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Regionally high rates of hybridization and introgression in German wildcat populations (*Felis silvestris*, Carnivora, Felidae)

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Abstract

While the western populations of the wildcat (*Felis silvestris silvestris*) in Germany come into contact with wildcats in France and Switzerland, the eastern distribution area is geographically completely isolated and consists of scattered subpopulations. To investigate population structure, evolutionary relationships and degree of hybridization with domestic cats we analysed the mitochondrial control region of 86 cats in combination with 11 microsatellite loci of 149 cats. According to our microsatellite data, German wildcats are divided into two separate populations corresponding to the western and eastern distribution areas. We found no indication of a further subdivision of the eastern population. German wildcat populations are genetically distinct from domestic cats in the main, but we identified 18.4% of the whole wildcat sample as being of hybrid origin, corresponding to 4.2% of the eastern and 42.9% of the western wildcat population, and 2.7% of the domestic cat sample. The mitochondrial haplotypes form a network of three connected clusters and reveal a high level of genetic diversity, especially within the eastern population. Our findings are explained at best in terms of continuous introgression between domestic cats and wildcat populations and differing degrees of recent hybridization in the various populations. Future conservation efforts should focus on preserving the existing gene flow between the isolated distribution areas, but also on preventing the spread of hybrids and limiting the habitat alterations that lead to increased contact with domestic cats. In conclusion we discuss possible evolutionary reasons for the still traceable genetic integrity of the wildcat despite its long history of interbreeding.

Key words: Population genetics – mammals – hybridization – conservation biology

Introduction

One European mammal that was formerly widespread but which is now seriously threatened in certain regions is the wildcat (*Felis silvestris*) [IUCN status: least concern (Red List 2008), EEC status: strictly protected species Appendix II, Stahl and Artois 1994]. Hundreds of years of intensive hunting and habitat loss have led to the extirpation of this species from most of its former range in many parts of Europe. Moreover, the extensive road network probably acts as a handicap to dispersal, thus limiting the gene flow and ultimately resulting in a hidden genetic structure within the European wildcat population (Eckert 2003; Mölich 2006; summary in Simon 2006). Recent advances over the past two decades in the development of molecular markers and mathematical techniques have led to better recognition of such correlations and ultimately to a new understanding of biogeographical patterns and processes. Highly polymorphic microsatellite markers in particular are powerful tools in detecting genetic variability and gene flow between populations and phylogenetic lineages (e.g. Lecis et al. 2006; Pierpaoli et al. 2003; Randi and Lucchini 2002; Wiseman et al. 2000). Another well-established molecular tool are the rapidly evolving sequences of the mitochondrial genome, which have been used to study a great variety of species (e.g. Barnett et al. 2006; Freeman et al. 2001; Pierpaoli et al. 1999; Randi et al. 2001). Considering both classes of molecular marker in combination and using sophisticated Bayesian clustering methods makes it possible to reliably identify gene transfer between different populations, subspecies or species, providing much needed insights into complex evolutionary processes and the delimitation of taxonomic entities (e.g. Randi et al. 2001; Oliveira et al. 2007, 2008; Vila et al. 2003). Endeavours to conserve biodiversity in the populous region of Central Europe are benefitting in particular from this renaissance of historical biogeography. Genetic studies also provide a solid and scientific background for decision-making in animal conservation, landscape planning and nature protection (Daniels and Corbett 2003; Daniels et al. 2001; Kitchener et al. 2005; Oliveira et al. 2007, 2008; Stahl and Artois 1994).

In Germany wildcats today inhabit two isolated and fragmented areas. The distribution area of the western population ranges from low mountain ranges east of the Rhine to the western banks of the Rhine contiguously to France and Switzerland, with the highest population densities occurring in the Eifel area in Rhineland-Palatinate and North Rhine-Westphalia (Raimer 1994). By contrast, the eastern distribution area is geographically isolated and is comprised of scattered populations in the Harz Mountains in Sachsen-Anhalt and the adjacent uplands of the Federal Lands Thuringia, Lower Saxony and Hesse (Mölich and Klaus 2003; Raimer 2006; Simon 2006). In Thuringia, the wildcat is restricted to scattered and limited areas in low mountain ranges surrounded by extensive cultivated landscape. Within these relict spots this shy species favours forests that are not intensively managed, and is often found in military training areas. The Harz Mountains in the north and the Hainich National Park in the west provide particularly appropriate habitat structure and are inhabited by stable or even increasing populations. Geographically speaking, distribution within Thuringia is divided into two clusters: the wildcats in northern Thuringia inhabit the southern slopes of the Harz Mountains, while the remaining specimens are dispersed over a chain of small subpopulations in the uplands around the Thuringian basin (Fig. 1, Görner 2000; Klaus 1993; Mölich and Klaus 2003; Piechocki 1990; Raimer 2006; Simon 2006).

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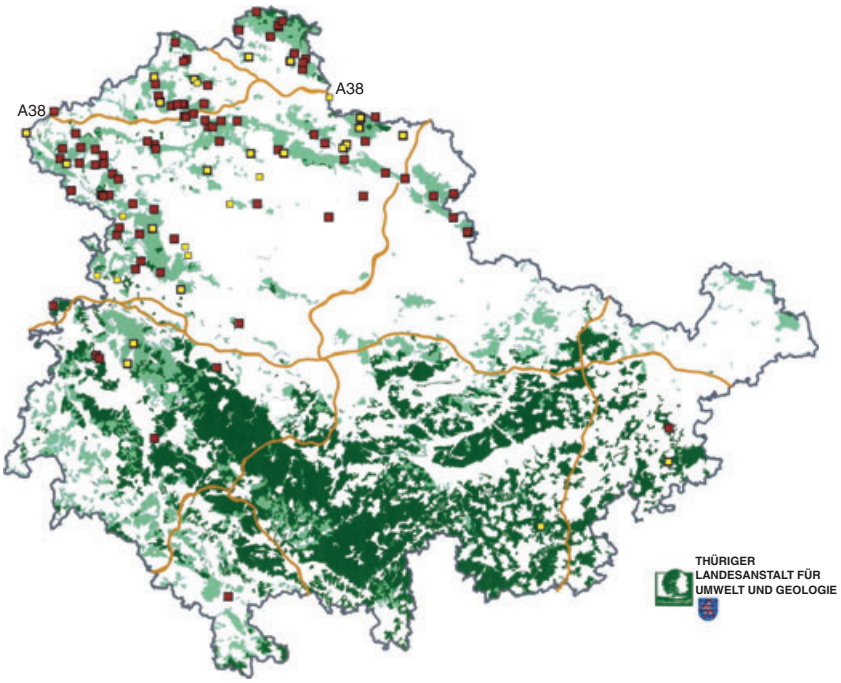

Fig. 1. Distribution of wildcats in Thuringia indicated by the localities of specimens used in the sample of this study, including the hybrid specimens (see Appendix S1 for detailed locality data and compare with Fig. 8 and Krüger et al. this volume)

Eckert (2003) found significant genetic differentiation between the German wildcat populations in different areas and interpreted this finding as the result of a long history of isolation and reduced gene exchange. Allocation of specific microsatellite and mitochondrial haplotypes revealed that the wildcats in the eastern distribution area were more closely related to each other than those in the western population. The smallest of all the populations in Eckert's sample from the Hainich area represented by only six specimens displayed a tendency towards reduced genetic variability in terms of nuclear markers. However, the rising number of casualties on roads between the small distribution areas in Thuringia over the last decade, especially in the south of the Harz Mountains, indicate dispersal between the isolated habitat spots in the eastern distribution area and suggest that wildcats are spreading into areas where there have been no signs of the presence of this species for over 80 years (personal observation at the Phyletisches Museum Jena, see also Piechocki 1990).

