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Genetic structure of wildcat (*Felis silvestris*) populations in Italy

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Abstract

Severe climatic changes during the Pleistocene shaped the distributions of temperate-adapted species. These species survived glaciations in classical southern refuges with more temperate climates, as well as in western and eastern peripheral Alpine temperate areas. We hypothesized that the European wildcat (*Felis silvestris silvestris*) populations currently distributed in Italy differentiated in, and expanded from two distinct glacial refuges, located in the southern Apennines and at the periphery of the eastern Alps. This hypothesis was tested by genotyping 235 presumed European wildcats using a panel of 35 domestic cat-derived microsatellites. To provide support and controls for the analyses, 17 know wildcat *x* domestic cat hybrids and 17 Sardinian wildcats (*F. s. libyca*) were included. Results of Bayesian clustering and landscape genetic analyses showed that European wildcats in Italy are genetically subdivided into three well-defined clusters corresponding to populations sampled in: (1) the eastern Alps, (2) the peninsular Apennines, and (3) the island of Sicily. Furthermore, the peninsular cluster is split into two subpopulations distributed on the eastern (Apennine mountains and hills) and western (Maremma hills and lowlands) sides of the Apennine ridge. Simulations indicated Alpine, peninsular, and Sicilian wildcats were isolated during the Last Glacial Maximum. Population subdivision in the peninsula cluster of central Italy arose as consequence of a more recent expansions of historically or ecologically distinct European wildcat subpopulations associated with distinct the Continental or Mediterranean habitats. This study identifies previously unknown European wildcat conservation units and supports a deep phylogeographical history for Italian wildcats.

Introduction

Cyclic glacial/interglacial climatic changes during the Quaternary significantly shaped the biogeography of plant and animal species in the northern Hemisphere. Major glacial refuges have been identified in Iberia, Italy, and the southern Balkans, supporting the classical Southern Mediterranean refuge model (Hewitt 2000; Weiss and Ferrand 2006). Two complementary phylogeographic models have been suggested as follows: (1) the existence of cryptic Northern refugia, putatively located in sheltered areas scattered in central Europe north of the Pyrenees and the Alps (Stewart and Lister 2001; Deffontaine et al. 2005), and (2) the occurrence of repeated colonization waves of species migrating into continental Europe from eastern European and Asian refugia (Bilton et al. 1998; Randi 2007;

Fløjgaard et al. 2011). In addition, research on paleopollen-reconstructed biomes and species distribution modeling identified a number of plant biodiversity hotspots scattered around the Mediterranean basin that correspond to putative late Pleistocene refugia (Médail and Diadema 2009), driving phylogeographic research to develop more complex hypotheses that include the occurrence of refugia in northern areas and of refugia-within-refugia (Gómez and Lunt 2007). These same refugia hypotheses have been proposed for different organisms. However, clear refugia are still less defined for species with wide geographical ranges, like carnivores (Johnson et al. 2004; Driscoll et al. 2007; Pilot et al. 2010; Davison et al. 2011), mainly due to the complex interlinks of past (natural) and recent (mainly anthropogenic) population dynamics. To this end, reconstructing the phylogeography of wildcat (*Felis silvestris*), which have both widespread and fragmented populations, might deepen our knowledge of the carnivores' biogeography in Europe.

Wildcats are widely distributed in three continents (Europe, Africa, and Asia), with at least five well-documented subspecies (Driscoll and Nowell 2010). Since the late Pleistocene, and before the dramatic population decline caused in the last centuries by deforestation and human persecution, the European wildcat subspecies (*F. s. silvestris*) was widespread across Europe. The fossil record documents a rapid expansion of the European wildcat throughout most of central and western Europe from the Late Glacial (ca. 15,000–9600 Y B.C.) to the Holocene (Sommer and Benecke 2006). Interestingly, fossils from the Last Glacial Maximum (LGM) were found not only in classical southern Mediterranean refuges in Spain and Italy but also in western and eastern Alpine peripheral regions (Sommer and Benecke 2006). Mild climatic conditions at low elevation and in coastal areas, plus the buffering effects of the Mediterranean Sea, allowed the persistence of temperate forest communities at the periphery of glaciated Alps (Willis and Van Andel 2004). During the LGM, patches of deciduous forest persisted at the tips of the western Alps (the Maritime Alps in Italy and southern France) and the eastern Alps (in Italy, Slovenia, and Istria; Tribsch and Schönswetter 2003; Szövényi et al. 2009). Those areas provided refuges for *Fagus*, *Betula*, *Populus,* and *Salix* trees (Petit et al. 2003; Magri et al. 2006; Maliouchenko et al. 2007), preserving habitat patches suitable to populations of forest-associated small mammals (e.g., *Clethrionomis*, *Apodemus,* and *Microtus*; Michaux et al. 2004, 2005; Fløjgaard et al. 2009) and their predators, including the European wildcat (Sommer and Benecke 2006). In the southern Italian peninsula, the LGM drove back European wildcat populations to coastal and mountain southernmost refugia, where rare and scattered fossils have been found in paleontological and archeological sites (Ragni et al. 1994).

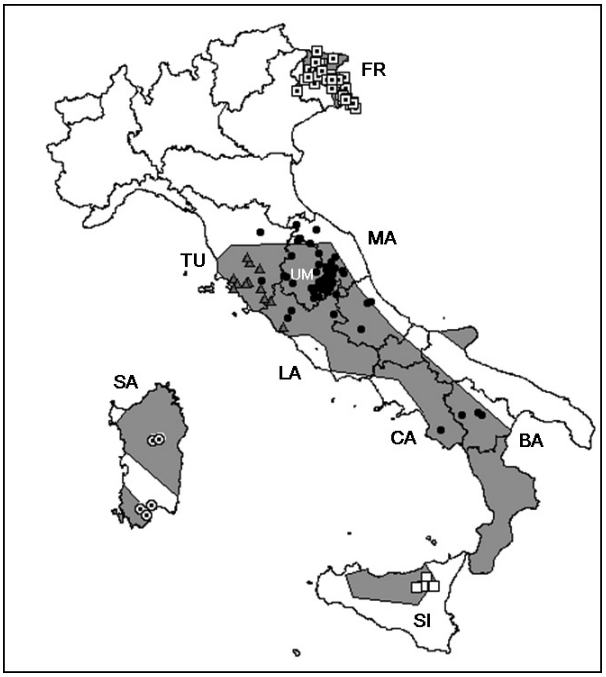

Figure 1. Sampling locations of European and Sardinian wildcats (*Felis silvestris*) used in this study. The gray areas indicate the approximate wildcat distribution ranges in Italy. Each symbol represents a population. Acronyms indicate the sampled regions: Friuli-Venezia Giulia (FR) in the eastern Alps; Tuscany (TU), Lazio (LA), Marche (MA), Umbria (UM), and Abruzzo (AB) in the central peninsula; Campania (CA); Basilicata (BA) in the southern peninsula; Sicily (SI); Sardinia (SA). The question mark indicates the probably extinct wildcat population in the western Alps – Ligurian Apennines.

