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POPULATION SURVEY OF THE EUROPEAN WILDCAT IN THE NATURAL BIOGENETIC RESERVE OF THE FORESTE CASENTINESI NATIONAL PARK (NOTHERN APPENINES)

INDAGINE GENETICA SULLA POPOLAZIONE DI GATTO SELVATICO EUROPEO NELLE RISERVE NATURALI BIOGENETICHE CASENTINESI (APPENNINO SETTENTRIONALE)

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Abstract. The European wildcat (*Felis silvestris silvestris*) is one of the most elusive carnivores in the Italian peninsula. It is mainly threatened by habitat fragmentation and especially hybridization with the domestic cat. Effective actions for its conservation are strictly related to a sound knowledge of its population parameters and genetic status. In this study we present a sistematic monitoring that provided an informative framework on the wildcat distribution in the Biogenetic Casentinesi Reserves inside the Foreste Casentinesi National Park, the northernmost border of its ascertained peninsular range. We applied an extensive camera-trapping protocol based on a systematic grid in order to approach the same capture probability for all the individuals in the study area. Furthermore, we used valerian-baited hair-traps to verify the reliability of this technique in attracting wildcat individuals, lead them to rub against the hair-traps in order to catch biological samples for genetic analyses. We collected a total of 99 wildcat pictures identifying a minimum number of 13 individuals (capture density 6.6/10 km^2) and detecting the presence of at least one domestic cat and a putative hybrid in the study area. The use of lures proved to be effective in increasing the capture probability of the target species in camera-trapping based studies ($p < 0,05$). However, only some individuals showed a clear rubbing behavior and left hair in the hair-traps, supporting the hypothesis of a different individual genetically-mediated reaction. Genetic survey identified seven individuals (four males and three females), all belonging to *Felis silvestrsis silvestris* population. Five of them showed putative introgressed mitochondrial haplotypes.

Riassunto. *Il gatto selvatico europeo* (Felis silvestris silvestris) *è uno dei carnivori più elusivi della penisola italiana. È principalmente minacciato dalla frammentazione dell'habitat e soprattutto dall'ibridazione con il gatto domestico. Le azioni efficaci per la sua conservazione sono strettamente legate a una solida conoscenza dei parametri di popolazione e del suo status genetico. Questo studio presenta i risultati di un monitoraggio sistematico finalizzato a definire la distribuzione del gatto selvatico nelle Riserve Biogenetiche Casentinesi, all'interno del Parco Nazionale delle Foreste Casentinesi, confine più settentrionale del suo accertato areale peninsulare. Abbiamo applicato un ampio protocollo di fototrappolaggio basato su una griglia sistematica, per avvicinarci alla stessa probabilità di cattura per tutti gli individui nell'area di studio. Inoltre abbiamo utilizzato trappole per peli con estratto di valeriana, per verificare l'affidabilità di questa tecnica nell'attrarre individui di gatto selvatico, inducendoli a strofinarsi contro le trappole, al fine di catturare campioni biologici per analisi genetiche. Abbiamo raccolto in totale 99 immagini di gatto selvatico, identificando un numero minimo di 13 individui (densità di cattura 6,6/10 km^2) e rilevando la presenza di almeno un gatto domestico e un ibrido putativo nell'area di studio. L'uso di esche si è dimostrato efficace nell'aumentare la probabilità di cattura delle specie bersaglio in studi basati su fototrappole ($p < 0,05$). Tuttavia, solo alcuni individui hanno mostrato un evidente comportamento di sfregamento e hanno lasciato i peli nelle trappole, supportando l'ipotesi di una diversa reazione geneticamente mediata dell'individuo. L'indagine genetica ha identificato sette esemplari (quattro maschi e tre femmine), tutti appartenenti alla popolazione di* Felis silvestrsis silvestris. *Cinque di loro mostravano aplotipi mitocondriali introgressi.*

Introduction

The wildcat *Felis silvestris* is a polytypic species comprising six morphologically, ecologically and genetically differentiated subspecies that inhabit Palearctic and Afrotropical Regions (see Driscoll *et al*. 2007 for details). In Europe, three of them coexist: the European wildcat (*Felis silvestris silvestris* Schreber, 1777), whose distribution is scattered throughout the continent; the African wildcat (*Felis silvestris libyca*, Forster 1780), living in the Mediterranean islands of Corsica and Sardinia, (Randi & Ragni 1991, Driscoll *et al*. 2007); and the domestic descendant of *libyca* North African cats, the domestic cat (*Felis silvestris catus*) that spread throughout the entire continent, as well as in the entire World.

