Genetic Identification of Wild and Domestic Cats (*Felis silvestris*) and Their Hybrids Using Bayesian Clustering Methods

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Crossbreeding with free-ranging domestic cats is supposed to threaten the genetic integrity of wildcat populations in Europe, although the diagnostic markers to identify ''pure'' or ''admixed'' wildcats have never been clearly defined. Here we use mitochondrial (mt) DNA sequences and allelic variation at 12 microsatellite loci to genotype 128 wild and domestic cats sampled in Italy which were preclassified into three separate groups: European wildcats (*Felis silvestris silvestris*), Sardinian wildcats (*Felis silvestris libyca*), and domestic cats (*Felis silvestris catus*), according to their coat color patterns, collection localities, and other phenotypical traits, independently of any genetic information. For comparison, we included some captive-reared hybrids of European wild and domestic cats. Genetic variability was significantly partitioned among the three groups (mtDNA estimate of $F_{ST} = 0.36$; microsatellite estimate of $R_{ST} = 0.30$; $P < 0.001$), suggesting that morphological diversity reflects the existence of distinct gene pools. Multivariate ordination of individual genotypes and clustering of interindividual genetic distances also showed evidence of distinct cat groups, partially congruent with the morphological classification. Cluster analysis, however, did not enable hybrid cats to be identified from genetic information alone, nor were all individuals assigned to their populations. In contrast, a Bayesian admixture analysis simultaneously assigned the European wildcats, the Sardinian wildcats, and the domestic cats to different clusters, independent of any prior information, and pointed out the admixed gene composition of the hybrids, which were assigned to more than one cluster. Only one putative Sardinian wildcat was assigned to the domestic cat cluster, and one presumed European wildcat showed mixed (hybrid) ancestry in the domestic cat gene pool. Mitochondrial DNA sequences indicated that three additional presumed European wildcats might have hybrid ancestry. These four cats were sampled from the same area in the northernmost edge of the European wildcat distribution in the Italian Apennines. Admixture analyses suggest that wild and domestic cats in Italy are distinct, reproductively isolated gene pools and that introgression of domestic alleles into the wild-living population is very limited and geographically localized.

Introduction

Closely related African wildcats, European wildcats, and domestic cats, widely distributed in Europe, Asia, and Africa (Nowell and Jackson 1996), form a distinct clade within *Felis,* the domestic cat lineage (Masuda et al. 1996; Johnson and O'Brien 1997). Speciation in Felis was recent, as suggested by morphological, biochemical, and molecular studies (Ragni and Randi 1986; Randi and Ragni 1991; Masuda et al. 1996; Johnson and O'Brien 1997). Randi and Ragni (1991) recommended that *Felis silvestris* Schreber, 1777, be regarded as a single polytypic species with three wild subspecies, the African (*Felis silvestris libyca*), European (*Felis silvestris silvestris*), and Asian (*Felis silvestris ornata*) wildcats, and a domesticated form (*Felis silvestris catus*) originating from north African and Near Eastern African wildcat populations (Wozencraft 1993; Clutton-Brock 1999).

The Sardinian wildcats belong to the *F. silvestris libyca* group (Ragni 1981; Amori, Angelici, and Boitani 1999) and originate from African wildcats which were introduced by Neolithic navigators into the island, as into Cyprus and Crete, about 6,000–8,000 years ago (Davis 1987) at an early stage of domestication, well before the domestication process was completed by the Egyptians about 4,000 years ago (Malek 1993). Historical evidence of tamed or early-domesticated cats was found at Etruscan and Greek archaeological sites from the beginning of the fifth and fourth centuries B.C. in Italy (Keller 1908; Ragni and Ragni 2001). Thereafter, the Romans probably spread domesticated cats throughout continental Europe and Great Britain (Clutton-Brock 1999).

Nowadays, domestic cats are distributed worldwide and virtually sympatric with European and African wildcats almost everywhere. Domestic cats and wildcats can interbreed and produce fertile offspring in captivity and in nature (Robinson 1977; Ragni 1993). The protracted coexistence of free-ranging domestic cats and wildcats lead one to suppose that interbreeding might be widespread and that ''pure'' wildcat populations would eventually no longer exist in parts of Europe (Suminski 1962; French, Corbett, and Easterbee 1988), the Middle East (Mendelssohn 1999), and South Africa (Stuart and Stuart 1991). Except for coat color variability, which is controlled by just a few genes (Robinson 1977), domestication did not drastically modify the morphology of cats. Therefore, morphological and morphometrical studies did not find evidence of diagnostic traits suitable for identify hybrids and/or introgressed cat populations (Balharry and Daniels 1998; Daniels et al. 1998). More-

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Table 1
Origin, Sample Size, and Identification of Cats (*Felix silvestris*) Included in this Study

Latin Name	Group[a]	ID	Geographic Origin[b]	Samples
F. silvestris catus.	Domestic cats	*Fca*	Italy: Umbria	12
			Italy: Emilia-Romagna	38
F. silvestris silvestris.	European wildcats	*Fsi*	Italy: Northeastern Alps	2
			Italy: Central Appennines and Tuscany Maremma	39
			Italy: Sicily	7
F. silvestris libyca	Sardinian wildcats	*Fli*	Italy: Sardinia	17
	African wildcats		South Africa[c]	6
F. silvestris × *catus*	Hybrids[d]	*Hy*	Captive	7

[a] Groups are based on phenotypic traits; see *Materials and Methods.*
[b] For a distribution map of wildcats in Italy, see figure 1 (Randi and Ragni 1991).
[c] Samples obtained thanks to Colleen O'Ryan, University of Cape Town, South Africa.
[d] Obtained in captivity.

over, the fear of widespread hybridization made uncertain any identification of ''pure'' wildcats to be used as references for taxonomy and for studies of population diversity (Balharry and Daniels 1998; Daniels et al. 1998).

Studies using allozyme electrophoresis, DNA analyses of nuclear genes, and mitochondrial sequences (Randi and Ragni 1991; Hubbard et al. 1992; Randi et al. 2000) indicated limited differentiation between wild-living and domestic cats, while the use of hypervariable nuclear markers (microsatellites) recently provided more stimulating results (Randi et al. 2000; Beaumont et al. 2001). Usually, microsatellites are variable enough to allow for the unequivocal identification of all the sampled individuals in a population. Thus, individuals can be used as units for clustering procedures, such as multivariate ordination of individual scores (Sneath and Sokal 1973, pp. 245–253), or genetic distance-based approaches (Bowcock et al. 1994). These methods are simple and intuitive, but evaluating the consistency and statistical significance of clusters, which must be identified visually, may be problematic. Therefore, these methods are more suited to exploratory data analysis than to precise statistical inference (Pritchard, Stephens, and Donnelly 2000). More efficient methods include a variety of maximum-likelihood assignment procedures (Paetkau et al. 1995; Rannala and Mountain 1997; Cornuet et al. 1999) and Bayesian clustering models (Pritchard, Stephens, and Donnelly 2000). In these procedures, individual genotypes can be assigned to populations irrespective of whether or not their potential source populations are known. The origin of individuals can be determined by calculating the probability of each individual multilocus genotype in each population, assuming that the individual comes from that population. Bayesian models aim to infer the structure of a data set by assuming that observations from each sample are random draws from unknown gene frequency distributions in which the marker loci are unlinked and at Hardy-Weinberg (HWE) and linkage (LE) equilibrium. Population structure within a data set is detected by the presence of Hardy-Weinberg and linkage disequilibrium and is modeled by assuming that the genotype of each individual is a mixture drawn at random from a number of different populations. The number of contributing populations can be estimated and, for a given number of populations, their gene frequencies and the admixture proportions for each individual are all jointly estimated. In this way, the sampled population is subdivided into a number of different subpopulations that effectively cluster the individuals. Then, individuals of a priori known or unknown origin may be assigned probabilistically to the subpopulations.

In this study, we analyzed mtDNA sequences and allele frequency variation at 12 feline microsatellite loci in Italian wild and domestic cats, with the following aims: (1) to estimate the extent of genetic differentiation between cats which were preclassified as wild and domestic using only morphological traits (prior phenotypic information), and (2) to infer the presence of genetically differentiated clusters assuming that all the samples may belong to a single indistinct ''population,'' independently of any prior classification, by means of multivariate ordination, interindividual genetic distances, and Bayesian clustering. Once distinct populations were identified, we used Bayesian methods to assign (or exclude) outlier individuals to the populations and infer their ancestry independently of any prior information.