Italy currently hosts at least three geographically distinct populations of the European wildcat (Fig. 1), which might represent the living remnants of Pleistocene refugial populations: (1) wildcats in the eastern Italian Alps (Friuli Venezia Giulia and Veneto), which are presumably connected with neighbor populations in Slovenia and Croatia (Lapini 1989, 2006); (2) a widespread population network that is distributed across the central and southern Italian peninsula (Ragni 2006); and (3) an insular population that has been confined to Sicily at least since the LGM (Pierpaoli et al. 2003). A remnant wildcat population in the western Alps-Ligurian Apennines might have gone extinct in the first decades of 1980s (Ragni et al. 2012). These three populations live in areas included in the Alpine, Continental, and Mediterranean biogeographical regions, which are characterized by different habitat types and climates (Schönswetter et al. 2005).

The existence of fragmented populations and putative multiple LGM refugia suggests that extant European wildcat populations in Italy have distinct phylogeographical

Table 1. Subspecies, sampling location, and sample size of genotyped cats (*Felis silvestris*) used in this study (see Fig. 1 and text for details).

Subspecies	Sampling locations	Years	*N*	Collectors
Domestic cats *F. s. catus*	Eastern Alps	2003–2009	7	L. Lapini
	Central Apennines and Maremma	2003–2009	64	E. Randi, A. Sforzi, A. De Faveri, B. Ragni, A. Giuliani
	Southern Apennines	2009–2010	3	E. Mallia
	Sardinia	2007–2010	3	R. Oliveira, M. Delogu
European wildcats *F. s. silvestris*	Eastern Alps	2003–2009	78	L. Lapini, B. Ragni, A. De Faveri
	Central Apennines and Maremma*	2003–2009	132	A. Sforzi, B. Ragni, A. Giuliani, A. Di Croce, L. Gentile
	Southern Apennines	2003–2010	11	B. Ragni, E. Mallia
	Sicily	2003–2009	14	S. Anile, B. Ragni
Sardinian wildcats *F. s. libyca*	Sardinia	2003	17	B. Ragni
Captive *silvestris x catus* hybrids	Captivity	2003	7	B. Ragni
Wild-living *silvestris x catus* hybrids	Eastern Alps	2006	5	L. Lapini
	Central Apennines and Maremma	2003	4	A. Sforzi, A. Giuliani, B. Ragni
	Sicily	2003	1	W. Trocchi

*One sample ID 1009 is a museum specimen collected in Maremma.

origins and are genetically differentiated (Ragni et al. 1994). This study tests this hypothesis by genotyping 235 presumed European wildcats, which were collected from their entire distribution range (excluding the vanished western Alpine wildcats), using a panel of 35 domestic cat-derived microsatellites. These samples showed the typical European wildcat coat color pattern (Ragni and Possenti 1996), but because of the possible presence of cryptic hybrids, which might be not morphologically recognized; Randi et al. 2001; Lecis et al. 2006; and before accurate genetic analyses, such as admixture analysis, any wildcat sample was considered a "putative wildcat". In order to support the identification of hybrids among the studied samples, 17 known European wildcat *x* domestic cat hybrids were also analyzed. Clustering and admixture analyses suggested that some putative wildcats were hybrids, and these cats were subsequently removed from the data set. Subsequent analyses of the identified putative wildcat hybrids will be considered in a forthcoming study on wildcat × domestic cat hybridization across Europe. To compare the extent of genetic diversity among European wildcat populations with divergence among subspecies, domestic cats (*F. s. catus*), and 17 Sardinian wildcats (*F. s. libyca*) were also evaluated. *F. s. libyca* is a subspecies that originated by anthropochorus introductions of predomesticated cats of African or Near Eastern origin into Sardinia and in other Mediterranean islands about 9.000–8.000 years ago (Ragni 2006; Vigne et al. 2012). The results provide a better understanding of the historical factors that shaped the European wildcat population structure in Italy, and lead to the identification of significant conservation units.

Materials and Methods

Sample collection and DNA extraction

DNA was isolated from 346 tissue, blood, and skin samples obtained from 77 free-living or house domestic cats, 235 putative European wildcats, 17 African wildcats from Sardinia (Sardinian wildcats), and 17 previously described *silvestris x catus* hybrids, collected from 2003 to 2010 (Table 1). Seven of these hybrids were obtained from controlled crosses (Ragni 1993); 10 wild-living hybrids, including a family of five full-sibs extracted from the uterus of a road-killed apparently pure *F. s. silvestris* female, that were genetically identified in other studies (Pierpaoli et al. 2003; Lecis et al. 2006) and reanalyzed here. The European wildcats were opportunistically collected from found-dead or trapped animals in the eastern Italian Alps ($n = 78$), central ($n = 132$) and southern ($n = 11$) Italian peninsula, and on the island of Sicily ($n = 14$; Fig. 1). Climate and habitat types in the Alps and in peninsular Apennines are different, with prevailing coniferous forests and snowy late autumn–winter–spring seasons in the first and broad-leaved forests, shorter snowy winter, in the second (Bransford 2009). The islands of Sicily and Sardinia have typical Mediterranean climate with mild winter, short raining spring and fall, long, dried, warm summer, and habitat dominated by evergreen sclerophyll wood, chaparral, and matorral features. The cores of the European wild cat distribution areas in the Alps and Apennines are at least 550 km distant. Ancient deforestation (beginning before the Roman times; Williams 2006) has continued and extended intensively for about three millennia, and recent heavy urbanization in

the lowlands and in the entire Po Valley is the major anthropogenic barriers which might have caused the fragmentation of extant European wildcat populations (Ragni 2006). Wildcats were phenotypically identified by collectors according to diagnostic coat-color traits (Ragni and Possenti 1996) and/or biometric indices (skull size and intestinal length; Schauenberg 1969, 1970, 1977), independent of any genetic information. Free-living domestic cats ($n = 39$) were randomly sampled within the range of wildcats. House cats ($n = 38$) were sampled from local catteries following veterinary rules (Abrams-Ogg and Schneider 2010). Samples were stored in 5 volumes of 95% ethanol (tissues and skins) or Tris/SDS buffer (blood; Longmire et al. 1997), and kept at −20°C. Total DNA was automatically extracted using a MULTIPROBE II^{EX} *Robotic Liquid Handling System* (Perkin Elmer Inc, Waltham, MA) and the QIAGEN DNeasy tissue and blood extraction kits (Qiagen Inc, Hilden, Germany).