The species' distribution range Italy covers the entire Apennines (Ragni *et al*. 1994), including Sicily (Anile *et al*. 2012a; 2014), with confirmed consistent populations in the first sectors of nothern Appenines (Foreste Casentinesi National Park, Velli *et al*. 2015).

Results of a national survey carried out by Cagnolaro (1976) in the '70 using indirect methods, compared with more recent findings (Agostini *et al*. 2010; Tedaldi 2012; Ragni *et al*. 2014; Velli *et al*. 2015) suggest a northwards wildcat expansion, likely promoted by suitable forested habitat corridors in protected areas along the Apennine ridge (Santolini *et al*. 2010). The European wildcat population in north-eastern Italy is genetically connected with the Dinaric-Balkan population (Mattucci *et al*. 2013).

Recent observations (Gavagnin *et al*. 2018) suggest the persistence of a north-western population in connection with a French source.

The European wildcat is a 'strictly protected' species included in Annex IV of the European Habitats Directive (92/43/CEE). It is included in Annex II of the Bern Convention, and it is classified as Least Concern by the IUCN (Driscoll & Nowell, 2010) and near threatened in the Italian Red List (Rondinini *et al*. 2013). Main threats are the loss of suitable habitat (Klar *et al*. 2009; 2012), human-caused mortality, in particular road kills (Nowell & Jackson 1996; Lüps *et al*. 2002; Schulenberg 2005; Krone *et al*. 2008) overgrazing by large game species (Lozano *et al*. 2007) and especially hybridization with the domestic cat (*Felis silvestris catus*; Randi 2008; Oliveira *et al*. 2008).

Reliable estimates of population abundance and trends are the key baseline data to assess the impact of threatening factors needed to outline sound conservation guidelines (see Council of Europe 1993). This is particularly relevant when dealing with expanding populations, in which the risk of hybridization increases (cfr. wolf, Galaverni *et al*. 2017).

The lower portion of the northern Apennines has been investigated in the last 10 years by a few studies (Agostini *et al*. 2010; Tedaldi 2012; Ragni *et al*. 2014; Velli *et al*. 2015) using different techniques, however an integrated survey of the Natural Reserves is still lacking.

In this work, we present the results of a non-invasive European wildcat survey carried out in the fully protected area of the Natural Biogenetic Reserve in the Foreste Casentinesi National Park aiming at investigating the presence of the subspecies in the northernmost area of its ascertained Apennine distribution by integrating hair-trapping, camera-trapping and scat collection methods.

Materials and methods

The study was carried out between July 2014 and July 2015. We selected a 20 km^2 wide area in the fully protected Sasso Fratino Natural Reserve (Fig. 1), one of the most biodiverse spots in the Foreste Casentinesi National Park. Following the monitoring protocol reported in Velli *et al*. (2015) and the information of previous surveys (2009-2013; Ragni *et al*. 2014) we applied a 1 x 1 km grid over the sampling area and systematically placed 21 raw pine sticks (60 x 4 x 4 cm), trying to uniformly cover the grid and placing, when possible, at least a lure in each square (Hupe & Simon 2007; Kéry *et al*. 2011, Hartmann *et al*. 2013; Steyer *et al*. 2013; Velli *et al*. 2015). Each picket was identified with a code, geo-localized and drenched with valerian (*Valeriana officinalis*) hydroalcoholic tincture (70%); in addition, longitudinally at the top of it, we made a hole of about 2 x 7 cm, and two smaller ones transversely on each side and filled them with valerian root powder to obtain a stronger, uniform and longer-lasting effect even during rainy days. Valerian has been proved to induce not only a significant investigative response from wildcats, but also to promote a strong rubbing behaviour (Monterroso *et al*. 2011) and has been used in several studies on wildcat (Kéry *et al*. 2011, Steyer *et al*. 2013; Velli *et al*. 2015). In order to catch as many hairs as possible, we scratched the surface of the wood applying a strip of Velcro tape. We equipped 17 of 21 sampling stations with a camera-trap. Cameras were tied to trees at about 2 m to the lured pickets and set on both video and photo modes, trying to get high quality pictures as well as behavioural informative videos. Each camera was equipped with a 4-GB SDHC card and was powered by four rechargeable AA batteries. Sampling stations were placed along the trails used for collecting faecal samples. We performed two different sampling sessions: the first one from 8th of July to 23th of October 2014 and the second one from 28th April to 29th July 2015. Sampling stations were visited each 7-10 days. During each session we replaced SD cards and batteries of camera-traps, collected possible hair samples and scratched the wood stick with an iron brush to remove any residual hairs, preventing contaminations in the following session. Finally, a new Velcro tape was applied. Hair samples were stored in silica envelopes while scats were conserved in 96% ethanol and frozen as soon as possible. Sample collection was performed using disposable gloves and flaming the forceps used to collect hair. A preliminary subspecies and individual identification were obtained by analysing coat colour patterns and body proportions of the animals

