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MICROSATELLITE MARKERS FOR THE ENDANGERED EUROPEAN MINK (*Mustela lutreola*) AND CLOSELY RELATED MUSTELIDS

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ABSTRACT

The European mink (*Mustela lutreola* L., 1761) is an endangered carnivore species whose populations suffered a severe decline during the last century. The genotyping of eight polymorphic microsatellite loci revealed a relatively low number of alleles per locus (2-8), as well as low levels of polymorphism (*Ho* and *He* values per locus were 0.49 and 0.54 respectively). Cross-specific PCR amplifications were successful in seven closely related mustelid species suggesting that these loci may be useful not only for assessing genetic variability in European mink populations but also for determining potential hybridization events between *M. lutreola* and other mustelid species.

The European mink (*Mustela lutreola* L., 1761) is a highly endangered semiaquatic carnivore species (IUCN Red List of Threatened Species, http://www.iucnredlist.org). Habitat loss, hunting, and competition with farm-released American minks largely contributed to a severe decline of European mink during the last century (Lode *et al*., 2001; Maran & Henttonen, 1995). The extant populations of European mink are restricted to southwestern France, northern Spain, Estonia, Belarus, Russia and Romania (Maran & Henttonen, 1995), and conform three genetically distinct (Western, Southeastern and Northeastern Europe) demes based on mitochondrial control region sequence data, and non-specific microsatellite allele size variation (Michaux *et al*., 2005). Recently, conservation and breeding programs were developed in order to maintain genetic diversity in wild populations, and to avoid the effects of inbreeding and reduction in reproductive fitness (Michaux *et al*., 2005).

The present study provides eight novel polymorphic microsatellites isolated from *M. lutreola*, which will allow examining population genetic structure of European mink, as well as assessing historical demography and potential genetic bottlenecks (Michaux *et al*., 2005). This genetic information will be decisive for improving conservation of wild populations of European minks, for monitoring breeding programs in captivity, and for studying hybridization events, which are relatively common among these carnivores (Kyle *et al*., 2003).

An enriched genomic DNA library of *M. lutreola* was constructed following the protocols of Hamilton *et al.* (1999) and Ostrander *et al.* (1992). Genomic DNA was extracted using a standard proteinase K/ phenol-chloroform procedure. Isolated DNA was partially digested with *Rsa*I (New England Biolabs), and ligated to the adaptor SNX (5'-CTAAGGCCTTGCTAGCAGAAG-3') (Hamilton *et al*., 1999). The digested DNA was incubated at 65ºC for 15 min with a biotin-labeled [di-]oligonucleotide probe. Microsatellite-containing products were isolated using streptavidin magnetic beads (Dynabeads), and PCR amplified using the SNX adaptor. PCR products were ligated into pGEM-T (Promega), and transformed into ultracompetent *Echerichia coli* XL-10 Gold cells (Stratagene). Recombinant clones were transferred onto nylon membranes (Hybond-N^+, Amersham), and hybridized with the biotin-labeled probes. Positive clones were sequenced using ABI PRISM 3700 and 3730 automated sequencers (Applied Biosystems) with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (V3.0).

A total of 153 positive clones were sequenced, and 26 (GenBank Accession No: EF093582-093585, EF093587-093591, EF380094-380110) of these contained simple GT dinucleotide repeats. Specific primer pairs were designed based on the microsatellite flanking region sequences to PCR amplify each locus. Only 11 microsatellites could be successfully amplified after PCR optimization. PCR assays with the remaining primers either rendered no product or resulted in numerous unspecific bands. PCR conditions consisted of an initial denaturing at 94ºC for 5 min, followed by 30-35 cycles of denaturing at 94ºC for 30 s, annealing at 52-60ºC (Table 1) for 45 s, and extending at 72ºC for 45 s with a final elongation step at 72ºC for 20 min. The PCR reaction included 0.2 µM of each primer, 0.33 µM dNTPs, 2mM $MgCl_2$, 1X reaction Buffer (160mM $(NH_4)_2 SO_4$, 670mM Tris-HCl (pH8.8), 0.1% Tween-20), 0.07 mg/ml BSA, 0.5 units of Bio*Taq* DNA polymerase (Bioline), and approximately 10-15 ng of template DNA, in a final volume of 15 µl. Microsatellite loci were tested on 41 European mink individuals, and polymorphism analyses were performed on ABI PRISM 3700 and 3730 automated sequencers (Applied Biosystems) as well as on a MegaBASE™ sequencer (Amersham-Pharmacia). Alleles were scored either using GeneMapper® software version 3.7 (Applied Biosystems) or MegaBASE™ Genetic Profiler Software Suite version 2.2.

Genotyping analyses revealed that only eight of the 11 assayed microsatellites were polymorphic (Table 1). One locus, MLUT11, was monomorphic whereas two showed moderate to high stuttering. The number of alleles of the polymorphic loci varied from 2 to 8. The average number of alleles per locus was 5.25, which is relatively lower compared to other species of the same genus such as the American mink (*M. vison*) or the stoat (*M. erminea*) with an average of 6.57 and 11.71, respectively (Fleming *et al.*, 1999). Genetic diversity was estimated based on observed (H_o) and expected (H_e) heterozygosities using Genetix 4.02 (Belkhir *et al.*, 2000). Mean H_o and H_e values per locus were 0.49 (ranging from 0.05 to 0.68) and 0.54 (ranging from 0.05 to 0.76), respectively. In addition, F_{IS} statistic estimations that detect deviations from Hardy-Weinberg equilibrium (HWE), and the linkage disequilibrium test were performed for each locus using the program GENEPOP version3.3 (Raymond & Rousset, 1995). Statistical significance was tested by running a Markov chain Monte Carlo (MCMC) consisting in 1000 batches of 2000 iterations each, with the first 500 iterations discarded before sampling. According to these analyses, three microsatellites (MLUT15,

MLUT20 and MLUT32) were in HW disequilibrium (*P*-value <0.05), Loci MLUT20 and MLUT32 showed heterozygote deficiency, whereas locus MLUT15 presented heterozygote excess. Analyses of genotypic linkage disequilibrium between loci showed only two significant pairwise comparisons, suggesting overall independence of the eight loci examined (*P*-value <0.05).

