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RFLP analysis of the mitochondrial DNA of otters (*Lutra lutra*) from Europe—implications for conservation of a flagship species

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Abstract

The distribution of otters (*Lutra lutra*) in Europe is largely fragmented, mainly due to historic anthropogenic causes. Nevertheless, in eastern Germany a large coherent area is inhabited by a viable otter population. In this paper, the mitochondrial genome of 81 otters is studied by RFLP analysis of PCR-amplified 1.5 and 2.5 kb long fragments, particularly to estimate the amount of genetic variability of European otters, and to identify possible geographical population subsets for conservation management. No restriction site polymorphism was detected within the control region (1.5 kb), but polymorphism of cleaving sites in the 2.5 kb fragment could be assigned to three haplotypes. Two occurred exclusively in a comparatively small area northwest and west of Berlin, which may be considered a region of increased genetic variability in otters. The low level of mtDNA variability in European otters might be due to genetic drift in postglacial founder populations with long-term low densities, and continuous historical overhunting.

Keywords: Otter; *Lutra lutra*; Genetic diversity; RFLP; Mitochondrial DNA

1. Introduction

Historically, Eurasian otters (*Lutra lutra*) were distributed in diverse habitats, ranging from the Arctic to the Mediterranean, and from Ireland in the west to the borders of Asia in Japan (Müller, 1981). Despite this historical wide distribution, surveys in the late 1900s found few remaining areas where otters are still common (Macdonald and Mason, 1991). Water pollution together with widespread habitat destruction have caused many populations in Europe to become smaller and more isolated. Such fragmented populations risk rapid loss of genetic diversity due to genetic drift and inbreeding, which might reduce their fitness and adaptive potential (Falconer and Mackay, 1996). However, a recent review has established that over large parts of their range otters appear to be recovering (J. W. H. Conroy, pers. comm.).

To date very little information is available on the distribution of genetic variability in European otters (Giulianelli and Laikre, 1994). Such data would help to identify genetic source regions, to delineate and to evaluate management units for otter conservation. The major aim of this study is to estimate the extent and distribution of mtDNA variability in otters from parts of Europe.

Eastern Germany is probably the only region among intensively cultivated landscapes in Europe to have otters in comparatively high densities and more or less continuous distribution. To enhance the natural spread of otters from this eastern German population into areas where they once occurred in fair numbers, Reuther (1992) suggested the development of a network of suitable habitats. In establishing such a network of habitat corridors, it would be important to identify centres of genetic diversity (genetic source populations), and to assign priority ranks to corridors of gene flow (Avise and Hamrick, 1996). On the other hand possible marked gene pool differences between regions should be considered for restocking programs (Van Ewijk et al., 1997).

In the present study we analysed the level of mtDNA variability in otters, because this genome compartment shows a rapid evolutionary rate, and is quite sensitive in detecting recent genetic bottlenecks due to lack of recombination effects. The maternal inheritance of mt DNA particularly provides a good tool for inferring

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phylogeographic relationships among populations (Avise, 1994).

2. Material and methods

Liver tissue samples of 81 otters from Germany, Austria, Hungary, and Scotland were studied (Fig. 1). German samples were allocated to six regional units according to potential physiographic barriers inhibiting geneflow. These included areas with few waterways and areas of dense human populations such as the environs of Berlin. In addition, separated tributary systems were used for establishing these regional units.

