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Sezione Biodiversità ed Analisi degli Ecosistemi

The European wildcat (Felis silvestris silvestris): study for a functional method of population research

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CHAPTER 3

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FINAL REMARKS

General issues

The European wildcat (*Felis silvestris silvestris*, Schreber 1777) is a medium-sized elusive carnivore. Apparently its aspect resembles a domestic tabby cat, with a general pelage pattern brown-grey or dark-grey. Actually the wildcat is somewhat larger than the domestic cat (Stahl and Leger 1992) and presents several diagnostic phenotypic features (Ragni and Possenti 1996; Beaumont *et al.* 2001; Kitchener *et al.* 2005) including a black *rhinarium*, a uniform colour pattern of auricular *pinnae*, four/five black occipital stripes, two short scapular bands, one long and sharp black dorsal line abruptly ending at the base of the tail that appears long and clavate with a black distal end and two/three black rings. In addition, a wildcat should present a white spot on the throat and black paws. An analysis of these signs together with cranial and intestinal indexes when available allow one to distinguish wildcat from domestic cat and could provide cues for hybrids detection (Ragni and Possenti 1996).

BIOLOGY

Habitat and diet

The European wildcat is mainly a forest animal (Schauenberg 1981) although it can be found in a large variety of habitats (Stahl and Leger 1992) if adequate shelter and prey availability are present (Virgos *et al.* 2002). In general, however, the ideal habitat are mosaic environments characterized by dense and protected patches (forests, dense scrublands etc..) alternating with open spaces like meadows, forest clearings or fallow lands where the wildcat can easily hunt (Easterbee *et al.* 1991; Lozano *et al.* 2003). Moreover, it avoids densely human populated areas (Easterbee *et al.* 1991; Klar *et al.* 2008; Lozano 2010). Considering the increasing density of humanized areas ecological corridors are now playing a crucial role in the connection between areas suitable by the wildcat (Klar *et al.* 2012). The wildcat diet consists mainly of rodents and rabbits on which it can behave as facultative specialist when necessary and preferring rabbits when available (Moleon and Gil-Sanchez 2003; Lozano *et al.* 2006).

Nevertheless, it can integrate its diet with birds, reptiles and occasionally insect and carcasses (Sunquist and Sunquist 2002). Usually, domestic cats are less specialized and more opportunistic regarding the feeding habits (Biro *et al.* 2005).

Mating and reproduction

Usually the *oestrus* falls in 5-9 days between December and February and the mating period between January and March, depending on the region and the age and health status of individuals. Gestation takes about two months and around April-May the mother give birth to litter ranging between one to four (until seven) helpless and blind cubs that disperse usually before the arrival of winter (after five or six months), although females may wait longer (Stahl and Leger 1992; Heptner and Sludskii 1992; Nowell and Jackson 1996; Daniels *et al.* 2002). Sexual maturity is reached usually around 300 days and the maximum life span (at least in captivity) is 15 - 21 years (Nowell and Jackson 1996; Sunquist and Sunquist 2002) even if in the wild it is not expected to exceed 13 -14 years (Heptner and Sludskii 1992).

Behaviour and spatial organization

The wildcat, unlike domestic cat (Macdonald *et al.* 1987), is a solitary animal, except in the mating season or when females are rearing kittens (Kilshaw 2011). The species is strongly territorial and patrol regularly the territory using trails and paths, marking it with faeces, urine and scratches and aggressively defending it from intruders (Corbett 1979; Biro *et al.* 2004). Home ranges can varies considerably from 175 ha to more than 2 000 ha, according to the season, sex and food availability with areas being larger for males and stable for females (Corbett 1979; Stahl *et al.* 1988; Liberek 1996; Biro *et al.* 2004; Bizzarri *et al.* 2010). It have been observed that home ranges can partially overlap (approximately 70%) especially between males and females of the same age class or between adults and juveniles (36%) (Stahl *et al.* 1988; Biro *et al.* 2004; Macdonald *et al.* 2010a). The dispersion range varies between male and female: in male reaching on average six kilometres of distance from native territory and in female generally not going beyond three or four kilometres (Tryjanowski *et al.* 2002). The wildcat is considered nocturnal, discrete and quite, moving mainly at dusk and night (Stahl and Leger 1992) but, in areas where human pressure is low or absent, it can take advantage also of diurnal hours (Kilshaw 2011). Wildcat generally avoids snow covered areas, descending at lower altitudes during snowy months and ascending back during warmer seasons to avoid the presence of man ad exploit the greater food availability (Liberek 2002; Mermod and Liberek 2002; Okarma *et al.* 2002).

TAXONOMY OVERVIEW

The wildcat, in the broad sense, is a small cat belonging to a lineage (Genus: *Felis*) that about six millions years ago diverged from other felid branches (Johnson *et al.* 2006; Werdelin *et al.* 2010). The genus includes four closely related species distributed across Africa and the Palearctic: the jungle cat (*Felis chaus* Schreber, 1777), the sand cat (*Felis margarita* Loche, 1858), the black-footed cat *(Felis nigripes* Burchell, 1824) and the wildcat (*Felis silvestris* Schreber, 1777). The modern wildcat probably is related to *Felis lunensis* Martelli, 1906, whose presence in Europe is known as early as the late Pliocene, about two millions of years ago (Kurtén 1965; Kurtén 1968; Kitchener 1991; Masseti 2010). Fossils records suggested that the transition to the modern wildcat may have occurred during the Pleistocene, probably between 450 000 and 350 000 years ago (Kurtén 1965; Yamaguchi *et al.* 2004b).

According to the last phylogenetic studies (Randi and Ragni 1991; Driscoll *et al.* 2007) the European Wildcat (*Felis silvestris silvestris* Schreber, 1777) should be considered the nominate subspecies of *Felis silvestris* together with other four subspecies: the African Wildcat (*Felis s. libyca* Forster, 1780), the Asian Wildcat (*Felis s. ornata* Gray, 1830), the Southern African Wildcat (*Felis s. cafra* Desmarest, 1822) and the Chinese Alpine Steppe Cat (*Felis s. bieti* Milne-Edwards, 1872) (Driscoll and Nowell 2010). However another taxonomic arrangement was proposed which considers as distinct species recently *F. silvestris, F. bieti* and *F .libyca* (including *F .l. cafra* and *F .l. ornata* as subspecies) (Kitchener and Rees 2009; Macdonald *et al.* 2010b). The Scotland population of *F. s. silvestris* has been long time isolated and shows several molecular and phenetic peculiarities (Pierpaoli *et al.* 2003; Driscoll *et al.* 2007) determining uncertainties on its belonging to the spp. *silvestris* or to an additional one, named *grampia* (Randi and Ragni 1991; Wilson and Reeder 1993). Actually, the nomenclature that sees the domestic cat as a subspecies of *Felis silvestris*, is wrong according to the rules of the International Code of Zoological Nomenclature (ICNZ) because *Felis catus* (Linaeus, 1758) is an older name vs. *Felis silvestris* (Schreber, 1777). This would result in a clear incongruence with the phylogenetic findings because one should be forced to consider all the wildcats as subspecies of Felis catus (so for example the European Wildcat should be named *Felis catus silvestris*). However, the International Commission on Zoological Nomenclature conserved the usage of the common use of *Felis silvestris* referring to the wildcat (Opinion 2027, ICNZ 2003). The debate on this taxonomic issue remains open due to the strong legal issues related to the conservation (see below) of the species and its sympatric co-existence with the domestic cat (Macdonald *et al.* 2010b). The wildcat is included, as strictly protected species, in Appendix II CITES (all *Felidae* species that are not present in Appendix I, excluding domesticated forms), Appendix II of the Bern Convention and Annex IV of the Habitats Directive (all considering *Felis silvestris*). Several national laws that deal with the protection of endangered species have adopted a nomenclature that considers only the species name without specifying how to

