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Is the western population of the European mink, (*Mustela lutreola*), a distinct Management Unit for conservation?

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Abstract

The European mink (*Mustela lutreola*) is one of the most threatened carnivores in Europe, with fragmented populations in Belarus, Russia and Romania, as well in south-western France and northern Spain. Many populations have become extinct recently, or are declining. We investigated mitochondrial DNA variation, using the complete D-loop region, and concentrating on the west European population. The aim was two-fold: to use the genetic information to advise on the conservation of European mink, and to begin to understand their history through the Pleistocene. Captive breeding and re-introduction programmes are underway, so it is particularly vital to know whether the West European population should be treated separately. We find that European mink probably colonised from a single refugium after the last glaciation. West European populations may be fixed for a single haplotype, also suggesting a common origin. Despite this evidence for gene flow, following the precautionary principle we suggest that mink from the three geographically separate populations (Romania, Eastern and Western Europe) should be managed separately, for the moment.

Keywords: *Mustela lutreola*; mtDNA; Conservation biology; Management unit; Phylogeography

1. Introduction

The European mink (*Mustela lutreola*) is one of the most threatened carnivores (Baillie and Groombridge, 1996). In the latter part of the 20th century, its distribution has fragmented, and populations continue to decline (Van Bree and Saint Girons, 1966; Camby, 1990; Maran, 1992; Tumanov, 1992; Sidorovich, 2000). Now, the species is extant in the eastern part of Belarus, in parts of Russia (Sidorovich, 2000; Wolsan, 1993) and in Romania, namely in the Danube delta (Gotea and Kranz, 1999). In contrast, in the west, it is reported in only seven departments of the south-westernmost part of France (Maizeret et al., 1995), as well as in the high valley of the river Ebra (Spain) (Ruiz-Olmo and Palazón, 1991).

In fact, it is possible that European mink were never distributed across most of western Europe during the Holocene. The fossil record is sparse (Davison et al., 2000), with the only confirmed records being an undated, probably Holocene, skull from Moscow District, another Holocene specimen from the Netherlands, material from the Polish site of Biskupin, and from the Romanian site of the 'La Adam' Cave (references in Davison et al., 2000). Although there is no direct evidence, there has also been some debate as to whether the western population is a recent introduction. The species was not recorded in France until the first half of the 19th century, when mink were already declining in Central Europe (de Bellefroid, 1999). One explanation for this is that local hunters or early naturalists did not distinguish between European mink and polecats (*M. putorius*).

Recently, many European species of mammals have been investigated using phylogeographic methods

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(Avise, 2000). In most cases, a strong geographic structure of the genetic variability has been found throughout Europe, with different lineages arising from putative refugia in Iberia (Spain and Portugal), Italy, the Balkans and the Caucasus (Michaux et al., 1996, 1998, 2003; Santucci et al., 1998; Taberlet et al., 1998 ; Libois et al., 2001; Hewitt, 1999). Yet some species, especially wide-ranging carnivores, exhibit little geographic structure between the proposed refugia. This seems to be true for mustelids especially, with low variation and few lineages reported in polecats, pine martens (*Martes martes*; Davison et al., 2001), otters (*Lutra lutra*, Cassens et al., 2000), and wolverines (*Gulo gulo*, Walker et al., 2001), though no mustelid has been intensively sampled across its whole range.

Understanding the population history of European mink is a key part in the conservation effort, with studies ever more urgent because of the continuing decline of the species, and captive breeding/reintroduction programmes that are already underway. Locally, the French restoration plan (Anonymous, 1999) has proposed that captive-bred individuals are released into the wild as a reinforcement measure. In this circumstance, the choice of the animals to be bred is of a great importance. Indeed, if the western population is genetically distinct from the eastern ones, and if the animals are locally adapted, outbreeding depression (Lynch, 1991) could result. The definition of precise "management units" for European mink (Avise, 2000), based on genetic markers is therefore of prime importance for the conservation of the species. On a European scale, the captive breeding programme has the stated aim to "*maintain in European Zoos and other breeding facilities a population capable to maintain 90% of its heterozygosity for 100 years*" (www.lutreola.ee/index.html). Captive-bred mink have already been released on Hiiumaa Island (Estonia). Thus, it is imperative that informed decisions are made regarding their management (in this case: restocking or reintroduction), based at least in part on genetic data.

A few studies have begun to investigate genetic variation in European mink, confirming that their superficial resemblance to American mink (*M. vison*) is a result of convergent evolution (Davison et al., 1999, 2000; Kurose et al., 2000; Hosoda et al., 2000). Mitochondrial studies have suggested that European mink is most closely related to the polecat (*Mustela putorius*) or to the steppe polecat (*Mustela eversmanni*), though the similarity may be a consequence of hybridization, in the late Pleistocene or Holocene (Davison et al., 2000). A nuclear DNA study seems to confirm this (Sato et al. in press).

