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Genetic distinction of wildcat (*Felis silvestris*) populations in Europe, and hybridization with domestic cats in Hungary

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Abstract

The genetic integrity and evolutionary persistence of declining wildcat populations are threatened by crossbreeding with widespread free-living domestic cats. Here we use allelic variation at 12 microsatellite loci to describe genetic variation in 336 cats sampled from nine European countries. Cats were identified as European wildcats (*Felis silvestris silvestris*), Sardinian wildcats (*F. s. libyca*) and domestic cats (*F. s. catus*), according to phenotypic traits, geographical locations and independently of any genetic information. Genetic variability was significantly partitioned among taxonomic groups (F_{ST} = 0.11; R_{ST} = 0.41; $P < 0.001$) and sampling locations (F_{ST} = 0.07; R_{ST} = 0.06; $P < 0.001$), suggesting that wild and domestic cats are subdivided into distinct gene pools in Europe. Multivariate and Bayesian clustering of individual genotypes also showed evidence of distinct cat groups, congruent with current taxonomy, and suggesting geographical population structuring. Admixture analyses identified cryptic hybrids among wildcats in Portugal, Italy and Bulgaria, and evidenced instances of extensive hybridization between wild and domestic cats sampled in Hungary. Cats in Hungary include a composite assemblage of variable phenotypes and genotypes, which, as previously documented in Scotland, might originate from long lasting hybridization and introgression. A number of historical, demographic and ecological conditions can lead to extensive crossbreeding between wild and domestic cats, thus threatening the genetic integrity of wildcat populations in Europe.

Keywords: assignment and admixture analysis, conservation genetics, *Felis silvestris*, genetic diversification, hybridization, wild and domestic cat

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Introduction

The wildcat (*Felis silvestris*) is a polytypic species with three wild subspecies, the African (*F. s. libyca*), European (*F. s. silvestris*) and Asian (*F. s. ornata*) wildcats, and a domesticated form (*F. s. catus*; Ragni & Randi 1986; Randi & Ragni 1991; Wozencraft 1993; Johnson & O'Brien 1997). African and European wildcats are closely related and split recently, probably towards the end of the Last Glacial Maximum, as suggested by palaeontological, biochemical and molecular data (Davis 1987; Randi & Ragni 1991; Masuda *et al*. 1996).

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Nowadays, the European wildcat is distributed in Europe in a number of fragmented populations threatened by destruction of their natural habitats, persecution and crossbreeding with free-ranging domestic cats (Nowell & Jackson 1996; Fig. 1). The Egyptians, from 8000 to 4000 years ago domesticated African wildcats. Thereafter, the Etruscans, Greeks and Romans spread domestic cats throughout continental Europe and Great Britain (Malek 1993; Clutton-Brock 1999). African wildcats at early stages of domestication were introduced, and became feral, on some Mediterranean islands by Neolithic navigators, probably less than 6000–4000 years ago (Ragni 1981; Davis 1987). The fossil record documents the presence of *F. s. libyca* in Sardinia at least by 3000 years ago (Vigne 1992). Therefore,

Europe hosts two wild subspecies of *F. silvestris*, the European and the African wildcats, and the domestic cat.

Domestic and wild cats can interbreed and produce fertile offspring both in captivity and in nature (Robinson 1977; Ragni 1993). Deforestation, the spread of agriculture and the eradication of large predators have probably favoured the expansion of free-ranging and feral cats, which are now distributed worldwide, often in sympatry with wildcat populations in Europe and Africa (Nowell & Jackson 1996). The protracted coexistence of domestic and wild cats raised the fear that widespread interbreeding would have led to genetic extinction, by hybridization and introgression (Rhymer & Simberloff 1996), of 'pure' wildcat populations in Europe (Suminski 1962), the Near East (Mendelssohn 1999) and South Africa (Stuart & Stuart 1991). Widespread introgression would also render uncertain any identification of 'pure' wildcat populations for use as reference populations in taxonomic studies (Daniels *et al*. 1998). The domestication process did not strongly modify the morphology of cats, except for coat colour variability, which is controlled by a few genes (Robinson 1977). Consequently, morphological studies were not able to show diagnostic traits suitable to identify hybridizing cat populations (French *et al*. 1988; Daniels *et al*. 1998). However, to avoid the risk of genetic pollution, hybridizing populations should be identified and monitored in wildcat conservation planning and management (Ragni 1993; Randi *et al*. 2001).

Beaumont *et al*. (2001) and Randi *et al*. (2001) used Bayesian clustering and admixture analyses (Pritchard *et al*. 2000) of multilocus genotypes to describe the genetic structure of cat populations and identify known or presumptive hybrids. Hybridization and introgression was widespread in Scotland (Beaumont *et al*. 2001), but only rare hybrids occurred in Italy (Randi *et al*. 2001). These findings strongly warn against any generalization: a number of different historical and / or (still unknown) ecological factors might account for different rates of crossbreeding and introgression. The genetic compositions of wild-living cat populations can be very different because hybridization can be locally rare or widespread. Cryptic hybrids, that is individuals that cannot be identified using only morphological traits, might be inadvertently included in sample collections, and should be identified using the appropriate molecular markers and statistical procedures.

In this study we analyse genetic variation at 12 feline microsatellite loci in samples of wild and domestic cats collected in Europe. We aim to: (i) estimate the extent of genetic differentiation between cats that were preclassified using morphological traits and sampling locations; (ii) assess the genetic structure in wildcat populations independently of any prior classification, using multivariate ordination and Bayesian genetic clustering; (iii) identify cryptic hybrids in European wildcat populations using admixture analyses.

Materials and methods

Sample collection and phenotypic identifications

A total of 130 domestic cats, 165 European wildcats, 16 African wildcats from Sardinia (Sardinian wildcats), and 25 known or presumptive European wild × domestic cat hybrids were obtained from various localities in Portugal, Belgium, Switzerland, Italy, Germany, United Kingdom, Slovenia, Hungary and Bulgaria. Cats were identified by collectors according to presence or absence of the 'wild' coat phenotype at the locus *Tabby* (Robinson 1977; Piechocki 1990; Ragni & Possenti 1996). Additional morphological criteria, such as the cranial and intestine indices, life-history traits and the stomach contents (Schauenberg 1969, 1977; Ragni & Randi 1986; French *et al*. 1988; Herrmann 1990; Piechocki 1990) were also considered in some cases. Sampling locations and a summary of criteria used for phenotypic identifications are shown in Fig. 1 and the Appendix I.