Beside the potentially detrimental effects of fragmentation on the gene pool of small populations, wildcats might be threatened genetically by their cousins the domestic cats (McOrist and Kitchener 1994; Daniels and Corbett 2003; Oliveira et al. 2007, 2008). The threat posed by the introgression of alien genes has increased dramatically in recent decades, especially in rare species, either as a result of the collapse of isolating barriers caused by habitat modification or as a consequence of the introduction of non-indigenous taxa (e.g. Gottelli et al. 1994; Rhymer and Simberloff 1996). Interbreeding between wildcats and domestic cats and regionally varying degrees of introgression of alien haplotypes from domestic cats into the gene pool of the European wildcat populations are reported in several parts of the distribution area. An essential question for the long-term survival of the wildcat is, therefore, its degree of hybridization with domestic cats. Wildcats have been in contact with domestic cats in their entire range since the Romans spread the domesticated form of *F. s. lybica* throughout Europe (Grant 1984; Lepetz and Yvinec 2002). Several studies have identified regions of extensive hybridization within the recent range of *F. s. silvestris* in Europe on the basis of various discriminant molecular markers and/or morphological traits. Stahl and Artois (1994) reported that interbreeding is a regular phenomenon and was 'mentioned as a potential or major danger in 11 out of 17 European countries'. Suminski (1962, 1977) takes the most radical position, denying the occurrence of wildcat populations in Europe at all in the light of the affiliation of the genepools of wild and domestic cats. A major role in the current discussion is played by the Scottish population, in which several authors have found an exceptionally high degree of hybridization resulting in a complete loss of genetic separation and finally the effective extinction of the 'pure' wildcat (French et al. 1988; Hubbard et al. 1992; Daniels et al. 1998; Beaumont et al. 2001; Daniels and Corbett 2003; Pierpaoli et al. 2003; MacDonald et al. 2004; Kitchener et al. 2005). An equally high percentage of hybrids is reported in Hungary and Bulgaria (Eckert 2003; Pierpaoli et al. 2003; Lecis et al. 2006). By contrast, populations from Belgium (Parent 1974), Italy (Ragni and Randi 1986; Randi et al. 2001; Pierpaoli et al. 2003; Lecis et al. 2006), Portugal and Spain (Oliveira et al. 2007, 2008), Germany (Piechocki 1990; Eckert 2003; Pierpaoli et al. 2003) and some other European countries (Pierpaoli et al. 2003) show no signs of significant amalgamation with domestic cats or only a low degree of hybridization. To date, no scientific explanation has been put forward of the nature of isolating barriers or, ultimately, of why interbreeding is confined to certain limited regions.

The aim of this study is to characterize the population structure, genetic variability and extant of isolation or migration of the wildcats in Germany, particularly in Thuringia, and to investigate the relationships between the different populations. Using a combined analysis of independent nuclear and mitochondrial markers we also want to identify the degree of present and historical hybridization between wildcats and domestic cats, including the introgression of domestic cat alleles or haplotypes into the gene pool of wildcats. Based on the results of recently published studies (Eckert 2003; Pierpaoli et al. 2003) we only expect a minor degree of admixture between

the two forms in the German populations. The results of such population genetics analyses of this enigmatic and threatened species not only help us to understand the population structure of wildcats in a European context, but are also relevant for decisions regarding its protection and long-term survival.

Material and Methods

Sampling and markers

The sample comprises a total of 82 domestic cats and 76 wildcats. The data set is not fully congruent because it was not possible to obtain both microsatellite and mitochondrial sequence data for all specimens. The study includes 41 wildcats from the federal state of Thuringia, in most cases accidental roadkills, identified *a priori* on the basis of morphological examinations in a parallel study (Krüger et al. this volume) (Appendix S1). In addition, numerous specimens were included from wildcat populations from other regions of Germany. Domestic cats – including three pedigree cats – were provisionally treated as one 'panmictic population' following Eckert (2003), Ruiz-Garcia (1999) and Todd (1977), but the pedigree cats in our sample were later excluded from further analyses (see below). Suspect individuals which could not be assigned to either wildcats or domestic cats with any certainty were intentionally not excluded from subsequent genetic analyses. The wildcats were assigned *a priori* to two groups on the basis of locality data and following previous studies of the population genetics of wildcats in Germany (Eckert 2003; Pierpaoli et al. 2003): western (including all specimens from the Eifel, the Palatinate Mountains and the one specimen from southwestern Hesse) and eastern, and later the latter group was divided into three subpopulations: 'Harz' (Harz Mountains) – 16 individuals (seven of them from the northernmost part of the Thuringian territory, see below), 'Hesse' (Hesse Uplands, Solling) – 7 individuals and 'Thuringia' – 25 individuals from the foothills around the Thuringian basin. Those from the southern Harz foothills north of the course of the highway A38 were allocated to the 'Harz' subpopulation (Figs 1 and 8, Appendix S1).

Tissue samples from muscle or liver stored in ethanol at −20°C were available. If not, coats from the museum collection, which were treated with Woguman FN but not tanned, provided small amounts of dried tissue (muscle, skin or cartilage) from the head. Total DNA was extracted and purified from about 25 mg of tissue following the protocols of the commercial kits [Dneasy tissue kit, Quiagen, Hilden, Germany; EZNA mini kit (Classic Line), Peqlab, Erlangen, Germany]. We selected ten dinucleotide and one trinucleotide (F115) microsatellite locus (Table 1) which were established on domestic cats (Menotti-Raymond and O'Brien 1995; Menotti-Raymond et al. 1999, 2003) and later assigned to wildcats (Hille et al. 2000; Beaumont et al. 2001; Paulus 2001; Randi et al. 2001; Eckert 2003). In addition, we sequenced a stretch of the mitochondrial genome corresponding to the control region between positions 16236 and 16955 of the *Felis silvestris* f. *catus* reference genome (Lopez et al. 1996; NCBI: NC 001700) using the primers CHF3 (5′-CTC CCT AAG ACT TCA AGG AAG-3′; Freeman et al. 2001) and CHR3 (5′-CCT GAA GTA AGA ACC AGA TG-3′; Tiedemann et al. 1996).

Laboratory protocols

Forward primers of the microsatellite loci were labelled with different fluorescent dyes (6-FAM™, NED™, VIC™) synthesized by Operon or ABI for parallel electrophoresis, while reverse primers were delivered by MWG Biotech AG, Ebersberg, Germany. To amplify microsatellites approximately 30–60 ng of purified DNA and 1 μl of each primer at a concentration of 10 pmol in a total volume of 30 μl on the basis of PCR Master Mix (Quiagen) was used following manufacturer's recommendations. After an initial denaturation step of 3 min at 92°C, 40 cycles followed of 45 s at 92°C, 45 s (specific annealing temperatures see Table 1), 30 s at 72°C and a final elongation step. Alternatively, multiplexing reactions were performed using the Quiagen Multiplex PCR kit with 5 pmol of each primer added to the reactions. Cycle conditions for the multiplexing reactions were initial denaturation at 95°C for 10 min, 35 cycles of 45 s at 95°C, 90 s at 57°C and 60 s at 72°C, followed by a final elongation step at 72°C. First, a six- and fourfold multiplexing was performed, combing primers FCA008, FCA031, FCA035, FCA045, FCA105, FCA223 and F115, FCA123, FCA124, FCA148 respectively. Finally, FCA126 was amplified eventually combined with one or more primers that had not worked in the previous reactions.

Amplification of mitochondrial DNA was performed using the hot start PCR AmpliTaq Gold-Kit (Applied Biosystems, Foster City, CA, USA) with 30–60 ng of purified DNA and 1 μl of each primer at a concentration of 10 pmol in a total volume of 30 μl following manufacturer's recommendations. PCR reactions was carried out with an initial denaturation step of 15 min at 94°C, 39 cycles of 90 s at 94°C, 75 s at 55°C, 90 s at 72°C and a final elongation of 10 min. The products were purified by precipitation with pure ethanol and ammonium acetate. Double-strand cycle sequencing was carried out using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions and with an initial denaturation step of 1 min at 96°C, followed by 25 cycles of 10 s at 96°C and 4 min at 60°C. The products were then purified with Centrisep®-Columns (Princeps Separations). In all cases PCR amplifications were performed with negative control reactions.

The PCRs were carried out on an Advanced Primus 96 (Peqlab) PCR machine. The success of the reactions was checked by visualizing the DNA of 5 μl PCR solution mixed with Roti-Load-DNA (Roth) or with 6× Loading Dye Solution (MBI Fermentas, St. Leon-Rot, Germany) on 1% agarose gel stained with ethidiumbromide. After an initial denaturation step (2 min, 94°C) electrophoresis of cycle sequencing products for sequencing and of microsatellites for identification of alleles was carried out on an ABI Prism 310 Genetic Analyser following the manufacturer's instructions using the POP6 gel (Applied Biosystems) and a size standard (GeneScan™-500, ROX Size Standard, Applied Biosystems).