For population genetic analysis, a cytochrome *b* fragment from 30 eastern European mink and seven Spanish animals has been sequenced previously, along with a more restricted D-loop fragment sample (Davison et al., 2000). The resulting phylogeny did not resolve the relationships of the species, probably due to hybridization and also because of low sequence variation. Whereas cytochrome *b* is useful in resolving some taxonomic groupings (Koepfli and Wayne, 1998; Kurose et al., 2000), its evolution is too slow (Flynn and Nedball, 1998) for intra-specific studies of mustelids.

In this study, we use the complete mitochondrial D-Loop region to investigate variation across a large part of the extant range of European mink, including the first samples from France and Romania. There were two main aims: to use the genetic information to advise on the conservation of European mink, and to better understand their history through the Pleistocene. Specifically, we would like to know whether the French/Spanish population should be managed separately from the Eastern populations.

2. Methods

2.1. Samples

A total of 43 European mink were studied, 23 from France, four from Spain, two from the Danube delta, three from Estonia, two from Belarus (Vitebsk) and nine from Russia (Tver and Pskov). The references and the geographic origin of these specimens are given in Table 1. They were compared with 10 polecats (*M. putorius*), two steppe polecats (*M. eversmanii*) and two black-footed ferrets (*M. nigripes*).

2.2. DNA methods

DNA was extracted from ethanol-preserved tissue as described by Sambrook et al. (1989). French samples were taken from the *Mustela* tissue collection of Dr. R. Rosoux, the GREGE samples (Groupe de recherches et d'études pour la gestion de l'environnement), and Romanian samples were provided by Dr. A. Toman. The remaining samples were described previously in Davison et. al. (2000). All samples were taken either from road-killed European mink (muscle) or from live specimens (ear piece) caught, marked and released in the wild.

The complete D-Loop was amplified using specific primers L0ML (5′-TAT TCT AAC TAA ACT ATT CCC TG-3′) and EML (5′-CTA TAG ATG TRT TTA TAA CCC-3′) designed by J.R. Michaux. A portion of cytochrome *b* (450 bp of the 5′ region) was also amplified by modifying the Universal PCR primers L7 (5′-ACC AAT GAC ATG AAA AAT CAT CGT T-3′) and H8 (5′-ACA TGA ATY GGA GGY CAA CCW G-3′) originally described by Kocher et al. (1989). Amplification reactions were carried out in 2 × 50 μl volumes including 25 μl of each 2 μM primer, 20 μl of 1 mM dNTP, 10 μl of 10× reaction buffer, 10 μl of purified

Table 1
Geographic distribution and references of *Mustela* tissues used for the experiments

Geographic origin:		Total No. of animals	(see Figs. 1 and 2)	Tissue sample numbers or Genbank access (for sequenced samples)
Mustela lutreola				
France	Bruges	2	F1	020500, 001G1
	Audenge	1	F2	60400
	Roquebrune	1	F3	003D
	St Martial sur le Né	2	F4	V1, V2
	Pont de Martrou	1	F5	VF4
	La Clisse	1	F6	VF5
	Crazannes	1	F7	VM8
	Orx	2	F8	003Orx, 004Orx
	Uzeste	1	F9	004C
	St Léger de Balson	1	F10	006C
	Ambleville	2	F11	003CHA, 004CHA
	Lachaise	2	F12	005CHA, 006CHA
	Tocanne St Apre	1	F13	B
	Villefranque	1	F14	C
	Belin-Beliet	1	F15	D
	St Médard en Jalles	1	F16	E
	Pont de Martrou	1	F17	VM6
	Blaye	1	F18	VF7
Spain	La Rioja	2	Sp1	Mulp 55, Mulp 56
	Navarra	1	Sp2	Mulp 50
	Gipuzkoa	1	Sp3	AF207725
Romania	Danube delta	2	Ro1	JRM-698, JRM-699
Estonia	Tallin	2	Est1	JRM-659, JRM-660
	Unknown locality	1	Est	AF207723
Bielorussia	Vitebsk	2	Bel	Mulu 3, AF207724
Russia	Tver	7	Rus1	AF207720, Mulp 8 to Mulp 13
	Pskov	2	Ru2	Mulp 6, Mulp 7
Mustela putorius				
Spain	La rioja	1	Sp1	AF207726
	Gipuzkoa	1	Sp3	Mulp 53
Portugal	Unkown locality	1	Por	MPU 27
Estonia	Unknown locality	2	Est	Mulp 2, Mulp 3
Russia	Unknown locality	1	Rus	Mulp 41
	Tver	1	Rus2	Mulp 35
?		1		AF207717
?		1		AF207718
?		1		AF068570
Mustela eversmannii				
Mongolia	E. Inner	1		M. Evers. 1
Serbia	North West region	1		M. Evers. 2
Mustela nigripes				
USA	Unknown locality	2		M. nigripes 1 and 2

water and 0.2 μl of 5 U/μ Promega Taq DNA polymerase. Approximately 200 ng of DNA extract (10 μl) was used per PCR amplification. PCR was performed using an MJ Research PTC100 thermal cycler, employing 33 cycles (20 s at 94 °C, 30 s at 50 °C and 1 min 30 s at 68 °C) with a final extension cycle of 10 min at 68 °C. PCR products were then purified using the Ultra-free DA Amicon kit (Millipore) and directly sequenced. Both strands were sequenced using a BigDye terminator (Applied Biosystems) sequencing kit on an ABI 310 (Applied Biosystems) automated sequencer.