consider the domestic form (e.g. Legge 157/92 in Italy, Real Decreto 139/2011 in Spain, UK Priority species list, 2010). Considering more than one species, opens up different interpretations of laws that deal with them. Indeed, for example, according to the last phylogenetic evidences (Randi and Ragni 1991; Randi *et al.* 2001; Driscoll *et al.* 2007), that better reflect the Biological Species Concept (BSC, Mayr 1970), wildcats and domestic cats should be considered the same species (*F. s. silvestris* and *F. s. catus*), thus constituting two distinct subspecies. In this way, most of the laws and regulations that would protect the wild cat would eventually paradoxically protect even its most important competitor and threat, including hybrids (Macdonald *et al.* 2010b). A similar case could be that of the wolf and domestic dog. In this perspective, the problem of nomenclature takes a more procedural and legal relevance rather than biological.

However, in this study we considered the wildcat as a subspecies of *Felis silvestris*.

DOMESTICATION PROCESS

Formally, domestication can be defined as "the process whereby a population of living organisms is changed at the genetic level, through generations of selective breeding, to accentuate traits that ultimately benefit the interests of humans" (Diamond 1999). Although a univocal definition is reductive, all processes of domestication related to animals involve modifications of physiology, morphology and behaviour (Price 1984; Hemmer 1990). Of course, the primary characteristic, common to all the domestics, regardless of the process of domestication, is the tolerance to human presence (Driscoll *et al.* 2009b). How can this have happened with the wildcat, and why? Wildcats are rather unsuitable for domestication. They are solitary, elusive and very territorial animals, obligate carnivores (Bradshaw *et al.* 1996) and have a low propensity to perform tasks even as mousers (Elton 1953). To try to explain this domestication is necessary to digress to the dawn of agricultural and sedentary civilization.

Although recent studies have identified a wider area, including Cyprus (Vigne *et al.* 2011; Vigne *et al.* 2012), the different archaeological, genetic and cultural evidences accumulated allow us to identify in the region of the Fertile Crescent (Breasted 1916) the primary area in which, between the thirteenth and the eleventh millennium B.P., have been developed the complex human-mediated evolutionary process that led to agriculture and domestication of many western domesticates (Davis 2005; Redding 2005; Dobney and Larson 2006; Driscoll *et al.* 2009b; Zeder 2006; 2008; 2011; 2012a; 2012b). This region, which extends from Turkish and Iraqi Mesopotamia to the Levant, during the terminal Pleistocene was a buxom land rich in fauna and vegetation (Bar-Yosef 1998; Clutton-Brock 1999). Populations of nomadic

hunter-gatherers (known today as Natufians) found here a place where the abundance of resources allowed limited movements for food supplies and this condition allowed the establishment of the first sedentary civilizations that began to accumulate and store resources since at least the twenty-first millennium B.P. (Tanno and Willcox 2006) and to build the first tools designed to transform and process wild grains (Bar-Yosef 1998; Zeder 2011). With the arrival of a cold and dry period, approximately 11 000 years ago, known as the Younger Dryas, the Natufian populations have probably accentuated the tendency to accumulate resources, thus pushing the trend to cultivation (Driscoll *et al.* 2009b). The accumulation of resources around anthropized nucleus led different opportunistic species, such as mice and other small rodents, to concentrate their activity centre around such settlements rich in food and free of most predators, becoming commensals of humans (Coppinger and Smith 1983; Auffray *et al.* 1988; Clutton-Brock 1999). These rodent populations began to spread as a pest becoming thus an abundant source of food for some predators, including some wildcats, which began themselves to exploit this environments by means of individuals genetically more tolerant to human presence, as suggested by the last findings on the cat genome (Montague 2014; Tamazian *et al.* 2014) that began to diverge from their "wild" relatives (Wandeler *et al.* 2003; Driscoll *et al.* 2009a). Therefore, unlike other processes of domestication (for example dog or barnyard animals) guided predominantly by artificial selection, in the case of the cat, at least in the initial stages, the starting engine was natural selection (Driscoll *et al.* 2009b). At this first stage of "commensalization" has probably followed a second step during which the presence of these cats was at first tolerated and then encouraged by villagers (Vigne *et al.* 2011). This becomes all the more likely, given the symbolic meaning that the cat took in some Neolithic societies, as suggested by numerous representations between 9500 and 8700 years ago (Helmer *et al.* 2004).

If the domestication of cereals in the region of Fertile Crescent involved independently different weed species in different villages from the south of Levant through Syria to the southern of Anatolia (Willcox 2005) we can hypothesize that the process of domestication of the cat reflects the geographical distribution of these settlements (Driscoll *et al.* 2009b). Genetic data support this hypothesis. Driscoll *et al.* (2007) have indeed identified in the analysed domestic cat populations five different mitochondrial lineages in common with the African wildcat (*Felis silvestris libyca*) that date back about 131 000 years ago (a value of an order of magnitude greater than the supposed age of domestication) suggesting a domestication process stretched out over time but likely originated in an single widespread event. This is supported not only by the monophyly of the two different subspecies coming from the same area, but also by the greater similarity between domestic cats and African wildcat with respect to this latter with other allopatric groups phenotypically more similar (as the Asian wildcat or Southern African Wildcat). This genetic structure is expected if one imagines early admixture events between initial domesticate and additional wild

female conspecifics spreading gene for domestication through the early Fertile Crescent agricultural area (Driscoll *et al.* 2009b).

After the Near East origin of domestication, first through the Phoenicians sailors and then through the Greek and Roman civilizations (Gippoliti and Amori 2006), cats subsequently became common in Europe and Asia by the 10th century, and were ultimately transported around the world on the major land and sea trade routes. The known breeds began to develop only from the eighteenth and nineteenth century (Zeder 2012a).

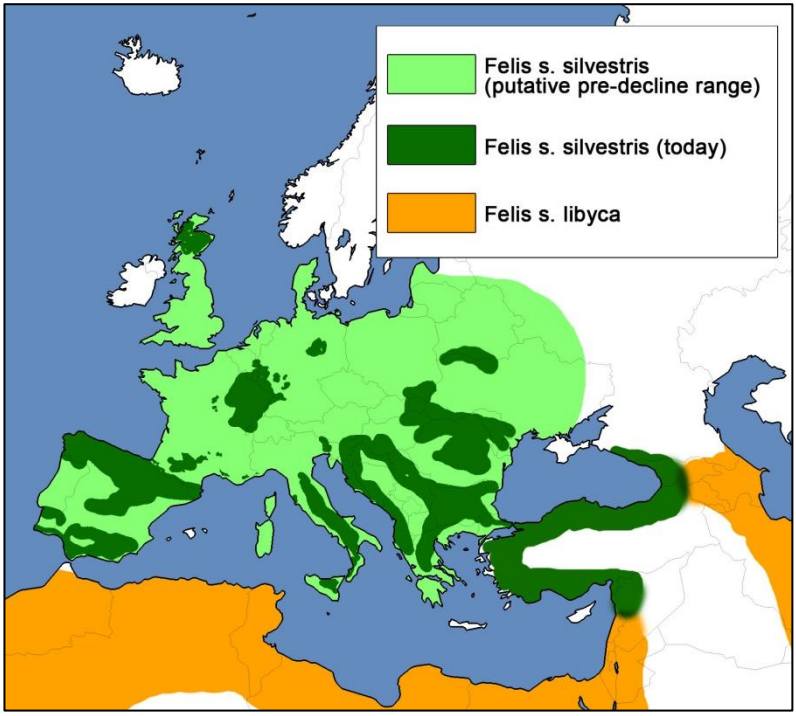
In any case, 97% of more of the 600 million of domestic cats existing today are random-bred house-cats which means that the vast majority of cats is not subjected to a prezygotic selection. Also these animals are perfectly self-sufficient and moreover, with the exception of some distinctive features (including also a greater intestinal length probably adapted to a less-strictly carnivorous diet), maintain a very similar morphology to that of the their wild relatives. The most significant changes can be found in the tolerance to humans, in the polyestrous and changes in the colour and quality of the coat. Furthermore, cat is the only animal to have developed a social behaviour in the domesticated form from the highly individualistic wild relative (Driscoll *et al.* 2009b).

