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Genetic variability in Danish polecats *Mustela putorius* as assessed by microsatellites

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Genetic variability and population structure was investigated in 83 European polecats *Mustela putorius* by means of six microsatellite markers. The samples came from two areas in Denmark, Østjylland and Thy, which are separated by the Limfjord. The genetic diversity ($H_e = 0.583$) found in the total sample was similar to those found in other mustelid species and carnivores in general. A heterozygote deficiency, probably due to a Wahlund effect, suggested a further substructuring of the Danish sample. Population genetic substructuring was investigated in three different ways: by means of the program STRUCTURE, Wright's F-statistics and by an assignment test. All the tests indicate a subdivision of the sample into two distinct groups, which is concordant with the two sampling locations, with an average genetic divergence of $F_{ST} = 0.126$ and $R_{ST} = 0.1692$. The higher genetic diversity found in the Thy population ($H_e = 0.578$), as compared to the Østjylland population ($H_e = 0.420$), could be explained by assuming two ancient waves of colonisation of the Danish peninsula. Tests for recent bottlenecks were conducted, and the results suggest no evidence of neither population decline nor expansion. Our study is the first one in which microsatellite markers are used on polecat samples, and one *locus* (mv54) was found to be diagnostic in distinguishing between American mink *Mustela vison* and European polecat.

Key words: assignment test, diagnostic locus, polecat, population structure, microsatellite DNA, Mustela putorius, STRUCTURE

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Habitat fragmentation is a major factor limiting the distribution of species in man-altered landscapes. Furthermore, fragmentation and habitat destruction have for some species led to a reduction in population size (e.g. Taylor, Sherwin, & Wayne 1994). Population subdivision, in turn, is expected to reduce within-population genetic polymorphism and to increase genetic differentiation. Loss of polymorphism can lead to reduced potential for adaptation, and, especially in combination with small population sizes, to inbreeding depression (Frankham 1995). From a conservation perspective, the need for further knowledge of the effects of habitat fragmentation on wildlife has never been greater than today. Carnivores are considered ecological indicators of the viability of other members of their communities ('umbrellas'; Wilcox 1984). Because carnivores occur at low densities and have relatively low mobility (compared to birds), habitat fragmentation can lead to genetic impoverishment (Wayne & Koepfli 1996). Small and medium sized carnivores are suitable species for analysing the genetic consequences of landscape fragmentation. In an extensive review, Bright (1993) classified several mustelids supposed to suffer with different intensity from the effects of habitat fragmentation into three groups based on their different ecological characteristics (Corbet & Harris 1991). Among these carnivore species, the European polecat *Mustela putorius* was suggested to be vulnerable to habitat fragmentation. The first group features the Eurasian otter *Lutra lutra*, the American mink *Mustela vison* and the pine marten *Martes martes*. Species belonging to this group should be the more vulnerable to the adverse effects of habitat fragmentation due to their relatively low densities, slow breeding, restriction to few habitats and often poor mobility. In a second group, the Eurasian badger *Meles meles* figures together with the polecat. Species belonging to this group should be somewhat vulnerable to habitat fragmentation and are mostly mosaic species with a wider habitat tolerance than the species in the first group. The weasel *Mustela nivalis* and the stoat *Mustela erminea* are classified in the third group. These species are not likely to be adversely affected by habitat fragmentation and can successfully exploit a wider range of habitats than species in the first and second groups (Bright 1993). Hence, the mustelids constitute an interesting and heterogeneous group consisting of species with different degrees of susceptibility to environmental perturbation. This heterogeneity is caused by the different strategies that the mustelids adopt in order to adapt to different habitats (Corbet & Harris 1991) and to their different sizes and shapes which are results of these adaptations (Moors 1980, Dayan & Simberloff 1994).

The polecat is a medium sized carnivore living in most parts of Europe, and it is thought to be declining in the central parts of its range. In certain parts of Europe, it is considered threatened (Engelhart, Behnisch, Hagenmaier & Apfelbach 2001, and references therein). According to the Danish game bag record, the number of polecats killed by hunting in Denmark during the last 60 years has been declining which suggests a decline in the population size (Hammershøj & Asferg 1999). In Denmark, habitat changes have taken place, and the polecat's habitats, such as moors, ponds and wetlands, have been greatly reduced due to canalisation of rivers and drainages (Jensen & Jensen 1972). Another possible reason that may have contributed to the decline observed in the bag record, is the increasing number of free-ranging American mink observed in Denmark during the last 30 years (Hammershøj & Asferg 1999). Mink and polecat share the same habitat and are presumably competing trophically and territorially, and the polecat appears to be competitively weaker than mink (Sidorovich 2000).