The newly determined sequences were compared with five European mink (AF207720, AF207721, AF207723, AF207724 and AF207725) and four polecat (AF068570, AF207717, AF207718 and AF207726) partial D-Loop sequences available in Genbank (Table 1) using the ED editor (MUST package; Philippe, 1993). The hypervariable C_nT_n region (Davison et al., 2000) and an 11 bp

minisatellite were excluded from phylogenetic analysis, because it was not possible to unambiguously align all individuals.

2.3. Analyses

The aligned sequences were analysed by distance (neighbour joining, NJ; Saitou and Nei, 1987), maximum parsimony (MP) (Fitch, 1971) and maximum likelihood methods. The General Time Reversible (GTR) model and Kimura two-parameter (K2P) estimator were used for the calculation of genetic distances. The GTR estimator was chosen as it is the more general model of sequence evolution which consider six parameters for its probability matrix corresponding to each possible substitution. To take into account differences of substitution rates across sites, the GTR analysis was performed assuming a gamma distribution at eight categories. The alpha parameter (Yang, 1996) and the proportion of invariant sites (I) were estimated with the maximum-likelihood method in PAUP 4.0b8. Maximum parsimony (MP: heuristic search; TBR branch swapping option) and maximum likelihood (ML: GTR model of sequence evolution) analyses were also conducted using PAUP 4.0b8 (Swofford, 1998). The robustness of inferences was assessed by bootstrap resampling (BP) (1000 random repetitions for MP and distance analyses, and 100 for ML).

A Bayesian approach to phylogeny reconstruction (Yang and Rannala, 1997; Huelsenbeck et al., 2001) was also used, implemented in MrBayes 2.01 (Huelsenbeck and Ronquist, 2001). Metropolis-coupled Markov chain Monte Carlo sampling was performed with four chains that were ran for 500,000 generations, using default model parameters as starting values. Bayesian posterior probabilities were picked from the 50% majority rules consensus of trees sampled every 20 generations, after removing trees obtained before chains reached apparent stationarity (''burn in'' determined by empirical checking of likelihood values).

A minimum spanning network was constructed using the MINSPNET algorithm available in the Arlequin 2.0 program (Schneider et al., 2000) as this method is very useful when sequences are closely related.

Nucleotide (π) and Haplotype (h) diversities, were estimated using the DNASP program (Rozas and Rozas, 1997). Calculations were performed on the main data matrix, including 57 animals.

3. Results

3.1. Sequences

The complete sequence of the mitochondrial D-Loop region was obtained for 38 European mink from 25 different localities, six European polecats from five regions, two steppe polecats and two black-footed ferrets (*M. nigripes*), used as outgroup (Table 1). These sequences have been deposited in the EMBL gene bank under accession numbers AJ548474 to AJ548496. They were aligned with the D-Loop sequences already available in GenBank. A 450 base pair fragment of the mitochondrial cytochrome *b* gene was also sequenced from 26 European mink from France and Spain.

3.2. Phylogenetic analyses

Cytochrome *b* sequences of different Mustelidae species: *Martes foina* (AF448245, ABO51250), *Martes martes* (AF448241, ABO51253), *Mustela vison* (ABO26106), *Mustela sibirica* (ABO51289, ABO51288), *Mustela putorius* (AF207716, AF207715), *Mustela nivalis* (ABO51272, ABO51271), *Mustela nigripes* (ARF068543), *Mustela lutreola* (ABO51263, ABO26105), *Mustela eversmannii* (ABO51261, ABO26102) and *Mustela erminea* (ABO51266, ABO51259) were used to determine the most suitable outgroup for the D-Loop analyses. Phylogenetic analyses (data not shown) performed on the basis of these sequences showed that *M. nigripes* is the most suitable outgroup to study mtDNA relationships within *M. lutreola* and *M. putorius*, as expected from earlier studies (Davison et al., 2000).

A first set of analyses was performed using partial D-Loop sequences, including those from Genbank. The aligned data matrix includes 57 *Mustela* (*lutreola, putorius, eversmannii* and *nigripes*) specimens and 254 sites, 40 of them variable and 27 phylogenetically informative. Thirty-one D-Loop haplotypes remained after removal of the C_nT_n array. The mean transition to transversion ratio is 2.29 and the nucleotide frequencies are: C 26.3%, T 30.5%, A 30.7%, G 12.5%. The Kimura two-parameter phylogeny (Fig. 1) shows two major clades: the first one corresponding to the polecat, *M. putorius,* and the second subdividing into two monophyletic groups of *M. lutreola* and *M. eversmannii*. The Bayesian, ML and MP (one most parsimonious tree, L = 60 steps; CI = 0.65; RI = 0.85) analyses yielded phylogenies of identical structure. Bootstrap values and Bayesian probabilities (BaP) resulting from theses analyses are indicated in Fig. 1. Unfortunately, the major groups are not well supported, with bootstrap support (BP) around 50% (Fig. 1).