Selection of molecular markers

Thirty-five domestic cat-derived dinucleotide microsatellites (33 autosomal and two X-linked: FCA240 and FCA651; Menotti-Raymond et al. 2003a,b) were chosen because of their wide chromosomal distribution and high heterozygosity in diverse domestic cat populations (Lipinski et al. 2008). Subsets of the Short Tandem Repeats (STRs) have been evaluated in other wildcat population genetic studies (Pierpaoli et al. 2003; Oliveira et al. 2008a, b; Eckert et al. 2009; O'Brien et al. 2009). These markers were amplified in eight multiplexed sets (Table S1) using the Qiagen Multiplex PCR Kit with forward tailed-primers fluorescently labeled with 6-FAM, NED, PET, or VIC dyes (Applied Biosystems, Foster City, CA). PCRs were performed in a total volume of 8 μL containing: 2.0 μL of DNA (20–40 ng), 1.0 μL of mix of primers and tails (10 mmol/L), 0.2 μL of 10 mg/mL bovine serum albumin, 4.0 μL of Qiagen master mix, and 0.8 μL of RNase-free water. Amplifications were performed in a GeneAmp® PCR System 9700 (Applied Biosystems) using the touchdown cycling profile: 95°C for 15 min, 30 cycles of 30 sec at 94°C, 90 sec at 62°C (decreasing 0.16°C per cycle to 57°C), 60 s at 72°C, eight cycles for the annealing of tails of 30 sec at 94°C, 90 sec at 53°C, 60 sec at 72°C, final extension of 10 min at 72°C, followed by a 4°C hold. The genotype of one museum sample (ID 1009; see Table 1) collected in Maremma was consistently obtained after four replicated PCRs, following a multitube approach designed for low-quality DNA samples (Taberlet et al. 1996). PCR products were analyzed in an ABI 3130 XL (Applied Biosystems) automated sequencer, and allele sizes were determined with GeneMapper 4.0 (Applied Biosystems). The power of markers to identify each unique genotype was evaluated calculating the probability of identity values (PID and PIDsibs; Mills et al. 2000; Waits et al. 2001) in GenAlEx 6.41 (Peakall and Smouse 2006). Individual genotypes were matched to exclude replicates. About 10% of randomly selected samples were independently replicated twice to assess the rate of allelic dropout (ADO) and false alleles (FA). Presence of null alleles was assessed with Microchecker (Van Oosterhout et al. 2004) with an adjusted *P*-value corresponding to $\alpha = 0.05$ after Bonferroni correction (Rice 1989).

Analysis of genetic variation

Population genetic analyses were performed within and among domestic, Sardinian and European wildcat subspecies, and in the European wildcat subpopulations in Italy, excluding all the admixed genotypes (putative hybrids; see below), which would have affected the estimates of allele frequencies in wildcats. GenAlEx was used to estimate allele frequencies, the number of private (N_P), average observed (N_A) and expected (N_E) alleles per locus, and the observed (H_O) and expected unbiased (H_E) heterozygosity for each locus and populations (Peakall and Smouse 2006). Allelic richness (N_{AR}) was computed with Fstat 2.9.3.2 (Goudet et al. 2002). Deviations of loci from Hardy–Weinberg (HWE) and linkage (LE) equilibrium in all populations, and the significance of the Weir and Cockerham's (1984) F_{IS} estimator was evaluated using the Guo and Thompson's (1992) Monte Carlo Markov Chains (MCMC) in Genepop 4.0 (Rousset 2008). Sequential Bonferroni corrections for multiple comparisons were used to adjust the significance levels. Single and multilocus *F*- (Weir and Cockerham's 1984) and *R*-statistics (Slatkin 1995) were estimated using Genepop and Fstat, respectively. The partition of genetic diversity within and between groups was obtained by analysis of molecular variance (AMOVA) on Euclidean pairwise genetic distances, using analogs of Wright's *F*-statistics as implemented in GenAlEx.

Population structure, assignment testing, and identification of hybrid cats

The genetic divergence and phylogeographic structure of the sampled wildcat populations were derived from the results of Bayesian cluster analyses implemented in Structure 2.3.1 (Pritchard et al. 2000; Falush et al. 2003). Structure was used to infer population structure and simultaneously assign the multilocus genotypes to their population (cluster) of origin. Each run of Structure was replicated five times, with 10^4 burn-in followed by 10^5 simulations, with or without the "admixture" model, with the correlated ("*F*") or the independent ("*I*") allele

frequencies models. The optimal number of clusters (K) was identified using ΔK and ΔF_{ST} statistics (Evanno et al. 2005) in CorrSieve 1.6.1 (Campana et al. 2011). For each selected K-value, we assessed the following: (1) the average proportion of membership (Qi) of the sampled populations to the inferred clusters, (2) the individual proportion of membership (q_i) to one or more than one (in case of admixed genotypes) of the inferred clusters, and (3) the 90% credibility intervals (CI) of the q_i values. We used Structure to perform the following analyses: (1) identification of hybrids in the European wildcats (using sample set A = European wildcats, domestic cats and known hybrids; n = 332), and in the Sardinian wildcats (using sample set B = Sardinian wildcats and domestic cats; n = 94); the admixed genotypes were identified at threshold $q_i = 0.90$ (based on admixture analyses of observed and simulated cat data sets; Randi 2008; Oliveira et al. 2008a) and subsequently removed from the data set; (2) genetic differentiation among the three cat subspecies (using sample set C = domestic cats, European and Sardinian wildcats, hybrids excluded; n = 295); and (3) inference on population substructuring in the European wildcats sampled in peninsular Italy (using sample set D = European wildcats only, hybrids excluded; n = 202). Details on the Structure options used in these analyses are indicated in Table 2.

Structure models are based on the assumption of Hardy–Weinberg and linkage equilibrium (HWLE) in the inferred cluster, which might be violated in empirical data sets (Pritchard et al. 2000). Therefore, independently, on explicit population models, patterns of genetic differentiation among cat subspecies (set C) and European wildcat populations (set D; excluding all the hybrids) were analyzed by a Discriminant Analysis of Principal Components (DAPC) in Adegenet (Jombart 2008). European wildcat genotypes (set D) were also depicted in an unrooted neighbor-joining tree using interindividual genetic distances (Nei and Tajima 1983) computed with Population 1.2.32 (Langella 2010).

Spatial analyses

The integration of both geographic and genetic information into spatial and landscape genetic models might improve the identification of weakly differentiated populations and yield accurate spatial locations of clusters and genetic barriers (Storfer et al. 2007). The spatial clustering of European wildcats in the Italian peninsula, excluding all the hybrids and seven cats without geographical coordinates, n = 105; set E, was determined by Spatial Principal Component Analysis (sPCA, Jombart et al. 2008). Connection networks among neighboring samples (individuals or populations) were defined through the inverse Euclidean distances algorithm (Jombart 2008). The spatial structure was described by spatial autocorrelations based on Moran's I (Moran 1948; Cliff and Ord 1981) and tested by nonparametric randomized regressions of allelic frequencies to global (U+) or local (U−) Moran's Eigenvector Maps (MEMs). For each MEM, a mean coefficient of determination R^2 was generated and the highest values

Table 2. Description of the cat sample sets used in this study. The known admixed cats include the captive hybrids (n = 7) and the previously identified (n = 10) hybrids.