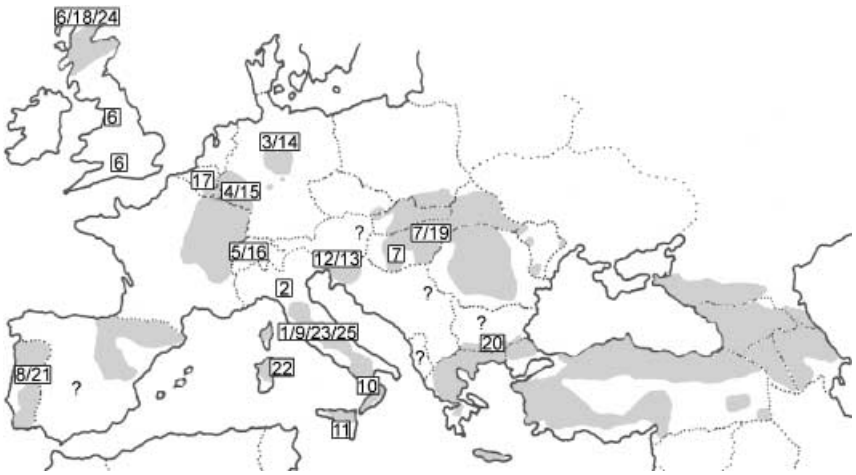

Fig. 1 Approximate distributions of European and Sardinian (in Sardinia) wildcat populations (grey areas). Numbers indicate the sampling locations of the wild and domestic cat populations used in this study (see Appendix I). Question marks indicate area of uncertain distribution of European wildcat populations (adapted from Nowell & Jackson 1996).

DNA extraction and microsatellite genotyping

Tissue and blood samples were obtained through veterinary practices (domestic cats) or from road-kills and local trapping projects (wildcats), and preserved in 100% ethanol (tissues) or in a Tris / sodium dodecyl sulphate buffer (blood). Total DNA was extracted using guanidinium–silica (from tissues; Gerloff *et al*. 1995), or salting-out procedures (from blood; Miller *et al*. 1988). Twelve microsatellites, originally isolated in the domestic cat (Menotti-Raymond & O'Brien 1995; Menotti-Raymond *et al*. 1999), were amplified by polymerase chain reaction and analysed using protocols described by Randi *et al*. (2001).

Analyses of genetic variation

Allelic frequencies, observed (H_O) and expected heterozygosity (H_E) were computed using the program GENETIX 4.02 (Belkhir *et al*. 2001; http://www.univ-montp2.fr / ~genetix / genetix.htm). Deviations from Hardy–Weinberg (HWE) and linkage (LE) equilibria, and the significance of Weir & Cockerham's (1984) estimator of F_{IS} were evaluated using GENEPOP 3.2a (Raymond & Rousset 1995; http://www.cefe.cnrs-mop.fr /). ARLEQUIN 2.0b2 (Schneider *et al*. 2002; http://anthropologie.unige.ch/arlequin) was used to evaluate the significance of genetic differentiation among the sampled groups by analysis of molecular variance (AMOVA) with analogues of F_{ST} and R_{ST} (Slatkin 1995).

Evidences of population bottlenecks were assessed using the *M*-ratio test (Garza & Williamson 2001; http://www.pfeg.noaa.gov / tib / carlos.htm). The probabilities of the observed *M*-values were estimated by simulations under the stepwise-mutation model (Kimura & Ohta 1978), or the two-phase mutation model (Di Rienzo *et al*. 1994), with 95% one-step mutations, average size of non one-step mutations $\Delta g = 3.5$, and $\theta = 4N_e\mu = 5$ or 10. Cornuet & Luikart's (1996) 'sign' and two-tailed 'Wilcoxon signed-ranks' tests of excess expected HWE heterozygosity (BOTTLENECK 1.2.02; Piry *et al*. 1999; http://www.ensam.inra.fr / URLB) were also used, as was the 'mode-shift' test, under a stepwise-mutation model or a two-phase mutation model with 95% one-step mutations.

Multivariate and Bayesian cluster analyses

Evidences of population distinction were obtained using two approaches. Patterns of differentiation were described by Factorial Correspondence Analysis (FCA; Benzécri 1973) of individual multilocus scores, computed using GENETIX. In this approach population clusters are identified graphically and it is not possible to assign quantitatively (probabilistically) the individuals to the clusters. Therefore, using FCA the identification of admixed individuals is uncertain.

In a second approach population structure was assessed using a model-based Bayesian procedure, implemented in the program STRUCTURE (Pritchard *et al*. 2000; http://pritch. bsd.uchicago.edu). This model was designed to identify the *K* (unknown) populations (genetic clusters) of origin of individuals, and simultaneously to assign the individuals to the populations with explicit estimates of their 90% confidence intervals. Individuals are probabilistically assigned to one cluster (the population of origin), or more than one cluster (the parental populations), if they are genetically admixed as a result of hybridization. STRUCTURE assumes that the neutral unlinked molecular markers are in HWE and LE in the populations, and that recent population admixture, migration or hybridization would probably produce departures from HWE and LE. STRUCTURE was run with the 'admixture model', and one to five repetitions of 100 000 iterations following a burn-in period of 10 000 iterations.

Population structure was assessed using the entire sample set ($n = 336$), and assuming that sampled cats belong to an unknown number of *K* genetically distinct clusters. Posterior probability values for *K* (log likelihood; ln L) were estimated assigning priors from three to seven (Randi *et al*. 2001; showed that a sample of European wildcats, Sardinian wildcats and domestic cats were split into at least three distinct genetic clusters). We chose the value of $K = 6$, which showed the highest likelihood, and then evaluated the average membership coefficients of predefined (sampled) cat groups to the six inferred clusters. Each sampled group was assigned to one cluster if its average proportion of membership was ≥ 0.80, or jointly to more than one cluster, if its average proportion of membership to each cluster was < 0.80.

Then, we assessed the individual proportion of membership (q_i) that is the average proportion of each genotype that is inferred to originate from each cluster using only genetic information. Individuals with $q_i \geq 0.80$ were assigned to one cluster, or jointly to more than one cluster, if the proportion of membership to each cluster was $q_i < 0.80$ (admixed cats). The threshold value of 0.80 was arbitrarily defined to be sure that at least 80% of an individual's genome is assigned to one or more than one inferred cluster.

Finally, the ancestry of cats inferred as admixed was investigated assuming that each genotype should belong to more than one of three clusters, the 'domestic cat', the 'Sardinian wildcat' or the 'European wildcat' cluster (see Randi *et al*. 2001). Therefore, we used STRUCTURE with $K = 3$ to compute cat ancestry, first using the available prior population information (phenotypic classification), and options USEPOPINFO = 1, POPFLAG = 1. In this way, each cat was forced to have its genotype assigned either to one of the three clusters, or, if admixed, in part to the current, first or second past generation in more than one cluster. In a second procedure individuals were assigned probabilistically

to the clusters, without using any prior information on individual classification (USEPOPINFO = 0). In a third procedure each individual was assigned probabilistically to the clusters, using prior information for all individuals except for cats which were identified as admixed after the first two procedures. These individuals were assigned to the clusters without using any prior information on individual classification.