The aim of our study was to investigate by means of microsatellite markers the level of polymorphism and pattern of genetic subdivision among polecats sampled from two areas in Denmark. We assumed that the polecat suffers from the effects of habitat fragmentation as suggested by Bright (1993), and that the bag record reflects a real declining trend produced by the fragmentation effect and/or mink presence. In particular, we focused on the genetic consequences of this hypothetical population decline, both on a temporal and on a geographical scale. We chose microsatellites as genetic markers, because a previous study conducted by Simonsen (1982), who used allozymes, did not find any genetic variation at 21 allozyme *loci* in 24 polecats from different localities in Denmark.

The above cited heterogeneity of the mustelid group is also reflected in their degree of nuclear genetic diversity. Microsatellites have been found to be polymorphic in most species of the mustelid family, e.g. the Eurasian badger (Bijlsma, Van de Vliet, Pertoldi, Van Appledorn

& Van de Zande 2000, Pertoldi, Loeschcke, Madsen & Randi 2000, Pertoldi, Loeschcke, Madsen, Randi & Mucci 2001a), the pine marten (Bijlsma et al. 2000), the American pine marten *Martes americana* (Kyle, Davis & Strobeck 2000), the Eurasian otter (Dallas & Piertney 1998, Dallas, Bacon, Carss, Conroy, Green, Jefferies, Kruuk, Marshall, Piertney & Racey 1999, Pertoldi, Hansen, Loeschcke, Madsen, Jacobsen & Baagoe 2001b, Randi, Davoli, Pierpaoli, Pertoldi, Madsen & Loeschcke 2003), the American mink (O'Connell, Wright & Farid 1996, Belliveau, Farid, O'Connell & Wright 1999, Fleming, Ostrander & Cook 1999), the North American wolverine *Gulo gulo* (Kyle & Strobeck 2001a), the fisher *Martes pennanti* (Kyle, Robitaille & Strobeck 2001b) and the European mink *Mustela lutreola* (Lodé 1999). Our study provides the first characterisation of the genetic structure of polecats.

Material and methods

Samples and study areas

Uniformly distributed over an area of approximately 1,200 km^2, 58 polecats were caught in traps in the northwestern part of Denmark (Thy), and 25 traffic killed individuals were collected from a more southeasterly study area (Østjylland; Fig. 1). The two study areas are separated by a large fjord area (Limfjorden).

Molecular analysis

The tissue samples (i.e. muscles) from the 83 polecats were stored at -20°C for further analysis, and total DNA was extracted from muscle and organ tissue using standard phenol/chloroform extraction (Sambrook, Fritsch & Maniatis 1989). Genotypes of eight microsatellite markers were determined by PCR, six markers (mvi54, mvi57, mvi87, mvi111, mvi114 and mvi232) were from O'Connell et al. (1996), mvi389 was unpublished (Genebank accession number U92534) and one marker (mvi022) originated from Fleming et al. (1999). The microsatellite markers utilised in Bijlsma et al. (2000) and in Pertoldi et al. (2001b) were also tested, but did not show any reliable PCR product.

The PCR reaction for all eight markers were carried out in a 6 μl volume using 0.3 U Taq polymerase, 1.5 mM $MgCl_2$, 1 μl of diluted DNA, 0.2 mM of each of the dNTP and 0.3 pmol primers, where one primer was fluorescently labelled with Cy5™. The PCR conditions were: 94°C for 3 minutes, 40 cycles with 94°C for 40 seconds, 56°C for 40 seconds and 72°C for 40 seconds. The PCR products were analysed directly without dilution on a 6% denaturing polyacrylamide gel (Reprogel™), and alleles were scored on an ALF automated sequencer (Pharmacia).