The D-loop sequences of French and Spanish mink are nearly identical, with variation restricted to the hypervariable C_nT_n array (either seven or eight thymine residues).

To confirm these results, a second set of analyses was performed, using the complete mitochondrial D-Loop region, with two *M. nigripes,* two *M. eversmannii*, six *M. putorius* and 38 *M. lutreola*. Again, all the French and Spanish European animals appear extremely similar.

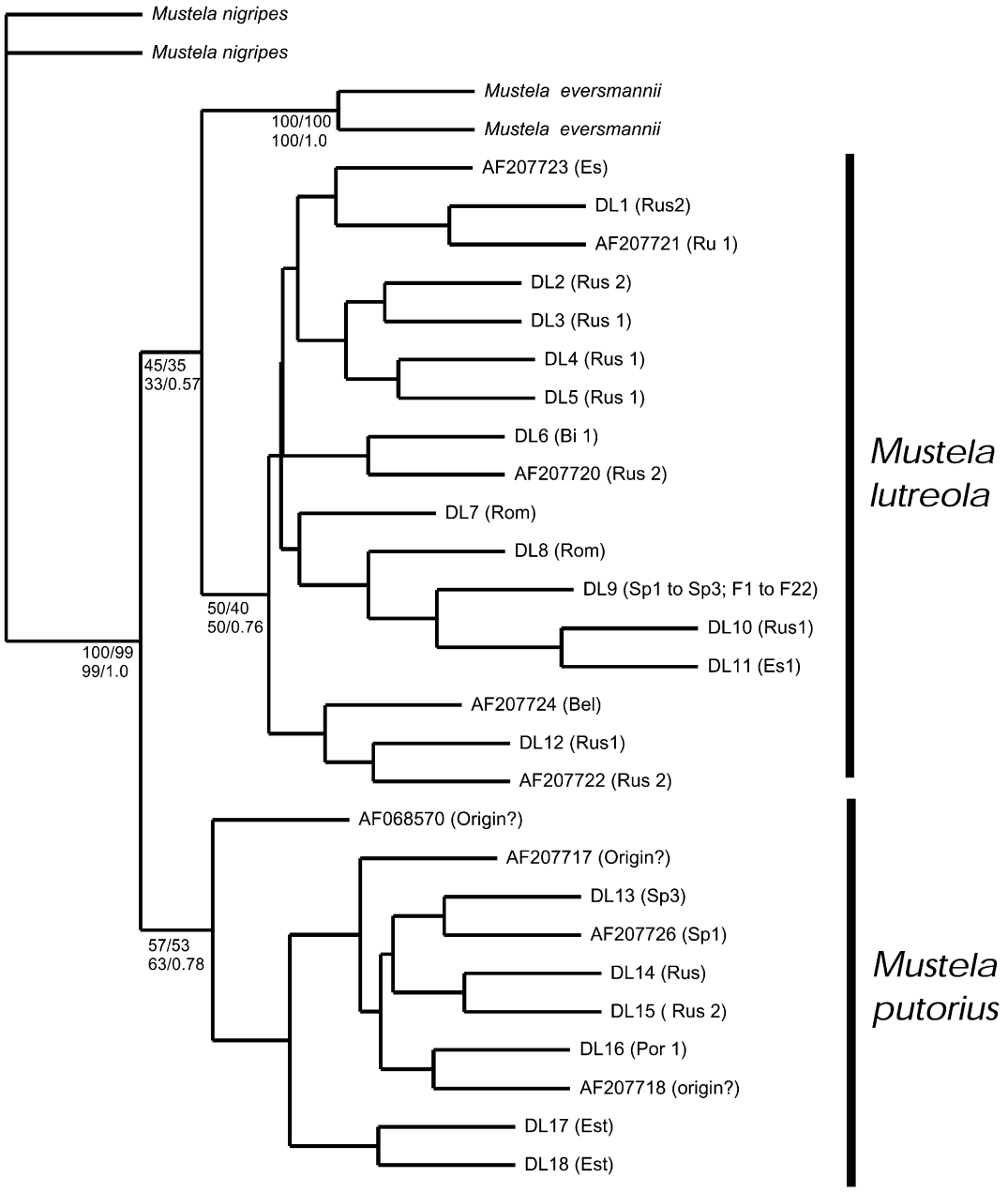

Fig. 1. Consensus tree derived from the analysis of a partial sequence of the mitochondrial control region for 57 European minks, polecats and black footed ferrets (used as outgroup). Each haplotype is identified by its accession number in GenBank or by the letters DL + a specific number. The locality codes (see Table 1) are also given for each haplotype. For each node supported by a least 50% of bootstrap value, the different robustness are indicated as followed: $\frac{\text{Neighbour joining/maximum likelihood}}{\text{Maximum parsimony/Bayesian probability}}$.