Results

Admixture analyses and identification of hybrid cats

All loci were polymorphic in domestic and wild cats and all individual genotypes were distinct. Bayesian analyses showed that the sample (n = 336) included at least six distinct populations (the highest likelihood was obtained with K = 6; average lnL of five repetitions ± one SE = –14 183 ± 18). The average proportions of membership of each sampled population in the six clusters (Table 1) showed that all the domestic cats grouped in cluster 6, the 'domestic cat' cluster, except for some of the cats from the UK, which were split between clusters 6 and 4. All European wildcats from Italy, the northeastern Alps, north and southwest Germany, Belgium, Bulgaria and Portugal were assigned to clusters 1, 2 and 3, the 'European wildcat' clusters. Wildcats from Switzerland, Hungary and the known hybrid cats were also partially assigned to these clusters. However, Hungarian wildcats were also partially assigned to the 'domestic cat' cluster 6. All the Sardinian wildcats grouped in the 'Sardinian wildcat' cluster 5, in which no other cats clustered. The known hybrid cats from Italy and Scotland (Beaumont *et al*. 2001; Randi *et al*. 2001), and the Scottish black cats, were split between clusters 4 and 2. Cluster 4 included also the majority of Scottish wild-living cats, and a proportion of Swiss wildcats and Scottish domestic cats. Accordingly, cluster 4 may be identified as a 'hybrid cat' cluster.

Admixed European wildcats were found in Italy (n = 2; Fsi228, Fsi284), Portugal (n = 2; Fsi293, Fsi325), Bulgaria (n = 1; Fsi207), Belgium (n = 1; Fsi194) and Switzerland (n = 2; Fsi177, Fsi182) (Table 2). There was no admixed Sardinian wildcat. The ancestry of admixed Italian wildcats was analysed previously (Randi *et al*. 2001): Fsi228 was assigned to the European wildcat cluster, while Fsi284 showed hybrid ancestry. The ancestry of the other admixed wildcats was inferred by computing the probability of each genotype to belong to more than one of three clusters, the 'domestic cat', the 'Sardinian wildcat' or the 'European wildcat' cluster. As a result of their highly admixed composition, any prior nongenetic identification of cats from the hybridizing Scottish and Hungarian populations would be problematic, and the samples from these populations were excluded.

Table 1 Bayesian clustering analyses for the pooled cat samples (336 individuals; 12 loci) performed using STRUCTURE (Pritchard *et al*. 2000)

Group	Population	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Domestic cats (*Felis silvestris catus*)	Italy	0.014	0.011	0.010	0.013	0.019	0.933
	Germany north	0.021	0.011	0.016	0.013	0.007	0.932
	Germany southwest	0.016	0.046	0.042	0.031	0.050	0.815
	Switzerland	0.009	0.024	0.016	0.008	0.012	0.930
	United Kingdom	0.009	0.016	0.016	0.166	0.075	0.717
	Hungary	0.015	0.034	0.049	0.012	0.011	0.878
	Portugal	0.006	0.009	0.006	0.012	0.025	0.942
European wildcats (*Felis s. silvestris*)	Italy	0.066	0.727	0.174	0.011	0.009	0.013
	Northeastern Alps, Slovenia	0.082	0.087	0.807	0.016	0.003	0.005
	Germany north	0.952	0.017	0.021	0.005	0.003	0.003
	Germany southwest	0.066	0.012	0.901	0.009	0.006	0.005
	Switzerland	0.118	0.039	0.527	0.302	0.008	0.006
	Belgium	0.028	0.064	0.864	0.033	0.006	0.005
	Scotland	0.009	0.007	0.008	0.966	0.005	0.005
	Hungary	0.028	0.279	0.425	0.065	0.008	0.195
	Bulgaria	0.041	0.571	0.301	0.011	0.007	0.070
	Portugal	0.032	0.249	0.612	0.078	0.016	0.013
Sardinian wildcats (*Felis s. libyca*)	Italy	0.005	0.009	0.006	0.004	0.967	0.010
Hybrids (*F. s. silvestris* × *F. s. catus*)	Italy, Scotland, captive	0.016	0.200	0.021	0.631	0.039	0.092

Average proportion of membership of each predefined population in each of K = 6 inferred clusters.

Table 2 Population assignment and inferred ancestry of admixed wildcats estimated using STRUCTURE

Samples	Cluster 1 Domestic cats	Cluster 2 European wildcats	Cluster 3 Sardinian wildcats
(a)			
Domestic cats	0.998	0.001	0.001
European wildcats	0.002	0.997	0.000
Sardinian wildcats	0.001	0.000	0.999
Fsi177 (Switzerland)	0.000–0.000–0.001	0.999	0.000–0.000–0.000
Fsi182 (Switzerland)	0.000–0.000–0.001	0.999	0.000–0.000–0.000
Fsi194 (Belgium)	0.000–0.000–0.000	0.999	0.000–0.000–0.000
Fsi207 (Bulgaria)	0.000–0.000–0.034	0.964	0.000–0.000–0.002
Fsi293 (Portugal)	0.000–0.000–0.001	0.999	0.000–0.000–0.000
Fsi325 (Portugal)	0.000–0.001–0.038	0.939	0.000–0.000–0.021
(b)			
Domestic cats	0.952	0.018	0.031
European wildcats	0.019	0.961	0.020
Sardinian wildcats	0.013	0.006	0.981
Fsi177	0.010	0.986	0.004
Fsi182	0.012	0.977	0.011
Fsi194	0.007	0.989	0.003
Fsi207	0.270	0.716	0.014
Fsi293	0.012	0.981	0.007
Fsi325	0.134	0.814	0.053
(c)			
Domestic cats	0.956	0.022	0.022
European wildcats	0.008	0.987	0.004
Sardinian wildcats	0.001	0.000	0.999
Fsi177	0.101	0.850 (0.575–0.998)	0.049
Fsi182	0.096	0.796 (0.500–0.944)	0.096
Fsi194	0.087	0.861 (0.633–0.997)	0.052
Fsi207	0.321 (0.050–0.603)	0.585 (0.291–0.869)	0.094
Fsi293	0.098	0.832 (0.567–0.995)	0.070
Fsi325	0.200 (0.002–0.490)	0.664 (0.367–0.923	0.136 (0.000–0.395)

The first three lines of each section in the Table report the average proportion of membership of each predefined population (domestic cats, European wildcats, Sardinian wildcats) in each of the three clusters. The other lines report the individual membership of each individual genotype to each of the three clusters. These values were computed: (a) using information on the prior classification of all individuals based on nongenetic characters; (b) without using any prior information on individual classification; (c) using information on the prior classification for all individuals except for the preselected admixed cats, which are assigned to the clusters with membership coefficients and and 90% probability intervals shown in the Table.

Individual cat ancestry was estimated first using prior population information. Results (Table 2a) showed that the six admixed wildcats were significantly associated to the 'wildcat' cluster 2 with individual $q_2 \geq 0.94$, and none of them had significant ancestry in any current or past generation of the other two clusters. Then cat ancestry was estimated without using any prior information (Table 2b). Sample Fsi207, a putative wildcat from Bulgaria, was assigned to both the European wildcat (with $q_2 = 0.716$) and the domestic cat clusters ($q_1 = 0.270$). Sample Fsi325, a putative wildcat from Portugal, was assigned to both the European wildcat (with $q_2 = 0.814$) and the domestic cat clusters ($q_1 = 0.134$). All the other admixed cats were assigned to the cluster with individual $q_2 > 0.98$. Finally, individuals were assigned to the European wildcat clusters using information on the prior classification for all individuals except for the admixed wildcats (Table 2c). All the admixed European wildcats were assigned to the European wildcat cluster 2 with $q_2 \geq 0.80$, except for samples Fsi207 (from Bulgaria) and Fsi325 (from Portugal), which were partially assigned to clusters 1 (domestic cats) and 2 (European wildcats). A consensus evaluation of these three admixture analyses suggests that samples Fsi207 (Bulgaria) and Fsi325 (Portugal) had hybrid ancestry.