Genetic variability and analyses of population genetic structure

Allelic frequencies, allelic diversity and the number of private alleles were estimated for each *locus* using the software FSTAT 2.9.1 (Goudet 1995; available at http://www.unil.ch/izea/softwares/fstat.html). Observed (H_o) and expected heterozygosities (H_E) of every *locus* were estimated using the program POP100GENE (Piry & Bouget 1999; available at http://www.ensam.inra.fr/URLB/). Deviations from Hardy Weinberg equilibrium (HWE) for each *locus* and each population were tested by the exact test (Guo & Thompson 1992) using the program GENEPOP 3.3 (Raymond & Rousset 1995; available at http://www.cefe.cnrs-mop.fr). All P values were assessed for significance using sequential Bonferroni correction (Rice 1989).

Bayesian admixture analyses

A Bayesian clustering procedure (implemented in the software STRUCTURE; Pritchard, Stephens & Donnelly 2000; available at http://www.stats.ox.ac.uk/~pritch/home.html) was used to infer the number of distinct genetic populations that were included in the sample set. The Bayesian model assumes K (unknown) populations, each of which is characterised by the allele fre-

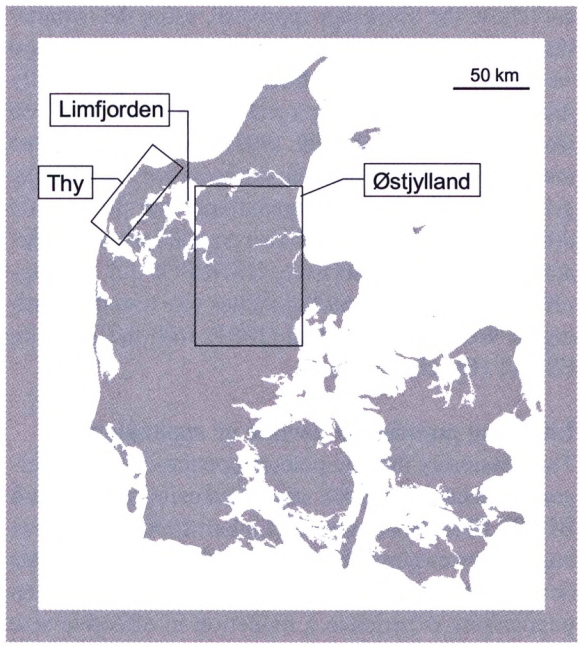

Figure 1. Location of the two sample sites, Thy and Østjylland, and the Limfjord area on the Danish peninsula of Jylland.

quencies at a set of independent *loci*. The model is based on the assumptions of HWE and linkage equilibrium. The likelihood of K is estimated from the allele frequencies. The highest likelihood value indicates the most likely number of populations in the sample. If polecats belong to more differentiated subpopulations, the likelihood of a partition in several panmictic clusters should depart from its expected distribution under the hypothesis of no structure (a single panmictic population).

Furthermore, we searched for the best possible (i.e. most likely) partition in several panmictic clusters of the whole polecat sample using a 'Simulated annealing' procedure (Castric, Belkhir, Bernatchez & Bonhomme 2002). The present ML-based algorithm (PartitionML) is available at http://www.univmontp2.fr/_genetix/partitionml.htm. The number of independent panmictic source populations (or clusters) assumed for a particular run of the program is set *a priori* by the user. To compensate for the fact that likelihood maximisation intrinsically favours partitions with more clusters, we utilised the Likelihood Ratio Tests to compare runs with different numbers of source populations (see Castric et al. 2002). The program PartitionML provides the opportunity to compare the likelihood of different configurations and is a fast and complementary alternative to other approaches to the same problem, such as for instance the STRUCTURE test.

We used the program GENECLASS 1.0 (Cornuet, Piry, Luikart, Estoup & Solignac 1999; available at http://www.ensam.inra.fr/URLB/geneclass/geneclass.html), and chose the 'Bayesian approach', using the 'simulation' option for testing whether or not individuals were 'accepted' in or 'rejected' from the two geographically distinct populations, using a probability threshold of 5% (for details see Cornuet et al. 1999). The populations that resulted from the assignment test were compared to the geographically distinct populations, and the genetic differentiation between the two populations was characterised by exact tests using GENEPOP 3.3. The significance of the F_{ST} and R_{ST} values was tested according to Weir & Cockerham (1984) with the program FSTAT (Goudet 1995).

