters were obtained from eight European countries (Table 1). DNA was extracted from blood and muscle with use of the conventional proteinase K/phenol/chloroform method and from serum with use of Chelex 100 chelating resin (Walsh et al. 1991). Teeth from two historical samples of animals captured in 1919 and the 1930s in northeastern Spain were fully crushed, and DNA was extracted with the DNeasy Tissue Kit (Qiagen), according to Iudica et al. (2001).

Amplification and Sequencing

The whole control region of the DNA isolated from blood and muscle was amplified with the primers L-Pro and H-Phe (Mucci et al. 1999). Because degradation of DNA was expected in teeth and serum samples, we amplified the 5' side of the control region, using internal primers MeCR2, MeCR3, MeCR4, MeCR5, and MeCR6 originally designed from Eurasian badger, Meles meles, sequences (Marmi 2004). Polymerase chain reaction (PCR) DNA amplification was performed with an initial denaturation for 2 min at 94°C, then 30–40 PCR cycles, including 94°C for 15 s, 55–56°C for 15 s, and 72°C for 15 s, and a final extension at 72°C for 5 min. The 15-s intervals were increased to 45 s when the DNA was of poor quality. Reactions were performed in a final volume of 25 μl (except for teeth and serum samples which were 15 μl, including 4–6 μl of extraction template), including 50–100 ng of target DNA, 16.6 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 0.01% Tween-20, 2.5 mM MgCl₂, 1.6 mM dNTPs, 4.25 pmol of each primer, and 0.85 units of EcoTaq DNA polymerase (Ecogen). The minisatellite, located at the 3' side of the L-strand, was also amplified in 29 individuals, with the same conditions and primers MeCR5 and H-Phe, and with annealing temperature increased to 64°C.

PCR products were purified by Gene clean (Qbiogene), but if they showed two bands after running in an agarose gel, each band was cut and extracted from the gel and purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). In this latter case, a new amplification was performed with the same PCR conditions, including 1.2 μl of purified DNA elute and negative controls, and sequenced in both directions. Purified products were sequenced with use of the Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing products were run on an ABI3100 automated DNA sequencer (Applied Biosystems). Sequences produced in this study and those published by Cassens et al. (2000; GenBank accession numbers AJ006174–AJ006178) and Mucci et al. (1999) were aligned with ClustalW (Thompson et al. 1994) and adjusted by eye. Two clear peaks of similar size in a single nucleotide position within the minisatellite were considered to be the result of heteroplasm.

Data Analyses

Nucleotide diversity π based on the Kimura 2-parameter model was calculated with Arlequin version 2.000 (Schneider et al. 2000). Mean number of nucleotide differences (d), (Nei and Kumar 2000) was estimated with MEGA version 2.1 (Kumar et al. 2001). Mantel's test correlating the number of nucleotide differences and geographic distances between countries was also performed. The DNAsp 3.51 program (Rozas and Rozas 1999) was used to estimate overall haplotype diversity (h). All sequences showed evidence of
having recently been derived from the same ancestral haplotype, and a minimum spanning network was obtained by hand. We constructed a neighbor-joining tree from minisatellite sequences with the MEGA version 2.1 program, using the Kimura 2-parameter model and a 1,000-replicates bootstrap. The DNA *mfold* program version 3.0 (SantaLucia 1998) was used to predict secondary structures in the minisatellite array.