Fig. 1: boundaries of the Sasso Fratino Integral Reserve in which was carried out the survey

(French *et al*. 1988; Ragni & Possenti 1996). The presence and shape of any additional sign on the pelage (Anile *et al*. 2012a), behaviour and body proportions were further considered. Each collected video of *Felis sp*. was analysed by three different independent operators. In order to investigate the reactions toward the bait we compiled an ethogram (Wells & Egli 2004; Ellis & Wells 2010) in which we listed seven different definitions that could cover the range of behaviours displayed: indifference (I), curiosity (C), facial marking (FM), strong interaction and rubbing (SI), only spray marking (SM), diffidence (D) and fear (F). If during the same shooting different behaviours occurred, we considered only the stronger one (i.e., if a cat displayed curiosity followed by facial marking and strong interaction, we considered only the "strong interaction" event) except for the urinary marking that could follow (or not) preview behaviours.

Genetic Analyses

DNA extraction was performed using the Blood&Tissue Kit® (Quiagen) protocol following manufacturer instructions. Furthermore, hair samples were processed adding to the digestion mix 20µl of dithiothreitol required to efficiently degrade the keratin skeleton of hairs (McNevin *et al*. 2005).

All samples were preliminarily analysed at the mitochondrial DNA (mtDNA) in order to discard samples belonging to non-target species using a 719 bp fragment of the mtDNA control-region (hence CR; sites 16236 - 16955) and primers CHF3 (5'-CTC CCT AAG ACT TCA AGG AAG-3'; Freeman *et al*. 2001) and CHR3 (5'-CCT GAA GTA AGA ACC AGA TG-3'; Tiedemann *et al*. 1996).

All samples belonging to the target species were then amplified at 10 autosomal microsatellite (STR) loci FCA23, FCA26, FCA43, FCA58, FCA77, FCA88, FCA96, FCA126, FCA132, FCA149 (Menotti-Raymond & O'Brien 1995, Menotti-Raymond *et al*. 1997) and one microsatellite locus SMCY-7 STR on Y chromosome showing a polymorphism that seems to be fixed with different alleles in the two subspecies studied (Luo *et al*. 2007, Nussberger *et al*. 2013). PCR conditions follow Velli *et al*. 2015 procedures. PCR products were analysed in an ABI 3130 XL (Applied Biosystems) automated sequencer, and allele sizes were determined with GENEMAPPER 4.0 (Applied Biosystems). We used a multiple tube approach with a minimum number of four replicates per sample in order to assess the rate of allelic dropout (ADO) and false alleles (FA) (Taberlet *et al*. 1999) The reliability value for each sample was assessed with RELIOTYPE (Miller *et*