Taken together, these data suggest that actually the most important process of domestication has developed over the last three centuries, and that might not yet be complete (Serpell 2014). The process is now under continuous development and also began to involve other species (such as the savannah, a hybrid between a Siamese cat and a Serval).

HISTORICAL AND RECENT DISTRIBUTION

Central Europe was probably the core of wildcat evolution (Macdonald *et al.* 2010b), as suggested by the continuous fossil records along the Pleistocene in this region (Kurtén 1965). In contrast fossils from Africa and Middle East are recorded only from the late Pleistocene (less than 130 000 years ago, Kurtén 1965). This gap can be interpreted in a rapid expansion of ancestral wildcats from Europe to southern regions and a consequent fast evolution that could have given rise to the steppe wildcat phenotype that, within 10 000 years would have spread eastward in Asia and southward in Africa (Yamaguchi *et al.* 2004a). This hypothesis was supported by recent phylogeographic analyses (Driscoll 2007) and the presence of fossil records of transitional forms of late Pleistocene resembling the middle Pleistocene forms in Europe (Kurtén 1965). Regarding the European Wildcat (*F. s. sivestris*), it experienced a subsequent rapid southward European shift during the Last Glacial Maximum (LGM) and probably recolonized central Europe only during the Late Glacial (c. 13 700 – 12 500 years ago) (Sommer and Benecke 2006). In historical times, the

European Wildcat was widely distributed throughout Europe except for the Fennoscandia region (Driscoll and Nowell, 2010).



Distribution of the two subspecies of wildcat (*F. s. silvestris* and *F. s. libyca*) in Europe and Mediterranean rim.

Between 1700s and 1900s the species suffered in Europe a severe decline because of the loss of habitat, hunting and persecution (Stahl and Artois 1994). Nowadays its distribution appear fragmented, and the wildcat range and population can be divided into eight main subrange (Nowell and Jackson 1996; Mitchell-Jones *et al.* 1999; Sunquist and Sunquist 2002). A first isolated nucleus still remain, even if scattered in subgroups, in central and western Europe (France, Germany, Belgium, Switzerland) (Peichocki 1990; Raimer 1994; Mitchell-Jones *et al.* 1999; Eckert *et al.* 2010; Say *et al.* 2012; Lozano and Malo 2012). It is considered extinct in Nederland (Nowell and Jackson 1996) and Austria (Spitzenberger 2005) even if there were recent contacts of putative wildcats caught with camera-traps near Limburg (Brouns 2014) and vagrants from north-east of Italy suggested an expansion trend towards Austria (Lapini and Molinari 2006). It highlights the Iberian population, well separated from

the rest of European range (Nowell and Jackson 1996, Mitchell-Jones *et al.* 1999; Lozano and Malo 2012). The population of the Italian Peninsula is also well distinct and roughly extends continuously from the Aspromonte massif to the northern Apennines (Ragni *et al.* 1994; Mattucci *et al.* 2013). The *eastern* population of Italian Alps belongs instead to the wide Balkan-east European nucleus that extends to the late Yugoslavian countries, south Romania, Bulgaria, Greece, Slovakia, Hungary Ukraine and southern Poland where the population is well settled (Mitchell-Jones *et al.* 1999; Bashta and Potish 2005; Heltai *et al.* 2006; Lozano and Malo 2012). Scotland represents possibly the most isolated area featured by the wildcat presence (Mitchell-Jones *et al.* 1999; Macdonald *et al.* 2010; Lozano and Malo 2012). A last wide population extends over the Turkish Peninsula and the Caucasus (Nowell and Jackson 1996; Macdonald *et al.* 2010; Can *et al.* 2011). Sicily is the only Mediterranean island occupied by a natural population of wildcat (Driscoll and Nowell 2010) and its population could be considered, as for other animals and plants, a layer of the Apennine one, while populations of island such as Sardinia, Corsica and Cyprus derived probably from early domestic individuals introduced in Neolithic times and became feral (Gippoliti and Amori 2006).

CONSERVATION CONCERNS

Hybridization and introgression of domestic cat alleles

Introgression of domestic cat (*F. s. catus*) into wildcat populations is possibly the most important and debated issue related to the conservation of the wildcat (Hubbard *et al.* 1992; Stahl and Artois 1994; Randi *et al.* 2001; Daniels and Corbett 2003; Oliveira *et al.* 2008; Hertwig *et al.* 2009). Hybridization occurs when individuals form genetically distinct populations interbreed (Rieger *et al.* 1991), or more precisely 'Successful mating in nature between individuals from two populations, or groups of populations that are distinguishable on the basis of one or more heritable characters' (Arnold and Burke 2004). If the first generation of hybrids (F1) is fertile and backcrosses to one or both parental populations than introgression or gene flow between the populations is said to occur (Rhymer and Simberloff 1996). In natural populations, admixture events are not rare and play a consistent role in evolution and adaptation (Allendorf *et al.* 2001; Barton 2001; Grant and Grant 2008). But human-mediated hybridization between domesticated and wild populations could imply modifications in the natural population structure, loss of local adaptations and is therefore a cause of concern especially for endangered species (Rhymer and Simberloff 1996; Allendorf *et al.* 2001;).

Nowadays domestic cats (*F. s. catus*) are worldwide distributed, with more than 600 million of individuals (O'Brien and Johnson 2007) and are listed among the 100 worst non-native invasive species in the world (Lowe et al 2000). Wildcats and domestic cats can interbreed producing fertile offspring in captivity and in nature (Ragni 1993; Beaumont *et al.* 2001; Pierpaoli *et al.* 2003) and the effects of this long-term sympatry between the two forms is mostly unknown and generated a complex debate (McOrist and Kitchener 1994; Daniels *et al.* 1998; Beaumont *et al.* 2001). Nonetheless, it is necessary to consider the possible consequences of such event. In general, if we assume that the today's wildcat genotypes identified as "pure" have the original genotype of the wildcat before any admixture event, two possible scenarios are possible: outbreeding depression or heterosis (hybrid vigour) (Frankham *et al.* 2002). The low fitness of hybrid progeny relative to either parent might arise, in the long term, due to the dilution of genes associated with local adaptation (genotype by environment interaction) or the disruption of co-adapted gene complexes (Fenster and Dudash 1994) that reflect epistatic gene action and the interaction among loci that enhances fitness (Falconer 1989). On the other hand increasing of heterozygosis, as consequence of hybridization, could lead to an improvement of the fitness, at least in the first generations (Hamilton 2009) In a scenario of outbreeding depression, F1 are supposed to be less fit than pure wildcat, and therefore backcrosses (F1 x wild) are less likely to occur, limiting introgression. But in case of heterosis, natural selection would facilitate backcrosses increasing introgression (Lozano 2012). Unfortunately, the long-term consequences of mixing populations of endangered or threatened species has not been adequately documented (Whitlock *et al.* 1995; Fenster *et al.* 1997; Fenster and Galloway 2000) and we cannot reject the hypothesis in which to an initial heterosis and introgression phenomenon follow an outbreeding depression scenario in an highly admixed population. In this case selection would operate to slowly reduce domestic cat alleles and increase the wildcat portion to the gene pool, but due to the disruption of earlier gene complexes, it would be much less likely to recover the original genetic pool of the wildcat (Whitlock *et al.* 1995).