They shared the same haplotype with the exception of the C_nT_n array (see earlier) and a variable (from 11 to at least 23) number of copies of an 11 bp minisatellite in the hypervariable region R. It was impossible to determine the exact number of minisatellite copies, when $n > 23$, due to sequencing difficulties. This region was not considered in any further phylogenetic analysis.

After removal of the C_nT_n array and the minisatellite repeats, the final data matrix involved 729 sites, of which 63 were variable and 41 phylogenetically informative. The neighbour joining tree is shown in Fig. 2, obviously similar in structure to the tree in Fig. 1. The group corresponding to *M. putorius* is well supported (BP values for NJ: 79%, ML: 59, MP: 80% and BaP: 0.99). The second group of *M. lutreola* and *M. eversmannii* appears less robust (BP values for NJ: 53%, ML: 50% and BaP: 0.55). Within this group, *M. lutreola* and *M. eversmannii* are monophyletic, with stronger support compared with the first analysis (Fig. 1).

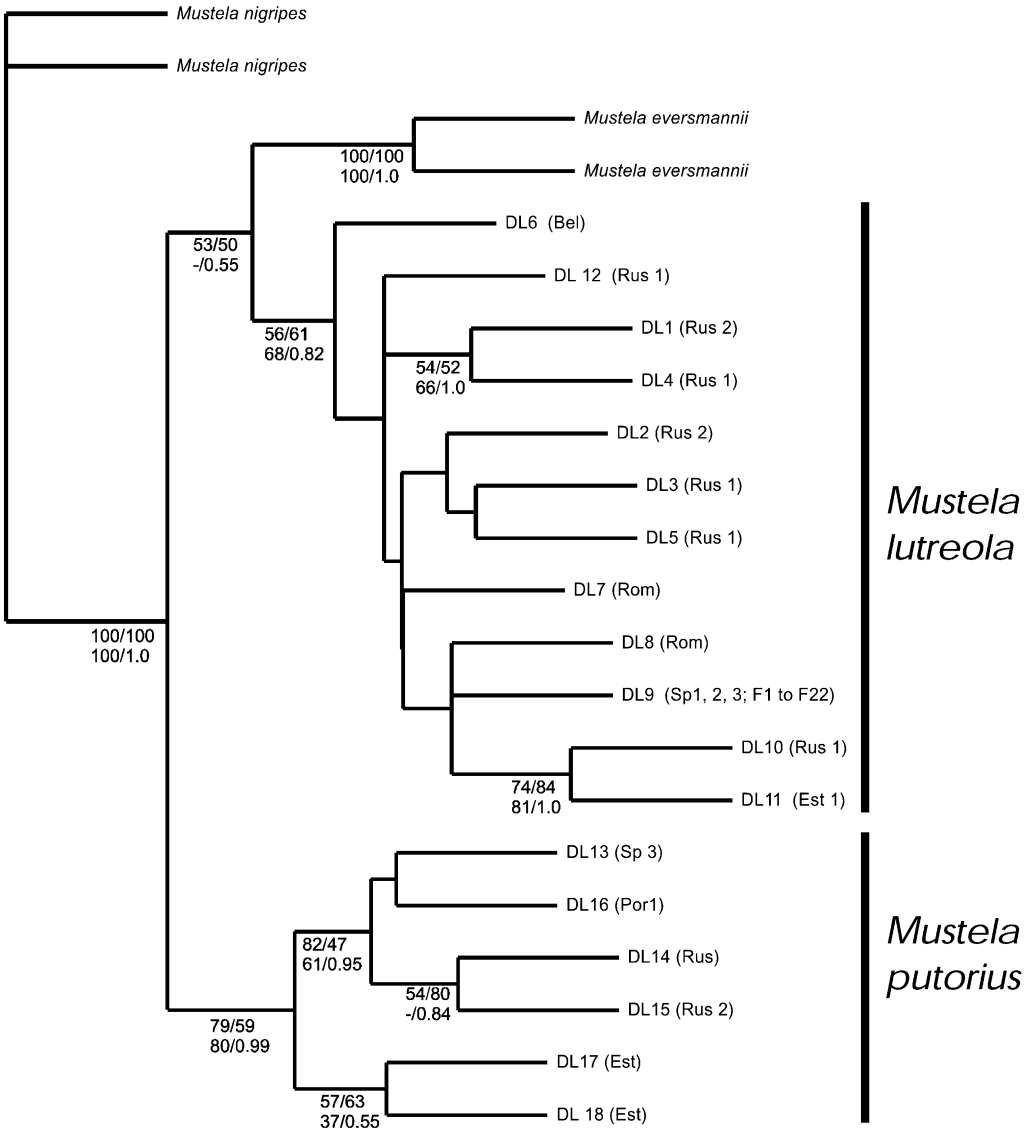

Fig. 2. Consensus tree derived from the analysis of the complete mitochondrial control region sequences for 37 European minks, polecats and black footed ferrets (used as outgroup). Each haplotype is identified by the letters DL + a specific number. The locality codes (see table 1) are also given for each haplotype. For each node supported by a least 50% of bootstrap value, the different robustness are indicated as followed: $\frac{\text{Neighbour joining/maximum likelihood}}{\text{Maximum parsimony/Bayesian probability}}$.