al. 2002). Using the match function in GENALEX 6.501 (Peakall & Smouse 2012) we detected individuals sampled more than once. Discrimination between the wild and the domestic subspecies was performed in STRUCTURE 2.3.4 (Pritchard *et al*. 2000), setting the genetic clusters K=2 (Oliveira *et al*. 2008; O'Brien *et al*. 2009). Analyses were based on 400 000 MCMC steps after discarding the first 40 000 steps as burn-in, under the admixture model with correlated allele frequencies (Hertwig *et al*. 2009; Eckert *et al*. 2010). 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 6.501 (Peakall & Smouse 2012). We used a panel of 77 free-living or house domestic cats, 235 putative European wildcats and 17 previously described *silvestris* x *catus* hybrids, collected in Italy from 2003 to 2010 and already analysed at 35 loci (Mattucci *et al*. 2013), as reference data set for calculation of PIDsibs (the probability of identity among siblings), chromosome Y subspecies assessment, mitochondrial and STRUCTURE analysis. Assignment threshold was set to qi > 0.8 for subspecies identification (Pierpaoli *et al*. 2003; Oliveira *et al*. 2008). We sequenced 877 bp (including the primers) of the mtDNA NADH dehydrogenase subunit 5 (hence ND5; nucleotides 13131 - 14007 mapped on the mitochondrial genome of the domestic cat; NCBI Reference Sequence NC001700), which, according to Driscoll *et al*. (2011), contains seven diagnostic SNPs discriminating European wildcats (*Felis silvestris silvestris*) and domestic cats (*Felis silvestris catus*). Sequences were amplified using PCR primers F2B (5'-TGCCGCCCTACAAGCAAT-3') and R3B (5'-TAAGAGACGTTTAATGGAGTTGAT-3') (Driscoll *et al*. 2011) and aligned using SEQSCAPE software v2.5 (Life Technologies). The mtDNA genome of the domestic cat (NCBI Reference Sequence: NC_001700), trimmed at the above-mentioned positions, was used as the reference sequence. In order to assign the samples to the correct subspecies lineage we analysed the ND5 sequences performing a median joining network using NETWORK 4.6 (Fluxus Technology Ltd).

Results

Camera trapping

Camera-traps worked for a total of 3220 trap-nights. We obtained a total of 99 *Felis silvestris* captures (269 events) with a capture frequency of 3.07% (Tab. 1). Based on size and proportion of the body, behaviour and coat colour marking patterns (Ragni & Possenti 1996), we identified 13 different individuals of *Felis silvestris silvestris,* and two individuals showing signs of possible hybridization (white paws and belly). Considering a study area of 20 km^2 we calculated a capture density of *Felis silvestris silvestris* of 6.6 individuals / 10 km^2 and a capture rate of 3.1/100 trap-nights.

Tab. 1: synoptic table resuming the genetic individual assignations using different genetic markers: microsatellites (STR genotype), mitochondrial DNA (mtDNA) and Y-chromosome haplotype (Chr Y). Individuals with question marks are samples that did not yielded reliable individual nuclear genotype but returned different mitochondrial haplotype.

ID genotype	ID samples	ID trap station	STR genotype	mtDNA	Chr Y
1	2005 (hair) 2006 (hair) 2007 (hair)	11 11 11	*F. s. silvestris*	D-intr	*F. s. silvestris*
2	2014 (hair) 2015 (hair) 2016 (hair)	12 12 11	*F. s. silvestris*	W	*F. s. silvestris*
3	2004 (hair) 2008 (hair) 2020 (hair) 2023 (hair)	11 11 11 11	*F. s. silvestris*	D-intr	*F. s. silvestris*
4	1981 (scat)	Fosso delle Macine	*F. s. silvestris*	D-intr	-
5	1983 (scat)	Pian del Pero	*F. s. silvestris*	D-intr	-
6	1994 (hair)	13	*F. s. silvestris*	D-intr	*F. s. silvestris*
7	2000 (hair)	11	*F. s. silvestris*	W	-
8(?)	1829 (scat)	Pian del Pero	-	W	-
9(?)	1831 (scat)	Pozza del Cervo	-	D	-