Sample set	Subspecies	Populations	*N*	Analyses
Set A	Domestic cats	Italy	332	Structure analyses
	European wildcats	Italy		K = 1–10
	Known hybrids	Italy		Admixture, *F* and *I* models
				Option *usepopinfo* not active
Set B	Domestic cats	Italy	94	Structure analyses
	Sardinian wildcats	Sardinia		K = 1–10
				Admixture, *F* and *I* models
				Option *usepopinfo* not active
Set C	Domestic cats	Italy	295	Structure analyses
	European wildcats	Italy		K = 1–10
	Sardinian wildcats	Sardinia		No-admixture, *F* and *I* models
				Genetic variability within and among populations
Set D	European wildcats	Eastern Alps	202	Structure analyses
		Maremma		K = 1–10
		Central and Southern Apennines		No-admixture, *F* and *I* models
		Sicily		Locprior model (Hubisz et al. 2009).
				Spatial analyses
Set E	European wildcats	Maremma	105	Spatial analyses
		Central Apennines		

were summed to obtain the test statistic (Jombart 2008). The results were graphically displayed as positive and negative eigenvalues, respectively, for global and local population structure. The spatial distribution of European wildcat clusters was determined in a Poisson-Voronoi tessellation of the sampling areas, using GENELAND 3.3.0 (Guillot et al. 2005b). Each run was replicated five times with 100 thinning followed by 10^5 MCMC iterations and with both "*I*" and "*F*" models. GENELAND was run first to estimate the optimal number of subpopulations (with *K* from 1 to 10). Then, the spatial structure was obtained by five replicated runs, with the previous parameter values and optimal $K = 5$ (see Results and Figure S1E). The level of uncertainty of spatial coordinates was set to 1.4 km, based on estimates of the average wildcat home ranges in the Apennines, Maremma, and Sicily (ca. 6 km^2; Anile et al. 2012; Bizzarri et al. 2010; Sforzi et al. 2010).

Estimates of population divergence time

Rough assessments of divergence times (in generations) between the European wildcat populations sampled in the eastern Italian Alps, peninsular Italy, and Sicily were obtained by simulations. Using EASYPOP 2.0.1 (Balloux 2001), three populations of size $N = 250$, 500 or 1000 were constructed (assuming sex ratio = 1:1 (Tryjanowski et al. 2002), 33 loci with free recombination and same mutation rate $\mu = 0.0001$ (Hille et al. 2000), single step mutation model (Sainudiin et al. 2004), maximum allele number $A = 20$, initial genetic variability set to randomly assign alleles: *var in = max*), which have diverged without gene flow for 50, 500, or 5000 generations. Each run was replicated 10 times. The simulated average number of alleles per locus, average heterozygosity, and F_{ST} were compared to the observed values, and the most likely combinations of population size and divergence times that might have produced the observed genetic diversity were identified.

Results

Genetic variability and identification of cat genotypes and subspecies

All 35 microsatellites were polymorphic, showing from eight (FCA453) to 22 (FCA045) alleles per locus, with the exception of FCA88 and FCA023, which were monomorphic in the Sicilian samples, and FCA035, which was monomorphic in the Sicilian and Sardinian samples (Table S1). The independent replication of 10% of the samples provided no evidence for genotyping errors (ADO and FA were equal to zero). Likewise, none of the 35 loci showed significant presence of null alleles. No identical genotypes were observed, and genotype pairs mismatched at a minimum of two loci. Genetic variation statistics are presented in Table 3. The low values of PID suggested that cats in the study were not highly related: PID = 1.5×10^{-34},

Table 3. Summary of genetic variability at 35 microsatellite loci (33 autosomal and two X-linked STR) in two cat sample sets split into three subspecies and six populations.

Samples	Subspecies	Populations	*n*	N_A	N_{AR}*	H_O	H_E	F_{IS}	HWE	LE	PID	PIDsibs
Set C	Domestic cat	Italy	77	11.3	6.9	0.65 (0.02)	0.75 (0.02)	0.11***	1/33	21/595	4.4×10^{-40}	6.2×10^{-15}
	Sardinian wildcat	Sardinia	16	6.4	5.8	0.66 (0.03)	0.70 (0.02)	0.05	0/33	1/595	1.5×10^{-34}	7.6×10^{-14}
	European wildcat	Italy	202	10.2	5.7	0.55 (0.03)	0.69 (0.03)	0.18***	20/33	105/595	1.5×10^{-34}	1.2×10^{-13}
Set D	European wildcat	Eastern Alps	74	7.6	2.5	0.57 (0.03)	0.64 (0.03)	0.09***	2/33	19/595	3.3×10^{-30}	1.9×10^{-12}
		Apennines	117	8.8	2.6	0.54 (0.03)	0.65 (0.03)	0.18***	11/33	10/595	6.4×10^{-32}	6.6×10^{-13}
		Sicily	11	3.6	2.1	0.46 (0.03)	0.50 (0.03)	0.13	0/33	0/595	2.4×10^{-20}	1.9×10^{-9}
Set D	European wildcat	Easter Alps	74	7.6	2.5	0.57 (0.03)	0.64 (0.03)	0.09***	2/33	8/595	3.3×10^{-30}	1.9×10^{-12}
		Maremma	23	6.0	2.4	0.51 (0.03)	0.62 (0.03)	0.18***	2/33	0/595	1.2×10^{-28}	4.5×10^{-12}
		Central and Southern Apennines	94	8.3	2.5	0.55 (0.03)	0.65 (0.03)	0.16***	9/33	2/595	2.4×10^{-31}	9.7×10^{-13}
		Sicily	11	3.6	2.1	0.46 (0.03)	0.50 (0.03)	0.13	0/33	0/595	2.4×10^{-20}	1.9×10^{-9}

Sample size = *n*; N_A = mean number of alleles per locus; N_{AR} = allelic richness estimated by rarefaction on sample sizes $n = 16$ (genotypes in Sardinia; set C) and $n = 11$ (genotypes in Sicily; set D); H_O, H_E = observed and expected heterozygosity; F_{IS} = Weir and Cockerham (1984)'s fixation index computed excluding the two X-linked loci (*** significant departures from HWE at $P < 0.001$ corresponding to $P < 0.00{,}028$ after Bonferroni correction for 33 independent comparisons computed excluding the two X-linked loci); HWE = number of loci out of Hardy–Weinberg equilibrium over the total; LE = number of pairwise loci comparisons out of linkage equilibrium over the total; PID = cumulative probability-of-identity; PIDsibs = cumulative Hardy–Weinberg-expected PID among full sib dyads; standard errors in parentheses.

Table 4. Analysis of molecular variance (AMOVA) for cat subspecies and European wildcat populations computed in GenAlEx, using Φ_{ST}.

Cat subspecies	Populations	Source of variation	Variance	% variation	PhiST	F_{ST}	R_{ST}
Domestic cat	Italy (n = 76)	Among groups	8.603	22.64%	0.226 ($P < 0.001$)	0.147	0.385
Sardinia wildcat	Sardinia (n = 16)	Within groups	29.402	77.36%			
European wildcat	Italy (n = 202)						
	Eastern Alps (n = 74)	Among groups	5.663	17.76%	0.178 ($P < 0.001$)	0.109	0.192
	Apennines (n = 117)	Within groups	26.209	82.23%			
	Sicily (n = 11)						

The F_{ST} and R_{ST} values were obtained over all loci with Genepop and Fstat, respectively.

Table 5. Genetic divergence among cat subspecies and European wildcat geographic populations. Lower triangular matrix: pairwise estimates of Φ_{ST} (Weir and Cockerham 1984); upper triangular matrix: pairwise estimates of R_{ST} (Michalakis and Excoffier 1996).