Sequence Analyses

Mitochondrial sequences of 48 wildcats and 38 domestic cats were aligned manually with BioEdit 7.0.5.2 (Hall 1999) and also compared with the domestic cat reference sequence from GenBank (NCBI-nr. NC 001700). After removing the tRNA coding sequence from the 5′

Table 1. Microsatellite loci, primers and their labelling, and the specific annealing temperature used for the genotyping

Locus	At °C	Dye	Forward primer 5′–3′	Reverse primer 5′–3′	nb.
FCA008	55	NED	ACTGTAAATTTCTGAGCTGGCC	TGACAGACTGTTCTGGGTATGG	13
FCA031	55	6-FAM	GCCAGGGACCTTTAGTTAGATT	GCCCTTGGAACTATTAAAACCA	13
FCA035	55	6-FAM	CTTGCCTCTGAAAAATGTAAAATG	AAACGTAGGTGGGGTTAGTGG	12
FCA045	55	VIC	TGAAGAAAAGAATCAGGCTGTG	GTATGAGCATCTCTGTGTTCGTG	15
FCA105	58	NED	TTGACCCTCATACCTTCTTTGG	TGGGAGAATAAATTTGCAAAGC	14
FCA123	60	NED	CCATTCCCTCCCTGTCTGTA	GCCTCAAGCCTCATTGCTAC	9
FCA124	58	6-FAM	CCTGAATGCTCCAATTTTCTCTC	CCTTCTATCCTTGCTGGCTGAA	10
FCA126	58	NED	TGACTTCAGGAAGGTTACTCAGC	GATGCTTAACTGCCTGAGCC	9
FCA148	58	VIC	CTGGGCACTAGGTGTGCAC	GGTCTTGGATTAGAACCGAGG	8
FCA223	60	VIC	CTCACACAAGTAACTCTTTG	CCTTCCAGATTAAGATGAGA	16
F115	50	VIC	ACTGCGAGAGGACTTTCGAA	CTTCTGACAGGCTTCCAGGTT	52

nb. = number of alleles.

end and a highly variable GC-rich region with questionable positions from the 3′ end we obtained a final alignment of the control region of 675 bp without ambiguously aligned positions except for repeat units in some specimens. The alignment contained one (one individual), two (one individual), three (10 individuals) or four repeats (73 individuals) of a sequence of 80 bp, each containing one or more polymorphic sites. Eckert (2003) treated these repeats as a possible result of PCR malfunctions and eliminated them from her analyses. However, comparable repeated sequences in the mitochondrial control region have been reported in several vertebrate taxa including fish (e.g. Stärner et al. 2004; Takagi et al. 2006) birds (e.g. Eberhard et al. 2001) and particularly mammals (e.g. Gemmell et al. 1996; Matson and Baker 2001; Nesbø et al. 1998; Purdue et al. 2006). To test the influence of these repeats on the outcome of our analyses we coded each repeat unit as present or absent and retained even the polymorphic positions, coded as missing, in specimens where the repeat was absent. In parallel analyses we omitted the repeat units completely. Gaps were included in the analyses and treated as a fifth character state. We used the software Phyde (http://www.phyde.de) to convert our alignments from the fasta to the nexus format. MODELTEST 3.7 (Posada and Crandall 1998) in combination with PAUPup (Calendini and Martin 2005) and PAUP 4.10 (Swofford 2002) was used to determine the best fitting model of sequence evolution, chosen on the basis of the Akaike information criterion (Akaike 1974). The software selected the K81uf + I + G model (AICc = 3391.7446, r = 0.9359) for the alignment including the repeat units, and the HKY + G model (AICc = 2838.8044, r = 0.2833) for the alignment excluding the repeat units. As these two models are not integrated in the program version of Arlequin used in this study we selected the most similiar Tamura-Nei model (see Excoffier et al. 2005).

Preliminary identification of the haplotypes present in the mitochondrial sequences was carried out using DNASP4 (Rozas et al. 2003). ARLEQUIN 3.01 (Excoffier et al. 2005) was used to estimate diversity indices for the different (sub)populations. The level of genetic differentiation between domestic cats and the various (sub)populations of wildcats was quantified by pairwise Φ_{ST} values (Weir and Cockerham 1984) and statistically tested with 10 000 permutations using ARLEQUIN 3.01. Genetic diversity within and among populations was analysed using AMOVA (Excoffier et al. 1992) with Arlequin 3.01 based on 10 000 permutations using the Tamura-Nei model (see above). The software package TCS (Clement et al. 2000) was used to reconstruct the parsimony network representations of the mitochondrial haplotypes. Finally, the number of private haplotypes was counted from the parsimony network yielded by TCS.

Genotyping

Microsatellite data from 76 wildcats and 73 domestic cats were tested pairwise with GENEPOP 3.4 (Raymond and Rousset 1995) to assess potential linkage disequilibria. Micro-Checker (van Oosterhout et al. 2004) was used to test for null alleles or other artefacts in the microsatellite data. Factorial correspondence analysis (FCA) and the calculation of allele frequencies were conducted using GENETIX 4.05.2 (Belkhir et al. 2001). ARLEQUIN 3.01 was used for the Hardy–Weinberg-Exact-Test, to estimate pairwise F_{ST} values and to analyse molecular variance (AMOVA) (see above). We analysed the genetic structure of the populations of wild and domestic cats as well as the affiliation of the individuals to these groupings with the Bayesian clustering method described by Pritchard et al. (2000) and recently updated in STRUCTURE 2.1 (Falush et al. 2003). To ensure comparability we used the allele frequencies correlated model, as this was the model used in other European studies on wild and domestic cats (cf. Oliveira et al. 2007) without prior population information. The most probable number of genetic clusters (K) in our sample was inferred by estimating the posterior probabilities for each K ranging from 1 to 5 without prior population information (Length of burnin period: 100 000; Number of MCMC reps after burnin: 100 000). The results were tested for congruency using 20 runs for each K value.

We carried out a similar procedure to assess the effectiveness of admixture analysis as introduced by Barilani et al. (2006) on the basis of the proposals of Pritchard and Wen (2003) and recently adopted for wild and domestic cats by Oliveira et al. (2007, 2008). Firstly we used STRUCTURE 2.01 without prior population information to estimate the assignment index (qi) to either the domestic or the wildcat cluster. The number of genetic clusters was set at two (K = 2) as we were only interested in admixture between domestic and wildcats and not among the various (sub)populations of wildcats. For K = 2, the admixture model with correlated allele frequencies among populations performed best (Length of burnin period: 100 000; Number of MCMC reps after burnin: 100 000). A subset of 30 individuals for each domestic and wild cat with an assignment index (qi) >0.95 for their respective cluster was randomly chosen to simulate parentals, F1, F2 and backcross hybrids using the software HYBRIDLAB (Nielsen et al. 2006). To set an appropriate threshold value for the assignment of individuals to the two population clusters or to the different hybrid classes, 100 simulated individuals for each hybrid class and the parentals were analysed in STRUCTURE 2.01 with the same settings as described above and no prior population information.

We used the Bayesian approach implemented in the software BAYESASS 1.3 (Wilson and Rannala 2003) to compute migration rates between the different populations including domestic cats. Locus F115 had to be removed from this analysis because the number of alleles exceeded the permitted limit for this software. We used 3 000 000 MCMC iterations with a sampling frequency of 2000 and a burn-in of 999 999. Convergence of MCMC algorithm was confirmed using different initial delta values of allele frequency, migration rate and inbreeding level in the range from 0.1 to 0.3. In addition, we used the software GENECLASS 2.0 (Piry et al. 2004) to detect first-generation migrants between wildcats and domestic cats as well as between eastern and western wildcat and domestic populations. The approach pioneered by Paetkau et al. (1995) was used to calculate likelihoods, with a default frequency for missing alleles of 0.01, and L = L_home/L_max (the likelihood of the individual genotype within the population where the individual was sampled divided by the maximum such likelihood observed for this genotype in any population) was selected for migrant detection. This method performs better than others when all source populations for immigrants are thought to be sampled (Paetkau et al. 2004). The probability that an individual is a resident was computed using the resampling algorithm of Paetkau et al. (2004) with 100 000 simulated individuals and a conservative type I error of 0.01.

The heterozygosity test integrated in the software BOTTLENECK 1.2.02 (Piry et al. 1999) was used to detect potential signatures of recent reductions in effective population sizes (demographic bottlenecks) in our wildcat data for western and eastern populations as well as the eastern subpopulations separately. We used a two-phase mutation model with 95% single-step mutations (and 5% multiple-step mutations) and a variance among multiple steps of 12 as recommended by Piry et al. (1999), again after removing locus F115. In addition, we tested our data with a stepwise mutation model. We used both sign test and Wilcoxon test as integrated in Bottleneck to check whether the expected heterozygosity (HE) under HWE significantly exceeded the heterozygosity expected at mutation-drift equilibrium (HEQ), as would be expected after a reduction of the effective population size (Cornuet and Luikart 1996; Luikart and Cornuet 1998). As recent immigration may mimic an increase in the population size and dilute the signal of a recent bottleneck (Cornuet and Luikart 1996), all analyses were also run after the hybrids identified with Structure had been removed from the dataset.

Results

Sequence analysis

The sequences of our sample (GenBank acc. no. GQ268232–GQ268316) differ from the sequence of the reference genome of the domestic cat in GenBank (Lopez et al. 1996, NCBI: NC 001700), with the result that there are relatively high genetic distances between this specimen and both the wildcats and domestic cats in our sample. The reference sequence, however, was excluded from further analyses because of the unknown

origin of this specimen both in terms of its locality data and its possible ancestry as a pedigree cat, something which could be responsible for the genetic distances observed (see below for problems associated with including pedigree cats). We regard the idea that nuclear copies of the control region sequence may be present in our data matrix to be implausible as all of our sequences differ from each other to an extent comparable to that found in previous studies on *F. silvestris*. Moreover our sequences are very similar to several other sequences of *F. silvestris* in GenBank.