Inferring population decline or expansion

The possibility that the polecat population had undergone a recent bottleneck was tested using the program BOTTLENECK (Cornuet & Luikart 1996; available at http://www.ensam.inra.fr/URLB). Bottleneck analysis developed tests based on the assumption that when populations experience a reduction in effective size they generally have higher heterozygosity than predicted by their allelic diversity, because allelic diversity is reduced faster than heterozygosity. We used the program BOTTLENECK to simulate the coalescent process of N genes under the infinite allele model (IAM), a stepwise mutation model (SMM) and a two-phase mutation model (TPM) with a 95% SMM. These three models were tested in order to produce the distribution of heterozygosity expected from the observed number of alleles given the sample size of each population under the assumption of mutation-drift equilibrium. This enabled calculation of the average expected heterozygosity and a computation of the P-value for the difference between observed and expected heterozygosity at each *locus*. To determine whether a population exhibits a significant number of *loci* with heterozygosity excess, we performed both a 'sign test' (Cornuet & Luikart 1996) and a 'Wilcoxon sign-rank test' (Luikart, Allendorf, Cornuet & Sherwin 1998). Luikart et al. (1998) argued that due to the probabilistic reduction in the number of rare alleles, bottlenecks might cause a characteristic mode-shift distortion in the distribution of allele frequencies, i.e. low numbers of low-frequency alleles. Because differentiation measures that use highly polymorphic *loci*, such as microsatellites, are very sensitive to the effective size of the populations in question, Hedrick (1999) recommended the use of statistical tests to identify bottlenecks that use other than lowered heterozygosity.

Intermediate bottlenecks were tested using the software M (Garza & Williamson 2001; available at http://www.pfeg.noaa.gov/tib/carlos.htm). The bottleneck tests made by Cornuet & Luikart (1996) can detect only severe and recent declines which have occurred within $0.2N_e$ to $0.4N_e$ generations, whereas the test made by Garza & Williamson (2001) could detect bottlenecks that have occurred within 100 generations.

The two tests for recent and intermediate bottlenecks were both tested for the whole population and for the two geographically separated populations in Thy and Østjylland.

Results

Application of mink primers to other mustelids

The genus Mustela is in an evolutionary perspective a recent group, probably only 10-15 My old with the polecat being approximately five My old (Binida-Emonds, Gittleman & Purvis 1999). The mink is considered to be the outgroup (e.g. Davison, Birks, Griffiths, Kitchener, Biggins & Butlin 1999). All primers developed for mink amplified well on polecat DNA (also if not all were polymorphic), and this probably reflects the limited divergence between the two species in the genus.

Table 1. Observed number of alleles per microsatellite locus, size ranges of the alleles and number of private alleles (Pa) in the populations of Thy and Østjylland. Outcomes of the tests for deviations from the Hardy-Weinberg equilibrium (HWE), observed heterozygosity (H_o) and expected heterozygosity (H_e) are given for the whole population and for each of the two polulations. A table-wide significance level test was applied, using the sequential Bonferroni technique (initial K = 20; Rice 1989).*** = P < 0.001.

Locus	No. of alleles	Range of allele size	Pa Study site; allele frequency	Thy + Østjylland (N = 83) H_o	Thy + Østjylland (N = 83) H_e	Thy (N = 58) H_o	Thy (N = 58) H_e	Østjylland (N = 25) H_o	Østjylland (N = 25) H_e
mvi389	1	109		-	-	-	-	-	-
mvi54	1	113		-	-	-	-	-	-
mvi57	7	102-112	(Thy; 0.0172, 0.0345, 0.0431)	0.385***	0.514	0.397***	0.573	0.360	0.372
mvi87	5	80-88	(Thy; 0.0259, 0.4483, 0.0086)	0.121***	0.522	0.172***	0.594	-	-
mvi111	9	90-116	(Thy; 0.0086) (Østjylland; 0.08, 0.02)	0.614***	0.703	0.621***	0.697	0.600	0.654
mvi114	6	76-86	(Thy; 0.095, 0.009)	0.690	0.731	0.741	0.719	0.560	0.546
mvi232	2	154-156		0.241	0.230	0.241	0.212	0.240	0.269
mvi022	6	272-282		0.626	0.710	0.672	0.666	0.520	0.735
Overall mean		4.63		***	0.583	***	0.578	n.s.	0.420

Genetic variability and analyses of population genetic structure

In the whole population, six out of eight *loci* were polymorphic, whereas mvi389 and mvi54 were monomorphic (Table 1). The average number of alleles was 4.63. Genetic diversity (H_e) ranged between 0.230 and 0.731, with an average of 0.583 across the six polymorphic *loci*. Deviations from HWE (heterozygote deficiency) were highly significant in three *loci* (mv57, mv87 and mv111; P < 0.0001). Overall, there was a highly significant deviation from HWE (F_{IS} = 0.221, P < 0.0001).