Bayesian analyses assigned a few other individuals to more than one cluster. Admixed domestic cats were found

Table 3 AMOVA in domestic and European wildcat samples (hybrid populations and individuals excluded)

Grouping	Source of variation	Per cent variation	F_{ST}	R_{ST}
(1) Three taxonomic groups; 14 populations*	Among groups	10.86	$F_{CT} = 0.11$	$R_{CT} = 0.41$
	Among populations	6.15	$F_{SC} = 0.07$	$R_{SC} = 0.06$
	Within populations	82.99	$F_{ST} = 0.17$	$R_{ST} = 0.44$
(2) Domestic cats; five populations†	Among populations	2.82	$F_{ST} = 0.03$	$R_{ST} = 0.01$
	Within populations	97.18		
(3) European wildcats; eight populations‡	Among populations	9.17	$F_{ST} = 0.09$	$R_{ST} = 0.11$
	Within populations	90.83		

*Fourteen populations (see Appendix I); †five populations (Italy, north Germany, southwest Germany, United Kingdom, Switzerland + Portugal); ‡eight populations (Italy, northeastern Alps, north Germany, southwest Germany, Switzerland, Belgium, Bulgaria, Portugal).

in Italy ($n = 2$) and southwestern Germany ($n = 7$). The two cats in Italy were house-living cats sampled in Bologna. The seven cats in Germany were road-kills, collected near or within villages, and four of them had domestic cat food in their stomach. These individuals probably represent genotypes produced by artificial selection and reproduction in captivity, which, in this study, are considered as domestic cats.

Genetic diversity in wild and domestic cats

In the following analyses we have excluded all cat samples from the hybridizing Scottish and Hungarian populations, plus the known or putative hybrid wildcats collected from the other localities. In this reduced sample set ($n = 245$) all loci were polymorphic, with nine to 21 alleles and an average of 14.2 alleles per locus. The values of observed and expected heterozygosity were $H_O = 0.428–0.875$ and $H_E = 0.600–0.881$, across all loci. Average values of heterozygosity were similar in wild ($H_O = 0.62$; $H_E = 0.75$) and domestic cats ($H_O = 0.70$; $H_E = 0.78$). Domestic and European wildcats showed H_O values lower than expected, with average F_{IS} values that were positive (0.102 and 0.181, respectively) and significantly different from zero ($P < 0.05$), and that were not in HWE. In contrast, when each wildcat population was analysed separately there was only one locus in wildcats from Belgium, and two loci in wildcats from Italy showing F_{IS} values significantly larger than expected ($P \leq 0.0005$). Thus, local wildcat populations were in HWE, with the possible exception of the Italian wildcats, which were sampled from distinct geographical areas (Apennines and Sicily) and that may represent distinct gene pools. After Bonferroni correction for multiple comparisons, 25 and two allelic combinations were not in LE ($P < 0.05$) in European wild and domestic cats, respectively. Sardinian wildcats were in HWE (average $F_{IS} = 0.003$) and LE.

A hierarchical AMOVA was performed by subdividing the reduced sample set into three taxonomic groups (European and Sardinian wildcats, domestic cats) and 14 geographical populations (the two domestic cat samples from Portugal and the three from Switzerland were pooled to avoid lower sample sizes). Relative variances among groups and among populations within groups were highly significant ($P < 0.001$; Table 3). Gene diversity among populations estimated using the number of different alleles ($F_{SC} = 0.07$) was similar to the corresponding estimate that was computed using the sum of squared allele size differences ($R_{SC} = 0.06$). In contrast, the diversity among groups estimated from $R_{CT} = 0.41$ was about four times higher than the corresponding estimate computed from $F_{CT} = 0.11$, indicating that mutational differences have contributed to differentiate the three taxonomic groups of cats.

Nonhierarchical AMOVAs were performed among five domestic and eight European wildcat populations, corresponding to the countries from where samples were collected (Table 3). The domestic cat populations were significantly differentiated using $F_{ST} = 0.03$ ($P < 0.001$), but not using $R_{ST} = 0.01$ ($P \leq 0.25$). The wildcat populations were significantly differentiated using both $F_{ST} = 0.09$ and $R_{ST} = 0.11$ ($P < 0.001$). Thus, genetic divergence among wildcat populations might be attributed both to different allele frequencies and different mutations at the studied loci.

Genetic diversification among nonhybridizing cat populations

The FCA plotting of the individual genotypes showed that European, Sardinian wild and domestic cats are distinct and plot into three separate areas of the multivariate space (Fig. 2b). Wildcats sampled in north Germany plot separately and are distinct from the other wildcat populations (Fig. 2a).

Bayesian clustering split the European wildcats sampled from nonhybridizing populations into four genetic clusters (ln L = –5194.1 ± 0.4; not shown), with compositions similar to the clusters shown in Table 1. Samples from north and southwest Germany, and Belgium were assigned to single clusters with $q_i = 0.81$, while the other populations

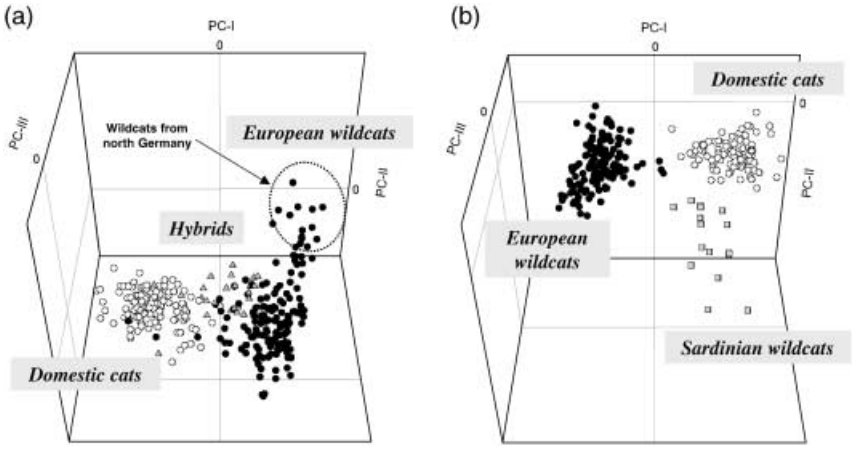

Fig. 2 Factorial correspondence analysis showing multivariate relationships among individual wild and domestic cat genotypes described using 12 microsatellite loci. PC-I, PC-II and PC-III are the first three principal correspondence factors. European wildcats, domestic cats and Sardinian wildcats are indicated with ●, ○ and, ▢ respectively. Known hybrid cats are indicated by △ in (a). The ellipse in (a) delimits the distribution of wildcats sampled from north Germany.

were split into more than one cluster. The two wildcat populations from Germany were distinct, being assigned to cluster 2 (north Germany) and cluster 3 (southwest Germany). Cluster 1 includes wildcat populations from southern Europe (Portugal, Italy and Bulgaria), while cluster 3 includes wildcats populations from central Europe (Alps, southwest Germany, Switzerland and Belgium). The Italian wildcats were split between clusters 1 and 4. Almost all the wildcats sampled in the central Apennines and Tuscany grouped in cluster 4, while all the wildcats from Sicily grouped in cluster 1. Wildcats sampled in the northeastern Italian Alps and Slovenia grouped in cluster 3. The geographical fragmentation of the Italian wildcats seems to be reflected in the partition of genetic variability.