Following DNASP4 our final alignment, excluding the repeats, comprised 434 positions containing 41 polymorphic sites, allowing us to identify 22 haplotypes. Including the repeats the final alignment was composed of 455 positions containing 44 polymorphic sites. Consideration of the repeat units resulted in a higher number of different haplotypes, especially in the domestic cats (Table 2). The pairwise differences between the various predefined groups and subpopulations only revealed significant Φ_{ST} values (Table 4) for the distinction between domestic and wildcats. The AMOVA explained most of the genetic variance among groups if the sample was structured into domestic versus wildcats (Table 3).

The maximum number of connection steps at 90% was calculated to be 13 for the parsimony haplotype network including the repeat units, and 15 and for the analyses excluding the repeat units. The resulting parsimony haplotype networks based on the two alignments (Figs 2 and 3) show three distinct clusters. However, there is no clear separation either between the wildcat populations or between wildcats and domestic cats. Three clusters are present, cluster A includes exclusively domestic cats while the wildcats are distributed in two distinct clusters. Cluster B includes both domestic cats and wildcats while cluster C includes exclusively wildcats from different populations. The haplotype H5 is present in domestic cats as well as in wildcats from the western group and from the 'Thuringia' and 'Harz' subpopulations, while H7 is present in wildcats from the whole distribution area (Figs 2 and 3).

Genotyping

The genotyping of 149 individuals from 11 polymorphic loci resulted in the identification of 8–52 alleles per locus and a total of 171 alleles. On the basis of 0.05 frequency criterion we identified 10 private alleles (2 western group, 8 domestic cats). Furthermore, a wildcat specific allele (Fca45) was found if the wildcats were treated as single population. The analysis of genetic linkage between loci only yielded a disequilibrium ($p < 0.05$, corrected according to Bonferroni) for the combination Fca105/Fca124, which are situated in close proximity to each other on the chromosome 8cM (Menotti-Raymond et al. 1999, 2003). In the wildcats, the Hardy–Weinberg-Exact-Test only revealed a significant deviation between expected (HE) and observed (H0) heterozygosity because of a deficit of heterozygotes after Bonferroni correction for the locus Fca123 in the western population and for Fca035 in the eastern population, which indicates no overall departure from Hardy–Weinberg Equilibrium for the wildcats. In domestic cats, however, three loci (Fca031, Fca035 and Fca123) displayed significant deviation from Hardy–Weinberg Equilibrium after Bonferroni correction. When the three pedigree cats were removed from the analysis only one locus (FCA035) deviate from Hardy–Weinberg Equilibrium, indicating that domestic cats including pedigree cats may not represent a panmictic population, as pedigree cats can be considered an isolated evolutionary lineage.

The results of the F-statistic procedures support the distinction between domestic cats and wildcats as well as that between western and eastern German populations. However, the predefined subpopulations within the eastern group did not differ significantly from each other (Table 4). In the AMOVA a division of the samples into three groups (domestic versus western versus eastern) got the highest percentage of variation among groups (Table 3). In congruence with this result, the parallel runs in Structure identified K = 3 as the most probable number of genetic clusters in our sample (Fig. 4). The FCA yielded two clusters corresponding to domestic and wildcats. The eastern wildcat populations cluster more closely

Table 2. Sequence diversity indices, values obtained including repeat units in brackets. *n* = number of individuals per population/subpopulation

Populations	*n*	Nucleotide diversity	Gene diversity	Haplotypes	Private haplotypes	Polymorphic sites
Eastern	38	0.021 (0.021)	0.866 (0.875)	17 (18)	8 (11)	24 (27)
'Thuringia'	23	0.019 (0.020)	0.881 (0.881)	14 (14)	7 (8)	22 (24)
'Harz'	12	0.018 (0.018)	0.758 (0.818)	5 (6)	1 (2)	16 (17)
'Hesse'	3	0 (0.005)	0.667 (0.667)	2 (2)	0 (1)	0 (3)
Western	10	0.012 (0.014)	0.956 (1.000)	8 (10)	6 (8)	19 (22)
Domestic	38	0.024 (0.027)	0.902 (0.984)	19 (29)	18 (26)	62 (72)
All		0.029	0.985	60		72

Table 3. Results of separate AMOVAs for different population structures for mtDNA and microstellite markers, including repeat units in brackets

	mtDNA				Microsatellites			
	Percent of variation				Percent of variation			
Population structure	Among groups	Among pops. within groups	Within pops.	ΦST	Among groups	Among pops. within groups	Within pops.	F_{ST}
Domestic versus wild cats	28.56 (32.65)	11.06 (9.10)	60.38 (58.25)	0.40 (0.42)	6.83 (6.05)	5.57 (6.47)	87.60 (87.48)	0.12 (0.13)
Domestic versus Western versus Eastern	24.37 (28.25)	11.97 (9.88)	63.66 (61.87)	0.36 (0.38)	11.79 (12.95)	−0.06 (−0.82)	88.26 (87.87)	0.12 (0.12)

All overall Φ_{ST} and F_{ST} values were significant based on 10000 permutations ($p < 0.05$).

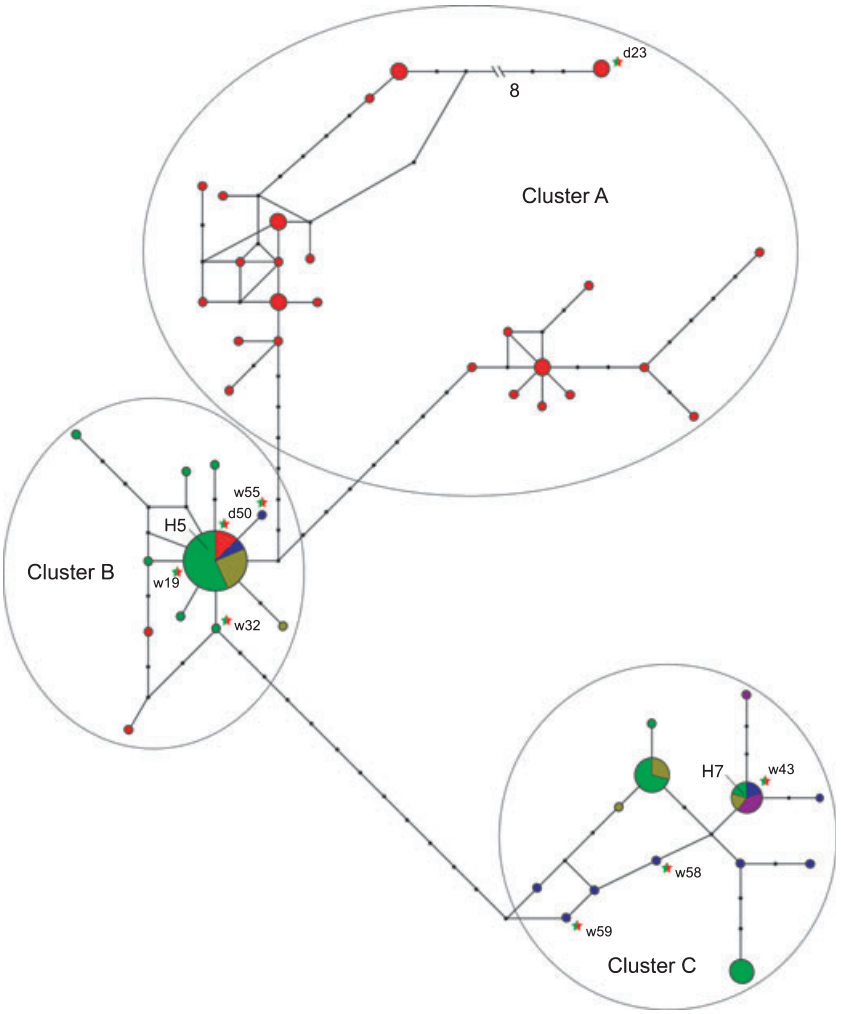

Fig. 2. Parsimony network of mitochondrial haplotypes. Repeat units of the sequences were included in the alignment. Colours correspond to the subpopulations: Domestic cats red, 'Thuringia' green, 'Harz' olive, Western blue, 'Hesse' purple. Hybrids identified by structure based on microsatellite data are marked by green/red stars

to each other than the western wildcats (Fig. 5). The individuals from the various wildcat populations identified by Structure (see below) as hybrids between domestic cats and wildcats are situated in the transition zone between the two clusters (Fig. 5). The three pedigree cats (d59, d64 and d60) lay outside the domestic cat cluster or on its margins.