This seems to be confirmed looking at the situation of the Scottish and Hungarian populations of wildcat that seem to have experienced a long-lasting introgressive hybridization (Beaumont *et al.* 2001; Pierpaoli *et al.* 2003; Lecis *et al.* 2006). In contrast research conducted in central and southern Europe showed limited rates of gene flow between domestic cat and wildcat (Randi *et al.* 2001; Pierpaoli *et al.* 2003). All these data should be taken with caution since the data come from studies that consider different sample sizes, environments contexts and markers. In order to better understand the problem further research is required, especially trying to standardize procedures and markers to have comparable data that could help in throwing light on this important conservational issue (Daniels and Corbett 2003).

Habitat alteration

Obviously, the habitat conditions play a key role in the conservation of the wild species and their alterations or destruction implies, of course, an important threat for the long-term stability of a population (Stahl and Artois 1994; Nowell and Jackson 1996). The European Wildcat is not an exception and the alteration and the loss of habitat are highlighted as important factors affecting its conservation (Council of Europe 1993).

Although the wildcat is considered mainly a forest species (Parent 1975; Schauenberg 1981; Mitchell-Jones *et al.* 1999) several studies demonstrated as the species prefers, were available, complex and composite environments (Easterbee *et al.* 1991; Lozano *et al.* 2003; Lozano *et al.* 2007; Monterroso *et al.* 2009). Therefore would be simplistic to analyse the risk considering only the loss of habitat, but it also necessary to take into account the consequences of its simplification and modification. Historically, deforestation contributed to the decline of the wildcats in Great Britain (Langley and Yalden 1977; McOrist and Kitchener 1994), but, for example, has been suggested that in Eastern Europe the replacement of deciduous forests with coniferous plantations affected negatively the wildcat population (Puzachenko 1993). In Mediterranean region it has been shown that scrublands offer an optimal source of shelter and prays (Lozano *et al.* 2003; Lozano *et al.* 2007; Monterroso *et al.* 2009) and the strategy of their removal to combat fire, especially in Spain and Portugal, represents a major threat for the wildcat as well as for other carnivore species (Lozano *et al.* 2003; Mangas *et al.* 2008; Sarmento *et al.* 2009).

Of course, urbanization and the construction of infrastructures (roads, highways, railways etc..) are a direct form of habitat destruction (Council of Europe 1993; Stahl and Artois 1994; Nowell and Jackson 1996), but additionally to this is worth taking into account that also the surrounding areas should be computed in the amount of lost habitat since the wildcat tends to avoid humanized area within a range going from 200 to 900 meters (Easterbee *et al.* 1991; Klar *et al.* 2008). Finally, we must not forget the indirect effects that other factors, in environmental terms, may affect the conservation of the species. It has seen, for example, that a high density of ungulates [the red deer (*Cervus elaphus)* and the wild boar (*Sus scrofa*)] in Monfragüe National Park reduced by several orders of magnitude the abundance of wildcats (Lozano *et al.* 2007) probably due to the loss of preys [in this case the wild rabbit (*Oryctolagus cuniculus*)] that are negatively associated with an high density of ungulates (Lozano *et al.* 2007). It is therefore necessary to consider that the management of ungulates, especially in game farms and hunting lands, whether directed towards an uncontrolled growth of the population may represent an additional risk to the wildcat (Lozano *et al.* 2007).

Man-induced mortality and diseases

Historically the wildcat has been considered a vermin and was regularly subject to eradication campaigns from which were obtained even their valuable furs (Langley and Yalden 1977; McOrist and Kitchener 1994). It could be argued that in the past the direct persecution has been the main factor of decline (and in some cases of local extinction) which acted on the distribution of the species (Lozano and Malo 2012). The Council of Europe (1993) reminded that the main factor of recovery for the wildcat population has been the limitation in the use of traps for carnivores in the second half of the last century. However, mortality data on persecution are relatively old and mostly related to the fur market. The legal protection enjoyed by the species in the last decades makes it much more difficult to find reliable data on the actual number of animals killed illegally. On one hand, the prohibition of hunting and the increasing environment awareness of society has definitely decreased the incidence of this threat. On the other hand, the killings are much more difficult to verify because they occur illegally and are kept hidden (Lozano and Malo 2012). A cause for concern comes from a study conducted in Serra de Malcata in which predator control is legally practiced (Sarmento *et al.* 2009). Today in this area the wildcat almost disappeared and although the author hypothesized several potential causes, the fact that between 1999 and 2001 on eight radio-collared wildcats, two were killed by gunfire has aroused great concern.

Information about wildcat road kills are scarce and fragmented and are mostly collected opportunistically. However, data collected between 1979 and 1993 in central Europe and Scotland suggested a lower impact than that caused by the direct persecution (Piechocki 1990; Muntyanu *et al.* 1993). The individuals more prone to the death for investment appear those released in front of reintroduction projects, which possibly tend to dispersion after the release and therefore undergo a greater chance of encountering streets (Nowell and Jackson 1996; PMVC 2003).

The incidence of the diseases on the wildcat populations is virtually unknown (Lozano and Malo 2012). The few available studies do not provide enough data that allow to extrapolate a statistics on the populations (Piechocki 1990; Ragni 1993). In any case, we know that the vast majority of the most common diseases are shared among felids and accompanied the evolution of these cats for a long time (Polani *et al.* 2010). Is then assumed that the wildcats are adapted to the presence of these pathogens and, as long as persists a sufficient genetic variability, are able to cope them without the population dynamics will be strongly affected. Moreover, solitary behaviour of the wildcat seems to further reduce the chances of infection and spread of the virus in the population (Leutenegger *et al.* 1999). A separate mention should be done for the feline immunodeficiency virus (FIV). This virus normally is not found among wildcats except that in some cases of contagion, particularly in France, (Fromont *et al.* 2000) and affects certain domestic cats by suppressing the immune system and causing the death of the animal. Disease transmission occurs primarily by bite and may have a

very long course, often allowing the animal to reproduce before symptoms occur. Furthermore, the frequency of FIV in domestic cats is low and so the likelihood of contagion to the wildcats population. Nevertheless, the disease is highly lethal and may represent a future threat, in a possible scenario of sympatry with feral cats (McOrist and Kitchener 1994)

CONSERVATION PRIORITIES AND TOOLS

The analysis of the threats reviewed above has highlighted the limits of quantity and quality of data available. Is therefore a priority to increase the knowledge about the species in terms of biology, biogeography and population dynamics (Lozano and Malo 2012). In particular, studies should be concentrated in those areas environmentally suitable to the species but on which its effective consistency is unknown and complementarily in those areas characterized by human disturbance where populations of wildcat were already detected (Macdonald *et al.* 2010b). This is because, as has been said, habitat destruction and human persecution has been a major factor in the reduction of the species (Langley and Yalden 1977; McOrist and Kitchener 1994). Is therefore necessary to identify and preserve these areas most suitable for the survival of this species. In a longer-term perspective, as has been proposed in some region of Europe (Macdonald *et al*. 2010b; Vogel and Mölich 2013), his knowledge would be useful for the realization of ecological corridors or Special Areas for Conservation (SACs) as planned by the EC Habitats Directive (92/43/EEC). In areas disturbed by a higher human pressure, it is important to verify how the wildcat could succeeds in adapt to this environment and face a greater likely invasiveness of its domestic conspecific (Macdonald *et al*. 2010b).