The two German wildcat populations showed significantly different values of H_E (north = 0.56 ± 0.14; southwest = 0.70 ± 0.12; $P < 0.01$), H_O (north = 0.56 ± 0.18; southwest = 0.67 ± 0.16; $P < 0.01$), and average number of alleles per locus (north = 4.0; southwest = 6.2; $P < 0.01$). To investigate the origin of low genetic diversity in the northern population, we performed two tests of population bottleneck. Sign, Wilcoxon and mode-shift tests (Cornuet & Luikart 1996) showed that both German populations were at mutation-drift equilibrium, independently of the mutation model used. The *M*-ratio test (Garza & Williamson 2001) showed that the southwestern populations had an average observed *M*-value (0.787) larger than the expected equilibrium values (*Mc* = 0.712 or 0.756, computed with $\theta = 4N_e\mu = 10$ or 5, respectively). In contrast, the northern population showed *M* (0.661) significantly smaller than *Mc* values (0.724 or 0.756). Thus, the *M*-ratio test indicates the occurrence of past demographic declines in the northern population.

Genetic composition of the hybridizing populations

An FCA plotting including all samples from the nonhybridizing and from the hybridizing populations, plus the known hybrids, showed overlapping spatial distributions of the individual genotypes of the domestic and wild cats (Fig. 2a). Known hybrids and some of the presumed wildcats or domestic cats from Hungary and Scotland plotted between the domestic and wild cats that were sampled from the nonhybridizing populations. The absence of any genetic gap between presumptive domestic and wild cats in hybridizing populations is dramatically exemplified by comparing the FCA of cats sampled from Germany (nonhybridizing; Fig. 3a) and Hungary (hybridizing; Fig. 3b).

An admixture analysis of cats from Hungary ($n = 46$), performed using STRUCTURE with $K = 2$ and including 86 domestic and 143 nonhybrid European wildcats as reference, showed that all reference domestic cats and European wildcats were assigned to cluster 1 with $q_1 \geq 0.80$ or with $q_1 \leq 0.20$, respectively (Fig. 4). Thirteen cats were assigned to both cluster 1 and 2 with $0.20 < q_1 < 0.80$: one cat, a domestic cat that was sampled in southwestern Germany, and 12 cats sampled from Hungary. Thus, the Hungarian cat sample included a number of admixed individuals, which could not be assigned exclusively to the domestic or to the wildcat clusters (for admixture analyses of the Scottish samples, see Beaumont *et al*. 2001).

A detailed analysis of the individual membership of the Hungarian cats to cluster 1 (domestic cats) and cluster 2 (European wildcats) allowed subdivision of the Hungarian cats into four groups (Table 4). Cats in the first group (n = 10) were assigned to the domestic cat cluster 1 with $q_1 \geq 0.96$ and narrow 90% probability intervals (range 0.800–1.000). All these 10 cats had been independently identified by collectors as domestic cats using both coat colour patterns and values of the intestinal index *II*, which ranged from 3.14 to 4.75. Thus, there was 100% concordance between the genetic assignment procedure and the morphological identification.

Cats in the second group were assigned to the domestic cat cluster 1 with $q_1 \geq 0.85$, but with much wider 90% probability intervals (range 0.160–1.000). All these 14 cats,

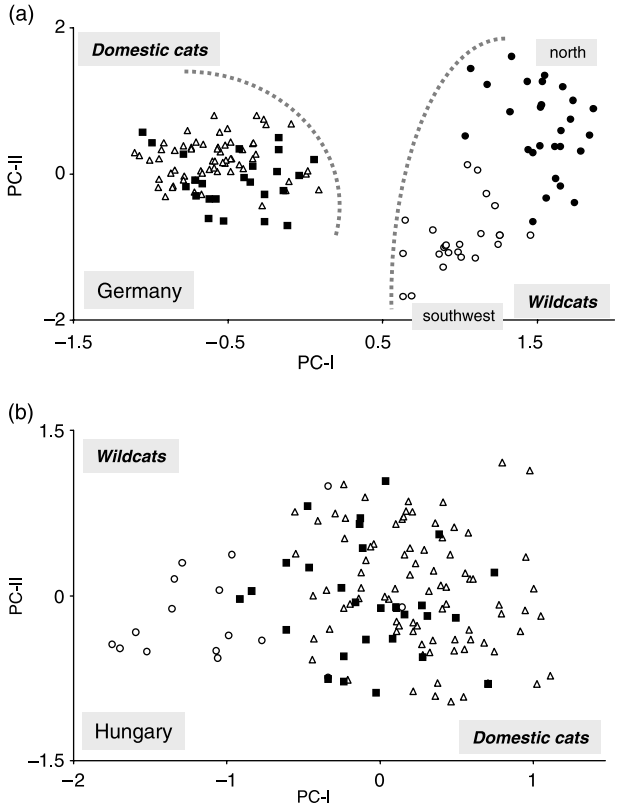

Fig. 3 Factorial correspondence analysis showing multivariate relationships among microsatellite genotypes of wildcats sampled in north Germany (●) and southwest Germany (○) (a) and in Hungary (b), and of domestic cats sampled in Germany (■), in Hungary (■), and in other countries in Europe (△).

except for two samples, were morphologically identified as domestic cats and showed values of intestinal index $II \geq 3.11$. One cat (sample no. 353), showing the lowest value of $II = 3.11$, was of dubious morphological identification. If this sample is excluded, the intestinal index values were $II \geq 3.53$. Another cat (no. 208), showing the larger 90% probability interval (0.160–1.000), was morphologically identified as a hybrid. In this group the concordance between genetic and morphological identifications was 86%, or 93% if the presumed hybrid is excluded.

The third group included eight genetically admixed cats, which were partially assigned to both clusters 1 and 2. These cats were assigned to cluster 1 with $0.71 \geq q_1 \geq 0.30$. They showed very large probability intervals (range 0.000–1.000) and intermediate values of $II = 3.22$–3.53. All these cats were morphologically identified as hybrids, with 100% concordance with the genetic assignment.

