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ORIGINAL PAPER

Preserving genetic integrity in a hybridising world: are European Wildcats (*Felis silvestris silvestris*) in eastern France distinct from sympatric feral domestic cats?

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Abstract We investigate the genetic profile of putative European Wildcats in north-eastern France, possessing the wildcat phenotype, but sampled in an area where they are sympatric with free-roaming domestic cats and, thus, are exposed to potential hybridisation. From a sample of 209 cats, the programme STRUCTURE clearly identified two distinct genetic clusters that corresponded to European Wildcats and domestic cats. The cats from these two clusters were clearly differentiated from each other ($F_{ST} = 0.16$). However, the genotypes of some individual cats were split between the two clusters, indicative of genetic admixture. Our analysis demonstrates that a genetically distinct population of cats that possess the European Wildcat phenotype persists in north-eastern France, but that there is a low, yet real, risk of hybridisation with sympatric domestic cats. These European Wildcats warrant conservation efforts to protect their genetic integrity.

Keywords Bayesian admixture analysis · Conservation genetics · European Wildcats · *Felis silvestris* · Hybridization · Domestic cats

Introduction

The identification of definable phylogenetic units is fundamental to the legislation and implementation of effective conservation management. Typically, these units take the form of species or subspecies designations (i.e., taxonomic groups), evolutionary significant units (ESU's, Ryder 1986) or isolated populations. However, defining such units unambiguously continues to challenge conservation policy-makers, particularly in cases where

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threatened species hybridise with sympatric, colonising or invasive species. The species complex *Felis silvestris* is a typical example of such difficulties. The most recent results indicate that European Wildcats (*F. s. silvestris*), African Wildcats (*F. s. libyca*), Asian Wildcats (*F. s. ornata*) and domestic cats (*F. s. catus*, of North and East African origin) are diverged subspecies of a single polytypic species (Driscoll et al. 2007). Domestication of the African lineage (*F. s. libyca*) is reported to have begun at least 9,000 years ago (Vigne et al. 2004) and although pelage characteristics of domestic cats have diversified into the multiple breeds now recognised, the basic morphology has remained remarkably consistent with that of their wild forbears (Randi et al. 2001). However, the human-mediated dispersal of the domestic cat pan-globally, the decline of wildcats population and the fragmentation of wildcat habitat (McOrist and Kitchener 1994) have increased the risk of hybridization of this subspecies with its wild congeners during the last century. In addition, the offspring of wild-domestic cats hybrids are fertile (Pierpaoli et al. 2003), facilitating continuing dilution of the wild genotype over progressive generations. In fact, hybridization has already been implicated in the extinction of localised populations of wildcats in central Europe (Suminski 1962) and the prevention of hybridization has been identified as the greatest priority for the persistence of the subspecies (Nowell 2008; Yamaguchi et al. 2004).

Wildcats are protected globally by the Convention on International Trade in Endangered Species (CITES Appendix II, UNEP-WCMC 2006) and in Europe by European Directive 92/43/EEC (EUROP 1992). However, only the Scottish Wildcat (*F. s. grampia*) is classified as Vulnerable by the World Conservation Union (IUCN), with all other wildcats classified as Least Concern (IUCN 2008). This inconsistency between the main conservation bodies on the status of wildcats in Europe highlights the confusion surrounding the subspecies and its conservation requirements. Given the problem of hybridization with domestic cats, there is a growing need to identify individuals and populations of European Wildcat in order to protect the genetic integrity of this subspecies. Indeed, it is unlikely that any European Wildcat does not possess at least some domestic cat ancestry (French et al. 1988), although such ancestry would be difficult to demonstrate without a considerable amount of data (Vähä and Primmer 2006; Randi 2008). The use of phenotypic traits such as pelage characteristics has proven unreliable for unequivocally identifying non-hybrid cats (Daniels et al. 1998; Hubbard et al. 1992; but see Kitchener et al. 2005). This is generally attributed to the recent divergence of the subspecies and the absence of domestication-induced morphological divergence (Randi et al. 2001). Studies in Europe have indicated varying degrees of hybridization between wild and domestic cats, suggesting that risk of hybridization is not uniform throughout the continent or across habitat types (Oliveira et al. 2008; Lecis et al. 2006; Pierpaoli et al. 2003; Eckert 2003; Randi et al. 2001; Beaumont et al. 2001) but the predominant factors determining such a geographical variability of hybridization remain unclear (Beaumont et al. 2001; Pierpaoli et al. 2003; Kitchener et al. 2005). Thus, studies of genetic introgression by domestic cats are required throughout the range of European Wildcats in order to facilitate a European-wide management plan and the efficient and effective targeting of conservation resources.