**Results and Discussion**

**Mitochondrial DNA Variability in the Eurasian Otter**

Sequences were obtained from the 5’ end of the control region, from 73 otters, resulting in a 361-bp final alignment. Five variable positions (1.39%) were identified, all of them transitions, resulting in seven haplotypes that we renamed H1–H7. Haplotype H1 was widely distributed throughout all countries (Table 1). None of the other haplotypes was found in two different countries. The minimum spanning network showed a shallow star-like feature that reflected the extremely low level of sequence divergence and the low frequency of rare haplotypes (Figure 1). The mean nucleotide diversity was \( \pi = 0.0006 \). This value was equal to \( \pi = 0.0008 \) in the United Kingdom, \( \pi = 0.0002 \) in Finland, \( \pi = 0.0037 \) in Russia and Belarus, and \( \pi = 0.00 \) for the rest of the countries. The overall haplotype diversity was \( b = 0.16 \) (SD = 0.06), much lower than the values obtained in other mustelid species whose mitochondrial variability across Europe has been explored, such as *Martes martes*, \( b = 0.76 \), data calculated from sequences of Davison et al. (2001; GenBank accession numbers AF336949–64, 68–69) or *M. meles*, \( b = 0.90 \) (Marmi 2004). The low number of mutations and the star-like feature of the network indicate a recent genetic divergence from H1. Mantel’s test showed no correlation between the number of differences and geographic distances (\( r = -0.093, p = .555 \)).

When we combined our sequences with those published by Mucci et al. (1999) and Cassens et al. (2000), a total of 250–251 bp could be aligned from 236 individuals, showing seven polymorphic sites (2.79%), six nucleotide substitutions, and one insertion, resulting in eight haplotypes. Haplotype Lut1 in the study of Cassens et al. (2000) matches our haplotype H1 and is shared by all Iberian otters and by 92% of individuals included in our study. Haplotype Lut3 from Cassens et al. (2000), dominant in some localities of eastern Germany, presented the same nucleotide substitution as haplotype H4 from Belarus (this study) and a haplotype named UK from English otters (Mucci et al. 1999). Overall haplotype diversity throughout all three studies was higher (\( b = 0.360 \)) than the value obtained from our sequences, mainly due to the high number of sequences proceeding from otters from eastern Germany with haplotype Lut3.

These results, expanding the distribution previously explored by Mucci et al. (1999) and Cassens et al. (2000), report extremely low levels of mtDNA variability in Eurasian otters. The variability in microsatellites is higher than in mtDNA in otters, with average, unbiased expected heterozygosity values of \( He = 0.62–0.71 \) in Scotland and \( He = 0.40–0.52 \) in southwestern England and Wales for 12 loci analyzed (Dallas et al. 2002). Average expected heterozygosity values were similar (\( He = 0.45–0.77 \)) in otters from eight European countries for 13 loci analyzed (Randi et al. 2003).

Haploidy, the low effective population size, reduced recombination, and differential mutation rates of mtDNA, as well as otter live history, such as the preferential dispersal by males, would explain differences in the patterns of genetic diversity between mitochondrial and microsatellite DNA. In addition, the highly variable ETAS domain is shorter (175 bp) in otter than in other carnivores, and this fact could contribute to the little variability observed in the otter mtDNA control region.

**Domain III Repeats**

The CSB domain of Eurasian otter contains a minisatellite composed of 22-bp repeat units. We considered that each repeat started with nucleotide sequence ACCTAGGG.

Twenty-seven individuals showed an array composed of 10 repeat units, resulting in a final array of 220 bp. The alignment of minisatellite repeat units showed the presence of two variable sites located at the eighth and 21st positions, resulting in five repeat unit haplotypes (a–e, Table 2). The first repeat unit \( a \) and the two last repeat units \( d \) were common for all sequences. Specimens L54 and L76 presented heteroplasm in their length, each one showing the presence of arrays composed by eight and 10 repeat units. The loss of two units could be a result of DNA slippage during replication.

Nineteen different haplotypes (A1–A19) were identified among 29 samples. Evidence of homogenization in most of
the array (e.g., L85 and L88 for \( b \) repeat, or L24, L34, L39, and L90 for \( c \) repeat) is important. These homogenizations and the constant size (220 bp) of the complete repeat region across Europe suggest the prevalence of cross-talk mechanisms leading to the generation of variability in these sequences. Using the \textit{mfold} program, we found that the minisatellite region can form secondary structures (Figure 2). The graphic representation of this folding shows stable stem structures and that the variable positions eighth and 21st of the minisatellite repeat units fall outside these structures in locations more prone to mutation. The accumulation of mutations in two variable positions and the regular pattern observed in many of the sequences is against the role of random point mutations.