Materials and Methods

Sample Collection and DNA Extraction

A total of 128 cat tissue and blood samples were collected from several localities in Italy and South Africa (table 1 and fig. 1). House-living domestic cats were obtained through veterinary practices or from road-killed animals collected in central Italy. Wildcats were collected mainly from road kills and local trapping projects in peninsular Italy (northeastern Italian Alps, central Apennines) in Sicily and Sardinia (for the distribution of wildcats in Italy, see fig. 1; Randi and Ragni 1991). Hybrids, obtained in captivity from controlled *silvestris* × *catus* crosses (Ragni 1993), as well as African wildcats from South Africa, were included in this study to provide reference groups. Tissues were preserved in 100% ethanol and blood in a Tris/SDS buffer (Longmire et al. 1988) and stored at −20°C. Total DNA was extracted from tissues using a guanidinium-silica protocol (Gerloff et al. 1995) and from blood using a salting-out procedure (Miller, Dykes, and Poleski 1988).

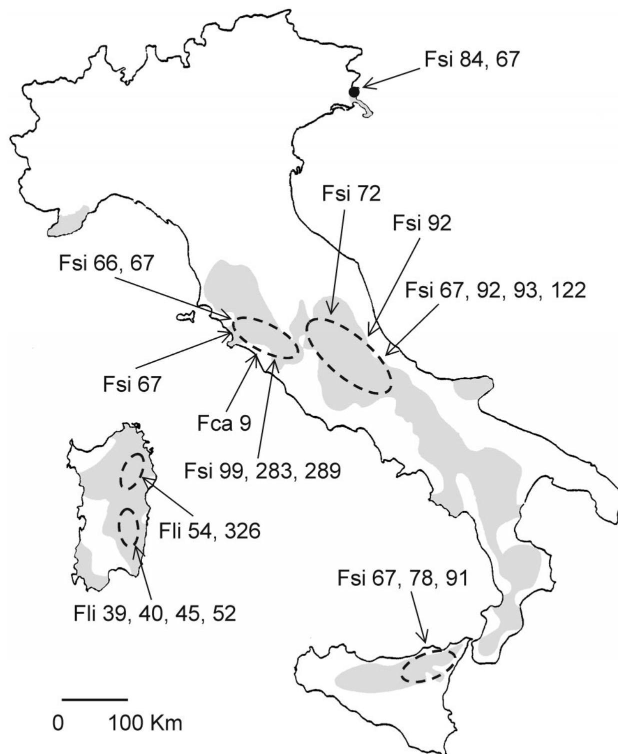

FIG. 1.—Map showing the distribution ranges of wildcats in Italy (in gray) and the locations of the sampled individuals and mtDNA haplotypes (for haplotype identification, see the appendix at the journal website [http://www.molbiolevol.org/]).

Characters Used in Nongenetic Classification

Almost all of the studied wildcats (except a few cats which were collected and identified by collaborators) were identified using the available procedures of morphological classification: coat-color pattern and markings system (Ragni and Possenti 1996) and cranial and intestinal indices (Schauenberg 1969, 1977; Ragni and Randi 1986). Cats were subdivided into three groups: European wildcats, Sardinian wildcats, and domestic house-living cats. All of the wildcats showed the typical wildcat coat color patterns (Ragni and Possenti 1996), and we did not observe wildcats showing phenotypical traits suggesting hybridization. The known hybrid cats kept in captivity were excluded from the analysis of allele frequencies, but their ancestry was investigated by clustering procedures and assignment testing. The African wildcats from South Africa were used for cluster analyses of mtDNA haplotypes and ordination of individual microsatellite genotypes, but they were excluded from analyses of genetic diversity within and among Italian cat groups, as well as from Bayesian assignment testing.

Mitochondrial DNA Sequencing and Microsatellite Genotyping

We PCR-amplified about 1,100 bp of mtDNA using the primers CATDL1 (5′-AAC ATC CGT TCA TCA CCA TCG GGC-3′) and CATDH1 (5′-GAA TAG CAC CCT GAC TGT CTG TGC G-3′), which match nucleotides 16068 and 191 of the domestic cat mtDNA (Lopez, Cevario, and O'Brien 1996) and include 107 bp of the 3′ terminal part of the cytochrome *b* gene, the entire tRNA-Pro and tRNA-Thr, and part of the 5′ hypervariable domain of the mtDNA control region. These primers were designed to flank a portion of the mitochondrial genome excluding the feline nuclear mitochondrial transposition (*numt*; Lopez et al. 1994). However, in a few cases, we amplified putative *numt* sequences which were divergent and phylogenetically basal to the true mtDNA sequences (detailed analyses of these findings will be reported in another paper). By comparing mtDNA and *numt* sequences, we designed the new primers FCAD16234H (5′-CCC TCC CTA AGA CTT CAA GGA AGA-3′, which binds at position 16234 within the tRNA-Thr), FCAD16460LMT (5′-GGG GTG AGT TGG TGG TTA ATA GAG-3′), and FCAD16460LNU (5′-GGG TTG AGT TGG TGG TTA ATA GGA-3′), which bind at position 16460 of the mtDNA and *numt* sequences, respectively. These primers, alternatively paired with FCAD16234H, amplified a fragment of ca. 230 bp of either mitochondrial or nuclear origin. Moreover, we have amplified an mtDNA fragment ca. 750 bp long, including the complete lysine tRNA, the complete ATPase 8, and the first 100 bp of ATPase 6, using primers ATP68H87 (5′-GGC TCA AAC CAT AGC TTC ATA CC-3′) and ATP68L94 (5′-GCA TAG GAA TTA GGG GGA CAG G-3′), which bind at positions 8502 and 9239, respectively.

PCRs were performed in 10-μl reactions (10 mM Tris-HCl, 2 mM $MgCl_2$, 50 mM KCl, 0.1 μg BSA, 0.5 U of *Taq* DNA polymerase, 2.5 pmol of each primer) with 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by 10 min of final extension, in a Perkin Elmer 9600 thermocycler. PCR products were purified with shrimp alkaline phosphatase and S1 exonuclease (U.S. Biochemicals) and sequenced using ABI Dye Terminators. Sequences were analyzed in an ABI 373 automated sequencer, corrected using the software SEQUENCE NAVIGATOR MT 1.0, and aligned using ClustalX (Thompson et al. 1997; ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). The alignments were edited using SE-AL 1.0a1 (http://evolve.zoo.ox.ac.uk/Se-Al/Se-Al.html).

Twelve microsatellites (listed in table 2) originally isolated in the domestic cat (Menotti-Raymond and O'Brien 1995; Menotti-Raymond et al. 1999) were PCR-amplified in 9-μl reaction volumes (10–20 ng of genomic DNA, 1.5 pmol of each primer, 100 μM of each dNTP, 1.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl [pH 8.8], 0.01% Tween-20, 1 μl DMSO, and 0.4 U of *Taq* DNA polymerase) using primers end-labeled with ABI dyes and 40 thermal cycles (94°C for 1 min, 50–55°C for 30 s, 72°C for 30 s, and 72°C for 10 min). PCR products were analyzed in an ABI 373 automated sequencer. Allele sizes were estimated using the Southern Local method with ABI software GENESCAN 2.1, and individual genotypes were determined using GENOTYPER 2.1.

Table 2
Description of the Microsatellite Loci Used and Summary of the Allelic Variation in the Studied Domestic Cat and Wildcat Samples

Locus ID[a]	Chromosomal Assignment[a]	No. of Alleles[b]	Size Range[c]	H_E[d] in *Fca*	H_E in *Fsi*	H_E in *Fli*
FCA008 . .	A1	15	119–151	0.861	0.794	0.719
FCA023 . .	B1	12	130–154	0.783	0.594	0.781
FCA026 . .	D3	14	136–162	0.827	0.820	0.684
FCA043 . .	C2	10	118–138	0.701	0.680	0.752
FCA045 . .	A1	19	144–163	0.836	0.830	0.760
FCA058 . .	A2?	9	209–231	0.619	0.742	0.732
FCA077 . .	C2	9	141–157	0.743	0.741	0.658
FCA088 . .	B3	12	102–126	0.865	0.519	0.757
FCA096 . .	A2	18	179–231	0.639	0.875	0.594
FCA126 . .	B1	12	113–151	0.793	0.740	0.785
FCA132 . .	D3	18	132–160	0.841	0.813	0.857
FCA149 . .	B1	10	120–138	0.760	0.678	0.717

[a] Locus identifications (ID) and chromosomal assignments are from Menotti-Raymond et al. (1999).

[b] Total numbers of alleles found in the studied domestic cats and wildcats.

[c] Allele size range in base pairs; including the primers.

[d] H_E is the expected heterozygosity (Nei 1987, eq. 7.39, p. 164) as computed using FSTAT.