Potential cross-species amplification of the eight microsatellites was tested on different species of the genera *Mustela* and *Martes* (Table 2). The locus MLUT08 could not be successfully PCR amplified in the mustelid species. Cross-species amplification revealed that these novel European mink polymorphic microsatellite loci could be used in population genetic studies of other mustelid species, and for determining hybridization between closely related species of mustelids (Kyle *et al.*, 2003).

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Table 1. Description of the eight polymorphic microsatellite loci isolated from European mink (*Mustela lutreola*) based on 41 individuals. H_o and H_e correspond to observed and expected heterozygosities, respectively., the F_{IS} (Wright's statistics) and the *P* value of departure from Hardy-Weinberg equilibrium (*P*) are listed for each locus. The forward (F) primers were labelled with fluorescent dye for detection (FAM, HEX, NED, VIC or PET) and a GTTT tail was added to the 5' end of reverse (R) primers in order to improve allele definition (Brownstein *et al*., 1996)

GenBank Accession no	Locus	Primer sequence 5'-3'	Tª	Repeat motif	Clone size (bp)	Nº alleles	Allele size range (bp)	H_o	H_e	F_{ISe}	*P*
EF093582	MLUT04	F: GGAGAGGAAAACTATACCTC R: CGTGTCTTGTATAGTTTTGTTCTCC	57ºC	$(GT)_{16}$	113	5	103-117	0.658	0.706	0.068	0.772
EF093583	MLUT08	F: GTCGTGAGTGTGGAGCATAGAG R: GTTTCACTCATCGTCATCCCTTCTT	60ºC	$(GT)_{12}$	150	4	152-158	0.613	0.599	-0.024	0.722
EF093585	MLUT15	F: GTGTGTTTTGTGTATGAGC R: GCATGTAAACAAAACCCATCATC	60ºC	$(GT)_{14}$	147	5	144-152	0.677	0.574	-0.181	0.001
EF093587	MLUT20	F: CTTATGGAGCAAAGTAACC R: GTTTGTTTCCATCTTCCATCAGG	52ºC	$(GT)_{18}$	134	8	122-146	0.600	0.757	0.209	0.032
EF093588	MLUT25	F: CTGGACCTCTATCAGTGTCC R: GTTTAAGCATATGCATCTTTGCC	55ºC	$(CA)_{15}$	133	6	129-147	0.568	0.690	0.180	0.314
EF093589	MLUT27	F: GCCGAATGTATTAATTACATGG R: GTTTCAGAGGTAATTTGGGAGAC	55ºC	$(GT)_8$ NN $(GT)_{15}$	189	2	198-200	0.050	0.049	-0.013	1
EF093590	MLUT32*	F: CAAGAGGGCGCGCAAAGAGCA R: GTTTGCTTAGGTGACTTACAGTTGAT	55ºC	$(GT)_{59}$	247	8	233-263	0.561	0.729	0.233	0.030
EF093591	MLUT35	F: GAGAGATTTTTTGGTAAACT R: GTTTCACCAAAGACACAATGACAGA	55ºC	$(CA)_8$ NNN $(CA)_3$ NNN $(CA)_{15}$	201	4	200-206	0.195	0.206	0.052	0.387

*The repeat motif has 8 transversions, nucleotide substitutions from G to C.

Table 2. Cross-species amplification data of microsatellite markers isolated from *M. lutreola* in eight closely related mustelid species. The number of individuals tested (N), the number of alleles (A) and the size range of the alleles (SR) are shown.

	MLUT04			MLUT15			MLUT20			MLUT25			MLUT27			MLUT32			MLUT35		
	N	A	SR	N	A	SR	N	A	SR	N	A	SR	N	A	SR	N	A	SR	N	A	SR
Mustela eversmannii	13	5	101-109	13	3	146-150	10	6	117-144	10	6	123-136	10	5	190-208	10	6	182-277	10	5	183-212
Mustela furo	3	1	101	3	1	151	3	2	124-138	3	4	129-136	3	2	200-202	3	1	236	3	2	200-201
Mustela sibirica	8	1	99	8	1	139	8	5	133-149	8	3	127-136	8	4	186-196	8	5	226-267	8	6	202-223
Mustela nivalis	2	1	99	2	1	146	1	2	131-134	1	2	125-127	1	2	198-200	1	1	222	1	2	195-202
Mustela vison	11	1	96	11	NA**		8	6	137-162	8	6	121-134	8	5	163-171	8	1	148	8	1	165
Martes martes	1	2	89-96	1	1	146	7	3	124-138	7	2	118-120	7	5	187-204	7	NA		7	1	162
Martes foina	7	1	94	7	NA		7	NA		7	1	118	7	2	187-195	7	NA		7	1	162

**NA=no amplification