All samples were derived from otters found dead (traffic casualities, fish trap accidents) between 1993 and 1996, and were provided to us by official nature conservation authorities or otter research units. Preparation of mitochondrial DNA was carried out according to the protocol of Lansman et al. (1981). We initially amplified the control region (1.5 kb) of 44 samples from all sampling regions with the two universal primers 5′-TCA AAG CTT ACA CCA GTC TTG TAA ACC-3′ and 5′-TAA CTG CAG AAG GCT AGG ACC AAA CCT-3′ (Kocher et al., 1989). In a second approach a 2.5 kb fragment was amplified in 46 German samples. This analysis included nine samples from the first 1.5 kb fragment amplification. The longer fragment, containing the control region and the entire cytochrome b gene, was amplified using two universal mammalian primers 5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′ (Irwin et al., 1991) and 5′-TAA CTG CAG AAG GCT AGG ACC AAA CCT-3′ (Kocher et al., 1989). Both fragments were amplified with the following PCR protocol: 2 μl mtDNA (10–50 ng) were used in a mixture with 0.3 mM of each dNTP, 1 mM $MgCl_2$, 1/10 of the end volume PCR buffer supplemented together with Taq polymerase of which we used 2 U and 0.5 μM of each primer in 100 μl Aqua dest. The samples were processed on a thermal cycler (Techne PHC-3) as follows: one cycle: 93°C for 5 min; 35 cycles: 93°C for 1 min, 51°C for 1 min, 68°C for 1 min 30 s; one cycle: 72°C for 10 min. Taq DNA polymerase was added right after the first cycle of denaturation. The shorter fragment was digested using seven restriction endonucleases: *Apa*I, *Xho*I (hexanucleotide-recognizing enzymes) and *Alu*I, *Cfo*I, *Hae*III, *Msp*I and *Rsa*I (tetranucleotide-recognizing enzymes). The 2.5 kb fragment was cleaved with eight tetranucleotide-recognizing enzymes: *Aci*I, *Alu*I, *Cfo*I, *Hae*III, *Mbo*I, *Msp*I, *Rsa*I, and *Tru*9I.

Seven microlitres of amplified mtDNA were used for each digestion. The conditions and buffers used were those recommended by the manufacturers (Boehringer-Mannheim, New England Biolabs). Samples were

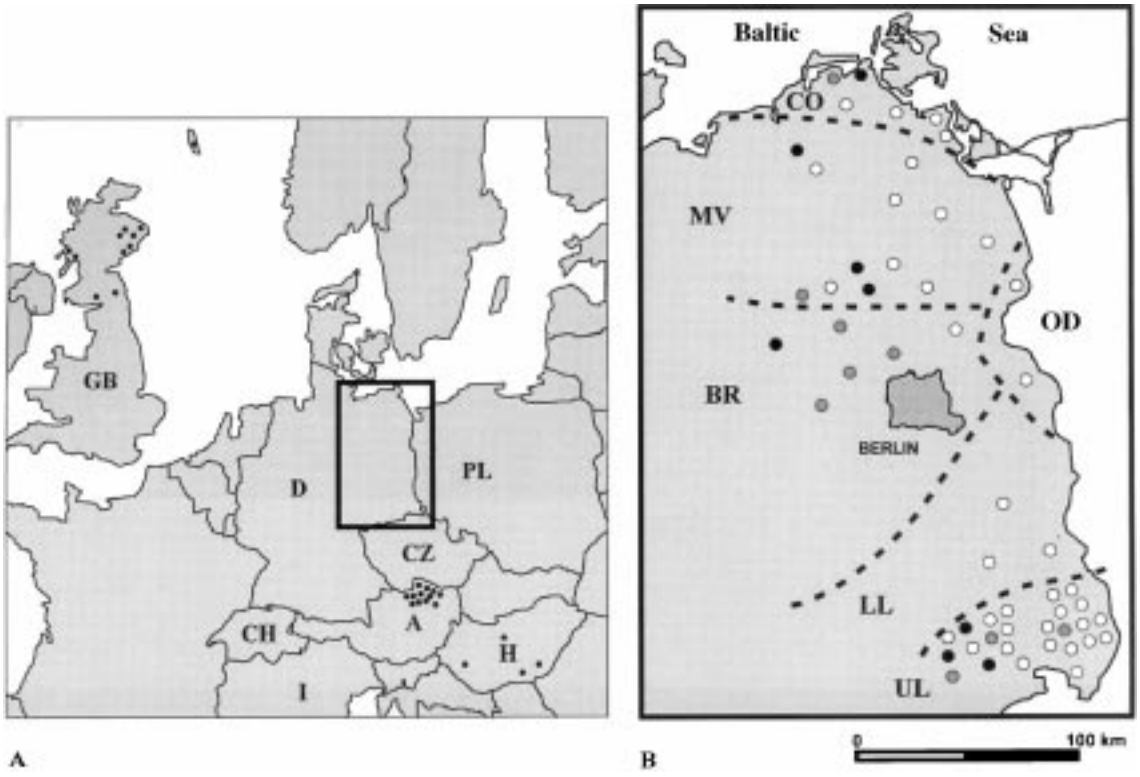

Fig. 1. Sample localities of 81 otters analysed from Europe (A) and a closer view of the focus study area in eastern Germany (B). Dotted lines separate regions based on physiographic characters and tributary systems. MV = Mecklenburg-Vorpommern, BR = Brandenburg, CO = Coastal region, OD = Oder region, LL = Lower Lusatia, UL = Upper Lusatia. ● 1.5 kb analysis, ○ 2.5 kb analysis, ◍ 1.5 and 2.5 kb analysis.