Cat subspecies	Populations	Domestic cat	Sardinia wildcat	European wildcat	
Domestic cat	Italy (n = 76)	–	0.170	0.408	
Sardinia wildcat	Sardinia (n = 16)	0.164	–	0.522	
European wildcat	Italy (n = 202)	0.222	0.265	–	
European wildcat	Populations	Eastern Alps	Peninsula	Sicily	
	Eastern Alps (n = 74)	–	0.195	0.309	
	Peninsula (n = 117)	0.165	–	0.097	
	Sicily (n = 11)	0.299	0.173	–	
European wildcat	Populations	Eastern Alps	Maremma	Central and Southern Apennines	Sicily
	Eastern Alps (n = 74)	–	0.207	0.197	0.309
	Maremma (n = 23)	0.185	–	0.020	0.106
	Central and Southern Apennines (n = 94)	0.173	0.055	–	0.103
	Sicily (n = 11)	0.299	0.210	0.179	–

All values are estimated at 35 microsatellite loci and are highly significant ($P < 0.001$).

PIDsibs = 1.2×10^{-13} in European wildcats; PID = 1.5×10^{-34}, PIDsibs = 7.6×10^{-14} in Sardinian wildcats; and PID = 4.4×10^{-40}, PIDsibs = 6.2×10^{-15} in domestic cats. The PID estimates excluded the 17 known hybrids and 37 newly identified admixed cats identified by admixture analyses (see below).

Allele richness, estimated by rarefaction for a sample size n = 16 (the number of successfully genotyped Sardinian wildcats), was similar in the European (5.7 ± 0.3) and Sardinian wildcats (5.8 ± 0.3), and slightly larger in the domestic cats (6.9 ± 0.3). Average values of heterozygosity were similar in the Sardinian (H_O = 0.66; H_E = 0.70) and domestic cats (H_O = 0.65; H_E = 0.75), and slightly lower in European wildcats (H_O = 0.55; H_E = 0.69). There were significant deficit of heterozygotes and departures from HWLE among European wildcats and domestic cats, suggesting population admixture or local substructuring. The Sardinian wildcats were in genetic equilibrium (Table 3).

The R_{ST} distances among the three subspecies were 2.6 times higher than the corresponding F_{ST} distances (R_{ST} = 0.385; F_{ST} = 0.147, on average), but R_{ST} distances were only 1.7 times higher among the three main European wildcat geographic populations sampled in the Alps, Italian peninsula, and Sicily (R_{ST} = 0.192; F_{ST} = 0.109, on average; Table 4). The genetic divergence between domestic cats and Sardinian (African) wildcats (Φ_{ST} = 0.164) was about 30% lower than between domestic cats and European wildcats (Φ_{ST} = 0.222–0.265), and between Sardinian and European wildcats (Φ_{ST} = 0.265; AMOVA; Table 5). The genetic divergence between the European wildcats in the eastern Alps and in Sicily (Φ_{ST} = 0.299) was higher than that between the domestic cats and the two wildcat subspecies (Table 5).

Population assignment and admixture analyses

The highest values of ΔK and ΔF_{ST} were obtained in Structure with K = 2 in both the European (set A) and Sardinian (set B) wildcats (Table S2 and Figure S1). Domestic cats (77 individuals with Q_I = 0.980 and

individual q_d ranging from 0.881 to 0.996) and European wildcats (202 individuals with $Q_{II} = 0.982$ and $q_w = 0.904–0.997$) were assigned to two distinct clusters either using the "*I*" or "*F*" allele frequency model. The Sardinian wildcats (16 individuals with $Q_{II} = 0.990$ and $q_w = 0.910–0.998$) and the domestic cats (average $Q_I = 0.993$ and $q_d = 0.950–0.999$) were also assigned to distinct clusters. At threshold $q_i = 0.80$, all the 17 known hybrids were confirmed as admixed. Moreover, 36 new admixed European wildcats (with individual q_w ranging from 0.127 to 0.888), and one new admixed Sardinian wildcat (with individual $q_s = 0.680$) were detected in sample set *A* and *B*, respectively. All these putatively admixed samples would have complicated the detection of population structure, and were, therefore, removed from the data set for subsequent analyses.

Genetic and spatial clustering of European wildcat populations

STRUCTURE analyses on sample set *D* (admixed genotypes excluded), with the "no-admixture" and "*I*" or "*F*" models, no prior information, showed that at $K = 2$ the European wildcats sampled in the eastern Alps clustered separately from the ones sampled in the Italian peninsula and in Sicily; at $K = 3$ also the European wildcats from Sicily clustered separately; and finally, the European wildcats split into four distinct subpopulations at $K = 4$, with the identification of a subpopulation with geographical distribution restricted to the plains and lower hills of Tuscany and Lazio Maremma. This pattern of population substructuring was confirmed using Hubisz et al. (2009) sampling location model (Fig. 2A; Table S3). GENELAND clustering (with the "*F*" model) splits the European wildcats in set *D* into five clusters ($K = 5$; see Figure S1E) including wildcats from (1) eastern Alps, (2) Mediterranean areas of Tuscany and Lazio Maremma, (3) central peninsular Apennines, (4) southern peninsular Apennines, and (5) Sicily (Figure S2). GENELAND analyses performed with only the Italian peninsular wildcats confirmed the existence of two clusters roughly separated by the Apennines ridge, namely grouping: (1) the European wildcat sampled from Tuscany and Lazio Maremma, on the western side of the ridge; and (2) the European wildcats from the Apennines (Emilia-Romagna, Umbria, Marche, and Abruzzo regions; Fig. 2B).

The DAPC scatter-plots confirmed a sharp distinction among the three cat subspecies (Fig. 3A) and among the four European wildcat subpopulations in Italy (Fig. 3B). The NJ clustering based on Nei's interindividual genetic distance showed also congruent results: wildcats sampled in the Alps, peninsula, and Sicily belong to three distinct clades (labeled 1, 2, and 3 in Fig. 4, respectively). However, wildcats sampled in the eastern (Apennines) and western (Lazio and Tuscany Maremma) sides of the central peninsula are only partially split into distinct subclades (*Fsi* E, *Fsi* W). Moreover, some wildcat sampled in