In a recent analysis of the current risks it was stated that the phenomenon of hybridization, except for some particularly affected regions (Scotland and Hungary), turns out to be a less urgent threat than has been used to think (Lozano and Malo 2012). Nevertheless, its incidence was crucial in contributing to the decline of the species where it has not been possible to quantify the size of the phenomenon in time to act accordingly (Macdonald *et al.* 2010b). To verify the current rates of introgression and succeed to keep it to an acceptable level, two actions are primarily is necessary. From a genetic point of view, it is fundamental to detect most reliable and efficient genetic markers to determine the population structure and the presence and depth of the admixture. For several species the combination of highly polymorphic microsatellite markers with sophisticated Bayesian clustering methods proved to be reliable for identifying population's structure (Randi and Lucchini 2002; Pierpaoli *et al*. 2003; Lecis *et al.* 2006), individual assignment and admixture analyses, and deep investigating complex evolutionary processes involving the subspecies (e.g. Randi *et al*. 2001, Vila *et al*. 2003; Oliveira *et al.* 2008,). With the advancement of sequencing techniques and the development of genomics projects that have allowed a more

thorough reading of the cat genome (Mullikin *et al.* 2010) has been possible to discover an increasing number of single nucleotide polymorphisms (SNPs), copies of previously undiscovered nuclear mitochondrial DNA (numt) (O'Brien *et al.* 2008) and define maps for the identification of genes controlling phenotypes of interest (cat radiation hybrid map, RH; Davis *et al.* 2009). The markers provided by genome research, in particular SNPs, have played a crucial role in the study of populations and selection processes (Allendorf *et al.* 2010; Ouborg *et al.* 2010) because of the large quantities in which they are present throughout the genome (Luikart *et al.* 2003; Wayne and Morin 2004), their evolutionary characteristics, easily describable by simple mutations models (e.g. infinite site model) (Vignal *et al.* 2002) and the easy comparability between studies, thanks to the standardization of genotype codes present in the databases. Great progress has also been made in the analysis of uniparental markers (mtDNA and Y chromosome) to increase the resolution of the analysis of gene flow and introgression (Hertwig *et al.* 2009). In particular have been developed suite of genetic markers that combining the most informative microsatellite with putative diagnostic mutations detected on mtDNA allow a more precise assessment of the degree of introgression between wildcats and domestic cats (Driscoll *et al.* 2011). In addition the results of other works analysing sequences from genes SMCY, SRY, ZFY UBE1Y provided numerous tools to elucidate the phylogeny of Felids and offered additional markers relating to the patrilineal history of the species (Pecon Slattery and O'Brien 1998; Pecon Slattery *et al.* 2000; Pecon-Slattery *et al.* 2004; Luo *et al.* 2007). The integration and comparison of these markers was until now only partly evaluated only in a few studies (Eckert *et al.* 2010; Driscoll *et al.* 2011; Nussberger *et al.* 2013) and is intended to develop reliable diagnostic protocols, even in contexts of non-invasive genetics.

The other important aspect to pursue is to keep monitoring the abundance of wildcat populations and detect the possible presence and consistence of feral cats. Has been seen, indeed, that reducing the wildcat population density as well as an increase in density of domestic cats increases the likelihood of hybridization (Stahl and Artois 1994). This is what probably happened in Scotland (Easterbee *et al.* 1991; McOrist and Kitchener 1994; Beaumont *et al.* 2001; Macdonald *et al.* 2010b) and what is happening in Hungary (Pierpaoli *et al.* 2003; Biro *et al.* 2005; Heltai *et al.* 2006). From this point of view it is therefore necessary to obtain abundant and qualitatively diversified information (non-invasive genetic, camera-trapping, radio-tracking etc..) whose integration may lead to a more complete understanding of the "in-field" scenario alongside the one described by genetics.

OBJECTIVES AND AIMS

The picture that emerges from what described above shows how the approach to the study and the preservation of the European Wildcat should be multidisciplinary. The different biological, genetic, taxonomic, phylogeographic and legal issues related to this species should all be considered in order to get to correct conclusions about its status and the actions that should be applied. In this context I carried out my PhD thesis which has been developed through the collaboration between the Science Department of Roma Tre University, the Foreste Casentinesi National Park and the conservation genetics laboratory of ISPRA in Ozzano dell' Emilia.

This work was structured primarily taking into account three main objectives tackled in three different sections:

1. Evaluate a standardized integrated protocol for non-invasive monitoring. In the first one, we studied a methodology that integrating the different non-invasive techniques might collect as much of diversified data (genetic, ecological, ethological, photographic, etc. ..) exploiting the least possible sampling effort. In this work we developed a protocol in which the sampling campaign was stratified simultaneously into three different layers: the camera-trapping layer, the scat-surveying and the hair-traps sprayed with valerian tincture (*Valeriana officinalis*). Using this method we were able to associate the genetic datum to the photographic one, identifying wildcats individuals, detecting hybridization traces, assessing the invasiveness of domestic cats and studying the reactions to the attractor. Alongside it was possible also to take advantage of the versatility of the camera-trapping in order to observe some aspects of the wildcat behaviour and the relationships with some potential competitors. This protocol has been chosen by the Biodiversity Department of the State Forestry Corps operating in the Foreste Casentinesi National Park as a tool for investigating the European wildcat in the integral Natural Reserve of Sasso Fratino.

Results of this research are summarized in the following paper.

Velli E., Bologna, M. A., Ragni B., Randi E. Non-invasive monitoring of the European Wildcat (*Felis silvestris silvestris* Schreber 1777): comparative analysis of three different monitoring techniques and evaluation of their combination. Submitted to *European Journal of Wildlife Research*.

2. Study more efficient genetic markers for assessing admixture and introgression between wildcat and domestic cat. In the second section, taking advantage of a fruitful collaboration with a ISPRA researcher, we performed a wide genetic investigation of novel loci to establish a set of diagnostic tools suitable for accurately detect levels of introgressive hybridization between European wildcats and domestic cats. 150 samples (including putative European wildcats, captive and

putative hybrids and domestic cats) from 15 different European countries and previously analysed at 31 microsatellites loci were analysed with an Illumina Infinium iSelect 63K Cat DNA Array from which SNPs were selected the 151 most informative. Results from Bayesian model-based computations of simulated and empiric genotypes showed that the entire set of 151 markers provided successful estimates of admixture, correctly assigning all parental, F1-F2 hybrids and first and second generation backcrosses. A direct comparison between most informative SNPs and STRs proved the outperforming power of SNPs on admixture detection and inferring admixed ancestries. To increase the temporal depth of such analysis and check for a possible hidden introgression were also analysed a total of 1269 bp of mtDNA (a coding portion of ND5 containing 7 putative diagnostic mutation and the Region of Control) and two markers on the Y chromosome (SMCY STR-7 and a SNP on the SRY gene). These markers allowed us to draw a phylogenetic history of the samples from which emerged two possible different transmission events that led to a mitochondrial haplotypes sharing between the wild and domestic population opening the way for further studies to better understand the phenomenon. Results were organized in a manuscript that is currently under preparation:

Results of this research are summarized in the following paper.