On the basis of the population structure K = 3 we tested the microsatellite loci for the existence of null alleles in the various populations. An analysis using Micro-Checker uncovered signs of null allels in the loci Fca008 and Fca126 in the western population, in the locus Fca035 in the eastern population, and in the loci FCA031, Fca035, Fca105, Fca124, Fca113, Fca223 in the domestic cats excluding the pedigree cats. These results indicate a surplus of homozygote individuals for these loci within the populations rather than null allels in our data, going by the inconsistent distribution of affected allels between the populations. Moreover, the test procedure with Micro-Checker found no evidence for scoring errors or large allele dropout.

The admixture analysis performed on simulated genotypes was able to exclude 100% of the parental individuals below a threshold value of the assignment index (qi) <0.79 for wild cats and qi <0.73 for domestic cats (Fig. 6). Using this threshold on the original data analysed by STRUCTURE 2.01 16 individuals were identified as belonging to one of the different hybrid classes (Figs 2, 3, 5, 7 and 8). However, on simulated data, 7 F2 (3.5%) and 94 (47%) backcrosses could not be distinguished from 'pure' domestic or wild cats respectively (Fig. 6). This conservative estimate of admixed individuals revealed 12 of 28 from the western population (42.9%) and two of 48 from the eastern population (4.2%), corresponding to 18.4% of the whole German wildcat sample, and two of 73 domestic cats (2.7%) to be hybrids (Fig. 7). The two eastern population hybrids originate from the 25 individuals (8%) of the group provisionally designated 'Thuringia'. Due to overlaps in the assignment indices (qi) of the different hybrid classes in the simulated data (Fig. 6), the individuals with a hybrid ancestry could not be assigned to any of the hybrid classes.

The migration rates (m) between populations, obtained using BAYESASS, are very low (0.0–0.09) (Table 5) in comparison to previously published studies (Wilson and Rannala 2003; Faubet et al. 2007; Lecis et al. 2008). The highest migration rate is from the eastern to the western group of wildcats (m = 0.0711–0.0925), followed by the migration rate from the domestic cats into the western group (0.0123–0.031) (Table 5). This indicates a very low but probably directionally biased gene flow. In agreement with these findings the assignment tests carried out with GENECLASS 2.0 only identified the domestic cat individual d50 as a first generation

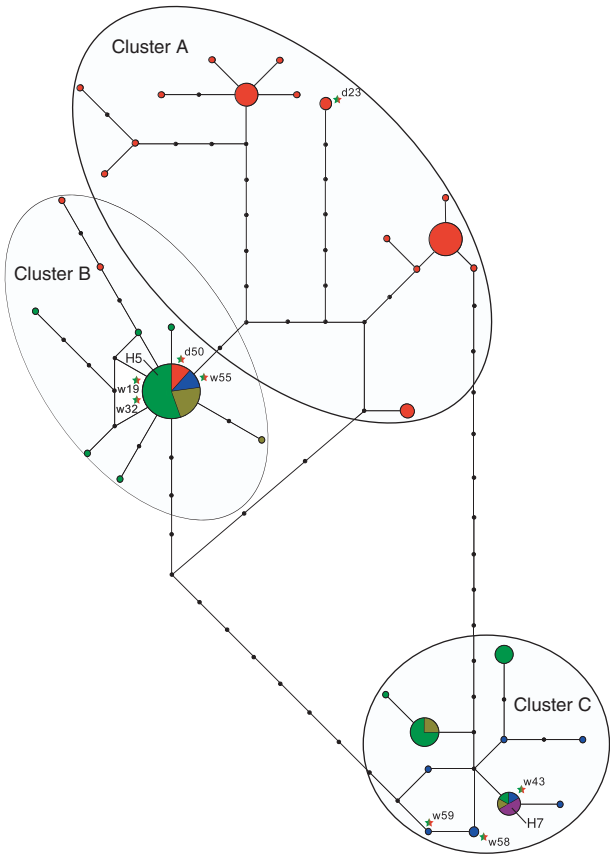

Fig. 3. Parsimony network of mitochondrial haplotypes. Repeat units of the sequences were excluded in the alignment. Colours correspond to the subpopulations: Domestic cats red, 'Thuringia' green, 'Harz' olive, Western blue, 'Hesse' purple. Hybrids identified by structure based on microsatellite data are marked by green/red stars

migrant with a type I error below 0.01. In Structure analyses this specimen was identified as a hybrid with an assignment index of 0.565 for the domestic cats cluster and 0.435 for the wildcat cluster.

No significant evidence of a recent decrease in effective population size in the sense of a bottleneck event could be detected in our wildcat data with the software Bottleneck. There was no statistically significant excess of HE over HEQ in eastern and western wildcats according to the various Bottleneck test procedures, regardless of whether the hybrid specimens pre-identified with Structure were included or not. Additional tests on the subpopulations 'Harz' and 'Thuringia', both of which were representatively sampled in our study for the specific purpose of providing a contrast to the 'Hesse' subpopulation, did not reveal a significant signature for such recent bottlenecks.

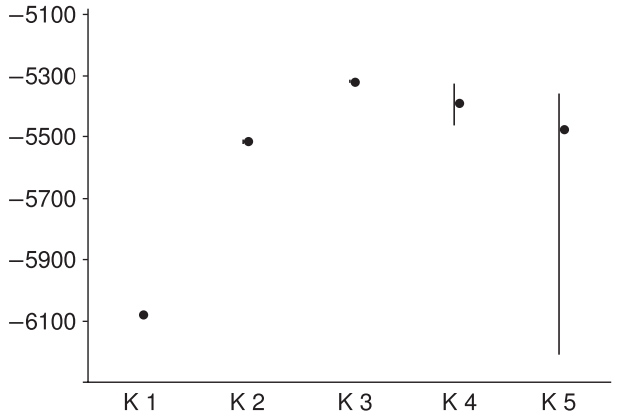

Fig. 4. Number of populations (K) expressed as the mean likelihood [log P (X/K)] and its range in 20 runs in the Structure software for each K value

Discussion

Population structure and genetic diversity

Our analyses of the microsatellite data yielded two clearly separate clusters, indicated by the highest F_{ST} values, which correspond to domestic cats and wildcats respectively. Moreover, both the AMOVA and the FCA supported a division of the western and eastern wildcats into two distinct populations (Fig. 5, Tables 2, 3 and 4). The resulting division of our sample into three distinct populations is also supported by the Bayesian analyses of the microsatellite data with Structure (K3, see Fig. 4). By contrast, the parsimony haplotype network based on the mitochondrial sequences failed to reveal a comparable population structure, coming up instead with three haplotype clusters, showing the wildcats in two admixed clusters and one cluster includes even domestic and wild cats (Figs 2 and 3). The statistical tests of the mitochondrial sequence data distinguished clearly between the domestic cats and the wildcats but not between the two wildcat populations. These incongruent findings, however, are probably influenced by recent and past admixture between the various lineages and the resulting dilution of a possible signal of population structure because of the introgression of mitochondrial haplotypes of domestic cats into the wildcat populations (see the discussion of Admixture analysis below).

We regard the hypothesis of two distinct wildcat populations in the eastern and western parts of the German distribution area (Fig. 8) to be the best-supported explanation of our data. The low migration rates detected with the microsatellite marker set between these western and eastern populations and the lack of unambiguous first generation migrants suggest a separation by distance that has resulted in a

Table 4. Estimates of pairwise genetic distance between populations based on mtDNA

	Eastern	'Thuringia'	'Harz'	Hesse	Western	Domestic
Eastern	–				n.s.	0.35 (0.39)
'Thuringia'	–	–	n.s.	n.s.	n.s.	0.30 (0.34)
'Harz'	–	n.s.	–	n.s.	n.s.	0.40 (0.42)
'Hesse'	–	n.s.	n.s.	–	n.s.	0.60 (0.58)
Western	0.11	0.09	0.13	0.12	–	0.52 (0.52)
Domestic	0.13 (0.12)	0.13 (0.13)	0.11 (0.10)	0.12 (0.12)	0.14 (0.13)	–

(Φ_{ST}) above diagonal and on microsatellites (F_{ST}) below diagonal, including repeat units in brackets. Only significant values are indicated ($p < 0.005$, Bonferroni-corrected for 10 independent comparisons, n.s. = non significant).