While it is recognised that cats with a phenotype matching that of European Wildcats occur in eastern France (Léger et al. 2008), it is not certain that these cats have a genetic profile distinct from that of sympatric free-roaming domestic cats, given the potential of hybridisation between the sub-species. In France, the degree of hybridisation between wild and domestic cats has never been assessed, and consequently, the genetic integrity of the European Wildcat in France is unknown. The distribution of the European Wildcat in France is discontinuous. Two endemic populations have been identified—one in the

Pyrénées on the French/Spanish border and another in the northeast of the country. More recently, evidence is accumulating that the European Wildcat is expanding its range southwards from the northeastern endemic region since the beginning of the 1980s (Léger et al. 2008). Our study focuses on the northeast endemic region and this newly colonised territory. Our objective here was not to know if there are still some "purebred" European Wildcats in France, nor to estimate the rate of introgression by domestic cat genotypes. Rather, our aim was to describe the situation of the wildcat in France, as has been done in others European countries (Oliveira et al. 2008; Lecis et al. 2006; Pierpaoli et al. 2003; Eckert 2003; Randi et al. 2001; Beaumont et al. 2001), and to determine if the cats in eastern France possessing the wildcat phenotype are indeed genetically distinct from sympatric free-roaming domestic cats, and thus are worthy of legal protection.

Materials and methods

Sampling

Free-living cats (both *F. s. silvestris* and *F. s. catus*) were collected as roadkill ($n = 175$) in Eastern France, where the presence of wildcats is strongly suspected or confirmed, from 1994 to 2006 by the French Hunting and Wildlife Office (Office National de la Chasse et de la Faune Sauvage, ONCFS). This sample was complemented by 34 live-trapped free-living cats from the same period in the same areas. The resulting 209 cats were classified into three a priori groups based on specific coat colour and biometric characteristics (cranial and intestinal indices) by specifically trained staff (Léger et al. 2008): (1) presumed wildcats ($n = 130$); (2) presumed domestic cats ($n = 44$); (3) unclassified cats ($n = 35$). Unclassified cases corresponded to cats whose pelage and morphologic characteristics were intermediate between wildcats and domestic cats, or cats whose carcass damage were too extensive to permit classification.

Microsatellite markers

Hair samples were collected from each carcass and from living cats and stored in individual envelopes. For each individual, 20–80 ng/µl of total genomic DNA was extracted using DNeasy Tissue Kits (Qiagen) from a sample of more than 50 hair bulbs. Selective amplification was carried out for 13 microsatellite loci divided into 3 PCR multiplexes by polymerase chain reaction (PCR). PCR were conducted in 10 µl volumes containing 5 µl of PCR Multiplex Master MIX (2x, Qiagen), 0.3 µl of each primer (10 mM) (one of the locus-specific flanking primers was labelled with a fluorescent marker), and 2 µl of the extraction product. PCR was conducted in 96-well microtitre plates using a Bioblock PTC 100 thermal cycler and the following program: 95°C/15 min, 30 cycles with 94°C/30 s, 57°C/1.30 min and 72°C/1 min denaturing, annealing and extension temperatures, respectively, and finally 60°C/30 min. The sizes of PCR amplified products were resolved by Genoscreen (http://www.genoscreen.fr/) using an Applied Biosystems 3730xl DNA Sequencing Analyzer. Microsatellite DNA fragments were independently amplified up to 10 times for the 13 microsatellite loci, from the same DNA extraction product of 7 individuals and in all cases the genotypes matched. All loci used in our analyses were polymorphic with a mean number of alleles of 11.23 [8–17] and a mean observed heterozygosity H_0 of 0.647 [0.258–0.789].