The fourth group included 14 cats which were assigned to cluster 1 with $q_1 \leq 0.15$, and that were genetically identified as wildcats ($q_2 \geq 0.85$). These cats showed II values ≤ 3.15. However, eight of these cats were morphologically identified as hybrids, so in this group the concordance between genetic and morphological identification of wildcats was low (43%).

Discussion

Genetic diversity in wild and domestic cat populations

Microsatellites are highly polymorphic in cats and have been used to describe microevolutionary distinction among populations (Hille *et al*. 2000; Wiseman *et al*. 2000), and detect domestic × wildcat hybridization (Beaumont *et al*. 2001; Randi *et al*. 2001). These studies showed concordant morphologic and genetic identifications of wild and domestic cats, indicating significant genetic divergence among cat gene pools. However, widespread introgression in Scotland (Beaumont *et al*. 2001) and the presence of cryptic hybrids in Italy (Randi *et al*. 2001), suggested that wild and domestic cats are not always reproductively isolated.

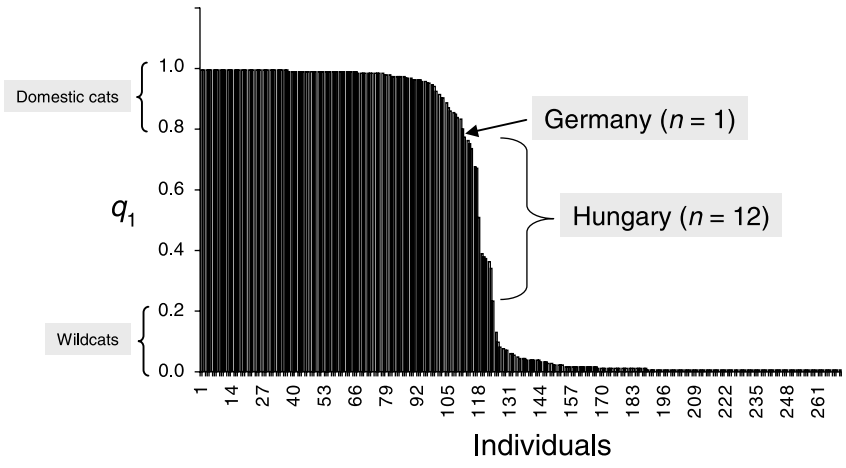

Fig. 4 Frequency distribution of the individual proportion of domestic cat genome (q_1) in 86 domestic cats ($q_1 \geq 0.80$), 143 nonhybrid European wildcats ($q_1 \leq 0.20$), and 46 cats collected in Hungary. One cat from Germany and 12 cats from Hungary showed intermediate q_1 values ($0.20 < q_1 < 0.80$). Individual values of q_1 in cats from Hungary are shown in Table 4.

Table 4 Assignment test and inferred ancestry of individual cats sampled from Hungary estimated using STRUCTURE

Sample ID	q_1*	q_2†	90% q_1 pi‡	90% q_2 pi§	*II*	*g*ID	*m*ID
First group: $n = 10$; $q_1 \geq 0.962$ (90% pi = 0.800–1.000); $II \geq 3.14$							
363	0.980	0.020	0.892–1.000	0.000–0.108	4.09	*Fca*	*Fca*
365	0.978	0.022	0.884–1.000	0.000–0.116	4.75	*Fca*	*Fca*
227	0.976	0.024	0.874–1.000	0.000–0.126	4.18	*Fca*	*Fca*
358	0.974	0.026	0.864–1.000	0.000–0.136	3.57	*Fca*	*Fca*
338	0.974	0.026	0.861–1.000	0.000–0.139	3.92	*Fca*	*Fca*
222	0.972	0.028	0.853–1.000	0.000–0.147	4.51	*Fca*	*Fca*
354	0.969	0.031	0.837–1.000	0.000–0.163	4.30	*Fca*	*Fca*
346	0.967	0.033	0.826–1.000	0.000–0.174	3.14	*Fca*	*Fca*
224	0.962	0.038	0.802–1.000	0.000–0.198	4.24	*Fca*	*Fca*
342	0.962	0.038	0.800–1.000	0.000–0.200	3.40	*Fca*	*Fca*
Second group: $n = 14$; $q_1 \geq 0.846$ (90% pi = 0.160–1.000); $II \geq 3.11$							
225	0.961	0.039	0.797–1.000	0.000–0.203	—	*Fca*	*Fca*
359	0.952	0.048	0.766–1.000	0.000–0.234	3.56	*Fca*	*Fca*
212	0.949	0.051	0.743–1.000	0.000–0.257	—	*Fca*	*Fca*
226	0.946	0.054	0.730–1.000	0.000–0.270	3.75	*Fca*	*Fca*
345	0.939	0.061	0.706–1.000	0.000–0.294	3.96	*Fca*	*Fca*
223	0.933	0.067	0.688–1.000	0.000–0.312	3.94	*Fca*	*Fca*
341	0.930	0.070	0.659–1.000	0.000–0.341	4.17	*Fca*	*Fca*
220	0.921	0.079	0.647–1.000	0.000–0.353	3.61	*Fca*	*Fca*
221	0.927	0.073	0.669–1.000	0.000–0.331	3.72	*Fca*	*Fca*
353	0.915	0.085	0.630–1.000	0.000–0.370	3.11	*Fca*	*Fca*?
339	0.906	0.094	0.615–1.000	0.000–0.385	3.53	*Fca*	*Fca*
213	0.895	0.105	0.541–1.000	0.000–0.459	3.74	*Fca*	*Fca*
219	0.851	0.149	0.504–1.000	0.000–0.496	4.15	*Fca*	*Fca*
208	0.846	0.154	0.160–1.000	0.000–0.840	3.68	*Fca*	*Hy*
Third group: $n = 8$; $0.713 \geq q_1 \geq 0.302$ (90% pi = 0.000–1.000); $3.22 \leq II \leq 3.53$							
367	0.713	0.287	0.318–1.000	0.000–0.682	3.53	*Hy*	*Hy*
340	0.628	0.372	0.256–0.997	0.003–0.744	3.30	*Hy*	*Hy*
360	0.612	0.388	0.068–1.000	0.000–0.932	—	*Hy*	*Hy*
361	0.590	0.410	0.072–1.000	0.000–0.928	—	*Hy*	*Hy*
347	0.541	0.459	0.147–0.921	0.079–0.853	3.28	*Hy*	*Hy*
366	0.442	0.558	0.033–0.788	0.212–0.967	3.31	*Hy*	*Hy*
350	0.348	0.652	0.000–0.712	0.288–1.000	—	*Hy*	*Hy*
356	0.302	0.698	0.000–0.769	0.231–1.000	3.22	*Hy*	*Hy*
Fourth group; $n = 14$; $0.148 \geq q_1 \geq 0.021$ (90% pi = 0.000–0.477); $II < 3.15$							
351	0.148	0.852	(0.000–0.477)	(0.523–1.000)	2.84	*Fsi*	*Hy*
343	0.107	0.893	(0.000–0.401)	(0.599–1.000)	3.06	*Fsi*	*Hy*
362	0.107	0.893	(0.000–0.418)	(0.582–1.000)	2.53	*Fsi*	*Hy*
211	0.094	0.906	(0.000–0.375)	(0.625–1.000)	2.46	*Fsi*	*Hy*
216	0.093	0.907	(0.000–0.403)	(0.597–1.000)	3.15	*Fsi*	*Hy*
349	0.069	0.931	(0.000–0.323)	(0.677–1.000)	2.71	*Fsi*	*Fsi*
209	0.069	0.931	(0.000–0.321)	(0.679–1.000)	2.63	*Fsi*	*Hy*
352	0.066	0.934	(0.000–0.322)	(0.678–1.000)	2.87	*Fsi*	*Hy*
215	0.052	0.948	(0.000–0.260)	(0.740–1.000)	2.46	*Fsi*	*Fsi*
210	0.049	0.951	(0.000–0.249)	(0.751–1.000)	2.76	*Fsi*	*Hy*
214	0.031	0.969	(0.000–0.162)	(0.838–1.000)	2.84	*Fsi*	*Fsi*
348	0.028	0.972	(0.000–0.146)	(0.854–1.000)	2.70	*Fsi*	*Fsi*
355	0.025	0.975	(0.000–0.130)	(0.870–1.000)	2.55	*Fsi*	*Fsi*
364	0.021	0.979	(0.000–0.110)	(0.890–1.000)	2.67	*Fsi*	*Fsi*