Analyses of Genetic Variation

The distributions of mtDNA haplotypes and microsatellite alleles were evaluated separately for domestic cats, European wildcats, and Sardinian wildcats. Differences in observed (H_O) and expected (H_E) heterozygosity between wild and domestic cats were tested with the Wilcoxon signed-ranks test. We used the programs GENEPOP 3.1d (Raymond and Rousset 1995; http://www.cefe.cnrs-mop.fr/), FSTAT 2.9.1 (Goudet 1995; http://www.unil.ch/izea/softwares/fstat.html), and ARLEQUIN 2.0b2 (http://anthropologie.unige.ch/arlequin) to compute values of standard genetic diversity indices and their variances, to compute pairwise estimates of F_{ST} (Weir and Cockerham 1984) and R_{ST} (Slatkin 1995), and to test pairwise linkage equilibria at all microsatellite loci over the three groups. Deviations from HWE were tested for all locus-population combinations and globally using the Markov chain method of Guo and Thompson (1992), implemented in GENEPOP. Significance levels were adjusted using the sequential Bonferroni method to take into account multiple tests on the same data set (Rice 1989). Differentiation between groups was also assessed by analysis of molecular variance (AMOVA; Excoffier, Smouse, and Quattro 1992), as implemented in ARLEQUIN, using analogs of F_{ST} and R_{ST} (Michalakis and Excoffier 1996) for microsatellite data and the estimator Φ_{ST} (Excoffier, Smouse, and Quattro 1992) for the mtDNA data. Pairwise F_{ST} values were computed to estimate genetic divergence among groups using ARLEQUIN.

Genetic Distances, Ordination Plots, and Clustering of Genotypes

The aligned mtDNA sequences were analyzed using PAUP* 4.0b2a (Swofford 1998). The best-fit maximum-likelihood model of DNA substitution was the HKY model (Hasegawa, Kishino, and Yano 1985) with among-sites substitution heterogeneity, which was selected by likelihood ratio tests among a suite of models of increasing complexity (Huelsenbeck and Crandall 1997). The values of shape parameter α of the γ distribution (Yang 1994) and the transition/transversion (Ti/Tv) ratios were estimated by the data set using maximum likelihood with the HKY model and four discrete-rate categories. Phylogenetic trees were obtained by neighbor-joining (NJ; Saitou and Nei 1987) with HKY+γ DNA distances, and maximum parsimony (MP) with unordered and equally weighted characters. Robustness of the phylogenies was assessed by bootstrap percentages computed using 1,000 random resamplings with replacement. A minimum-spanning network among mtDNA haplotypes was constructed using ARLEQUIN.

Interindividual microsatellite genetic distances, including the 1 − proportion of shared alleles (DPS; Bowcock et al. 1994) and deltamu ($\Delta\mu^2$; Goldstein et al. 1995) distances, were estimated with MICROSAT 1.5d (http://human.stanford.edu/microsat/microsat.html). Distance matrices were then used to construct NJ trees with the program NEIGHBOR in PHYLIP 3.5c (http://evolution.genetics.washington.edu/phylip.html). In addition, individual genotypes were ordinated in a multidimensional space by principal-component analysis (PCA) using the program PCAGEN (http://www.unil.ch/izea/softwares/pcagen.html).

Bayesian Clustering, Genetic Admixture Analysis, and Population Assignment

Pritchard, Stephens, and Donnelly (2000) described a Bayesian clustering method (implemented in the program STRUCTURE; http://www.stats.ox.ac.uk/~pritch/home.html) which uses multilocus genotypes to infer population structure and simultaneously assign individuals to populations. This model assumes that there are K populations (where K may be unknown), each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned probabilistically to populations, or jointly to two or more populations if their genotypes indicate that they are admixed. This method can be used to detect the presence of cryptic population structure and to perform assignment testing. Pritchard, Stephens, and Donnelly's (2000) model assumes HWE and LE among the unlinked marker loci. Departures from HWE and LE lead the population to be split into subpopulations, to which individuals are assigned, and those with admixed ancestries are assigned to more than one source population. In this study, the posterior probabilities of K (i.e., the likelihood of K as a proportion of the sum of the likelihoods for different values of K) are estimated assuming uniform prior values on K between 1 and 5 (option MAXPOPS = 1–5). The presence of structure in the data set is revealed by the increasing likelihood of the data. The results presented in this study are based on 100,000 iterations, following a burn-in period of 10,000 iterations.

Table 3
Genetic Diversity Indices for Cats Grouped According to Phenotypic Criteria (see *Materials and Methods*)

Genetic Markers	Genetic Diversity Indices	*Fca*	*Fsi*	*Fli*[a]
mtDNA[b]	No. of genotyped cats	46	40	17
	No. of haplotypes	24	17	6
	No. of polymorphic sites (transitions/transversions/indels)	43 (28/2/14)	27 (25/1/1)	16 (14/0/2)
	Mean no. of pairwise differences (SD)	5.74 (2.80)	5.75 (2.81)	6.38 (3.18)
	Gene diversity (SD)	0.92 (0.03)	0.91 (0.03)	0.77 (0.07)
	Nucleotide diversity (SD)	0.008 (0.004)	0.008 (0.004)	0.009 (0.005)
Microsatellites[c]	No. of genotyped cats	49	48	17
	No. of private alleles	6	3	4
	No. of pairwise differences (SD)	9.13 (4.23)	8.56 (3.99)	8.36 (3.97)
	Observed heterozygosity (SD across loci)	0.70 (0.08)	0.60 (0.16)	0.71 (0.09)
	Expected heterozygosity (SD across loci)	0.76 (0.08)	0.71 (0.10)	0.70 (0.07)
	Average value of F_{IS}[d]	0.094 ($P \leq 0.05$)	0.179 ($P \leq 0.05$)	0.027 (NS)

[a] Estimates of genetic diversity include only African wildcats form Sardinia.
[b] Mitochondrial DNA sequences were 692 bp long on average.
[c] Twelve loci.
[d] Departures from Hardy-Weinberg equilibrium were tested using the average values of F_{IS}.

A Bayesian assignment procedure is implemented in STRUCTURE, where individuals are assigned probabilistically to one or more predefined subpopulations using or not using prior population information.

Results

Analysis of Genetic Variation: mtDNA Sequences

We sequenced about 700 bp of mtDNA control region (CR), ATPase 8, and ATPase 6 from 108 cat samples. These sequences comprised 46 haplotypes in the Italian cats and 3 distinct haplotypes in the South African wildcats, defined by 69 variable sites showing 70 nucleotide substitutions and 17 indels that were limited to the CR (an alignment of variable nucleotide positions and all the individual haplotypes and their EMBL accession numbers are reported in an appendix that can be retrieved at the journal website [http://www.molbiolevol.org/]; an alignment of the complete sequences can be retrieved at ftp://ftp.ebi.ac.uk/pub/databases/embl/align, accession number: ALIGN_000094). Haplotypes were not shared among the three cat groups, except for haplotype Fca9, which was found in three domestic cats and three European wildcats. A summary of mtDNA variation is shown in table 3. Gene diversity was slightly lower in Sardinian wildcats than in European wildcats and domestic cats, while the mean number of pairwise sequence differences was higher among Sardinian wildcats. Sequence divergence among haplotypes was <1% on average, suggesting recent time to the most recent common ancestor in the three groups.

Mitochondrial DNA variability was significantly partitioned among the three groups (Φ_{ST} = 0.39; $P <$ 0.001; AMOVA). Pairwise divergence between domestic cats and Sardinian wildcats (Φ_{ST} = 0.22) was 50% lower than that between these two groups and the European wildcats (Φ_{ST} = 0.42–0.44; table 4), in accordance with current hypotheses suggesting that the domestic cat is derived from African wildcats, the *libyca* group, to which the Sardinian wildcat belongs. However, because the sample used in this study is a subset of the full geographic distribution of domestic cats and wildcats, we are unable to make reliable estimates of genetic divergence to infer divergence times between *F. silvestris libyca* and *F. silvestris silvestris* and the age of domestication.

Table 4
Genetic Divergence Among Wild and Domestic Cats Grouped According to Phenotypic Criteria

(*a*)

Groups	mtDNA	*Fca*	*Fsi*	*Fli*
Fca. . . .	Linearized F_{ST}	—	0.73	0.29
Fsi		0.42	—	0.79
Fli	F_{ST}	0.22	0.44	—

(*b*)

Groups	Microsatellites	*Fca*	*Fsi*	*Fli*
Fca. . . .	R_{ST}	—	0.32	0.14
Fsi		0.13	—	0.38
Fli	F_{ST}	0.10	0.15	—

NOTE.—(*a*) mtDNA sequence divergence was estimated using about 700 bp. Upper triangular matrix: linearized F_{ST} (Slatkin 1995); lower triangular matrix: pairwise estimates of F_{ST} (Weir and Cockerham 1984). (*b*) Values of pairwise F_{ST} and R_{ST} (Michalakis and Excoffier 1996) were estimated at 12 microsatellites. All values were highly significant ($P < 0.001$) as tested by randomization (10,000 permutations) using ARLEQUIN.