Mattucci F, Velli E, Lyons LA, Alves PC, Oliveira R, Randi E Combining use of most informative autosomal SNPs with uniparental markers (mtDNA and Y-chromosome) for the assessment of hybridization in European wildcats (*Felis silvestris silvestris*). In prep.

3. Clarify the phylogeographic history of the species in Europe. The second work mentioned above has shown how different haplotypes characterized by typical domestic polymorphisms appeared with good frequency in the wild population. So in this last one part we provided the preliminary results of a phylogenetic and phylogeographic analysis sequencing 669 bp of the subunit 5 of NADH dehydrogenase of 717 samples of European wildcats, domestic cats and putative hybrids collected from 14 different locations in Europe previously analysed using 31 microsatellite loci. In this work, we have deepened the phylogeography of wildcat in Europe and shed light on the causes that led to the cytonuclear discordance on several individuals. Further analyses are underway for the definition of effective sample size and migration rates between the groups identified

Results of this research are summarized in the following paper.

Velli E., Bologna M., Mattucci F, Oliveira R., Randi E. Phylogeography of the European Wildcat (*Felis silvestris silvestris*) in Europe: today structure and historical inferences on species biogeography. In prep.

In conclusion, this work aims to propose a multidisciplinary approach to deal with the problems of conservation of the wildcat defining the most efficient and standardized tools possible that could mediate a reasonable operational effort with a high quantity and quality of data.

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NON-INVASIVE MONITORING OF THE EUROPEAN WILDCAT (*FELIS SILVESTRIS SILVESTRIS* SCHREBER 1777): COMPARATIVE ANALYSIS OF THREE DIFFERENT MONITORING TECHNIQUES AND EVALUATION OF THEIR COMBINATION.

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ABSTRACT

European wildcat is threatened by habitat fragmentation, illegal or incidental killings and hybridization with free-ranging domestic cats. Conservation projects should be based on sound knowledge of the patterns of wildcat distributions and population structure. This information is, however, scanty, mainly because of the species' elusive behaviour. We tested the efficiency of a protocol that integrates the use of non-invasive genetic identifications and camera photo-trapping for wildcat monitoring. The study was carried out in the Foreste Casentinesi National Park, a protected area in the central Italian Apennines where wildcat presence has been recently detected. DNA samples were extracted from the scats collected during the survey and hair tufts trapped by baited sticks, and individual genotypes were identified using 10 autosomal microsatellites, mtDNA and Y-chromosome markers. Thirty-second long video clips were obtained from 20 camera-trap stations associated to the hair-traps. Results confirmed the presence of wildcats in the study area. We identified between 6 and 9 wildcat individuals. Some of them showed anomalous coat colour patterns and genetic signals of possible hybridization. We further detected five domestic cats individuals sharing part of wildcats territories. In one cases, we filmed an individual leaving hair samples that were successfully genotyped allowing us to compare genetic and photographic information. We found individual variations in the response to the valerian attractor. We compared and evaluated the pro and cons of these monitoring methods, concluding that the efficiency of wildcat detection and the quality of data would be considerably increased by using them simultaneously.

Keywords: *Felis silvestris*, non-invasive genetics, wildlife monitoring, camera-trapping, olfactory attractant, genetic markers

INTRODUCTION

The European wildcat (Felis silvestris silvestris Schreber, 1777) has a wide but partially fragmented distribution throughout Europe (Driscoll and Nowell 2010; Lozano and Malo 2012). The species lives in a variety of habitat types that range from scrub-pastures (Lozano et al. 2003; Monterroso et al. 2009; Lozano 2010) to forest patches intermixed with open fields (Klar et al. 2008; 2012) and, more marginally, in coniferous forests with rich undergrowth (Easterbee et al. 1991; Lozano et al. 2003). 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 and 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 and 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. 2008a; 2008b). Driscoll and Nowell (2010) reported a decreasing global population trend. However some local populations appear stable, despite poorly known distribution ranges (Lozano et al. 2013). Projects for restoring ecological corridors are underway to counteract the effects of habitat fragmentation and facilitate the connection of isolated populations (Vogel and Mölich 2013). The species' distribution range in the Italian peninsula covers the entire southern and central Apennines (Ragni et al., 1994) (Figure 1). 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) suggest a northwards wildcat expansion, sustained by suitable forested habitat corridors in protected areas along the Apennine ridge (Santolini et al. 2010). However, we cannot reject the alternative hypothesis, even if improbable (Ragni et al. 1994), that undetected wildcat populations persisted in the past, scattered at low density in these regions. The European wildcat population in north-eastern Italy (Angelici and Genovesi 2003) is connected with the Dinaric-Balkan population (Mattucci et al. 2013). Recent observations (Bologna and Cristiani 2012) suggest the persistence of a north-western isolated population, even if the probable draining of the French source (Stahl and Artois 1994) makes its consistency uncertain. In Sardinia the ssp. libyca Forster, 1780 is widely distributed. However, in Italy, the knowledge about the European wildcat distribution range, population dynamics and conservation status is largely inadequate.
Reliable estimates of population abundance and trends are the key baseline data to assess the impact of threatening factors and to outline sound conservation guidelines (see Council of Europe 1993). Wildcat monitoring is largely based on direct sightings (Hartmann et al. 2013), camera and live trapping (Bizzarri et al. 2010a; Can et al. 2011; Kilshaw and Macdonald 2011; Anile et al. 2012a), radio tracking (Monterroso et

al. 2009; Bizzarri et al. 2010b), scat surveys (Lozano et al. 2003; Lozano et al. 2013; Anile et al. 2014) and, opportunistically, through the occurrence of road kills (O'Brien et al. 2009; Hartmann et al. 2013). However, low-density populations have been mostly monitored through non-invasive techniques. Consequently only a few studies have succeeded in monitoring the populations of the European wildcat using non-invasive techniques such as camera-trapping (Can et al. 2009; Kilshaw and Macdonald 2011; Anile et al. 2012a), scats-surveys (Lozano et al. 2013) or hair trapping using valerian baits (Hupe and Simon 2007; Kéry et al, 2011; Steyer et al 2013). Each method has some technical drawbacks, e.g. uncertain individual identification and hybrid detection (i.e. camera-trapping), genotyping errors and underestimation of the population (i.e. scats surveys; Mondol et al. 2009; Marucco et al. 2011) and variable responses to odorous bait (Kilshaw and Macdonald 2011; Monterroso et al. 2011; Anile et al. 2012b). It is therefore necessary to integrate different methodologies trying to balance the pros and cons of each. Recently Anile et al. (2014) assessed the population density of the wildcat on the Etna volcano (Sicily) using both camera-trapping and genetic analyses of faecal DNA, while no multi-method attempt has been carried out in low-density areas of the Apennines. We tested and evaluated the integration of three non-invasive sampling techniques, basing on camera-trapping and genetic analyses of DNA obtained by both surveys for scats and hair-trapping with valerian lure. This approach was applied in the northern Apennines, where the species occurrence has been confirmed only recently (Ragni et al. 2014), with the aim of assessing its feasibility and effectiveness for the monitoring of low-density wildcat populations..