*Individual membership to cluster 1; †individual membership to cluster 2; ‡90% probability interval of individual assignment to cluster 1; §90% probability interval of individual assignment to cluster 2.
II = intestinal index; *g*ID = individual identification based on genetic assignment testing; *m*ID = individual identification based on morphological data; *Fca* = domestic cats; *Fsi* = European wildcats; *Hy* = hybrid cats.

Results of this study confirmed that wild and domestic cats are genetically distinct in central and southwest Europe, but that they are extensively admixed in the sampled locations in Hungary. Therefore, assessing hybridization and identifying the causes of breakdown of reproductive isolation are of paramount importance for wildcat conservation in Europe.

Domestication apparently did not reduce the levels of genetic diversity in domestic cats as compared to wildcat populations. Wild and domestic cats showed both distinct private alleles and different allelic frequencies at microsatellite loci (see Randi *et al*. 2001). Genetic variability was significantly different between wild and domestic cats, and R_{ST} (0.41) was larger than F_{ST} (0.11), suggesting that mutational differences might have contributed to the differentiation of wild and domestic cats. Mutational differences might originate from admixture of multiple genetically differentiated African wildcat sources, which were not sampled in this study, or from past episodes of gene flow between domestic and European wildcats. A more comprehensive sampling of wildcats, and particularly of the widespread African wildcat populations, is needed to reconstruct the genetic structure and infer the origins of domestic cat populations.

Genetic structure in nonhybridizing European wildcats

Bayesian clustering showed that wildcats in southern and central Europe were assigned to distinct clusters, suggesting that European wildcats include genetically differentiated subpopulations. The strong membership of wildcats from the Alps, southwestern Germany, Switzerland and Belgium to cluster 3 (q_3 from 0.52 to 0.85), further suggest that there has been a protracted gene flow among populations in central Europe, with the notable exception of wildcats living in the Solling region in north Germany.

The wildcats sampled in north Germany were distinct from all the other wildcat populations (Fig. 2), they were significantly less variable than wildcats in southwest Germany, and showed instances of significant bottleneck effects [as assessed using the procedure of Garza & Williamson (2001)]. Historical information documents the occurrence of recent and strong demographic declines of all the wildcat populations in Germany. During the 19th century a campaign to eradicate the wildcats in Germany led populations to decline to a few dozen individuals at the beginning of the 20th century. During the first quarter of the 20th century only a few small populations survived in parts of the Eifel, Hunsrück and Pfälzerwald, southwest of the Rhine-valley, and in the Harz and Solling mountains. The southwest population is currently part of the large central European wildcat population, and should be genetically connected with wildcats in northwest France, Belgium and Luxembourg (Hille *et al*. 2000). In contrast, wildcats in Harz and Solling underwent dramatic population declines in the recent past (Haltenorth 1957), and are still geographically isolated from all the other populations. Genetic drift as a result of strong population decline might have contributed to the diversification of the two wildcat populations in Germany. Wildcats from Germany, and particularly the samples from Solling, were not intermediate between European wildcats and domestic cats when plotted (Fig. 2), and did not show any instance of hybridization using Bayesian admixture analyses. Therefore, these data do not support the hypothesis that the distinction of wildcats from Solling is the result of past or current crossbreeding with free-ranging domestic cats.

Genetic composition of the hybridizing cat populations in Hungary

Admixture analyses allowed the detection of three cryptic hybrids (in Portugal, Italy and Bulgaria) that were not previously identified using morphological markers. These findings confirm results published by Randi *et al*. (2001), suggesting small rates of crossbreeding in Italy and other European countries. We did not find admixed wildcats in Sardinia, although larger sampling could reveal additional cases of hybridization in the island and in other regions in Europe.

Beaumont *et al*. (2001), and results from this study, showed that introgression was widespread in wild-living cats in Scotland and Hungary (Table 4 and Fig. 4). Free-ranging domestic and wild-living cats in Scotland include a composite assemblage of admixed genotypes, which probably originated through a protracted process of hybridization and introgression (Beaumont *et al*. 2001). The Scottish black cats also showed hybrid ancestry, in agreement with previously published morphometric data (Kitchener & Easterbee 1992).

The genetic structure of hybridizing cats in Scotland and Hungary is, apparently, similar: wild-living cats in both regions show a variety of coat colour patterns ranging from 'wild' to 'domestic-like' phenotypes, with intermediate phenotypes that are difficult to identify (French *et al*. 1988; Daniels *et al*. 1998; Beaumont *et al*. 2001). The Hungarian cat sample includes a number of admixed individuals, with ancestry in both the domestic and wild cat gene pools. These findings strongly suggest that the studied cat populations in Hungary hybridize extensively, either currently or in the past.

Hungarian cats include intermediate individuals, which can be identified as hybrids using both genetic and morphological traits. However, using a $q_{(0.80-0.20)}$ criterion, more than 50% of cats that were genetically identified as 'pure' wildcats still show morphological markings of hybridization (see the fourth group of cats in Table 4). If morphological identifications were correct, we conclude

that either the $q_{(0.80-0.20)}$ criterion is not stringent enough, or that 12 microsatellite loci are not enough for detecting past hybridization.

Multivariate and model-based statistical approaches to population and individual identification have their own weaknesses. Multivariate, as well as distance-based methods are graphical representations of similarity, and do not allow quantitative assignments of the individuals to the populations (Pritchard *et al*. 2000; Randi *et al*. 2001). Model-based methods, such as STRUCTURE, assume HWE and LE, which may not hold in reference populations, and in particular in domesticated taxa. A few (10–15) hypervariable markers with high expected heterozygosity may be able to cluster efficiently samples of 15–20 individuals from populations that were reproductively isolated for less than 20 generations, or that showed F_{ST} values as low as 5% (Rosenberg *et al*. 2001, 2002). However, it is still not known how departures from HWE and LE might affect the efficiency of individual assignment and the identification of admixed ancestry.