Analysis of Genetic Variation: Microsatellites

We determined the individual genotypes at 12 loci in 127 cat samples. All microsatellites were polymorphic, showing 9–19 different alleles per locus (average 13.17 ± 3.61), and values of H_E ranging from 0.519 to 0.875 in domestic cats and wildcats (table 2). The allele frequency distributions varied across loci, and some of them (i.e., FCA58, FCA126, and FCA132) showed marked differences between wild and domestic cats (fig. 2). At a threshold frequency of 5%, chosen to reduce

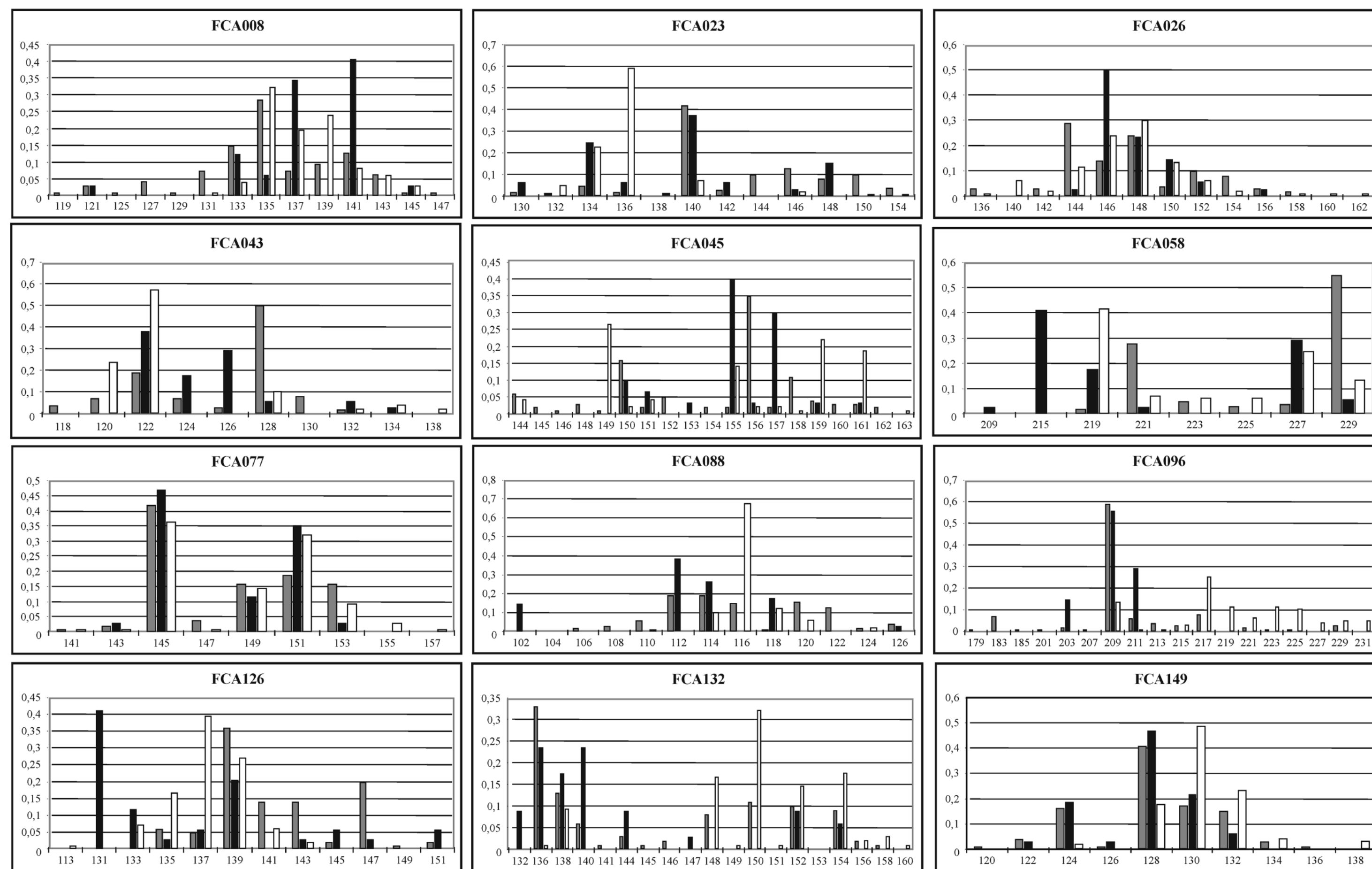

FIG. 2.—Histogram illustrating the frequency distributions of microsatellite lengths of Italian wildcats (in white), Sardinian wildcats (in black), and domestic cats (in gray).

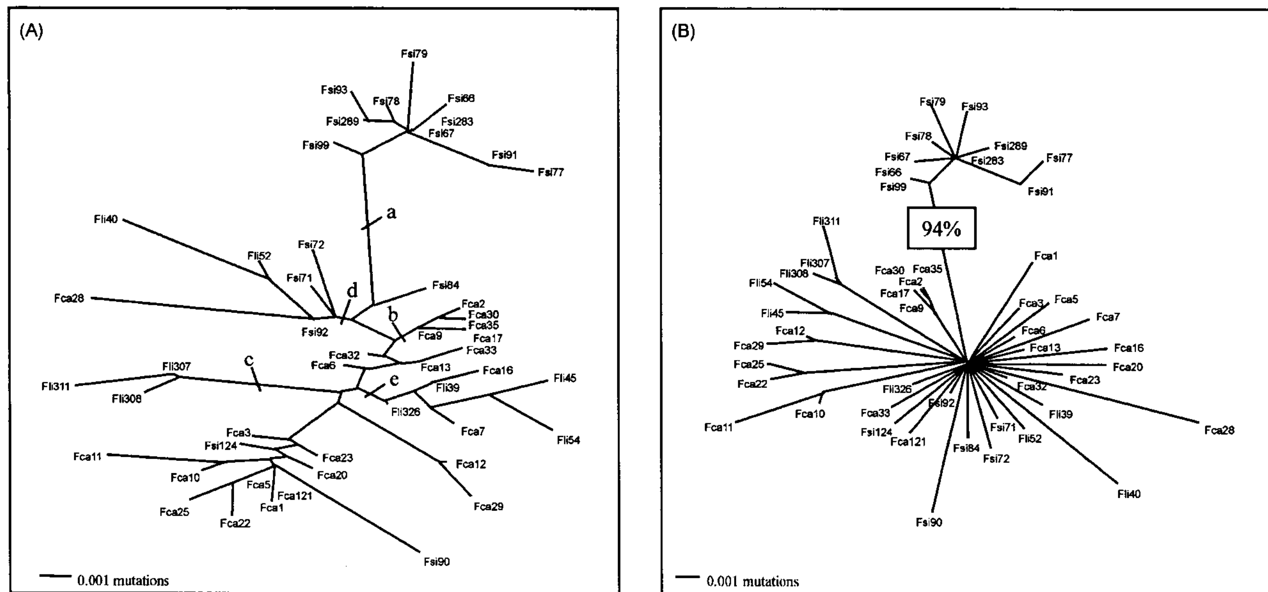

FIG. 3.—*A,* Unrooted neighbor-joining tree of European wildcat (Fsi), Sardinian and African wildcat (Fli), and domestic cat (Fca) mtDNA haplotypes (see also fig. 1 and the appendix at the journal website [http://www.molbiolevol.org/]) computed using the program NEIGHBOR in PHYLIP 3.5c with the HKY+γ model of nucleotide substitutions (letters indicate clusters which are described in the text; see *Results*). *B,* The same neighbor-joining tree showing only the internodes with bootstrap support >50%. Branch lengths are proportional to the number of nucleotide substitutions estimated using the HKY+γ model.

the effects of sampling error, there were 13 private alleles, 6, 4, and 3 of which were present in domestic cats, Sardinian wildcats, and European wildcats, respectively (table 3). The microsatellite loci either are on separate chromosomes or are spaced enough on the same chromosome (Menotti-Raymond et al. 1999) to allow for independent allele recombination (table 2). Pairwise allelic combinations were in LE at all loci over the three groups except in three cases in European wildcats and two cases in domestic cats ($P < 0.05$, Bonferroni-corrected for 396 comparisons).

A summary of microsatellite variation is shown in table 3 (full individual genotypes are available at http://www.molbiolevol.org/). The average number of pairwise allelic differences and average H_O and H_E were not significantly different among the three groups. European wild and domestic cats showed significant deficits of heterozygotes at 5 and 2 of 12 loci, respectively ($P < 0.05$, Bonferroni-corrected), and overall, they were not in HWE. Average F_{IS} values were significantly positive in European wildcats ($F_{IS} = 0.179$; $P < 0.05$) and domestic cats ($F_{IS} = 0.094$; $P < 0.05$). On the contrary, the Sardinian wildcats were in HWE (average $F_{IS} = 0.027$; NS). Domestic cats do not belong to a single breeding population and are expected to deviate from HWE as consequence of inbreeding within reproductively separated populations. Deviations from HWE due to heterozygote deficit in European wildcats may indicate local inbreeding and the presence of geographical population structuring in Italy (Wahlund effect; Hartl and Clark 1989; pp. 282–296).