digested with a two-fold excess of enzyme for 2–3 h. Fragments were separated electrophoretically in a mixture of 1% SeaKem agarose and 3% NuSieve agarose for better resolution of smaller fragments. Fragment lengths were determined using a 100 bp ladder as size marker.

The resulting fragment patterns were used to create mtDNA haplotypes, separately for the two amplicons. Nucleotide diversity (π) was calculated based on restriction sites (see e.g. Nei and Li, 1979) between all haplotypes, and haplotype frequencies were used to calculate haplotype diversity (Nei, 1987) for each sampling region, respectively. In addition, netto nucleotide diversities (Nei, 1987) were calculated between all regions to obtain indices of pairwise genetic differentiation. Netto nucleotide diversity is based on a correction for within-region polymorphism, whereas brutto nucleotide diversity refers to overall variability in the respective pairs of regions (see e.g. Avise, 1994).

3. Results

RFLP-analysis of the 1.5 kb mtDNA stretch in 44 individuals from four European countries yielded only one haplotype (Table 1). Subsequent screening of the 2.5 kb amplicon in 46 individuals from six designated regional samples from Germany yielded a total of 48 fragments, which could be assigned to three different haplotypes. Only one (*Alu*I) out of the eight restriction endonucleases was informative in producing these haplotypes (A, B, C, see Table 2). All other enzymes produced monomorphic restriction fragment patterns among all individuals. 89% of all screened otters showed the standard haplotype A, 4% the haplotype B, and 7% the haplotype C. Haplotype B could be deduced from the standard haplotype A by two additional restriction sites, and haplotype C by the absence of one restriction site. Pairwise nucleotide diversity values (Nei and Li, 1979) between the three haplotypes were as follows: A vs B = 0.0062; A vs C = 0.0032; B vs C = 0.0094. There was no clear pattern of divergence of nucleotide diversity values and geographic distances. The geographic distribution of the three haplotypes is depicted in Fig. 2. Values of haplotype and nucleotide diversity are listed in Table 3 separately for all regional samples. Pairwise netto and brutto nucleotide diversity values are given in Table 4.

Table 1
Restriction patterns for haplotype A in the 1.5 kb mtDNA fragment from 44 otters from central Europe and Scotland[a]

Enzyme	Recognition sequence	Haplotype	Fragment sizes (bp)
*Alu*I	AGCT	A	320, 270, 240, 210, 200, 100
*Apa*I	GGGCCC	A	1100, 420
*Cfo*I	GCGC	A	800, 250, 200, 150
*Hae*III	GGCC	A	800, 400, 180
*Msp*I	CCGG	A	1000, 380
*Rsa*I	GTAC	A	420, 380, 280, 250, 150
*Xho*I	CTCGAG	A	1100, 350

[a] Total fragment length can vary due to small (< 100 bp) undetected fragments or measurement error of fragment length.

4. Discussion

This study indicates a low level of genetic variability in the mitochondrial genome of otters across large areas of central Europe and Scotland. We used mtDNA to detect spatial distribution of genetic diversity, because this gene pool compartment shows an increased rate of evolution. Hence, it is fairly sensitive for historical population developments. Its (almost exclusive) maternal inheritance without recombination effects provides a good prerequisite for detecting phylogeographic patterns (e.g. Avise, 1994). However, we emphasize that this molecular marker system represents only a small portion of the total genome diversity, and no direct conclusion regarding the nuclear gene pool variability can be drawn (e.g. Cronin, 1993; Moritz, 1994). The mitochondrial control region is known to exhibit relatively high genetic diversity in many vertebrate species (Avise, 1994). Thus, we initially focused our analysis on this part of the mtDNA. Despite a reasonable sample size including otters from different provenances spread over large geographic distances in Europe we could not detect any variability in this segment of the mtDNA. The currently found paucity of variation in the control region complies with the results of Cassens et al. (in press), who found little sequence variability in control regions of otters from various parts of Europe.