To confirm absolutely the lack of variation among all the French/Spanish European mink, we sequenced a 450 bp fragment of the mitochondrial cytochrome *b* gene in 23 French and three Spanish animals. All the sequences obtained were identical to a shorter sequence available in Genbank (AF207725; haplotype C11; Davison et al., 2000).

3.3. Genetic distances between the mustelids

The levels of genetic divergence (distance analysis, K2P estimator) observed between the four *Mustela* species are summarised in Table 2. The values range from 3.2 to 4.7% K2P distance.

3.4. Genetic structure

Nucleotide (π) and haplotype (h) diversities were calculated for each population of *M. lutreola* (Table 3)

Table 2
Mean levels of genetic divergence (in%) between the studied *Mustela* species, obtained in the distance analysis (K2P estimator)

	Mustela nigripes	*Mustela eversmannii*	*Mustela putorius*
Mustela eversmannii	4.7		
Mustela putorius	3.4	4.1	
Mustela lutreola	3.9	3.5	3.2

Table 3
Genetic variability observed within the three populations of *M. lutreola* and the one of *M. putorius*

		Sample size	Number of haplotypes	Genetic divergence within each group (% K2P distance)	Nucleotide diversity (Pi)	Standard deviation	Haplotype diversity (Pi)	Standard deviation
M. putorius		10	10	2.4	0.0108	0.0015	0.978	0.054
M. lutreola	Russian + bielorussian populations	14	11	1.5	0.0197	0.0025	0.978	0.035
	Romanian population	2	2	0.4	0.0039	0.0019	1	/
	Western european population	27	1	0	0	/	1	/

Animals from Russia and Belarus have a high nucleotide and haplotype diversity, compared to the French and Spanish samples, which share a single mitochondrial type. The mismatch distribution of pairwise differences shows a signature of population growth (Luikart et al., 2001), a bell-shape, similar to that found in *M. putorius* (Fig. 4). The network analysis (Fig. 3) shows a similar distribution of the samples.

4. Discussion

4.1. The phylogeography of European mink

As previously observed by Davison et al. (2000), the intraspecific genetic variability of *M. lutreola* is low (see Table 3). This is consistent with other European carnivores, especially mustelids, like wolverines (Walker et al., 2001), otters (Cassens et al., 2000; Morales, 2002), polecats, pine martens (Davison et al., 2001) and wolves (Vila et al., 1999). However, the low variation contrasts with the situation in other mammals, such as brown bears (*Ursus arctos*) (Taberlet et al., 1998), hedgehogs (*Erinaceus europaeus*) (Seddon et al., 2001), shrews (*Sorex araneus*, *S. minutus* and *Crocidura suaveolens*) (Taberlet et al., 1994, 1998; Bilton et al., 1998), and the rodents *Apodemus sylvaticus* (Michaux et al., 2003; Libois et al., 2001), *Myopus schisticolor* (Federov et al., 1996), *Arvicola* sp. (Taberlet et al., 1998), *Microtus agrestis* and *Clethrionomys glareolus* (Jaarola and Tegelström, 1995; Bilton et al., 1998). All of these species are characterised by divergent mtDNA lineages which are distributed over distinct geographic areas.

The absence of highly divergent lineages in the European mink, as well as a mismatch distribution that is consistent with an expanding population (Fig. 4), is an indication that the present-day population is the result of a colonisation from a single refugium, after the last glaciation. Unfortunately, the majority of the southern central European populations of the mink have long since become extinct (Youngman, 1982; de Bellefroid and Rosoux, 1998), and the analysis was limited to two Romanian animals. This, coupled with a poor fossil record, means that a more accurate reconstruction of the postglacial recolonisation is not possible.

However, the phylogeny supports earlier work (Davison et al., 2000), suggesting that European mink and steppe polecat are closely related, based on the mitochondrial D-loop region. This is in contrast to a recent nuclear gene phylogeny. Sato et al. (in press) compared mustelid cytochrome *b* (complete sequence) and nuclear interphotoreceptor retinoid binding protein phylogenies. Overall, the three phylogenies are congruent (including D-loop also), with the exception of the placement of European mink, which appears more closely related to a *M. itatsi–M. sibirica*–M. *putorius* clade in the nuclear phylogeny.