Microsatellite variability was significantly partitioned among the three groups ($F_{ST} = 0.13$; $R_{ST} = 0.30$; $P < 0.001$; AMOVA), suggesting that phenotypic classification reflects significant genetic differences among cats. The estimated R_{ST} distances were more than two times F_{ST}, suggesting that cats differ in distributions of both allele frequency and allele size. Domestic cats and Sardinian wildcats showed the lowest pairwise F_{ST} and R_{ST} values (table 4), which, once again, supports current hypotheses on domestication.

Clustering of mtDNA Sequences and Ordination Plot of Individual Cats

Phylogenetic clustering of mtDNA sequences produced very similar NJ or MP trees. The unrooted NJ tree (fig. 3*a*), obtained using the best-fit substitution model (HKY with γ-rate heterogeneity; $\alpha = 0.75$ and Ti/Tv = 29, as estimated from the data), showed a number of lineages joining haplotypes which were found in more than one group, except lineage **a**, which included only European wildcat haplotypes. Lineage **b** is mainly a domestic cat lineage but includes haplotype Fca9, which was found also in three European wildcats (see the appendix at the journal website). Haplotypes from South African wildcats were distinct (lineage **c**), and the haplotypes from Sardinian wildcats split into two different lineages, including also domestic cats and European wildcats (lineages **d** and **e**). Phylogenetic signal from these sequences was weak, and most of the clades were not supported after 1,000 bootstrap replications (fig. 3*b*), suggesting that mtDNAs of cats diversified rapidly. Weak phylogenetic resolution makes it difficult to use these mtDNA sequences to infer group distinction, divergence times, and eventual hybridization. However, mtDNA analyses indicated that (1) European wildcats sampled in Italy harbor at least two distinct

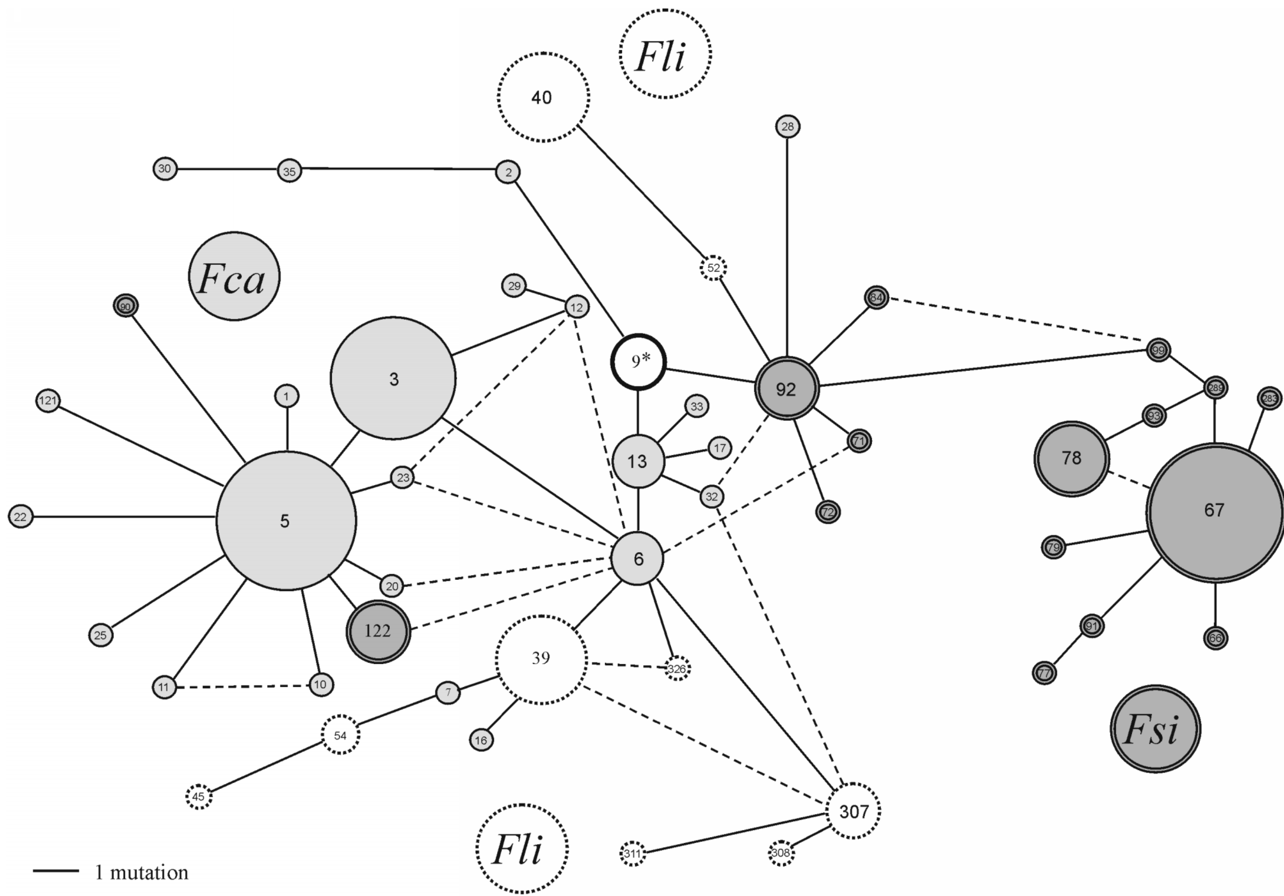

FIG. 4.—Minimum-spanning network of European wildcat (Fsi, dark-gray circles), Sardinian and South African wildcat (Fli, empty circles), and domestic cat (Fca, light-gray circles) mtDNA haplotypes, computed using ARLEQUIN and the number of observed nucleotide substitutions as a measure of interhaplotype genetic distance. Circle size is proportional to the observed frequency of each mtDNA haplotype, numbers within circles refer to single haplotype numbers as listed in the appendix at the journal website (http://www.molbiolevol.org/), the bold circle represents haplotype Fca9 shared between wild and domestic cats. Continuous lines indicate the preferential minimum-spanning network connections among haplotypes; broken lines indicate possible alternative connections among haplotypes.

haplotype groups (lineage **a** and the other haplotypes) diverging by about 2%, and (2) wildcats of African origin include at least three different lineages, thus suggesting that Sardinia was colonized perhaps more than once and certainly not with cats originating from South Africa.

Additional information was obtained by a minimum-spanning network of the mtDNA haplotypes (fig. 4), which supported the existence of (1) three distinct African wildcat lineages; (2) two distinct European wildcat lineages, stemming from haplotypes Fsi92 (corresponding to lineage **d** of the NJ tree; see fig. 3) and Fsi99 (lineage **a**), respectively; and (3) two main groups of domestic cat lineages, the main one of which stems from haplotype Fca5 and includes the European wildcat haplotypes Fsi90 and Fsi122 (the geographical distribution of mtDNA haplotypes in the sampled Italian wildcats is drafted in fig. 1).

The NJ tree clustering interindividual microsatellite distances (DPS = 1 − proportion of shared alleles; Bowcock et al. 1994) is shown in figure 5. Most European and African wildcats and domestic cats split into different clusters, showing, once again, that phenotypic classification reflects genetic differences. However, there were some outlier cats which were apparently placed into the "wrong" clusters: (1) most of the hybrid cats (*Hy*) plus *Fli*311 (from South Africa), *Fca*28, and *Fca*32 joined the European wildcat clusters; (2) the European wildcat *Fsi*284 joined the domestic cat cluster; (3) wildcats from South Africa and Sardinia grouped separately; however, Sardinian *Fli*46 and *Fli*326 joined the domestic cat cluster; and (4) domestic cats *Fca*28, *Fca*30, and *Fca*32 grouped outside of the domestic cat clusters. The main clusters, joining at the central polytomy shown in figure 5, had bootstrap values <20%. Thus, distance-based trees were poorly resolved and of limited utility in identifying hybrids and assigning individuals to populations.