For those reasons the rates of hybridization in European wildcats could be higher than suggested by the available data. Future research should define validated morphological markers, accounting for geographical variability among wildcat populations, to be used to identify 'pure' or hybridizing wildcats, in correlation with additional genetic markers and improved statistical procedures.

Conclusions

Hybridization with translocated or domesticated populations is increasing the risks of genetic deterioration and extinction of wild-living populations of terrestrial and acquatic vertebrates (Rhymer & Simberloff 1996; Allendorf *et al*. 2001). Determinants and outcome of both natural and anthropogenic hybridization processes are various and complex, and an understanding of them is important for the study of the evolution of natural populations and for practical conservation biology. The availability of highly polymorphic molecular markers, and of novel statistical procedures aimed to investigate population structure and individual ancestry based on the distributions of multilocus genotypes, promise to be helpful in investigating the structure and dynamics of hybridization.

We do not know what peculiar historical or ecological factors have differentially affected the population genetic structure of wild-living cats in Scotland, Hungary, and in other regions in Europe. Wild-living cats in Scotland with both 'tabby' (the wildcat phenotype) and 'nontabby' (the domestic cat phenotypes) pelage, and significantly distinct microsatellite allelic frequencies, showed similar kinship structure, home-range size, activity pattern and habitat use (Daniels *et al*. 2001). Contrasting demographic trends arising from the concomitant consequences of deforestation and wildcat persecution, and the spread of agriculture that probably favoured the expansion of free-ranging domestic cats, might have fostered crossbreeding between rare wildcats and widespread domestic cats in some areas in Europe. Thus, historical, rather then local ecological, factors could explain the occurrence of admixed wild-living cat populations in parts of Europe.

We suggest that rates of hybridization could locally increase if wildcat populations strongly decline as a result of direct persecution and / or habitat destruction and artificialization, in rural areas with a widespread and abundant presence of domestic cats. Unpublished radiotracking data from southwest Germany (M. Herrmann, personal communication) suggest that wildcats (n = 12) spent more than 90% of their time in forest, and did not range more than 1500 m from the closest large patch of forest. In contrast, domestic cats have almost never been seen in that forest. Thus, coexistence of patches of forest and villages in the same agricultural landscape could favour contacts between wildcats (that should not move too far from forest patches) and domestic cats (that should not enter into the forest patches).

The wildcat is legally protected by national law in most European countries, and internationally by the Bern and Washington (CITES) conventions, and under the European Directive 92 / 43 / EEC. The results of this study strongly urge that hybridization be considered as a priority threat to the conservation of wildcat populations in Europe. Non-hybridizing wildcat populations in Iberia, Germany and Italy have high conservation value, and should be actively protected by preserving the integrity of their natural habitat. Specific measures aimed to limit the diffusion of free-ranging domestic cats should be considered with high priority in wildcat conservation projects.

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This research was conducted at the Conservation Genetics Laboratory, Italian Institute of Wildlife Biology (INFS) within an ongoing project on population and conservation genetics of carnivores in Italy and Europe. Massimo Pierpaoli is a researcher at INFS, with main interests in the conservation and noninvasive genetics of large carnivores. Mathias Herrmann is head of OEKO-LOG, a field-research company. Karsten Hupe is a doctoral candidate at the Institut für Wildbiologie und Jagdkunde, Universität Göttingen, and is responsible for a research project on wildcats in Lower Saxony. Margarida Fernandes works at the Institute of Nature Conservation in Portugal, and has been involved in wildcat biology studies since 1990. Bernardino Ragni is head of the Vertebrate Zoology Section at the Animal Biology and Ecology Department, University of Perugia, and has projects on conservation biology of terrestrial vertebrates, particularly carnivores. Lazlo Szemethy and Zsolt Birò, researchers at St Stephen University, have main interests in behavioural ecology, management and conservation of wild carnivores and ungulates in Hungary. Ettore Randi is head of Conservation Biology and Genetics at INFS.

Appendix I

List of cat samples used in this study. Criteria used for taxonomic identifications: A = coat colour pattern (Ragni & Possenti 1996; Daniels *et al*. 1998), B = life history (Herrmann 1990), C = intestinal index (Schauenberg 1977), D = cranial index (Schauenberg 1969; Ragni & Randi 1986), E = sampling area (Ragni 1993), F = DNA analyses (Beaumont *et al*. 2001; Randi *et al*. 2001). Populations with the same identification (ID) were pooled in population genetic analyses. Numbers refer to the sampling locations that are mapped in Fig. 1

Taxonomic group (Latin name and sample size)	Geographic origin and sampling location	Prior taxonomic information	Sample size	Sample collectors
Domestic cats (*Felis silvestris catus*; *n* = 130)	1 – Italy: Umbria	A, B	11	B. Ragni
	2 – Italy: Emilia-Romagna	A, B	38	E. Randi
	3 – Germany: Solling (north)	A, B, C, D	6	K. Hupe
	4 – Germany: Rheinland-Pfalz (southwest)	A, B, C, D	18	M. Herrman
	5 – Switzerland	A, B	3	M. Liberek
	6 – Great Britain: Scotland, Surrey, Yorkshire	A, B, C, D	23	A. Kitchener
	7 – Hungary	A, B, C, D	29	Z. Biro
	8 – Portugal	A, B	2	M. Fernandes
European wildcats (*F. s. silvestris*; *n* = 165)	9 – Italy: Central Apennines and Tuscany	A, B, C, D	38	B. Ragni, A. Sforzi
	10 – Italy: Southern Apennines (Calabria)	A, B, C, D	1	B. Ragni
	11 – Italy: Sicily	A, B, C, D	7	B. Ragni
	12 – Northeastern Alps: Italy	A, B, C, D	2	B. Ragni
	13 – Northeastern Alps: Slovenia	A, B	2	D. Huber
	14 – Germany: Solling (north)	A, B, C, D	27	K. Hupe
	15 – Germany: Rheinlan-Pfalz (southwest)	A, B, C, D	24	M. Herrman
	16 – Switzerland	A, B	6	M. Liberek
	17 – Belgium	A,B	19	R. Pirlot
	18 – Scotland	A, B, C, D	3	A. Kitchener
	19 – Hungary	A, B, C, D	17	Z. Biro
	20 – Bulgaria	A, B, C, D	6	F. Suchentrunch
	21 – Portugal	A, B	13	M. Fernandes
Sardinian wildcats (*F. s. libyca*; *n* = 16)	22 – Italy: Sardinia	A, B, C, D, E, F	16	B. Ragni
Hybrids (*n* = 25)	23 – Italy: Captive	A, B, C, D, F	7	B. Ragni
	24 – Scotland	A, B, F	17	A. Kitchener
	25 – Italy: Tuscany	A, B, C, D, F	1	A. Sforzi