Data analysis

We used the programme STRUCTURE v2.1 (Pritchard et al. 2000) to (a) identify population substructure among the samples in terms of the number of K clusters and (b) to assign the proportion of each individual's genotype derived from each of these K clusters. STRUCTURE uses Bayesian Monte-Carlo Markov Chain sampling to identify the optimal number of clusters for a given multi-locus dataset by minimising departures from Hardy–Weinberg and linkage equilibrium expectations, without needing to identify population subunits a priori. We used 500,000 generations, of which the first 20,000 were discarded as burn-in, and applied the admixture model with correlated allele frequencies due to the possibility of hybrid individuals being present in the sample set. We simulated the dataset for $K = 1$ through to $K = 6$ and performed 20 STRUCTURE runs for each value of K. We then employed the methods of Evanno et al. (2005) and Garnier et al. (2004) to assess the optimal value of K (i.e., the optimal number of clusters in the dataset).

Simultaneously, STRUCTURE calculates the proportion (q_{ik}) of each genotype of individual samples that is derived from each of the K clusters. Individual samples can have membership in multiple clusters, but membership coefficients (q_{ik} values) sum to unity across clusters. Thus, the values of q_{ik} can be effectively used to identify first generation hybrid or admixed animals if their q_{ik} value is split between different clusters.

Power of admixture analysis and threshold determination

The proportion of parental and admixed (hybrid) individuals in a given sample is very sensitive to the threshold value of q_{ik} used to assign each individual in a given cluster (Vähä and Primmer 2006). Therefore, we assessed the power of admixture analysis on simulated genotypes to avoid false classification of admixed or parental individuals (Barilani et al. 2007; Oliveira et al. 2008). Two subsamples consisting of the 30 individuals showing the highest q_{ik} values for the "wild" and "domestic" clusters, respectively (see Sect. "Results") were created to generate samples for four classes (parental, F1 and F2 populations, and backcross hybrids) using the function *hybridize* of the R package *adegenet* (Jombart 2008; R Development Core Team 2008). By taking only the highest q_{ik} values, we excluded possible admixed individuals from the parental populations used to simulate F1, F2 and backcross hybrids. From these two initial subsamples of wildcat and domestic parental populations, we simulated 100 genotypes of each of the four classes, replicated 10 times. The simulated genotypes were used to carry out admixture analysis with STRUCTURE with $K = 2$ and using the admixture model with correlated allele frequencies and no a priori information on population structure. The proportion of simulated genotypes correctly assigned was used to assess the efficiency of the admixture analysis to detect the different classes of parental, and F1, F2 and backcross admixed genotypes, and to choose reliable threshold q_{ik} values to assign individuals into different genetic clusters.

Genetic differentiation between inferred groups of cats

Once all cats were assigned as wildcat, domestic cat or admixed, the number of alleles per locus, observed heterozygosity and heterozygote deficit from Hardy–Weinberg expectations (HWE measured by *Fis* values) was estimated in each of these three groups using GENETIX 4.05 (Belkhir et al. 2000). We also tested for the significance of the *Fst*-statistic in the complete data set and for each pairwise combination using the *G*-test with 999

permutations (Goudet et al. 1996) implemented in the R package *adegenet* (Jombart 2008). We then tested linkage disequilibrium between loci-pairs separately for each group to avoid a Wahlund effect (Wahlund 1928) using the permutation test ($n = 999$) implemented in GENETIX 4.05.

Results

The results of analysis with STRUCTURE without a priori information on sample phenotypic classification are presented in Fig. 1. The variation of the Ln P(D) values with the number of inferred clusters K (Fig. 1a) indicated that $K = 2$ is the most likely number of clusters, which was confirmed by both the Evanno et al. (2005) and Garnier et al. (2004) methods (Fig. 1b, c). We therefore estimated the proportion of membership of each predefined phenotypic group into these two genetic clusters. STRUCTURE differentiated well between presumed wildcats ("wildcat" cluster) and presumed domestic cats ("domestic" cluster); the unclassified cats being more evenly distributed between the two identified clusters (Table 1; Fig. 2) suggesting that both wildcats, domestic and admixed cats coexist in our sample.