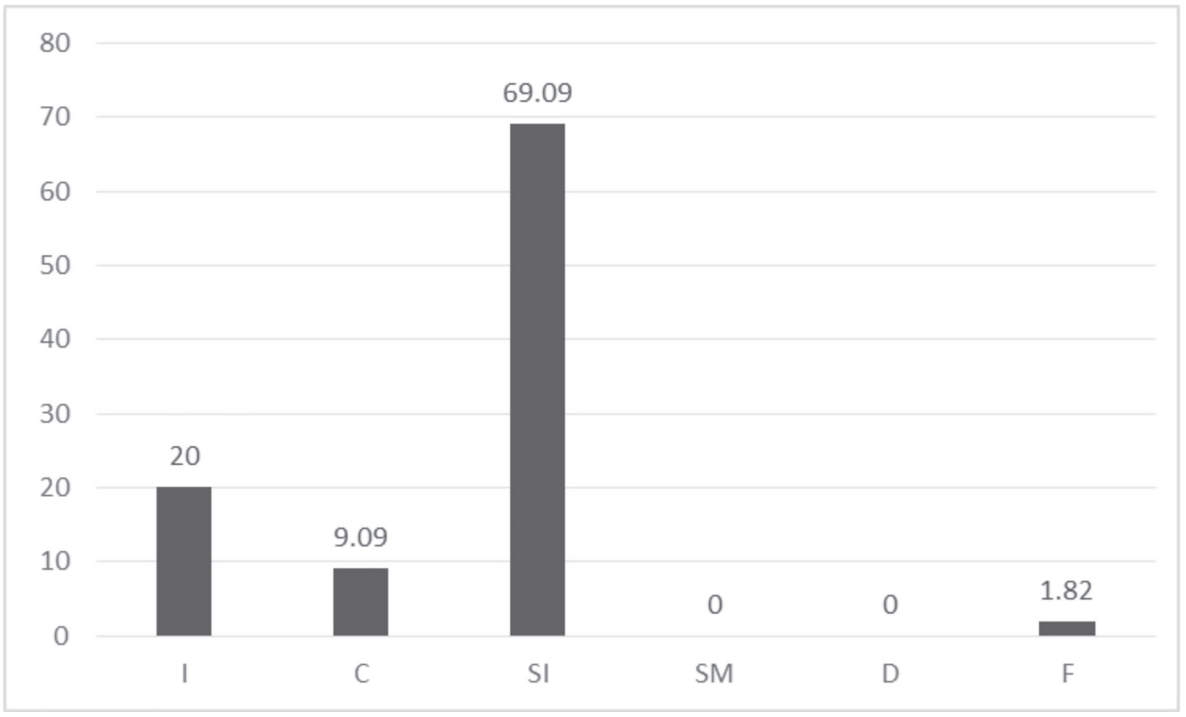

Fig. 2 - istogram showing the proportion of different behaviours towards the lure among all sampled individuals. indifference (I), curiosity (C), facial marking (FM), strong interaction and rubbing (SI), only spray marking (SM), diffidence (D) and fear (F).

Activity patterns of wildcats in the study area were mainly nocturnal (more than 90% of capture between 9:00 pm and 5:00 am). In 20% of cases the individuals showed no interest (I) for the lures and a total of 69.09% individuals acted out the expected behaviour, leaving hair samples (SI) (Fig. 2). Two individuals didn't show any interest toward the lures, while nine scratched on the picket leaving hair-samples (FM or SI). To investigate the effectiveness of valerian tincture in increasing capture probability, we compared the capture mean rates for three sites used in both 2009-2013 (mean = 2.66 ± 1.76, without lures) and 2014-2015 (mean= 29.57 ± 29.20, with valerian scent lure) monitoring programs. Fisher's test confirmed a high significant (*p-value* = $1{,}99 \times 10^{-7}$) influence of valerian lures in increasing capture probability, although this effect was likely not evenly distributed among wildcat individuals.

Genetic analyses

We collected 54 non-invasive samples (39 hair samples and 15 scats). Preliminary mitochondrial screening identified 11 samples belonging to non-target species that were discarded for subsequently analyses. Of the remaining 43 *Felis* samples, we successfully genotyped the 32.4% (n = 14), of which the majority (n = 12) deriving from hair trapping. Ten autosomal loci yielded a value of PIDsib = 0.0001. Absence of samples presenting more than two alleles guaranteed that no contamination occurred among samples. We identified at least seven different individuals, all belonging to the *Felis s. silvestris* subspecies (Tab. 1). According to the chr-Y analyses, four of them were male, with a wildcat associated Y-haplotype. We calculated a capture density of 3.5 individuals / 10 km^2. Regarding the mitochondrial analyses of ND5 subunit, the samples fell into two main different haplogroups (W, D) that, according to the previous assignation of reference samples, correspond respectively to the wildcat haplogroup (W) and the domestic haplogroup (D). Combining the information derived from the three types of markers (STR, mtDNA and SMCY-STR) we were able to confirm as pure wildcats the individuals with concordant attribution through the different markers. We also detected five individuals with putative introgressed mitochondrial haplotypes (D-intr) as they were featured by a nuclear genotype attributed to the wildcat (qi > 0.8) but a mtDNA haplotypes presenting all the domestic / *Felis s. lybica* cat polymorphisms. In six occasions (Tab. 2) we were able to associate the genotype to the putative phenotype captured by the camera-traps.