MATERIALS AND METHODS

No animals were physically captured and manipulated during the work. All the information gathered is based on non-invasive methods.

Study Area

Based on available information on the presence of wildcats obtained by published records, forest rangers, wildlife technicians and preliminary field surveys (Cagnolaro et al. 1976; Agostini et al. 2010) we identified a 2800 Ha large area within the northern sector of the Foreste Casentinesi National Park (Emilia Romagna, Italy) (Figure 1). The area ranges from 600 to 1150 m.a.s.l.. The mean annual temperature is 14 °C ± 5°C, and the annual rainfall is between 380 and 680 mm. This area is the northernmost location of the Apennine where the presence of the European wildcat has been recently assessed (Ragni 2003; Ragni and Petruzzi, 2010; Ragni et al. 2014). The Foreste Casentinesi National Park (36,000 Ha) features over 29,000 Ha of mixed

woodlands (Fagus sylvatica, Quercus cerris, Quercus pubescens, Fraxinus excelsior, Castanea sativa; with introduced coniferous such as Pinus nigra, Picea abies etc.) spaced out with wide pasturelands, grasslands and clearings. Furthermore with fully-protected centuries-old forests and the rich hydrographic network this area represents the ideal environment for the species on the Apennine hogback (Santolini et al. 2010). The ecosystem's complexity and the preservation level of the Casentinesi Forests supports a rich mammal community, including several ungulates and carnivores. Human density is low (about 4 residents per km2) and tourism strictly regulated..

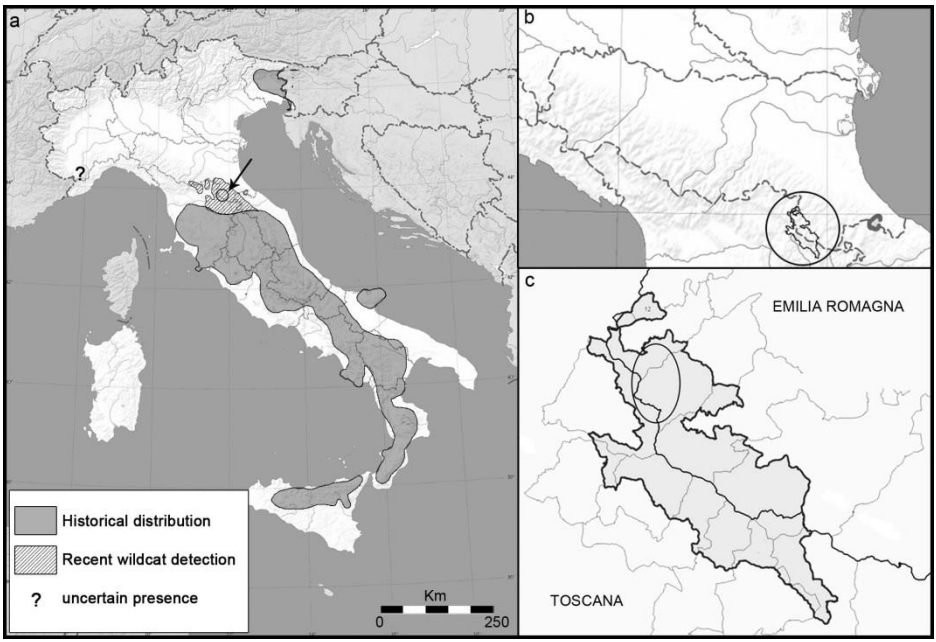

Figure 1. Study area, located in the northern portion (c) of the Foreste Casentinesi National Park (b), northern Apennines (a)

Genetic sampling protocol

To collect both hair and scats samples we designed a grid 4 x 7 km widely partitioned in 28,1x1km large cells (Figure 2). We systematically placed a total of 45 raw pine sticks (60 x 4 x 4 cm), trying to cover the grid uniformly and placing, when possible, at least a lure in each square (Hupe and Simon 2007; Kèry et al. 2011;

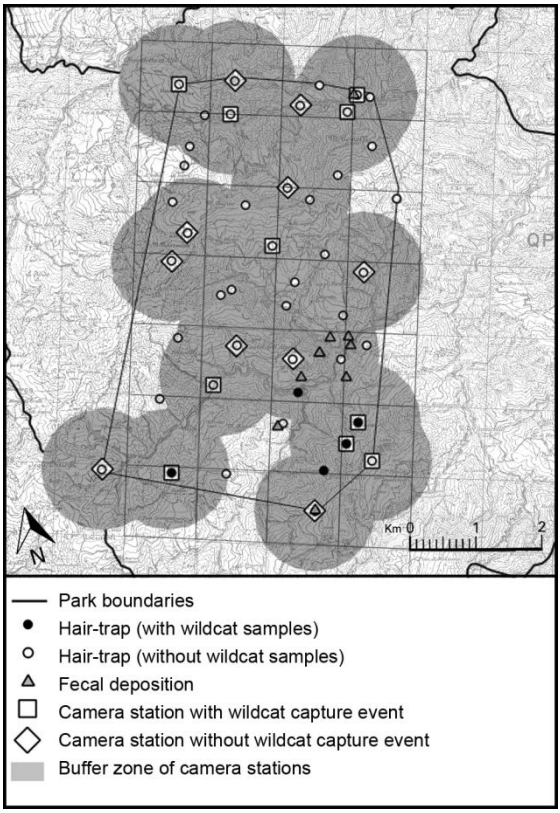

Figure 2. The sampling area in the Foreste Casentinesi National Park with the buffer area (grey) and the minimum convex polygon on which the calculations of the sampling areas were based. The legend shows the typologies of sampling sites (hair-traps, camera stations and wildcat faecal depositions)

Hartmann et al. 2013; Steyer et al. 2013). 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). In order to catch as many hairs as possible, we scratched the surface of the wood while also applying a strip of bi-adhesive tape. We selected a total of about 25 km of trail linking the trap-stations that were covered by walk to collect scat samples, with a total effort of 17 two-days sampling sessions and 425 km of trails walked. For capture density calculation, we traced a minimum convex polygon (MCP) considering the outermost pickets (22.4 km2, Figure 2). Considering the smallest observed 95% Kernel area used by a wildcat in central Italy (277,71 Ha for an adult male, Bizzarri et al. 2010b) we further added to the MCP a buffer of 939 m (for a total of 42,3 km2).

Sampling period extended from November 19th 2012 until June 24th 2013, although heavy snowfalls between January and February 2013 forced us to stop the surveys for about 30 days. Inspection of lure sticks was carried out every 7–10 days. Attached hairs were removed with forceps and stored in an envelope with silica gel to keep

samples dry and to avoid degradation of DNA. In order to prevent contamination, after each sampling event forceps were flamed and lure sticks were scratched with an iron brush to remove any residual hairs while a new tape strip and valerian lure were applied. Scats were collected using sterile disposable gloves. Samples were stored in ethanol 96% and frozen as soon as possible. Only samples that seemed to be recent and well hydrated were collected..

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 previously subjected to mtDNA analysis in order to discard samples belonging to non-target species.

In all analyses contamination risks were minimized using a laboratory dedicated to the pre-polymerase chain reaction (PCR) handling of non-invasively collected samples (Taberlet *et al.* 1999). Negative and positive controls were run alongside all reactions to monitor possible cross contamination during extraction and amplification.