The simulation study provided important insights into the threshold values most appropriate for assigning individuals into the three groups (wildcat, admixed and domestic). Parental simulated genotypes and F1, F2, backcross admixed simulated genotypes were obtained from the 30 individuals having the higher q_{ik} values for the "wild" cluster (i.e., $q_{ik} > 0.98$) and from the 30 individuals showing the higher q_{ik} for the "domestic" cluster (i.e., $q_{ik} > 0.953$) in the previous STRUCTURE admixture analysis. None of the simulated F1 and F2 individuals showed a membership proportion q_{ik} greater than 0.863 and 0.893, respectively, for the "wildcat" parental cluster (Table 2). However, our analysis did not allow us to distinguish between wildcat parent and wildcat X F1 backcross individuals, as the maximum value of membership proportion (q_{ik}) for the "wildcat" cluster ranged from 0.954 to 0.989 over the 10 simulations (Table 2). Consequently, we define wildcats as all specimens having a membership proportion (q_{ik}) for the "wildcat" cluster higher than 0.893, albeit acknowledging that these wildcats might be either wild or backcrossed cats. Similarly, none of the simulated F1 and F2 individuals showed a membership proportion (q_{ik}) higher than 0.753 and 0.910, respectively, for the "domestic" parental cluster (Table 2), and again domestic X F1 backcross individuals

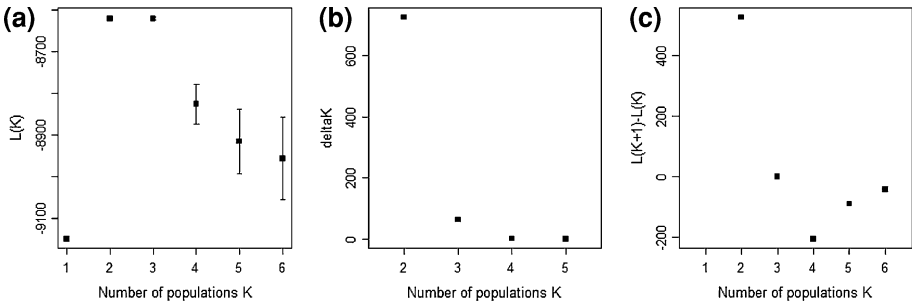

Fig. 1 (**a**) Mean (±SD) of *Ln P(D)* over 20 *STRUCTURE* runs for successive K values. Variations of (**b**) ΔK as calculated by Evanno et al. (2005) and (**c**) $L(K + 1) - L(K)$ as calculated by Garnier et al. (2004) for successive K values

Table 1 Proportions of membership in the two inferred genetic clusters for the three pre-defined groups of free-living cats in Eastern France (presumed wildcats, presumed domestic cats and unclassified)

	"Domestic" cluster	"Wild" cluster
Presumed wildcats	0.106	0.894
Unclassified	0.391	0.609
Presumed domestic cats	0.877	0.123

Fig. 2 Posterior probability assignments into "wildcat" (*dark grey*) and the "domestic cat" (*light grey*) clusters of the 209 cats in relation to their a priori classification: presumed wildcat, presumed domestic cat, and unclassified. Each cat is represented by a *vertical bar* divided into two depending on their proportion of membership (q_{ik}) in either genetic cluster inferred by STRUCTURE (Software DISTRUCT 1.1, Rosenberg 2004)

Table 2 Mean and range over 10 replicates of the simulation procedure for the maximum value of proportion of membership (q_{ik}) of simulated hybrids (Wild X F1 backcross, F1, F2, Domestic X F1 backcross) in both wild and domestic parental clusters

	Wild parental cluster	Domestic parental cluster
Wild X F1 backcross	0.980 [0.954–0.989]	0.512 [0.426–0.646]
F1	0.758 [0.654–0.863]	0.698 [0.655–0.753]
F2	0.827 [0.788–0.893]	0.839 [0.778–0.91]
Domestic X F1 backcross	0.484 [0.404–0.558]	0.989 [0.981–0.994]

could not be distinguished from domestic parents (membership proportion q_{ik} for the domestic cluster ranged from 0.981 to 0.994, Table 2). Therefore, the specimens defined as domestic cats were those having a membership proportion q_{ik} for the "domestic" cluster greater than 0.91. Overall, 116 cats were defined as wildcats and 38 as domestic cats on a genetic basis (Table 3). The remaining 55 cats corresponded to admixture between wildcat and domestic cat gene pools and may be considered hybrids.