4.2. The history of French and Spanish European mink

In contrast to the situation in other species of mammals, where the southern-most refugial populations have the greatest diversity (Michaux et al., 2003), European mink mtDNA diversity is highest in East European populations, with an almost complete lack of variation in French and Spanish animals. While this needs to be confirmed using nuclear markers, such as microsatellites, it suggests that a few individuals (possibly a single female) established this population, possibly as an anthropogenic introduction. However, an early Holocene origin is equally conceivable, not least because the D-loop mutation rate is completely unknown in this group. Leptokurtic dispersal from a refugium, where a few long-distance migrant individuals establish populations in advance of a colonisation wave, is expected to produce patches of relative genetic homogeneity such as those reported here (Ibrahim et al., 1996).

4.3. Implications for the conservation of European mink

Avise (2000) defined the concept of a Management Unit as "*any population that exchanges so few migrants with others as to be genetically distinct from them normally will be demographically independent at the present time*". In this way, he noted that "*even shallow matrilineal subdivisions can be relevant to conservation efforts*". Certainly, specific haplotypes are found in the East European and French and Spanish populations at different frequencies (insufficient samples were investigated in Romania), with a single haplotype predominating in France and Spain.

Ideally, this mtDNA study should be augmented by an analysis of nuclear markers and fitness related phenotypic differences (Hedrick, 1999), before defining Management Units. In the meantime, the captive breeding program is proceeding. Following the precautionary principle, we recommend that mink from the three geographically separate populations (France/Spain, Romania, Eastern Europe) should be managed separately, at least for the

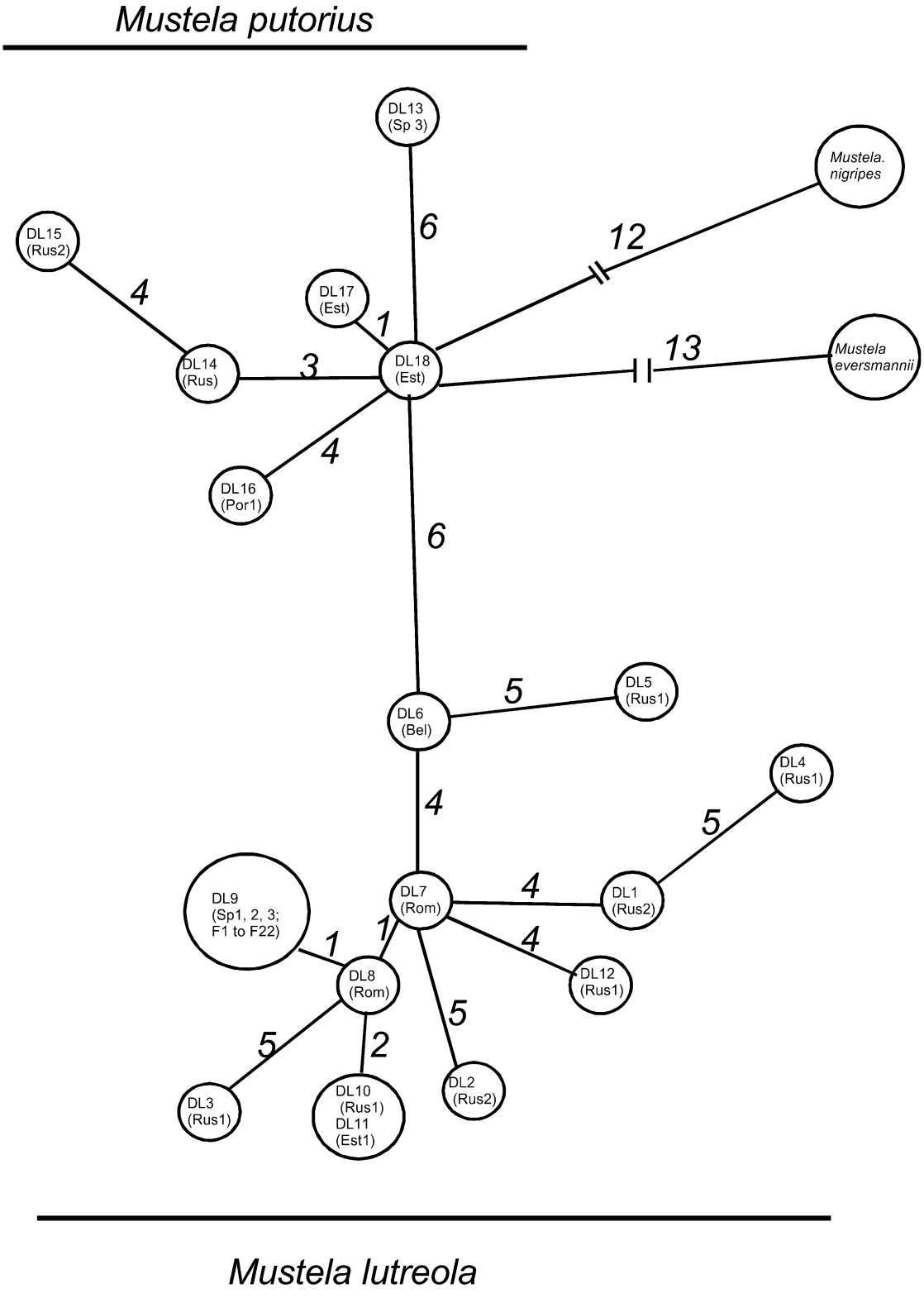

Fig. 3. A minimum spanning network constructed using mitochondrial control region sequences. The identity of the haplotypes (see Fig.s 1 and 2) and their geographic origin (see Table 1) are indicated. Numbers in italics correspond to the mutational steps observed between haplotypes.