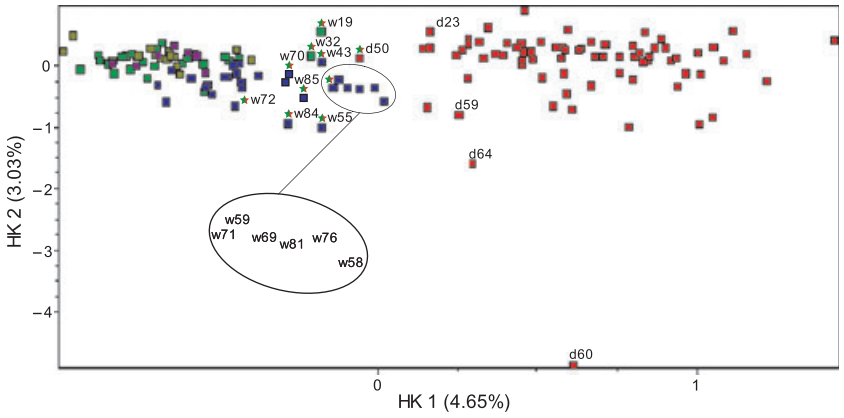

Fig. 5. Factorial correspondence analysis of the microsatellite allele data. Colours correspond to the subpopulations: Domestic cats red, 'Thuringia' green, 'Harz' olive, Western blue, 'Hesse' purple. Hybrids are marked by green/red asterisks

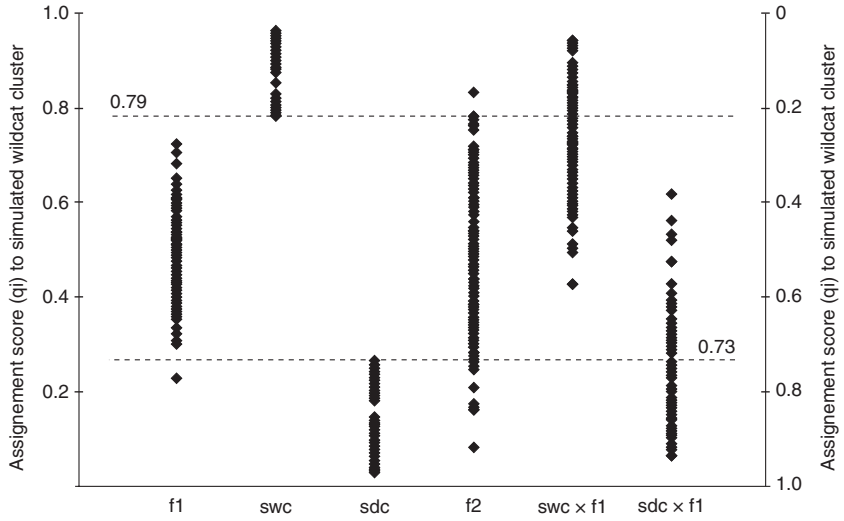

Fig. 6. Results of the simulation analysis with Hybridlab. Swc = simulated wildcats, sdc = simulated domestic cats, f1 = first generation hybrids, f2 = second generation hybrids, swc × f1 and sdc × f1 = backcrosses. Individuals were regarded as hybrids below a threshold of the assignment value (qi) < 0.79 for wildcats and below qi < 0.73 for domestic cats

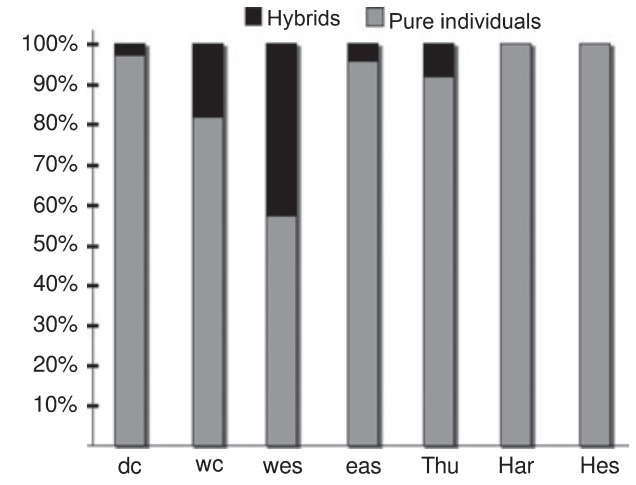

Fig. 7. Proportional distribution of hybrid individuals in the different predefined groups and subpopulations. Dc = domestic cat, wc = wild cat, wes = western, eas = eastern, thu = 'Thuringia', har = 'Harz', hes = 'Hesse'

very low or even interrupted gene flow between the wildcats in these areas. The higher but still very low migration rate from the eastern into the western population could be interpreted as a sign of a directionally biased dispersal from east to west. Pierpaoli et al. (2003) reported a similar population structure even on the basis of a much smaller sample size and named the clusters in Germany 'north' and 'southwest'. The western population has contact with the large distribution area of the wildcat in central Europe and is therefore probably only part of a larger genetically connected population (Pierpaoli et al. 2003). By contrast, the eastern population is geographically isolated from other wildcat populations and consists of numerous scattered habitat spots that only harbour relatively low numbers of individuals (Raimer 2006; Simon 2006). However, our preliminary allocation of the individuals of the eastern population to the geographically defined subpopulations 'Hesse', 'Harz' and 'Thuringia' is not supported by indices of genetic differentiation based on our data. This finding indicates that the wildcats inhabiting the fragmented eastern distribution area are not genetically distinct from each other (Fig. 8, Table 4). Moreover, we did not find any evidence of a hidden population structure within the eastern and western wildcat populations, but rather of significant gene flow within the two populations which we hypothesize to be mediated by a high level of dispersal (Fig. 4).

Intense hunting pressure in the first half of the last century probably led to a dramatic decline in the wildcat populations in Germany, and ultimately to the survival of a reduced number of individuals in isolated 'retreat' areas (Raimer 2006; Simon 2006). Eckert (2003) found the genetic diversity of

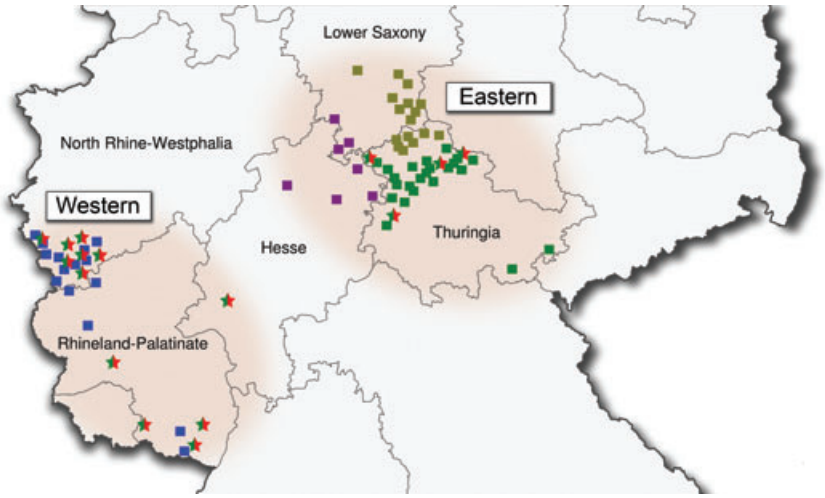

Fig. 8. Localities of the specimens analysed in this study and their allocation to the different populations and subpopulations. Eastern: eastern population, western: western metapoplulation, 'Thuringia' green, 'Harz' olive, Western blue, 'Hesse' purple. Hybrids are marked by green/red asterisks. For locality data pertaining to the domestic cats see Appendix SI

microsatellite alleles to be lower than that of mitochondrial markers in the German wildcat populations. She concluded that inbreeding caused by a dramatic decline in population size had led in the long term to the loss of genetic diversity, particularly in the small and isolated 'boundary' populations without contact to the recent main distribution area of the wildcat. Pierpaoli et al. (2003) also confirmed the tendency towards low genetic variability in their 'Germany north' population, which corresponds to our eastern population, on the basis of a sample of 27 individuals exclusively from the Solling area in Hesse. These findings were interpreted as an indicator of genetic bottleneck effects caused by the eradication of the wildcat populations in Germany in the past and the geographical isolation of these populations today. In contrast to these previous studies, our study of a large sample uncovered no evidence in recent wildcat populations of either bottleneck events in the past or reduced genetic diversity because of genetic drift or inbreeding.

Furthermore, the genetic diversity (indicated by high gene diversity, the presence of several private haplotypes and high sequence divergence) of the mitochondrial control region of the wildcats of the eastern population is even higher than that of the western population (Figs 2 and 3, Table 2). Randi et al. (2001) found a mitochondrial gene diversity comparable to our results, but only among populations from the whole area of Italy, including the isolated form from Sardinia. We hypothesize that there are several possible factors, probably linked, behind our observation of this high genetic diversity. Firstly, the reason that the temporal fluctuations in the wildcat populations in the past reported by several authors (Piechocki 1990; Mölich and Klaus 2003; Raimer 2006; Simon 2006) did not result in population bottleneck events that left traces in the markers used in this study may be because the effective number of individuals was, in fact, not as low as previously supposed. Similiarly to the recent situation within the eastern distribution area the dispersal-mediated gene flow might never have been interupted despite the fact that the remaining wildcats inhabited a fragmented habitat (see below). Alternatively, an accelerated genetic drift could have taken place within the probably small and temporarily isolated relict populations which resulted in the establishment of alternative haplotypes in different places. The complete protection afforded to wildcats during subsequent decades led to a significant increase in population size, recolonization movements, and finally the spread and genetic exchange of haplotypes by dispersal that provided the prerequisite for the recent high genetic diversity, especially of mitochondrial markers, observed in this study and which has also been described by Eckert (2003). Moreover, the repeated introgression of mitochondrial haplotypes from domestic cats into the wildcat populations, followed by their spread and diversification, could also have played a major role in bringing about the high genetic diversity of the German wildcat populations (Figs 2 and 3, and see discussion of Admixture analysis).