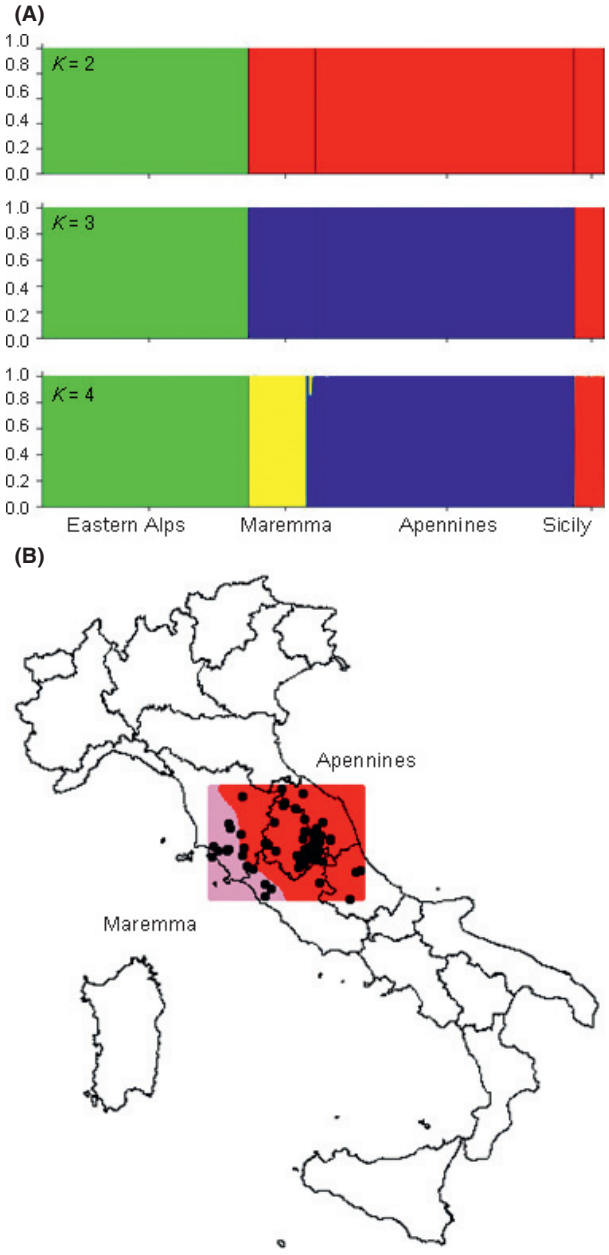

Figure 2. (A) Population clustering obtained in STRUCTURE (with the "sampling location prior model" and assuming $K = 2$, 3, or 4 genetic clusters) of European wildcats sampled in the eastern Alps, Maremma (areas in Tuscany and Lazio Maremma in the western Italian peninsula; Apennines (areas in Marche, Umbria, Abruzzo, Campania, and Basilicata regions), and in Sicily. Each cat genotype is represented by a vertical bar split in *K* colored sections, according to its relative assignment to the *K* genetic clusters. (B) Maps of posterior probability of European wildcats sampled in central Italy (Maremma and central Apennines) and assigned to two spatial clusters identified by GENELAND.

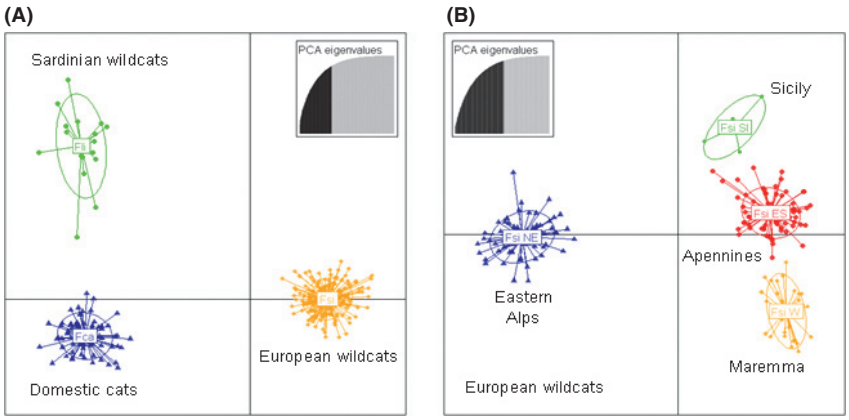

Figure 3. Scatterplot of a Discriminant Analysis of Principal Component (DAPC) obtained with ADEGENET showing genetic distinctions among: (A) three cat subspecies (set *C*, including domestic cats, Sardinian wildcats, and European wildcats); (B) all European wildcat subpopulations in Italy (set *D*), including samples from the eastern Alps, Maremma (areas in Tuscany and Lazio Maremma in the western Italian peninsula), Apennines (areas in Marche, Umbria, Abruzzo, Campania, and Basilicata regions), and in Sicily. The barplots in the inserts show the proportion of genetic diversity described by each Principal Component (PCA eigenvalues). In all the plots, the first PC describes 86.81% and 85.61% of the genetic diversity, respectively, among cat subspecies and populations.

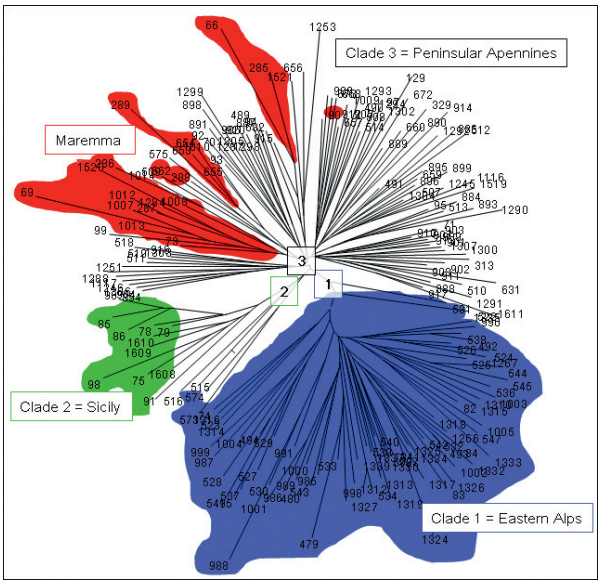

Figure 4. Neighbor-joining tree clustering the pairwise Nei and Tajima (1983) genetic distances among individual multilocus genotypes of the European wildcats sampled in Italy. The European wildcat subpopulations and the main clades are indicated.

the southern Apennines (*Fsi* S) are closely linked to the Sicilian clade, suggesting shared ancestries.

The sPCA analysis of the four European wildcat subpopulations showed a significant correlation between genetic and geographic distances (*P*-value = 0.047; Mantel test with 999 permutations), which revealed a global structure mainly explained by the first global principal component λ_1. The spatial genetic pattern is visualized in the interpolated gradient map of individual scores (Fig. 5A) and by the individual scores (Fig. 5B). A deep separation occurred along an east–west direction between the European wildcats sampled in the Lazio and Tuscany Maremma and those distributed in the eastern side of the central peninsula (Apennines).

Estimates of population divergence time

Results from EASYPOP simulated populations indicated that a combination of large population size (N = 500 or 1000 breeding individuals) and long divergence times (5000 generations) produced the values of genetic parameters that are most closely correspond to the observed (Fig. 6). Simulated values with N = 1000 and population divergence protracted for 5000 generations (A = 11; H_O = 0.63; F_{ST} = 0.21), compared well with the corresponding observed values (average allele number per locus in the Alpine and peninsular wildcats A = 9; average heterozygosity H_O = 0.57; average divergence between the Alpine and peninsular wildcats F_{ST} = 0.165). Simulations showed also that at smaller population sizes, the effects of genetic drift were too strong as compared to the observed values (F_{ST} values were close to 0.59 and 0.39, allele number were 5 and 8 with n = 250 and 500, respectively, after 5000 generations). Moreover, 50 or 500 generations of independent evolution were not sufficient to produce the observed values of A, H_O, and F_{ST} (for instance, with n = 1000, after 50 or 500 generation the simulated values of F_{ST} were 0.003 and 0.030, respectively, much smaller than the observed F_{ST} = 0.09). Assuming that generation time in the European wildcat is 2–3 years, the estimate divergence time between populations in the eastern Alps and in the