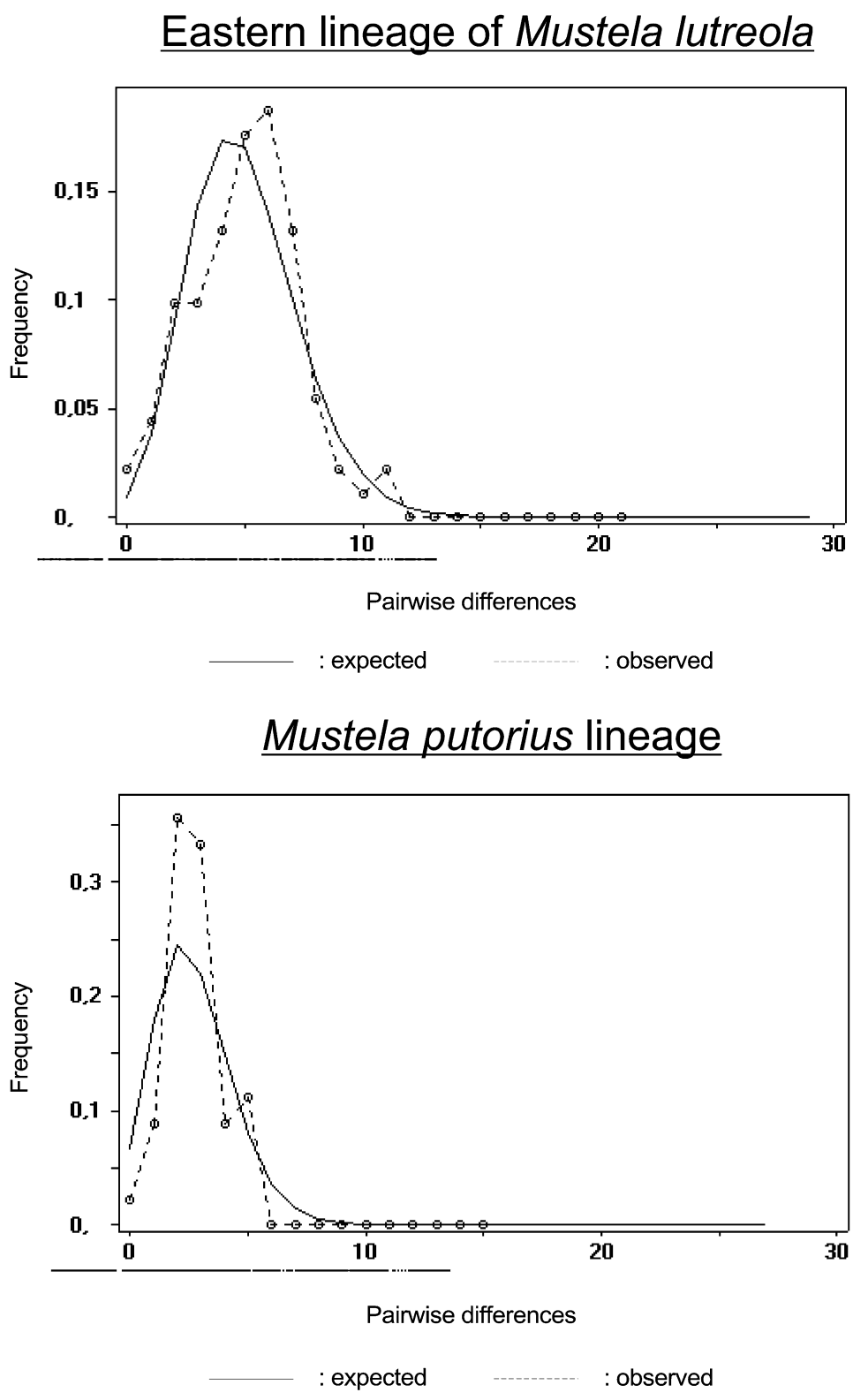

Fig. 4. Mismatch distribution for mtDNA types from the eastern genetic lineage of *Mustela lutreola* and the *M. putorius* group. The expected frequency is based on a population growth-decline model (for the groups, respectively: θ initial = 0, 2.4, 0 and 0, θ final = 1000, τ = 4.4, 9.4, 2.7 and 1.6), determined using the DNASP v3.5 program (Rozas and Rozas, 1997).

moment. It is also possible that the animals are locally adapted, so that outbreeding depression (Lynch, 1991) could result from some mating combinations, especially once the animals were released into the wild.

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