The result of PCA of individual genotypes is shown in figure 6. Individual scores were plotted onto the two principal axes (PC-I and PC-II), which cumulatively explain 19% of the total genetic diversity. This plotting showed a clear separation between domestic cats (placed on the right side of the plot) and European wildcats (placed on the left side of the plot) on PC-I. South African and Sardinian wildcats were distinct, roughly intermediate, and in part overlapping the domestic and European wildcat distributions. Wild and domestic cats

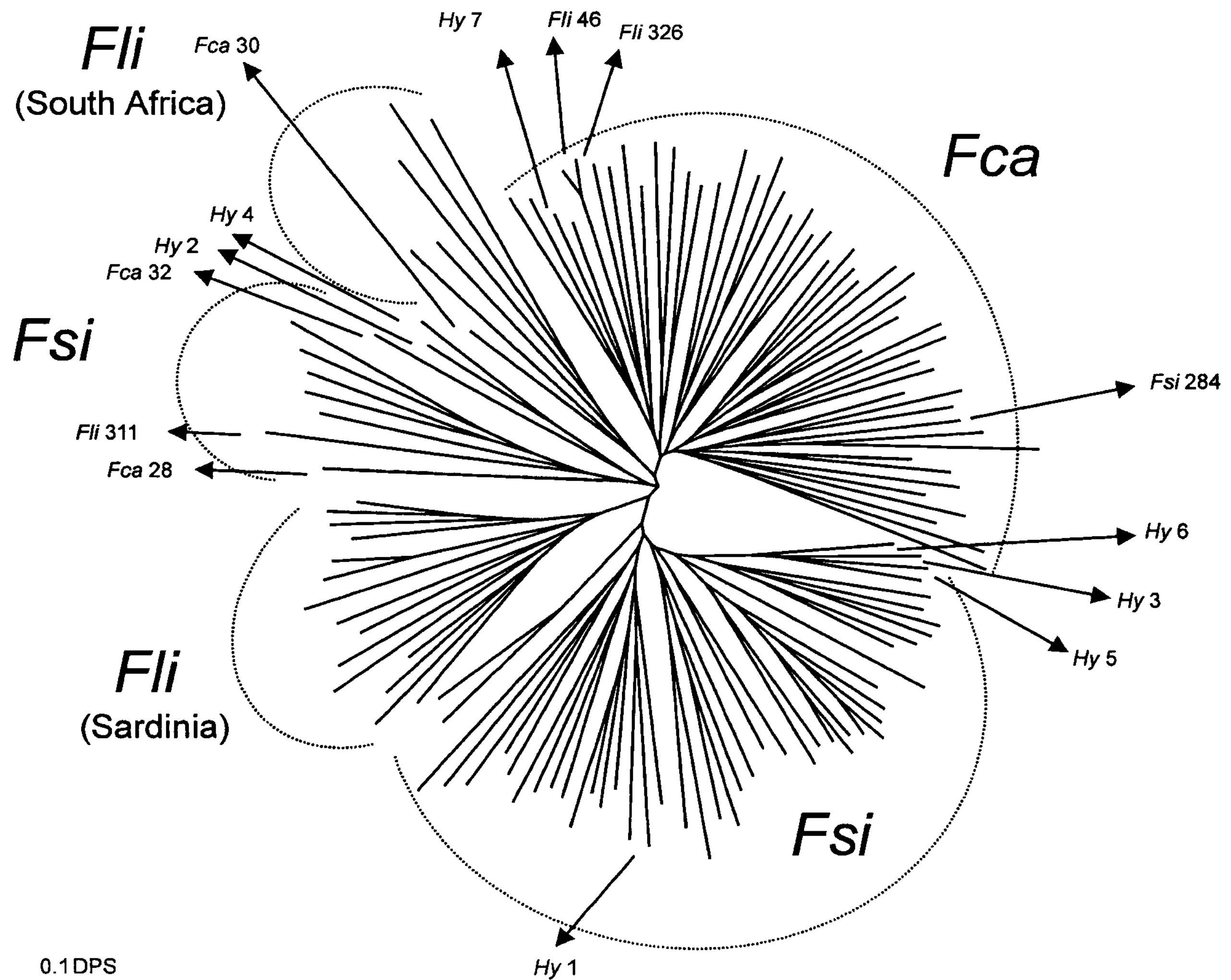

FIG. 5.—Unrooted neighbor-joining tree of individual European wildcats (*Fsi*), Sardinian and South African wildcats (*Fli*), and domestic cats (*Fca*) computed using interindividual pairwise DPS microsatellite distances (Bowcock et al. 1994). Each branch represents a single individual. European wildcats (*Fsi*), Sardinian and South African wildcats (*Fli*), and domestic cats (*Fca*) group mainly in the indicated clusters. Outliers and individuals which appear to be misclassified are indicated by arrows and labels (see table 1). The tree was constructed using the program NEIGHBOR in PHYLIP 3.5c.

were almost totally overlapping on PC-II and all the other axes as well. Except for sample *Hy*4, which was intermediate between wild and domestic cats, all of the hybrid cats were included within the distribution of European wildcats or domestic cats (*Hy*7). Some, but not all, the individuals that were misplaced in distance-based clustering were also misplaced in PCA analysis (e.g., *Fli*326 and F*si*284). However, score distributions were widespread and, without using prior population information, it would be difficult to use PCA to identify hybrids and assign the outlier individuals to the populations.

Genetic Admixture Analysis

For this analysis, we used STRUCTURE (with mtDNA and microsatellite data set and excluding the South African wildcats) with two modeling approaches. First, we assumed uninformative priors on all K; that is, we assumed that before applying the model to the data, all samples belonged to a hypothetical single ''population'' (option USEPOPINFO = 0). The probability of the number of populations (K) for the pooled data was estimated, without using prior population information, by fixing prior values of $K = 1$–5 (option MAXPOPS) and comparing the Ln likelihood of the data. If the hypothetical single ''population'' is admixed and includes more than one subpopulation, then the likelihood of the data will increase with K (Pritchard, Stephens, and Donnelly 2000). Results of this analysis are reported in table 5. The Ln probability of the data was minimum with $K = 1$ population (Ln = −6,172.1) and maximum with $K = 3$ populations (Ln = −5,518.5), thus suggesting that the pooled cat ''population'' was heterogenous and may contain at least three genetically distinct groups.

We therefore estimated the proportion of membership (q) of each predefined group into three clusters representing the ''cryptic'' genetic populations (table 5), that is, the average proportions of genotypes in each predefinite group that are inferred to come from each

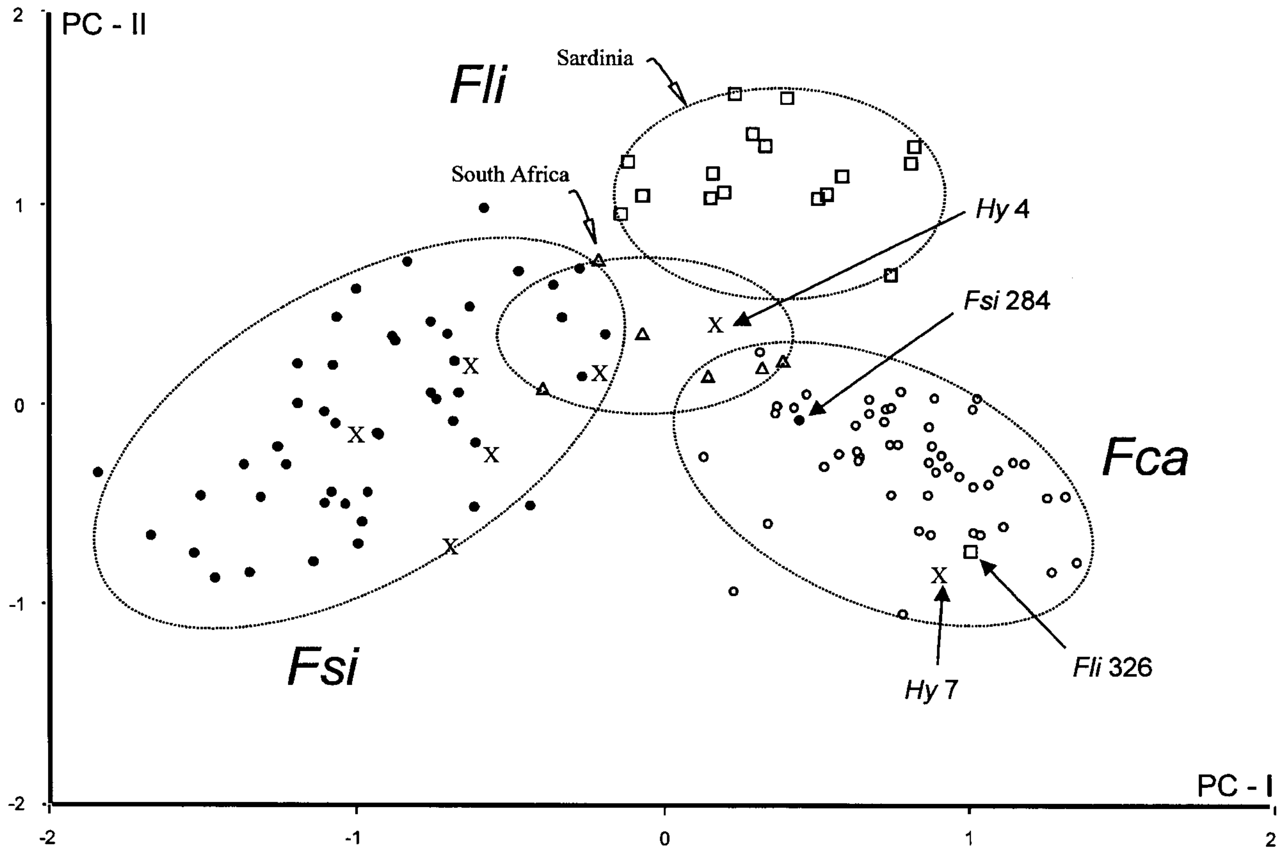

FIG. 6.—Scores of individual European wildcat (*Fsi,* filled circles), Sardinian (*Fli,* squares) and South African wildcat (*Fli,* triangles), and domestic cat (*Fca,* open circles) microsatellite genotypes plotted on the first two axes (PC-I, PC-II) of a principal component analysis performed using PCAGEN. Outliers and individuals which appear to be misclassified are indicated by arrows and labels. Captive-reared hybrids (*Hy*) are indicated by X's.