In a second step we focused our analysis on the otters from eastern Germany, which are spread more or less continuously over an area extending from the seaboard of the Baltic Sea to the border of the Czech Republic in relatively high densities. To increase the chance of detecting genetic variation we included the total cytochrome b gene into the RFLP analysis. Although we obtained a reasonable number of restriction sites (40), the presence of only three haplotypes indicated little genetic diversity in this increased mtDNA segment. Restriction patterns of haplotype B varied considerably in length in the second amplification of the 2.5 kb fragment. Missing fragments of about 500 bp in total could not be detected, which might be due to the fragmentation of a tandem repeat unit (Lunt et al., 1998) into small (< 100 bp) undetectable pieces occurring only in this haplotype.

Interestingly, out of eight otters that were analysed using both the short (1.5 kb, only control region) and

Table 2
Restriction pattern for haplotypes A, B and C in the 2.5 kb mtDNA fragment from 44 otters from central Europe and Scotland[a]

Enzyme	Recognition sequence	Haplotype	Fragment sizes (bp)
*Aci*I	CCGC	A,B,C	700, 500, 450, 330, 245, 150, 120
*Alu*I	AGCT	A	1058, 320, 270, 220, 210, 200, 100
	AGCT	B	668, 320, 270, 220, 210, 200, 100, xxx
	AGCT	C	1058, 480, 320, 210, 200, 100
*Cfo*I	GCGC	A,B,C	930, 820, 370, 250, 150
*Hae*III	GGCC	A,B,C	840, 650, 370, 250, 150
*Mbo*I	GATC	A,B,C	630, 600, 300, 250,230, 210, 190
*Msp*I	CCGG	A,B,C	1000, 900, 700
*Rsa*I	GTAC	A,B,C	600, 420, 400, 370, 350, 120
*Tru*9I	TTAA	A,B,C	1058, 420, 320, 230, 150, 130, 110, 100

[a] Total fragment length can vary due to small (< 100 bp) undetected fragments or measurement error of fragment length. xxx = undetected fragments.

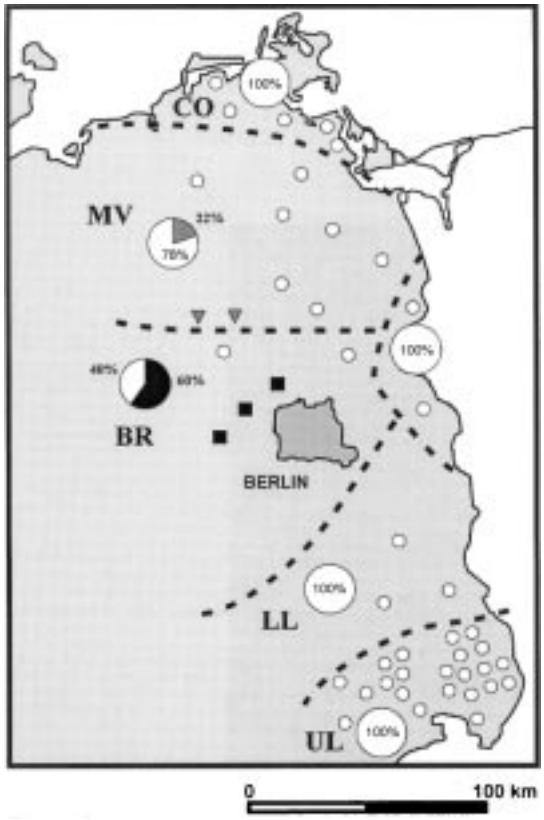

Fig. 2. Geographic distribution of haploptypes found in the 2.5 kb analysis in eastern Germany. Dotted lines indicate regional separation. Exact locations of haplotypes B (▼ Zootzen See, Klein Haßlow) and C (■ Klietzer See, Lüwenberg, Fehrbellin). ○ haplotype A.