None of the phenotypically classified wildcats were assigned as domestic cats based on their genotypes, and vice versa. However, 31 presumed wildcats and 15 presumed domestic cats were assigned as genotypic "hybrids". Twenty-eight unclassified cats were assigned to either the wild or domestic cat genetic clusters. Finally, the proportion of genetically assigned wildcats displaying the wildcat phenotype was 76.2% (99/130).

Group differentiation and linkage disequilibrium

Considering the three groups defined using STRUCTURE (wildcat, domestic and admixed cat), allele frequency distributions varied across loci with sometimes marked differences

Table 3 Assignment of 209 cats from the three a priori phenotypic groups based on specific pelage and morphologic characteristics (Presumed wildcats, Presumed domestic cats, Unclassified) into the three genetic groups based on STRUCTURE analysis (wildcat for individuals having $q_{ik} > 0.893$ for the "wild" cluster, domestic cat for individuals having $q_{ik} > 0.91$ for the "domestic" cluster, and admixed for individuals having intermediate q_{ik} values)

A priori group	q_{ik} for the "wild" cluster > 0.893	Intermediate q_{ik} values	q_{ik} for the "domestic" cluster > 0.91	*n*
Presumed wildcats	99	31		130
Unclassified	17	9	9	35
Presumed domestic cats		15	29	44
n	116	55	38	209

between wildcat and domestic cat groups. There were 10 and 5 private alleles with a more than 5% frequency in the wild and domestic groups, respectively. Mean number of alleles per locus was similar in the three groups (8.77, 8.15 and 9.30 in the wildcat, domestic and admixed groups, respectively). Average values of observed heterozygosity were also similar across loci in wildcat ($H_0 = 0.69 \pm 0.12$), domestic ($H_0 = 0.66 \pm 0.19$), and admixture ($H_0 = 0.74 \pm 0.12$) groups. Observed heterozygosity was lower than expected under HWE in the three groups (multi-locus $Fis = 0.06$, 0.11 and 0.06 for wildcat, domestic and admixture groups, respectively, $P < 0.01$). However, we had no information on the local population substructure of these three groups, and such heterozygote deficiencies may be largely attributable to a Walhund effect.

There was no evidence of extensive linkage disequilibrium between pairs of loci (all $P > 0.006$, Bonferroni corrected $\alpha = 0.0007$) within each group. However the number of marginal ($P < 0.05$) was higher in the admixture and domestic groups than in the wildcat group (12, 10 and 5 out of the 76 values, respectively). Menotti-Raymond et al. (1999) reported that the loci used in this study are on separate chromosomes or at least 11 CM from each other, so physical linkage can then be ruled out. Thus, linkage disequilibrium, if it occurs, is likely the result of hybridization or admixture.

The overall genetic differentiation between the three groups was significant ($Fst = 0.083$, $P = 0.001$). The wildcat and domestic cat showed a relatively high pairwise *Fst* value of 0.16 ($P = 0.001$), much higher than the pairwise *Fst* values they showed with the admixture group (wildcat-admixture $Fst = 0.033$, $P = 0.004$, and domestic–admixture $Fst = 0.055$, $P = 0.003$).

Discussion

STRUCTURE clearly identified two genetic clusters within the dataset, without enforcing any a priori information on cat phenotype. The two inferred clusters clearly corresponded to a European Wildcat cluster and a domestic cat cluster, confirming both the relative efficiency of biometric traits to characterise European Wildcats and the genetic distinction between the two species (Beaumont et al. 2001; Randi et al. 2001). Nevertheless, results obtained with the genetic method are both more precise and more conservative. First, the genetic method facilitated classification since 28 of the 35 phenotypically unclassified cats were defined as wildcat or domestic cat based on their genotype suggesting, unsurprisingly, a higher discriminative power for the genotypes than for the phenotypes. Second, some

specimens classified as either wildcat or domestic cat based on their phenotype were defined as admixed based on their genotype. This suggests that the genetic method is more conservative than the use of phenotype because miss-classification of a given specimen as wildcat or domestic cat is reduced. Overall, 174 cats were classified as presumed wildcat or domestic cat based on phenotypic characteristics; whereas only 154 cats were assigned to the "wildcat" or "domestic" clusters by STRUCTURE.