Table 5
Bayesian Clustering Analyses for the Pooled Cat Samples (121 individuals; 13 loci) Performed Using STRUCTURE (Pritchard, Stephens, and Donnelly 2000)

(*a*)

K	Ln Probability of the Data
1	−6,172.1
2	−5,704.2
3	−5,518.5
4	−5,547.7
5	−5,536.0

(*b*)

	CLUSTER		
POPULATION	I	II	III
Fca	0.970	0.012	0.017
Fsi	0.028	0.963	0.009
Fli	0.069	0.007	0.925
Hy	0.203	0.734	0.063

NOTE.—(*a*) Probability of the number of populations (*K*) for the pooled cat samples. (*b*) Proportion of membership of each predefined population in each of the three inferred clusters.

population. Cluster I grouped the domestic cats (with proportion of membership $q_1 = 0.97$), cluster II grouped the European wildcats (with $q_2 > 0.96$), and cluster III grouped the Sardinian wildcats (with $q_3 > 0.92$), in complete accordance with the nongenetic classification and independent of any prior population information. Interestingly, the hybrid cats had memberships in both the European wildcat cluster II ($q_2 > 0.73$) and the domestic cat cluster I ($q_1 > 0.20$); that is, the hybrids could not be joined into a single cluster, but they were split between two clusters in accordance with their admixed ancestry.

The assignment of individual cats was inferred by STRUCTURE either without (USEPOPINFO = 0) or with (USEPOPINFO = 1) using prior population information. In the first case, domestic cats and European and Sardinian wildcats are probabilistically assigned to cluster I, II, or III. In the second case, we force sampling of all cat genotypes from one of the three different clusters, and STRUCTURE estimates the probability for each sample of having an ancestry in the other groups, either in the sampled generation or in the first or second past generations (q-values were computed with prior intergroup "migration" rate = 0.01; in this context, "immigrant" means "hybrid"). Probabilities of membership are the posterior values of q_i (i = 1, 2, 3) for each

Table 6
Population Assignment and Inferred Ancestry of Individual Cats Estimated Using STRUCTURE

POP	Samples	Cluster I (*Fca*)	Cluster II (*Fsi*)	Cluster III (*Fli*)	mtDNA
0	*Fca* (n = 46)	0.92	0.05	0.07	
	*Fca*28	0.87	0.06	0.07	Fca
	*Fca*32	0.84	0.07	0.09	Fca
	*Fca*35	0.80	0.03	0.17	Fca
	Fsi (n = 46)	0.05	0.93	0.06	
	*Fsi*228	0.18	0.80	0.02	Fsi
	*Fsi*284	0.78	0.21	0.01	—
	Fli (n = 15)	0.01	0.02	0.97	
	*Fli*46	0.11	0.01	0.88	Fli
	*Fli*326	0.99	0.01	0.00	Fli
1	*Fca* (n = 46)	0.98	0.01	0.01	
	*Fca*28	0.98	0.00—0.00—0.01	0.00—0.00—0.01	
	*Fca*32	0.97	0.00—0.00—0.01	0.00—0.00—0.02	
	*Fca*35	0.91	0.00—0.00—0.01	0.00—0.00—0.07	
	Fsi (n = 46)	0.01	0.98	0.01	
	*Fsi*228	0.00—0.00—0.01	0.92	0.00—0.01—0.06	
	*Fsi*284	0.03—0.78—0.11	0.08	0.00—0.00—0.00	
	Fli (n = 15)	0.00	0.00	1.00	
	*Fli*46	0.00—0.00—0.01	0.00—0.00—0.00	0.99	
	*Fli*326	1.00—0.00—0.00	0.00—0.00—0.00	0.00	

Note.—The probability (q) of each genotype to belong to each of the three clusters was computed either without using prior population information (POP = USEPOPINFO = 0) or using information on the prior classification based on nongenetic characters (POP = USEPOPINFO = 1). With POP = 1, STRUCTURE estimates the probabilities of each cat to have an ancestry either in the sampled or first or second past generations (q values computed with prior migration rate = 0.01).

individual, that is, the proportion of each individual genotype originating in one or in more than one cluster. Results (reported in table 6) showed that with USEPOPINFO = 0, cluster I grouped 94% (i.e., 46/49) of domestic cats with individual values of $q_1 \geq 0.92$. Only three cats (preclassified as domestic cats) had individual $q_1 < 0.90$, that is, *Fca*28, *Fca*32, and *Fca*35, which were nevertheless significantly associated with the domestic cat cluster I. Cluster II grouped 96% (i.e., 46/48) of European wildcats with $q_2 \geq 0.93$. Two cats had $q_2 < 0.90$, that is, *Fsi*228 (nevertheless significantly associated with cluster II) and *Fsi*284, which was associated in part with the domestic cat cluster I ($q_1 = 0.78$) and in part with cluster II ($q_2 = 0.21$). Cluster III joined 88% (15/17) of preclassified Sardinian wildcats with $q_3 \geq 0.97$. One cat, *Fli*46, was significantly associated with cluster III, with $q_3 = 0.88$, while *Fli*326 was unambiguously associated to the domestic cat cluster I, with $q_1 = 0.99$.

In the second modeling approach, we assumed that samples should belong to one of three predefined ''groups''—domestic cats, European wildcats, and Sardinian wildcats (excluding the South African samples)—and asked the program to assign the individuals and infer the ancestry of hybrids and outlier cats (USEPOPINFO = 1; table 6). In this case, all domestic cats were assigned to cluster I with individual values of $q_1 \geq 0.91$ and had no significant ancestry in the other clusters or past generations. All the European wildcats were associated with cluster II with $q_2 \geq 0.92$ except *Fsi*284 (a juvenile male found dead by road casualty in Tuscany Maremma, central Italy), which had a significant ancestry in the first past generation of cluster I (the domestic cat cluster) and was therefore probably a hybrid. The value of the cranial index of *Fsi*284 (=2.54) fell within the range of *silvestris,* while its intestinal index (=3.05) was near the upper value for *silvestris,* with 3.15 being the discriminant value between *silvestris* and *catus* (Ragni, Lapini, and Perco 1989). The coat color and marking patterns of *Fsi*284 were out of the *silvestris* range and showed a larger than usual amount and distribution of white-spottings that overcame the putative recessive homozygous condition at locus ''S,'' that is, the typical condition of *silvestris* phenotypes and genotypes (Robinson 1977; Ragni and Possenti 1996). Additional information from the mtDNA sequences was not particularly useful in identifying *Fsi*284 and *Fli*326. We could not produce readable sequences from *Fsi*284, while *Fli*326 showed a unique haplotype which was not shared with any other cat and was not obviously associated with any domestic or wildcat mtDNA lineages (figs. 3 and 4).

All of the Sardinian wildcats had $q_3 \geq 0.99$ (cluster III) except *Fli*326, which was assigned to the domestic cat cluster I with $q_1 = 1.00$ in the sampled generation. The mtDNA haplotype of *Fli*326 was unique, not shared with other cats or clearly associated with any wild or domestic lineages (figs. 3 and 4). Although we cannot definitely exclude the possibility that this cat is a hybrid, mislabeling and wrong phenotypical classification are the most plausible explanations. In conclusion, we found 1 cat in the 114 studied (0.9%) that was genetically assigned to a different group if compared with the morphological preclassification.

We excluded the two outlier cats *Fsi*284 and *Fli*326 from the data set and assumed that all the other cats definitely belonged to three genetically distinct populations (clusters I, II, and III), which can be used as a

Table 7
Inferred Ancestry of Hybrid Cats Estimated Using STRUCTURE

Samples	Cluster I	Cluster II	Cluster III	mtDNA
Fca (n = 48)	1.000	0.000	0.000	—
Fsi (n = 47)	0.000	1.000	0.000	—
Fli (n = 16)	0.000	0.000	1.000	—
*Hy*1	0.140	0.590	0.271	Fsi
*Hy*2	0.263	0.514	0.223	—
*Hy*3	0.112	0.790	0.098	Fca
*Hy*4	0.359	0.289	0.352	Fca
*Hy*5	0.151	0.781	0.068	Fca
*Hy*6	0.183	0.708	0.109	Fca
*Hy*7	0.817	0.101	0.082	—

NOTE.—The probability of each genotype to belong to each of the three clusters was computed using information on the prior classification based on phenotypic characters for *Fca, Fsi,* and *Fli.* STRUCTURE estimates the probabilities of each of the unassigned cats to belong to each of the three clusters (reference putative parental populations). Outlier cats *Fsi*284 and *Fli*326 were excluded from this analysis (118 individuals, 13 loci).

reference for further testing of the assignment of the known hybrids to putative ancestral populations. Assignment was performed using STRUCTURE with prior information on the reference populations only, and not for the hybrid cats to be assigned. In this case, the q values of all reference cats were 1.00 (table 7), while all hybrids always had q values lower than 0.82. All of the known hybrids could be exactly identified as cats having admixed ancestry in more than one cluster.