The neighbor-joining tree failed in resolving phylogenetic relationships among repeat array haplotypes, with no branches showing bootstrap values above 50%, and no further arrays were sequenced. Thus, we could not extract any relevant phylogeographic information from the comparison of minisatellite sequences across countries. Only similarities are evident in populations from Extremadura (L24, L34, L39, and L90) or Asturias (L33 and L89). In otters the analysis of the mini satellite variability might be informative for individual identification as seen in other species (Matsuhashi et al. 1999), for instance in combination with genetic markers such as microsatellites.

### Pleistocene Refugia

Climatic fluctuations during the Quaternary are responsible for local extinctions and reduced genetic variability in European fauna (Hewitt 1999). The widespread monomorphism and lack of phylogeographical structuring in our study supports a single genetic lineage colonizing Europe from a single refugium after the last glaciation, as has been suggested for \textit{Martes martes} and \textit{Mustela putorius} (Davison et al. 2001). The lack of structuring in mtDNA and haplotypes shared over long distances is not an exception in wideranging carnivores (Vila` et al. 1999). This phenomenon could be the result of leptokurtic dispersal, where there is a rapid and long-distance dispersal of individuals to set up colonies far ahead of the main dispersing population. For the otter, this dispersal would be favored in wet habitats and periods. However, our study does not support the role of the Iberian Peninsula as a refugium for otters. We detected most mutations in eastern Europe, that is, in otters from Russia and Belarus. This result could suggest an otter colonization of Europe from the Balkans or western Asia as has been suggested for other species (Bilton et al. 1998; Desmesure et al. 1996). The reduced number of mutations and eastern samples involved in this study prevent any firm conclusion on this issue but prompt further research with additional samples from the Balkan Peninsula and eastern Europe.

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### Table 2

<table>
<thead>
<tr>
<th>Repeat array haplotype</th>
<th>Country (sample code)</th>
<th>Repeat units (( 5' \rightarrow 3' ) of L-strand)</th>
<th>1st</th>
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<td>A1 Belarus, Verhnedvinsk (L85, L88)</td>
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<td>A2 Finland, Hyrynsalmi (L56)</td>
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<td>Finland, Pylväärä (L60)</td>
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<td>A4 Finland, Puolanka (L44)</td>
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<td>United Kingdom, Walsh (L92)</td>
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<td>A5 Finland, Ruukki (L54)</td>
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<td>A6 Finland, Viljiesi (L55)</td>
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<td>A7 Sweden, Testeboom (L71)</td>
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<td>A8 Norway, Tromsö (L77)</td>
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<td>A9 United Kingdom, Scotland (L80)</td>
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<td>A10 United Kingdom, Scotland (L84)</td>
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<td>A11 Spain, Asturias (L26)</td>
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<td>A12 Spain, Asturias (L33)</td>
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<td>A13 Spain, Asturias (L89)</td>
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<td>A14 Spain, Extremadura (L37)</td>
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<td>A15 Spain, Extremadura (L35, L38)</td>
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<td>A18 Portugal, Alentejo (L17)</td>
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<td>A19 Norway, Nordland (L76)</td>
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Each letter represents a repeat unit with the following sequences: \( a = \text{ACGTACGTATACACGCACACTC} \), \( b = \text{ACGTACGTATACACGCACACCC} \), \( c = \text{ACGTACGTATACACGCACACCG} \), \( d = \text{ACGTACGTATACACGCACACGC} \), \( e = \text{ACGTACGTATACACGCACACGC} \), \( y = \text{ACGTACGTATACACGCACACGC} \), \( z = \text{ACGTACGTATACACGCACACGC} \). Y represents C/T and is found in \( y-z \) heteroplasmic sequences.

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with other molecular markers in addition to mtDNA. The low genetic variability observed indicates that this colonization must be quite recent, maybe even subsequent to the main glacial period of the Pleistocene, with otter populations recovering only after two more recent episodes of drought, 13,000 and 10,000 years ago (Starkel 1991), which may have been critical for otter survival.

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References


Figure 2. Example of folded secondary structure of the complete minisatellite array for individual L92. Triangles indicate variable sites in the 8th and 21st positions of each repeated unit.


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