In the Thy population, 10 private alleles (mean frequency ± S.E. = 0.07155 ± 0.042) were observed, and in the Østjylland population, two private alleles were observed (mean frequency = 0.05 ± 0.03). In the population of Østjylland, the *locus* mvi87 was found to be monomorphic, whereas in the Thy population it was polymorphic (see Table 1). In the Thy population, three *loci* showed highly significant heterozygote deficiency (mvi57, mvi87, mvi111; P < 0.0001), whereas in the Østjylland population, none of the *loci* showed significant deviation from HWE (see Table 1).

The proportion of correctly assigned individuals in the two geographically distinct populations was high: 88% for Østjylland and 95% for Thy, whereas 11% of the individuals were not assigned to either of the two populations.

The exact test for differences in allele frequencies between the two populations showed a highly significant differentiation (P < 0.001). Similarly, the pairwise F_{ST} and R_{ST} values between the two populations were highly significant (F_{ST} = 0.126, P < 0.001; R_{ST} = 0.169, P < 0.001; FSTAT permutation procedure).

Bayesian admixture analyses

The Bayesian clustering procedure STRUCTURE maximised the likelihood of the data given that the model split the whole polecat sample into two genetically distinct populations (K = 2; Ln = -1207).

The PartitionML software confirmed the results obtained by the STRUCTURE software. A partition with K = 2 was significant, whereas partitions with K = 1 and K = 3 were not significant.

Inferring population decline or expansion

The tests used to evaluate the effects of recent fluctuations in population size and bottlenecks were conducted on the whole population and on the two populations separately. None of the tests detected significant fluctuations (results not shown). All tests for intermediate bottlenecks showed a higher average M value (0.820) and none fell within the range of 0.6-0.7 associated with a known history of bottlenecks (Garza & Williamson 2001). However, these results should be interpreted with caution, as the sample sizes of local populations are smaller than the thresholds suggested by Cornuet & Luikart (1996) and Garza & Williamson (2001).

Discussion

Genetic variability and analyses of population genetic structure

The two studied Danish polecat populations were not genetically depauperate. There is an acknowledged large difference in mutation rate between microsatellites and allozymes (Hedrick 1999). Consistent with this, genetic variability was found using microsatellites, whereas the allozyme investigation performed by Simonsen (1982) showed no variability at all. Using micro-

satellites, we have shown in our study that genetic variability in Danish polecat populations exists. The average H_e of 0.583 for the whole population is quite similar to the results reported from other Danish and Dutch mustelid investigations using microsatellites. In Danish otters, an average H_e of 0.425 was observed (Pertoldi et al. 2001b), and in pine martens from the Netherlands and badgers from Denmark, H_e averages of 0.566 and 0.418, respectively, were found (Bijlsma et al. 2000).

The genetic substructuring we found in the whole population using the programs STRUCTURE and PartitionML suggests that the most likely explanation for the HWE deviation in the whole population is the pooling of different populations with a distinct allele frequency within each population (Wahlund effect; Wahlund 1928). The programs STRUCTURE and PartitionML detected no further substructuring within each of the two populations. The capacity to detect substructuring by using these two programs increases with the amount of *loci* investigated (Pritchard et al. 2000, Castric et al. 2002). Therefore, given the low amount of loci investigated in our study, we can not exclude the possibility that the program was not able to detect further substructuring. The significance of the F_{st} and R_{st} values confirm that the two sampled polecat populations are significantly differentiated according to both the frequencies and the size of microsatellite alleles. However, strong quantitative conclusions about the genetic distance can not be drawn as measurements of genetic divergence tend to show inverse correlations with levels of population polymorphism (Paetkau, Waits, Clarkson, Craighead & Strobeck 1997, Hedrick 1999). In fact, our estimates of population polymorphism are in some way affected by the population size. The present allelic composition we observed in the polecat population could hardly have been caused by polecats escaped from farms, as the numbers of polecat farms and farmed polecats in Denmark have always been very small (Hammershøj & Asferg 1999). The assignment test suggested that some connection existed between the two separate polecat populations during the study period, even though the Limfjord, by acting as a barrier, has limited the gene flow. Theoretically, one migrant per generation is sufficient to keep populations genetically homogeneous (Wright 1931).Other scientists (e.g. Whitlock & McCauley 1999, Vucetich & Waite 2000) have, however, shown that one migrant per generation is not sufficient in structured and fluctuating populations, so we can not rule out the possibility that some gene flow does take place between the two populations, but the action of drift and mutation which produces genetic divergence between populations probably exceeds the homogenising effect of migration. The heterogeneity in the heterozygosity values and allele numbers, which were found to vary among *loci*, also supports the possibility that gene flow restriction and population size fluctuations have somehow influenced the detected genetic structure. In fact, genetic drift is expected to create gaps in within-population distributions of microsatellite allele sizes.