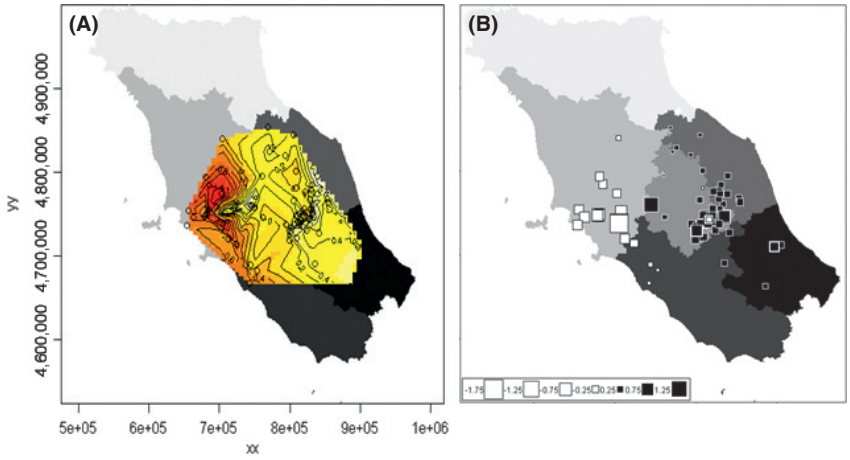

Figure 5. Spatial Principal Component Analysis (sPCA, obtained with ADEGENET) of European wildcats sampled in the central Apennines. (A) Interpolation of the individual genotype scores. The contour lines quantify the degree of genetic differentiation among individuals; circles represent the individual genotypes. (B) Assignment of individual genotypes to their population of origin. Black and white squares represent individual genotype scores on the first principal component (the only significant PC, represented by λ_1 and explaining a significant proportion of spatial structuring). Large white squares indicate individuals with high negative scores; large black squares indicate individuals with highly positive local scores; square dimension is proportional to the degree of differentiations (high for large squares, low for small squares). Letters indicate the sampled regions.

Italian peninsula should correspond to 10,000–15,000 years, correlating with the end of the LGM.

Discussion

In this study, using an extensive sample size genotyped by a higher number of microsatellite loci than in previous works (Pierpaoli et al. 2003; Lecis et al. 2006), we describe the main patterns of genetic subdivision of the European wildcat populations distributed in Italy. The cat samples from Italy show values of genetic diversity comparable to other wildcat or domestic cat populations in Europe (Pierpaoli et al. 2003; Oliveira et al. 2008a,b; Eckert et al. 2009; O'Brien et al. 2009). Despite risks of genetic erosion in the Sardinian wildcats due to founder effects during the historical introduction process, and in the European wildcats due to long-term population decline and fragmentation, these populations are not genetically depleted. All populations showed allelic richness and heterozygosity comparable to random-bred cat populations from the region. The known wildcat x domestic cat hybrids were easily distinguished in the population assignments ($q_i = 0.80$), providing valid controls and a threshold limit for hybridization detection in the remaining wildcats.

Within Italy, this new data set shows a subdivision of European wildcats in at least four genetically distinct subpopulations, as it is concordantly supported by model-based, multivariate, or distance-based clustering procedures. The European wildcats in the eastern Alps, in peninsular Italy, and in Sicily are sharply differentiated. Further, the wildcats in the central Italian peninsula are split into two distinct groups; the first one distributed in the Apennine mountain-hills; the other one in the Maremma hill-plain and coastal areas. This eastern–western subdivision of European wildcats in the central peninsula is clearly described by landscape genetic methods. The assignment of individual genotypes to these two subpopulations is always unambiguous, with the exception of one sample (ID 1009), a museum specimen collected in Maremma, that was partially assigned to the western peninsular subpopulation by both STRUCTURE and sPCA. However, a labeling error cannot be excluded for this sample, which was typed from DNA extracted from a museum skin.

This pattern of population structure is concordant with the Pleistocene biogeographical framework of the Italian peninsula, and is congruent with the distribution of fossil cats before, within and after the LGM (Sommer and Nadachowsky 2006). The main population subdivision: eastern Alps, peninsular Italy, and Sicily, fits well within a scenario of LGM isolation of European wildcat populations in Mediterranean refuges in southern Italy, on the island of Sicily, and in Cisalpine refuges around the borders of the south-eastern Alps. The subdivision in the central Italian peninsula might be the consequence of more recent expansions of historically or ecologically distinct European wildcat subpopulations associated to distinct habitat types. In particular, populations in the western sector of the range might have experienced periods of isolation and local adaptation to a peculiar Medi-

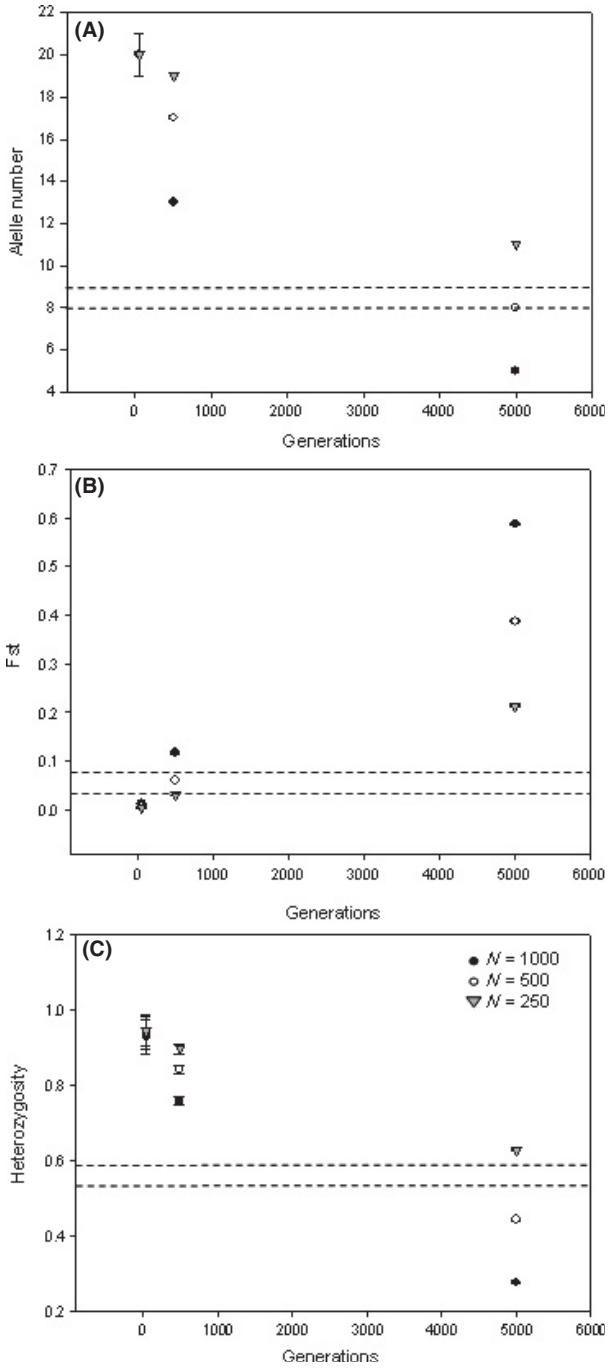

Figure 6. Plot of the average number of alleles per locus (A), F_{ST} values (B) and observed heterozygosity (C), computed simulating two populations of different size (N = 250, 500, and 1000), that were allowed to evolve independently at 33 unlinked autosomal microsatellite loci for 50, 500, and 5000 generations. Horizontal gray blocks identify the average number of alleles per locus (A = 8.77 ± 0.29), the average heterozygosity (H_O = 0. 57 ± 0.03) observed in European wildcats sampled in the Alps and peninsular Apennines, and the observed F_{ST} (F_{ST} = 0.09) value between them. Dots indicate the average values (± their standard errors) obtained from 10 replicate simulations of each parameter settings in EASYPOP.

terranean-type habitat known as Maremma. The central Apennines and the Maremma regions, although parts of the same latitudinal range, represent two distinct bioclimatic and ecological regions (Piovesan et al. 2005). The Apennines are characterized by temperate-fresh summer–autumns and cold-fresh snowing–raining winter and springs. Deciduous-broad-leaved forests and pastures are prevalent in the mountain habitats used by European wildcats. On the contrary, climate in Maremma (the western-coastal part of Tuscany and Latium regions) is influenced by the Tyrrhenian Sea and presents dry and warm spring and summer, temperate and relatively raining autumn and winter, with Mediterranean vegetation composed by sclerophyll and evergreen forest, maquis and garriga (Sforzi and Ragni 1997).