In this study, 76.2% (99/130) of cats with a wildcat phenotype were assigned to the "wildcat" genetic cluster and, consequently, represent a genetic entity distinct from that of sympatric domestic cats. This proportion may, nevertheless, be underestimated because we used the maximum q_{ik} value of 0.893 obtained from simulation analyses as the threshold value to assign cats to the "wildcat" cluster. Nevertheless, we cannot assert that "pure-bred" wildcats persist in eastern France because our data does not allow us to discriminate between wild and backcrossed cats. The proportion of wild phenotype cats assigned to the "wildcat" cluster by STRUCTURE is lower than that found in an Italian (98%, Randi et al. 2001), Sardinian (100%, Randi et al. 2001), Bulgarian (83%, Randi 2008), Belgian (95%, Randi 2008) or Portuguese (86%, Oliveira et al. 2008) samples, but it is higher than the percentage of non-hybrid individuals found in a Scottish (Beaumont et al. 2001) and Hungarian (Pierpaoli et al. 2003) samples. These results clearly indicate differences in intensity of genetic introgression across Europe. Nevertheless, caution must be exercised in comparing these studies. Sampling (and in particular a priori parental group sampling) and type and number of genetic markers used in the different studies varies greatly and may explain, in part, differences in reported genetic diversities and genetic structures between wildcats and domestic cats. *K*-value parameter and assignment processes and, consequently, the proportion of cats assigned as admixed or "purebred" with STRUCTURE are sensitive to genetic diversity and the genetic structure (Vähä and Primmer 2006; Randi 2008). In addition, the number of clusters has been defined using STRUCTURE without using a priori population information in some studies (this study; Lecis et al. 2006), whereas it is constrained by the authors in others (Beaumont et al. 2001; Randi et al. 2001; Pierpaoli et al. 2003).

The relatively large number of private alleles found in the wild and the domestic cats groups, the low linkage disequilibrium highlighted in the wildcat group and the relatively high genetic structure ($Fst = 0.16$) reported between the domestic cats and the wildcat groups of our study, together suggest that the north-eastern French population of European wildcats has maintained its genetic identity and integrity. Similar results have been found for the Italian, German and Portuguese Wildcat populations, where hybridization is expected to be sporadic (Oliveira et al. 2008; Randi 2008). However, the relatively low linkage disequilibrium in the admixed group, as well as the similar genetic diversity observed within wild and domestic cat groups is consistent with the general idea of a long history of hybridization (Chakraborty and Weiss 1988; Briscoe et al. 1994) experienced by the French European wildcat in the last century when the number of wildcats was low and the population was highly fragmented by deforestation and human persecution whereas, at the same time, domestic cats were widespread and expanding their range (Léger et al. 2008).

To conclude, we have highlighted the existence in eastern France of individuals we refer to as European Wildcats and which may have high conservation value. These individuals had both wildcat phenotypic characteristics and genotypes clearly different from those of free-roaming sympatric domestic cats, even though some domestic cat ancestry cannot be ruled out. The relatively high frequency of these wildcats found in our sample, as well as their population genetic profile show that cross-breeding with free-roaming domestic cats

is rare in the north-eastern French population of European Wildcats. Although conditions seem then to warrant specific conservation efforts for the European Wildcat population in this area of France, more ecological information is necessary before the establishment of any efficient measures for conservation. Respective ecological niches of wildcats, admixed individuals and free-roaming domestic cats are poorly described. In particular, if feeding habits seem to be largely overlapping (Biró et al. 2004), differences in habitat requirement may occur (Klar et al. 2008) and may better describe hybridization dynamics in these subspecies.

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