The Thy population is not in HWE as significant heterozygote deficiencies are found at several *loci*. However, given that the investigated Thy area is relatively small compared to the polecats' dispersal capacity (Corbet & Harris 1991), we expect the contribution of the Wahlund effect on the HWE disequilibrium to be less important. We therefore suggest that the heterozygote deficiency within the Thy population could be a consequence of inbreeding due to the small population size which increases mating among relatives. Mating among relatives should be more frequent in smaller than in larger populations as polecats would have access to a smaller number of unrelated mates. Experimental and field studies have suggested that N_e/N can be as low as 0.1-0.2 (e.g. Frankham 1995). Metapopulation dynamics that involve frequent extinction-recolonisation events, as suggested by Hedrick (1996), can also contribute to reduce N_e.

Additionally, for many mammal populations the main impacts of roads demonstrated so far have been caused by increased disturbance and mortality (Blandford 1987). Traffic mortality can change the genetic composition of a population, and heterozygote deficiency can also be caused by systematic removal of a specific age class or gender. Hence, systematic removal of adult males, which have a higher risk of being killed due to their relatively higher patrolling activity as compared to females, can produce heterozygote deficiency. In our study, the ratio of traffic killed males:females in the Østjylland area was 21:4, and so was male biased. Analysis of a large number of *loci* will increase the power of detected population substructures because each *locus* will contain an independent history of the population dependent on the amount of random drift, mutation and migration that has taken place.

The higher genetic diversity found in the Thy population ($H_e = 0.578$) as compared to the Østjylland population ($H_e = 0.420$) could be explained by assuming two waves of colonisation of the Danish peninsula of Jylland after the last pleistocenic glaciation: the first wave could have colonised both Thy and Østjylland, whereas the second wave could have colonised only Østjylland. However, fragmentation of populations alone can lead to fixation of alternate alleles in different populations,

and more importantly, loss of rare alleles (Hedrick & Miller 1992).

Inferring population decline or expansion

Despite the lacking detection of significant recent or intermediate population fluctuations, we can not exclude that fluctuations have occurred. The main reason for this is the limited number of polymorphic *loci* screened, and on which the bottleneck test is highly dependent. Furthermore, the model assumes HWE (Cornuet & Luikart 1996) which was not obtained within the proposed populations. Lastly, the program is also quite conservative and rarely shows a bottleneck in natural populations. Another problem associated with the bottleneck tests is the relatively short-time window in which these tests detect possible population changes.

Diagnostic locus for genetic tagging

The monomorphic *locus* mv54 used in our study was found to distinguish mink from polecat. The allele length in all 83 polecat samples was 113 bases long, whereas the alleles from the 164 mink samples were scored as 95, 125, 127, 129 and 131 bases (T.B. Møller, C. Pertoldi, A.B. Madsen & V. Loeschcke, unpubl. data). This diagnostic *locus* could, when utilised in connection with field investigations where scats are collected, become a relatively quick method for quantification of the abundance ratio between these two sympatric species in different areas. Our study can not confirm that habitat fragmentation over the previous 50-60 years has caused a major decline in the genetic diversity of the polecat, despite a decline in the polecat population.

Future directions

The lacking evidence of a demographic decline in the polecat populations, as suggested by our results, should be further investigated by a temporal analysis based on extraction of DNA from canine teeth of individuals from museum collections to assess if there are any indications of a historical population decline (Pertoldi et al. 2001b). To gain further information, microsatellite analyses should be carried out, in particular to investigate how widespread the polecat is compared to the mink. To look further into the effects of fragmentation on the polecat populations, it is necessary to increase both the number of *loci* investigated and the sample size, and to expand the area of investigation.

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