The observed genetic diversity within and among the European wildcat populations in the Alps and in the Italian peninsula might have been jointly generated by large population size (N = 500 or 1000 breeding individuals) and long divergence times (5000 generations). Thus, both observed and simulated genetic parameters suggest that extant European wildcat populations in Italy did not undergo deep historical declines of their effective population sizes, and that genetic divergence among populations cannot be explained by recent fragmentation, but by extended periods of isolation without gene flow (in the order of 5000 generations). A scenario of ancient isolation in LGM Alpine and Mediterranean refuges is further supported by the observed genetic divergence between European wildcats in the eastern Alps and in Sicily, which was larger than that between the domestic cats and the two wildcat subspecies. Both the R_{ST} and the F_{ST} distances between the domestic cats, the European wildcats and the African wildcats from Sardinian support previous studies that domestic cats are more closely associated with North African subspecies of wildcats than the European subspecies (Driscoll et al. 2007; Lipinski et al. 2008). The phylogeography of the European wildcat might be further refined by expanding the samples to include other populations, and by expanding the markers by the use of genome-wide SNPs scans. For instance, the European wildcat population in the eastern Alps might be in contact with neighboring populations in Slovenia and Croatia. An integrated data set, including Italian plus Slovenian and Croatian samples will help to better delineate the phylogeography of European wildcats, and to assess if wildcats in the eastern Alps have been isolated in the hypothesized LGM refuge, or originated by post-glacial expansions of south Balkan source populations.

The integration of STR and SNP data sets, or the analysis of genome-wide SNP scans, will also accomplish the need to develop more realistic phylogeographic events (Nussberger et al. 2013). The poorly known mutation mechanisms, and the rapid molecular evolutionary rates

of STR loci, with the consequent risk of homoplasy, might complicate the reconstruction of the dynamics of populations that have been genetically isolated for thousand of generations. In contrast, the simpler mutation mechanism of SNPs, and the possibility to select nucleotide substitutions in different regions of the cat genome, might help to reconstruct ancient evolutionary events (Miller et al. 2012). In this study, we did not use mtDNA sequences because of the following: (1) the extensive presence of nuclear-mitochondrial copies (*numts*) in the cat genome (Antunes et al. 2007), (2) the uncertain distinction among domestic and wildcats mtDNA haplotypes (but see: Driscoll et al. 2011). In these conditions, we feel that any mtDNA phylogeographic reconstruction of populations which might have hybridized in the recent past, or that are still hybridizing, is risky, because mtDNA haplotypes of undetected domestic origin might confuse the evolutionary reconstructions of the European wildcat populations.

Conclusions and conservation perspectives

Results in this study add novel details to the reconstructions of the European wildcat population structure in Italy, contributing to better identify significant conservation units that are relevant for wildcat conservation strategies. The discovery of distinct refugial populations dictates the need of conservation plans focusing on the priority to guarantee long-term survival of both population networks. The western versus eastern population subdivision in central Italy might be related to peculiar processes of local adaptations to different habitat types, which need to be better understood. The ongoing transition from conservation genetics to conservation genomics will help to answer both theoretical and practical wildcat conservation issues. Whole- or wide-genome screening might identify mutations showing sharp frequency changes among populations, indicative of functional divergence and adaptation to variable ecological conditions and/or domestication. The discovery of selected loci will clarify the evolutionary dynamics of local adaptations in wildcats in the widest contest of comparative ecological genomics (Martin et al. 2003). Wildcat populations are threatened by hybridization with free-ranging domestic cats. The discovery of novel diagnostic molecular markers will also help to identify hybrid individuals and areas of genetic introgression which greater precision and efficiency (Nussberger et al. 2013).

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Plot of Log probability [L(*K*)] as a function of *K* averaged over five independent runs of STRUCTURE. The Y-error bars are standard deviation and *K* is the assumed number of genetic clusters. Each plot represents a sample set: (A) sample set *A* (European wildcats, domestic cats and known hybrids); (B) sample set *B* (Sardinian wildcats, domestic cats and known hybrids); (C) sample set *C* (domestic cats, European and Sardinian wildcats); (D) sample set *D* (European wildcats only). (E) Inference of the number of genetic clusters in the study area: posterior distribution of the number of populations estimated using GENELAND.

Figure S2. Maps of posterior probability of European wildcats identified by GENELAND. Samples are split into five clusters ($K = 5$): (1) eastern Alps; (2) Maremma; (3) central peninsular Apennines; (4) southern peninsular Apennines; and (5) Sicily.

Table S1. Description of 35 microsatellite loci used to genotype the cat samples (*Felis silvestris*) analyzed in this study. Locus identifications (ID) and chromosome assignments are from Menotti-Raymond et al. (2003a,b); the asterisk indicates imperfect dinucleotide microsatellites showing some intermediate alleles; primer tails were labelled to fit the design of eight multiplex sets. The allelic range (in base-pairs) and the observed number of alleles at each locus (N_A) are reported. F_{IS} = Weir and Cockerham (1984)'s fixation index for each locus in cat subspecies (data set E) and European wildcat populations (data set F) were computed excluding the admixed genotypes (see Results); *** significant departures from Hardy-Weinberg

equilibrium at $P < 0.001$ ($P < 0.00{,}028$ after Bonferroni-correction for 35 independent comparisons). F_{IS} values at X-linked loci FCA240 and FCA651 were computed only in females (*N*f, number of females in samples). M, monomorphic loci in wildcats sampled in Sardinia and Sicily.

Table S2. Identification of the number of *K* clusters in STRUCTURE analyses of cat samples (see Table 2). Optimal *K* values (in bold) were identified by the maximum increase (ΔK) of the mean *Ln* posterior probability (Mean lnPD) and of the mean F_{ST} values (ΔF_{ST}) between subsequent analyses. NA, not analysed.

Table S3. Average proportion membership (Q_i) of wildcat populations obtained by STRUCTURE with $K = 2$–4 and the "localities as prior model", using: the three cat subspecies (sample set *C*); European and Sardinian wildcats (domestic cats excluded); European wildcats (Sardinian wildcats excluded), sampled in eastern Alps, Maremma, central and southern Apennines and Sicily (sample set *D*, see Fig. 2A)