Tab. 2: table resuming the phenotype-genotype association integrating the information from camera trapping and non-invasive genetic survey

ID trap station	ID individual from camera trapping	ID genotype
2	A-B-F	
4	C-E	
7	G	5/8
8	G	5/8
9	N	
10		9
11	A-H-I-L-O	1/2/3/7
12		2
13		6
14	D	
15	L	4
16	M	

Discussion

In this work we applied a multidisciplinary non-invasive methodology to monitor the presence of the European wildcat in the Natural Biogenetic Reserve of the Foreste Casentinesi National Park. Integrating camera trapping, scat survey and hair-trapping with valerian lures we were able to collect information about the minimum consistency of wildcat population, impact of free ranging domestic cats and possible presence of hybrids. We detected 13 different wildcat individuals through camera trapping with a capture rate of 3.1/100 night-traps and seven different wildcat genotypes from non-invasive genetic monitoring. Two putative hybrids were identified using camera traps. Although none genotype showed sign of recent hybridization, five individuals showed domestic mitochondrial signature. Capture density showed different values depending on the used technique: using camera-trapping we calculated 6.6 individuals / 10 km^2 that is almost two times the values coming from genetic monitoring of 3.5 individuals / 10 km^2. Capture densities were comparable with a similar study carried out in similar ecological conditions (4.5 individuals / 10 km^2 for camera trapping and 2.6 individuals / 10 km^2 for non-invasive genetic; Velli *et al*. 2015) and also capture rates (1.8/100 trap-nights, Can *et al*. 2011; 2.3/100 trap-nights, Kilshaw & Macdonald 2011; 2.9/100 trap-nights, Anile *et al*. 2012b; 3.1/100 trap-nights, Velli *et al*. 2015). Genotyping success rates (32.4%) was slightly lower but still comparable with previous works (36%, Anile *et al*. 2014; 37.2%, Velli *et al*. 2015). The use of an integrated monitoring tool allowed us to collect heterogeneous data by using single sampling effort and to offset the weaknesses of each method. In particular, camera trapping is one of the most functional methods for monitoring several species (Silveira *et al*. 2003) and in our study contributed to detect possible hybridization events that would have been left unveiled using only non-invasive genetics. However, it may suffer from overestimation errors, in particular in the abundance estimate reached with capture-recapture methods, especially in studies of elusive animals with few identification marks in low-density areas (Foster & Harmsen 2012). Non-invasive genetics monitoring, instead, provide a more accurate individual detection and a deeper insight on population ancestry, although it suffers from genotyping errors and low success rates. The application of integrated monitoring techniques allows to avoid the risk of an underestimation of the population due to the heterogeneity in the response to the bait. A total of 69.09 % of detected wildcat positively reacted to valerian lures. This result slightly differs from other similar application of this method. A study by Monterroso *et al*. (2011) highlights that only 11.5% of the wildcats identified showed an investigative behaviour towards the bait. While Velli *et al*. (2015) found that 20.6% of individuals reacted with the expected behaviour, leaving hair samples on the picket. A work by Anile *et al*. (2012b) in Sicily, found no reactions at all. Monterroso *et al*. (2011) also evidenced the greater attractive power of the lynx (*Lynx lynx*) urine. However, in order to avoid any interference in the spatial behaviour and in other ethological features of the target-species, we preferred not to use such intensive substance belonging to a species not present in the study area. The integration of non-invasive genetic sampling with the camera

trapping allows to successful associate the genetic data with the picture of an individual.

Current results suggest the presence of a well established population in the study area, reinforcing the hypothesis of a northward expansion of the subspecies (Ragni *et al*. 2014; Velli *et al*. 2015). Nonetheless, the presence of putative hybrids, in a fully protected area such as the Sasso Fratino Natural Reserve arises concern about the long term conservation of the subspecies, often sharing the territory with its domestic counterpart, in a medium anthropized environment such as the northern Apennines. However, mitochondrial results should be taken with caution since domestic cats and their strictly related progenitors, the North African wildcats, *Felis s. lybica*, share most of the mitochondrial variation (Driscoll *et al*. 2007). Hence, the presence of putative domestic polymorphisms in European wildcat individuals might derive from haplotype sharing with ancient populations of *Felis s. lybica*. Further studies are currently underway.

In conclusion, in this study we improved the knowledge on the population dynamics of the European wildcat living in an expanding area such as the northern Apennines. We provided a preliminary insight on its genetic status by using an integrated protocol of different monitoring techniques. Since the European wildcat areal seems to keep advancing northward, future study aimed at ascertaining the presence/absence of the subspecies even in other areas of northern Apennines are, therefore, strongly recommended.

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