An important factor in the high level of genetic exchange, particularly within the eastern population, is likely to be the

Table 5. Migration rates (m) as mean values of the posterior probability distribution between the different populations under the assumption of two or three populations (K) calculated in five parallel runs with BayesAss 1.3 with varying parameter values (delta values of allele frequency, migration and inbreeding between 0.1 and 0.3)

K	Population from	Population to			
		Eastern	Western	Domestic	Wildcats
3	Eastern		0.0711–0.0925 (0.0371–0.0461)	0.0069–0.0075 (0.0058–0.0063)	
	Western	0.0045–0.0050 (0.0053–0.0068)		0.0034–0.0040 (0.0038–0.0045)	
	Domestic	0.0038–0.0042 (0.0043–0.0051)	0.0123–0.0131 (0.0115–0.0129)		
2	Wildcats			0.0090–0.0094 (0.0060–0.0069)	
	Domestic				0.0061–0.0073 (0.0060–0.0069)

Intervals of standard deviation given in parentheses.

presence of mountain ranges, which used to serve as hideaways for relict populations but which today have the highest densities of wildcats and may act as source populations (Haltenorth 1957; Piechocki 1990; Götz and Roth 2006). The hypothesis of a panmictic eastern wildcat population is supported by observations that a growing number of specimens are accidentally being killed on roads in transition areas between scattered subpopulations in Thuringia over the past two decades. Moreover, the first signs that wildcats have returned to certain areas after their presumable extirpation also indicate that this species has been expanding its range recently (Piechocki 1990; Raimer 2006; Simon 2006).

The high genetic diversity of domestic cats [presence of 18 (26 if repeat units were included) private haplotypes, high variability in the repeat units within the control region, high number of polymorphic positions, high gene diversity] was found in a comparable manner in previous studies (Driscoll et al. 2007; Pierpaoli et al. 2003; Randi et al. 2001; compare with Figs 2 and 3, Table 2). The descent of domestic cats from several independent genetic lineages of different local populations of the Steppe cat (including *F. s. lybica* and partly *F. s. ornata*) in North Africa and the Near East, and/or genetic traces of repeated crossing with different subspecies of *F. silvestris,* and the long history of breeding in different parts of the world are all factors which explain why a highly diverse gene pool is observed in this form (Pierpaoli et al. 2003; Driscoll et al. 2007). The deviation of our sample of domestic cats from the Hardy Weinberg equilibrium is explained at best by the presence of a Wahlund effect caused by an underlying cryptic population structure. Pedigree cats seem to represent separate evolutionary lineages because of their long history of numerous generations of isolated breeding, inbreeding and artificial selection and should therefore be excluded from population genetics analyses of wildcats and free ranging domestic cats.

Admixture analysis

Gene transfer between taxonomic entities is an important phenomenon, not only in the study of evolution and speciation, but also in conservation biology when the introgression of alien genes is disrupting the gene pool of a threatened species (e.g. Adams et al. 2003; Gottelli et al. 1994; Ward et al. 1999). Our admixture analysis of the microsatellite data revealed a total of 18.4% of all specimens morphologically identified *a priori* as wildcats and 2.7% of the domestic cats to belong to one of the simulated hybrid classes (Fig. 7). The threshold applied, calculated on the basis of simulated hybrids with the HYBRIDLAB software, is rather conservative because some of the simulated hybrids were not recognized by Structure (Fig. 6). The effective number of specimens with hybrid origin in our sample may therefore be even higher. It has to be stressed, however, that our sample of domestic cats is biased because we considered numerous specimens from the collection of the Phyletisches Museum Jena that were brought to the museum as wildcats or suspect individuals. The percentage of hybrids among the whole population of German domestic cats is therefore probably lower than in our sample although our hybrid estimate is conservative.

Our analyses yielded evidence not only of recent hybridization but also of past introgression from domestic cats into the western and eastern wildcat populations and *vice versa*. The heterogeneous composition of the mitochondrial haplotype cluster B in particular (Figs 2 and 3), which as well as the widespread haplotype H5 also includes private wildcat haplotypes, indicates that admixture between the lineages already took place in the past. Five (six repeat units included) of seven (eight) private haplotypes of the subpopulation 'Thuringia', for instance, are closely related to H5, probably descended from this haplotype, diversified secondarily (cluster B) and contribute significantly to the high diversity of haplotypes in wildcats (Figs 2 and 3). Randi et al. (2001) and Driscoll et al. (2007) also found shared mitochondrial haplotypes in the domestic cats and wildcats of different populations. The resulting haplotype network, the presence of a 'wrong' mitochondrial haplotype in some individuals that are assigned to the domestic cats or the wildcats but carry a haplotype of the other form, and the observed incongruences between the signal of the independent nuclear and mitochondrial markers regarding population structure and assignment in our study can also be interpreted as a reliable indicator of an occasional gene flow between wildcats and domestic cats during their long history of coexistence in Europe (see also Driscoll et al. 2007). One of the most far-reaching conclusions that can be drawn from these findings is that distinctions between wildcats and domestic cats based solely on mitochondrial markers, as used in field studies in Germany and Switzerland (Thomas Mölich, personal communication; Nussberger et al. 2007; Weber et al. 2008), is unfortunately not reliable in the populations of Central Europe too.

While our microsatellite data show equal levels of genetic differentiation between domestic cats and wildcats and eastern and western wildcat populations the statistical tests of the mitochondrial sequence data revealed a greater distance between domestic cats and wildcats than between the two wildcat populations. We believe that this observed discrepancy between the signals of mitochondrial and nuclear markers could be caused by different life history patterns of the two sexes. Male domestic cats as well as male wildcats intrude, more often in comparison to the rather philopatric females, into the territories of the other form resulting in more frequent matings between them and the resident females. This sex specific behaviour could cause a biased introgression of both differently inherited genetic markers (maternally versus biparentally). An alternative explanation could be the divergent evolution rates of the markers used in this study. According to the phylogenetic mtDNA tree and the dating of splitting events within *F. silvestris* proposed by Driscoll et al. (2007), the cluster containing the subspecies *F. s. lybica* and *F. s. ornata* and the domestic cat has been clearly separated from the lineage of the European wildcat *F. s. silvestris* for a long time. Despite the long lasting admixture between the lineages that occurred over centuries of sympatry certain haplotypes of wildcats and domestic cats carry conserved substitutions informative for this ancient divergence which result in a pronounced genetic distance between recent wildcats and domestic cats. This explanation is supported by the two distinct clusters in the parsimony networks which consist exclusively of domestic cat and wildcat haplotypes respectively (Figs 2 and 3). The more rapidly evolving microsatellites, on the other hand, lost such information after a few generations (Vähä and Primmer 2006).

Numerous previous studies in various distribution regions have shown that using several different genetic marker systems permits better recognition of domestic cats and wildcats and of the degree of admixture between them (French et al. 1988;

Hubbard et al. 1992; Hille et al. 2000; Beaumont et al. 2001; Daniels and Corbett 2003; Pierpaoli et al. 2003; MacDonald et al. 2004; Kitchener et al. 2005; Oliveira et al. 2007, 2008). In the case of microsatellites the number of selected loci seems to be crucial to the resolution and robustness of the resulting hypotheses about genetic structure and hybridization. Hille et al. (2000) showed successfully that a marker system of only eight unlinked microsatellite loci is sufficient to distinguish between domestic cats and wildcats in the Eifel area. Subsequent authors selected eight to twelve loci (Beaumont et al. 2001; Daniels et al. 2001; Randi et al. 2001; Eckert 2003; Pierpaoli et al. 2003; Koskinen et al. 2004; Oliveira et al. 2007, 2008), while recent publications recommend the use of significantly larger sets of microsatellite loci (Koskinen et al. 2004) or even the additional consideration of y-chromosome markers (Vila et al. 2003). The advantages of using linked loci as the source of information in admixture analyses is a matter of some controversy (Falush et al. 2003; Lecis et al. 2006; Vähä and Primmer 2006; Oliveira et al. 2008). Our study combines microsatellites with mitochondrial markers, which is considered an established alternative approach to increasing genotyped loci (French et al. 1988; Hubbard et al. 1992; Beaumont et al. 2001; Daniels and Corbett 2003; Pierpaoli et al. 2003; MacDonald et al. 2004; Kitchener et al. 2005; Driscoll et al. 2007; Oliveira et al. 2008). In this case the more rapidly evolving microsatellites can be used as indicators of recent interbreeding events a few generations ago, as Vähä and Primmer (2006) have shown in their simulation study. By contrast, the maternally inherited mitochondrial sequences even of the relatively fast-evolving control region preserve traces of historical splitting events and gene transfer between genetic lineages because of their slower rate of evolution (Gottelli et al. 1994; Ward et al. 1999; Adams et al. 2003).