Table 3
Haplotype and nucleotide diversity for otters from six regions in eastern Germany

	Haplotype diversity	Nucleotide diversity (π)
UL	0.00	0.0000
MV	0.39	0.0021
CO	0.00	0.0000
BR	0.60	0.0015
LL	0.00	0.0000
OD	0.00	0.0000

Table 4
Netto nucleotide diversity between otters from regions in eastern Germany (above diagonal), and brutto nucleotide diversity for pairs of otter regions (below diagonal) in percent. — indicate values of zero

	UL	MV	CO	BR	LL	OD
UL		0.0306	—	0.1152	—	—
MV	0.1378		0.0306	0.1458	0.0306	0.0306
CO	—	0.1378		0.1152	—	—
BR	0.1920	0.3298	0.1920		0.1152	0.1152
LL	—	0.1378	—	0.1920		—
OD	—	0.1378	—	0.1920	—	

the long (2.5 kb, control region and cytochrome b gene) mtDNA fragments, three otters varied in their restriction patterns only in the long 2.5 kb fragment (Tables 1 and 2). This suggests that nucleotide substitutions occurred only in the cytochrome b gene, which is known to be a usually rather conserved element in vertebrate mitochondrial DNA (Anderson et al., 1981; Avise, 1994). The low level of nucleotide substitution in the mitochondrial control region could be due to stabilising selection. Since we only analysed the 2.5 kb mtDNA fragment from German otters, more variability might be found in otters from diverse regions across Europe when screening particularly the cytochrome b gene.

Nevertheless, one should take into account that mtDNA variability may be considerably reduced particularly during long-lasting low population densities (Avise and Hamrick, 1996). In this case mtDNA markers

are of less use for detecting within-population polymorphism, as compared to recombining nuclear markers, such as structural gene loci or microsatellite DNA (Avise, 1994).

The very low level of mitochondrial RFLP found in our study could also be explained by generally low gene pool diversity in mustelids, as has been suggested by Wayne and Koepfli (1996) based on some allozyme studies (but see Hartl et al., 1988). Other possible explanations for the low level of mtDNA variability in central European and Scotish otters might be genetic drift in postglacial founder populations with long-term low densities, and continuous historical overhunting.

The phylogenetic divergence among all three haplotypes is quite low, as demonstrated by changes in only one to three cleaving sites (Table 2). This suggests either a postglacial colonization of central Europe by otters after the retreat of the inland ice sheet (some 18,000–16,000 years bp, Lowe and Walker, 1997) from only one major refuge area, or invasion into central Europe from geographically distant refugia inhabited by otters with very similar gene pools. In the latter case pleistocene otters should already have had a rather uniform mtDNA.

In Germany, the spatial distribution of the two additional mtDNA haplotypes is restricted to an area northwest and west of the city of Berlin, touching the southern edge of the Mecklenburg lake district ('Mecklenburger Seenplatte'). Hence, this region encompassing ca. 10,000 km^2 can be viewed as an area of increased genetic diversity in otters. In fact, the nucleotide diversity recalculated for all otters we studied in this area, disregarding the initial grouping into two sample regions 'Brandenburg' and 'Mecklenburg-Vorpommern', amounts to $\pi = 0.0033$ as compared to $\pi =$ zero for all other regions in Germany. The nucleotide diversity is even higher ($\pi = 0.0044$), when calculated for the minimum convex polygon spanned across the area including all haplotypes B and C. In contrast, in the fishpond district of Upper Lusatia (UL, see Fig. 1), a region of high otter density, we found no variability in our molecular marker systems, although almost 50% of our samples were derived from this region. The paucity of mtDNA variability in this region may reflect a founder effect after the demographic bottleneck by the middle of this century (Kubasch, 1996).

According to our results, the area between Berlin and the southern edge of the natural lake district of Mecklenburg ('Mecklenburger Seenplatte') may represent a region of increased genetic diversity in European otters. From the genetic conservation point of view, we emphasize the need to implement legislative and practical measures of habitat protection in this region. Such conservation measures should also consider waterways radiating from this region and connecting with more remote areas suitable for otters, in order to enhance the spread of genetic diversity by otters migrating along such habitat corridors (Reuther, 1992). Our study did not reveal any strong genetic subdivision among the initially detailed regions in eastern Germany. Apparently, neither physiographic changes across regions nor separate tributary systems influence the spatial distribution of mtDNA variability, and no specific population units can be discriminated. Our results suggest that there is no need for geographical subdivision into regional management units when planning a network of habitat corridors. However, we emphasize that nuclear gene pool markers such as allozymes, microsatellite DNA, or chromosomes could reveal some spatial substructuring. Hence, we recommend the application of further molecular markers to obtain a complete picture of geographical partitioning of genetic variability in European otters.

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