We chose not to additionally perform analyses using the Rannala and Mountain (1997) and Paetkau et al. (1995) methods. The first method performs significance tests for each individual and for each degree of relationship, thus necessitating some correction for multiple comparisons. In their analysis of a data set previously analyzed using the Rannala and Mountain (1997) method, Pritchard, Stephens, and Donnelly (2000) suggest that their method would give comparable results once such a correction was made. However, it is not clear how to best make the correction when there are many comparisons, since, for example, the Bonferroni procedure is known to be very conservative. Paetkau et al.'s (1995) method considers only first-generation hybrids and is not appropriate for analyzing this data set.

Discussion

Wildcat populations in Eurasia and Africa are often sympatric and potentially hybridizing with domestic cats. Introgression might have occurred for centuries and blurred any distinction between wild and domestic gene pools (Suminski 1962). If a priori known ''pure'' wildcats do not exist, there will be no reference wildcat population to be used for estimating the rate of crossbreeding between wild and domestic cats (Daniels et al. 1998). Domestication produced obvious changes in cats' behavior and coat colors. However, behavioral repertoires are plastic and can rapidly adapt to variable ecological conditions (Todd 1978). Coat colors are controlled by a few genes, and wild-living cats homozygous for coat color genes of domestic origin could automatically be scored as ''domestic.'' Alternatively, natural selection against coat color phenotypes of domestic origin might rapidly reconstruct wild-type phenotypes in wild-living hybrid cats. Under these conditions, therefore, it has been somewhat problematic to unequivocally define morphological criteria that may identify pure wildcats (Balharry and Daniels 1998; Daniels et al. 1998). However, a set of diagnostic morphological traits described by Ragni and Possenti (1996) appear to be good predictors of the genetic distinction between domestic and wild-living cat groups, as suggested by results obtained in this and other recent studies (Beaumont et al. 2001).

In this study, we preclassified cats sampled in Italy using exclusively morphological markings, which, combined with lifestyle traits (behavior of domestic cats) and geographical origins (the wildcats sampled in Sardinia), allowed a clear-cut subdivision of three groups—European wildcats, Sardinian wildcats and domestic cats—corresponding to the three nominal subspecies of *F. silvestris* which are present in Italy: *F. silvestris silvestris, F. silvestris libyca,* and *F. silvestris catus.* Genetic diversification between preclassified cats was as follows: $\Phi_{ST} = 0.39$ (as derived from mtDNA sequence divergence), $F_{ST} = 0.13$, and $R_{ST} = 0.30$ (as derived from microsatellite allele frequency or allele size variability), meaning that about 30% of the total genetic diversity was distributed among groups and that nongenetic classification identified groups that were genetically differentiated. All mtDNA haplotypes (with the exception of haplotype Fca9, which was shared between three domestic cats and three European wildcats; see the appendix at the journal website) were completely sorted among domestic cats and wildcats. The microsatellite R_{ST} distances were more than two times as large as F_{ST}, suggesting that divergence between wild and domestic cats cannot be explained by different distributions in allele frequencies alone; there must also be a shift in the mean allele length, implying divergence over a longer period, rather than recent drift (Slatkin 1995). Moreover, the existence of many ''private'' alleles, some of which are at relatively high frequencies in domestic cats or wildcats, clearly suggests that wild and domestic cats are genetically differentiated and that there is little gene flow among them. Therefore, the hypothesis that frequent crossbreeding with free-ranging domestic cats might have strongly polluted the gene pool of wild populations (Suminski 1962) was not confirmed by these data.

Genetic differentiation among cats was fully recognized by multivariate and NJ clustering procedures, which, without using prior population information, split the cats into separate clusters largely corresponding to the three nominal subspecies (figs. 5 and 6). However, these procedures led to the identification of a number of outliers, which were assigned differently from the nongenetic classification and apparently joined the ''wrong'' clusters. The known hybrid cats were poorly identified in both NJ and PCA procedures, which did not offer objective criteria for assigning individuals to populations.

By contrast, the Bayesian procedure jointly assigns a probability to the number of populations and to the membership of each individual in each population, allowing extraction of precise quantitative information from the data set. The multilocus genotypes from individual cats fit the genetic model (i.e., the assumptions that genetic markers are independent in HWE and LE) better if samples are split into at least three distinct populations. When samples were assigned to three inferred clusters without using any prior population information, the domestic cats and the European and Sardinian wildcats were assigned to clusters I, II, and III, respectively, with the average proportion of individual memberships $q > 0.90$ and without significant ancestry in the other clusters. Only two cats (*Fsi*284 and *Fli*326) among the many putative outliers which were apparently ''misplaced'' in the multivariate or distance-based clustering procedures had significant ancestry in other clusters (table 6). When ancestry of the outlier cats was further investigated using prior population information, results suggested that putative European wildcat *Fsi*284 may be a hybrid with statistically significant ancestry in the first past generation of the domestic cat population, while the Sardinian wildcat *Fli*326 had about 100% probability of belonging to the sampled generation of the domestic cat population. Therefore, *Fli*326 may represent a case of a misidentified or mislabeled sample.

The European wildcats used in this study were sampled from across the entire species' distribution range in Italy. Only 1 (*Fsi*284) of 48 genotyped European wildcats had admixed ancestry and was probably a hybrid with the domestic cat. Moreover, three additional putative European wildcats (*Fsi*70, *Fsi*73, and *Fsi*285) showed mtDNA haplotype Fca9, which was shared with three domestic cats. In both minimum-spanning network and NJ trees, haplotype Fca9 appears to be related to other domestic cat haplotypes and not to wildcat haplotypes (figs. 3 and 4). Therefore, although mtDNA haplotypes did not convey strong phylogenetic information, it is probable that Fca9 is a domestic cat haplotype. Nevertheless, the Bayesian assignment procedure classified *Fsi*70, *Fsi*73, and *Fsi*285 as European wildcats. The putative hybrid cat *Fsi*284 might derive from recent crossbreeding, while cats *Fsi*70, *Fsi*73, and *Fsi*285 could have a more ancient ancestry with domestic cats. Cats *Fsi*70, *Fsi*285, and *Fsi*284 were collected in Tuscany Maremma, on the Thyrrenian (western) coast of central Italy, and cat *Fsi*73 came from a central Apennines area geographically very close to Maremma. These localities map on the northernmost edge of the zoogeographical range of *F. silvestris silvestris* in Italy (fig. 1), which is thought to have been stable from the end of the last glaciation (Ragni et al. 1994), and were historically densely settled by humans and by potentially free-ranging domestic cats.

These findings suggest that despite a long period of sympatry and syntopy, hybridization is negligible and is limited to particular areas at the geographical and ecological edges of the wildcat distribution in central Italy. However, more samples, and probably more microsatellite loci, should be analyzed to obtain quantitative estimates of the rate of crossbreeding in the Italian wildcat population. The microsatellites used in this study are widely spaced on the same chromosome or on separate chromosomes (Menotti-Raymond et al. 1999). Backcrossing of first-generation hybrids into the wildcat population will dilute the proportion of domestic parental genotypes through the generations, and linkage disequilibrium will be negligible after a few generations of backcrossing. Therefore, except for the introgressed nonrecombining mtDNA, evidence of episodic hybridization in the past might have been lost, and the identification of past hybridization might require an exponentially increasing number of molecular markers (Goodman et al. 1999). Thus, the existence of distinct groups of wildcats (European and African wildcats) does not necessarily mean that we have identified ''pure'' populations with no introgression, but rather that we have identified cats that show little evidence of recent domestic cat ancestry.

Results of Bayesian admixture analyses validate the morphological protocols used to identify wild and hybrid phenotypes of *F. silvestris* and have implications for the conservation of wildcat populations in the Mediterranean region (Stahl 1993; Nowell and Jackson 1996). Despite national and international protection in most European countries, the wildcat is threatened and declining throughout most of its range due to habitat destruction, direct persecution, accidental killing, transmission of viral diseases, and possible hybridization with feral cats. To enforce legal protection, it is therefore important to improve a set of morphological, behavioral, and molecular traits diagnostic for the wildcat and to map the regional distribution of ''pure'' wildcat populations, which must be protected with high priority.

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