Our study provides a convincing demonstration of the enormous potential of the combined marker systems in distinguishing statistically pure and admixed individuals and unearthing ancient gene flow. Moreover, we regard the Bayesian method integrated in Structure combined with the admixture model of the same software to be the best mathematical solution available so far to the complex problem of the long-term intraspecific admixture in *F. silvestris*. However, to obtain a reliable distinction between the different hybrid classes and improve recognition of backcrosses, we would advise using a higher number of microsatellite markers or even a genomic approach, as discussed by Oliveira et al. (2008).

According to the most plausible interpretation of our results, German wildcats are genetically distinct from domestic cats, but carry significant traces of a long history of ancient introgression. Furthermore, occasional interbreeding still take place, with the result that individuals with hybrid ancestry, including first and second generation hybrids and backcrosses, are present in all populations but to a regionally varying degree. These findings contradict our null hypothesis that reticulate evolution plays a minor role in German wildcat populations. The surprisingly high percentage (42.9%) of individuals with hybrid ancestry in the Eifel/Palatinate population contrasts starkly to previous studies on German wildcat populations (Hille et al. 2000; Eckert 2003; Pierpaoli et al. 2003). This high rate of hybridization observed here in comparison to the population from the eastern part of the German distribution area is probably linked to differences in habitat structure that lead to more frequent contact with feral cats. However, this striking finding needs further investigation in terms of ecological background and population genetics on the basis of a broader sample including specimens from the neighbouring populations in France and Switzerland. A direct comparison with the ecological situation in the eastern distribution area in Germany, which harbours a low percentage of hybrids, could shed light on the nature of barriers between domestic cats and wildcats in Central Europe.

An alternative explanation for the presence of shared haplotypes, especially haplotype H5, in wildcats and domestic cats could be that some haplotypes are highly conserved in comparison to others and, therefore, represent an ancient heritage already present in the common ancestor of *F. s. silvestris* and *F. s. lybica* (the latter subspecies being regarded to be the ancestor of the domestic cat, which could also include lineages of *F. s. ornata* according to Driscoll et al. 2007). A persistence of ancestral polymorphisms in genetic markers across species has been found in numerous evolutionarily young species, e.g. of cichlid fishes, which are morphologically differentiated but closely related (Moran and Kornfield 1995; Verheyen et al. 2003). In the case of *F. silvestris* the divergence between the Asian/African lineages and the European wildcat lineage occurred between 20 000 and 155 000 years ago according to estimations based on a molecular clock approach, allozyme electrophoresis data and morphology (Driscoll et al. 2007; Randi and Ragni 1991; Yamaguchi et al. 2004). The oldest remains of tamed cats have been dated to be 9500–9200 years old (Vigne et al. 2004), which could be interpreted as the beginning of domestication. In our opinion the assumption that conserved haplotypes in the fastest evolving region of the mitochondrial genome exist unaltered in several different populations of *F. silvestris* over a period of at least 20 000 years is not very plausible. Moreover, if certain haplotypes did not evolve at all while others accumulated numerous informative substitutions, this would radically challenge the usability of the mitochondrial control region for population genetic studies, because such heterogenous substitution rates would lead to highly biased information content.

Conclusions for conservation

The phenomenon of interbreeding between wildcats and domestic cats, or at least of introgression in certain regions of Europe is the subject of a long-standing and controversial debate (Beaumont et al. 2001; Biro et al. 2005; Daniels et al. 1998; Daniels and Corbett 2003; Eckert 2003; Fernandez et al. 1992; French et al. 1988; Hubbard et al. 1992; Kitchener et al. 2005; Lecis et al. 2006; MacDonald et al. 2004; McOrist and Kitchener 1994; Oliveira et al. 2007, 2008; Piechocki 1990; Pierpaoli et al. 2003; Ragni and Randi 1986; Randi et al. 2001). Several factors which promote the reproductive interaction of the two forms have been discussed, of which the low population densities of wildcats in comparison to the more numerous free-ranging domestic cats may be crucial. In addition, the extensive loss of forests that has created a mosaic-like landscape structure and resulted in small and fragmented wildcat populations may have intensified wildcat contact with domestic cats and led to a break-down of isolating barriers, especially in landscapes that were originally forest-dominated. Radical deforestation in Scotland and perhaps also in the native wildcat localities in Hungary has made the wild forest-cat secondarily into a field-cat and forced

it to use the same resources as feral domestic cats (Stahl and Artois 1994; Randi et al. 2001; Lecis et al. 2006). A comparable situation which probably also favoured hybridization with domestic cats prevailed for long periods in recent centuries in numerous regions of Germany, when the percentage of forest coverage was significantly lower than today and the wildcat populations were probably smaller because of habitat loss and extensive hunting pressure.

Today the areas inhabited by wildcats in Thuringia and adjacent regions are mainly small and isolated from each other by cultivated landscape, but they provide sufficiently structured and closed forests to house viable wildcat populations. Studies, including radio telemetry tracking (Mölich and Klaus 2003), on the biology of these populations and those from other localities (Naidenko and Hupe 2002) showed a low level of overlap between the territories of domestic cats and wildcats because the latter spends most of its time in the forest, in contrast to feral domestic cats. However, a study using photo traps in the Jura mountains of Switzerland clearly showed that free ranging domestic cats advance far into the forests and indeed often come into contact with wildcats (Darius Weber, personal communication), leading to undesirable liaisons and ultimately to a minimal rate of recent hybridization at least in all populations investigated so far.

If the wealth of evidence of a long history of habitat alteration and fragmentation and of coexistence and interbreeding with domestic cats is taken into account, the question arises of why and how 'typical' wildcats that can be identified genetically and morphologically still exist after hundreds of years of sympatry with their tamed cousins (Krüger et al. this volume). In our opinion specific selective pressures in natural habitats must privilege wildcat morpho- and genotypes, and *vice versa,* certain traits displayed by domestic cats resulted in better adaptation to cultivated landscapes and the vicinity of humans. One underlying mechanism that might be responsible for these differences in the fitness of the two forms for life in their respective habitats could be a differential response to pathogens or parasites. This hypothesis is based on the observation of high rates of infection in wildcats with viruses which are highly pathogenic and lethal after a few months in domestic cats (Leutenegger et al. 1999). Evolutionary mechanisms such as these that maintain the genetic and morphological integrity of most wildcat populations in Europe and prevent complete amalgamation with the wildcat's domestic relatives appear even more likely when one considers that in many European countries both forms have coexisted for hundreds of years and that the likelihood of interbreeding was possibly even greater in the past than it is today, as mentioned above.

'Pure' wildcat populations without at least genetic traces of past introgression no longer seem to exist in most parts of the wildcat's distribution area in Central Europe, so conservation efforts and legal protection should focus on saving the local 'functional' wildcats (see also Daniels and Corbett 2003). This means that the protection of sufficiently large areas of suitable habitat must become a priority to maintain environmental conditions that favour typical wildcat morpho- and genotypes and to allow the wildcat to realize their ecological niche. In the light of the risk of interbreeding, 'corridors' intended to promote outbreeding that connect geographically isolated habitat spots within the eastern and western distribution areas, as well as the two areas themselves, must be planned with care. In contrast to the hypothetical threat posed by the loss of genetic variability in the scattered subpopulations, a phenomenon which is not supported by our data, increasing levels of admixture with domestic cats seem to be present a more acute risk to the long term survival of wildcats. Extensive cross-breeding could, in the long term, reduce the fitness of the wildcat populations by destroying their specific selective advantage in their primary habitat. Although the desire to maintain genetically healthy populations through dispersal-mediated genetic exchange between them is a worthy one, we need to be sure that accelerated exchange via connecting corridors between regions with vastly differing percentages of specimens of hybrid ancestry makes sense. The planning and design of such corridors must take account of the fact that contact with feral cats needs to be discouraged to reduce the length of time wildcats are forced to spend within cultivated landscapes populated by numerous free ranging cats.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Appendix S1. Specimens used in this study and the data collected from the specimens. Coll. nr. = collection number of the Phyletisches Museum Jena, x = data present, ms data = microsatellite data, mt data = mitochondrial data.

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