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 7 diagnostic SNPs discriminating European wildcats (*Felis silvestris silvestris*) and domestic cats (*Felis silvestris catus*). This sequence was amplified using PCR primers F2B (5'-TGCCGCCCTACAAGCAAT-3') and R3B (5'-TAAGAGACGTTTAATGGAGTTGAT-3') (Driscoll *et al.* 2011). In addition, we sequenced 719 bp of the mtDNA control-region (hence CR; sites 16236 - 16955) using 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). Each 10 µL PCR reaction contained 2 µL of DNA , 0.8 µL of 10X Taq Buffer advanced with self-adjusting Mg^{2+} (5Prime), 0.80 µL of 0.2% bovine serum albumin (Sigma-Aldrich), 0.36 µL of 2.5 mM dNTPs (5Prime), 0.15 µL of each 10 mM primer solution (Bionordika), 0.04 µL of 5U/µL HotStart Taq polymerase (5Prime) and 5.70 µL of purified water. PCRs were performed in a Veriti Thermal Cycler (Life Technologies) with the following thermal profile: 94°C for 15 min for initial denaturation and Taq activation, followed by 50 cycles of 30 s at 94°C, 60 s at 55°C and 60 s at 72°C. The PCR cycling was followed by a final extension for 10 min at 72°C. PCR products were stored at 4°C and then purified by exonuclease digestions (1 µL of EXO-SAP per samples, incubated at 37° C for 30 min, then at 80° C for 15

min). The purified amplicons were Sanger-sequenced. Each 10 µL reaction contained 1 µL of amplified DNA, 1 µL of BigDye 1.1 (Life Technologies), 0.2 µL of either the forward or reverse primer and 7.8 µL of purified water. Sequencing was performed in a Veriti Thermal Cycler with 25 cycles of 10 s at 96° C, 5 s at 55° C, 4 min at 60° C and a storage at 4° C. Sequences were cleaned from unincorporated label nucleotide by precipitation adding to each PCR product a 12 µL mix composed by 2 µL of NaOAc 3M and 10 µL of purified water. Then were added 50 µL of 100% EtOH. The mix was then centrifuged at 12500 rpm for 10 min. The supernatant was extracted and eliminated manually by using a transfer pipette. The precipitate was washed with 70 µL of 70% EtOH and centrifuged at 12500 rpm for 7 min. The supernatant was eliminated and the precipitate was left to dry in the dark. The purified product was combined with 10 µL of Hi-DI formamide (Life Technologies) and denatured for 3 min at 95°C. Products were separated on an ABI 3130 DNA Analyzer. Sequences were aligned using SEQSCAPE software v2.5 (Life Technologies). The sequence of full mtDNA genome of the domestic cat (NCBI Reference Sequence: NC_001700), trimmed at the above-mentioned positions, was used as the reference sequence. The seven known variants were tagged on the reference sequence and all sequences were analysed to find all the other variants and also checked by eye. The exported sequences were trimmed using BIOEDIT v7.1.11 respectively into equal sequences of 671 bp (positions 13243 – 13913) to maintain full-length, high-quality sequence data across all samples. Haplotypes, genetic diversity and basic statistics were extrapolated using DNAsp v5.10.01.

Then BLAST database (NCBI) were queried with the CR sequences in order to verify which ones belonged to the *Felis silvestris*.

In order to assign the samples to the correct subspecies lineage we analysed the ND5 sequences performing a median joining network using NETWORK v4.6 (Fluxus Technology Ltd).

The samples were then amplified at 10 autosomal microsatellite loci FCA23, FCA26, FCA43, FCA58, FCA77, FCA88, FCA96, FCA126, FCA132, FCA149 (Menotti-Raymond and O'Brien 1995, Menotti-Raymond *et al.* 1997) and one microsatellite locus SMCY-7 STR on Y chromosome that present a polymorphism that seems to be fixed with different alleles in the two subspecies under study (Luo *et al.* 2007, Nussberger *et al.* 2013). The markers were amplified in reactions of 8 µL of total volume containing: 0.8 µL of 10X Taq Buffer advanced with self-adjusting Mg^{2+} (5Prime), 0.80 µL of 0.2% bovine serum albumin (Sigma-Aldrich), 0.36 µL of 2.5 mM dNTPs (5Prime), 0.2 µL of of the 10 mM primer solution (Bionordika), 0.04 µL of 5U/µL Taq polymerase (5Prime) and 5.80 µL of purified water. PCRs were performed in a Veriti Thermal Cycler (Life Technologies) with the following thermal profile: 94°C for 2 min for initial denaturation and Taq activation, followed by 10 cycles of 40 s at 94°C, a touch-down step of 30 s from 60°C to55°C decreasing 1°C per cycle and 30 s at 72°C. The PCR cycling was followed by a final extension for 10

min at 72°C. PCR products were analysed in an ABI 3130 XL (Applied Biosystems) automated sequencer, and allele sizes were determined with GeneMapper 4.0 (Applied Biosystems). Working with non-invasive samples, we decided to perform 5 duplex reaction in order to minimize primer interactions and PCR errors. 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) . Using RELTOYPE (Miller *et al.* 2002) we determined the reliability value for each samples and checked if further replicates were needed to reach the threshold value of 95%. Using the match function in GENALEX 6.501 (Peakall and Smouse 2006) we detected individuals sampled more than once. Discrimination between the wild and the domestic subspecies was performed in STRUCTURE (Pritchard *et al.* 2000) version 2.3.4 and setting the genetic clusters K= 2 (Oliveira *et al.* 2008a; 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 and Smouse 2006). 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), stored at -20 °C at the Institute for Environmental Protection and Research (ISPRA, Ozzano dell'Emilia, Bologna, Italy) 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.* 2008a).

Camera-trapping protocol

Ten camera traps (five Multipir12® and five Multipir12- HD®) with one passive infrared/motion front trigger sensor and two lateral preparation sensors were tied to trees at about 2 m to the lured pickets. According with the manufacturer's instructions the delay between the detection of the sensors and the triggering of the camera was of about one second. Cameras were set on video mode with a video length of 30 s and an interval between consecutive shots of 60 s. Each camera was equipped with a 4 GB SDHC card and was powered by 4 rechargeable AA batteries. In order to avoid any interaction between the animals and the camera we chose an infrared flashlight of 940 nm. Due to technical issues cameras were placed on March 28th (one was placed on 8th April). After 45 days, all cameras (except one that was withdrawn on 8th April due to malfunctioning) were simultaneously shifted to be associated to other hair-traps in order to cover at least 20 sampling stations until 24th June. Overall we placed a total of 20 camera-trap stations with an average trapping effort of 43

nights each and a total of 819 trap-days, from 28th March to 24th June 2013.. According to the observed 95% Kernel area considered above (see genetic sampling protocol), we spaced the camera-traps (mean distance = 1039m ± 368m) as to cover a continuous area, including non-monitored areas too small to host a whole wildcat home range . Consequently, individuals with a home range greater than 277.7 Ha were exposed to a greater number of traps (Otis et al. 1978; Karanth and Nichols 2002). Thus, to perform capture density calculations, we considered a total monitored area of 30,4 km2 (the grey area in figure 2).

We based the subspecies and the individual identification on coat colour patterns and body proportions of the animals (French et al. 1988; Ragni and Possenti 1996). In particular, according to the quality of the videos, we considered the number and the distance of tail rings, the proportional length of the dorsal stripe respect to the body and the presence and the shape of any additional sign on the pelage. Furthermore, we considered the behaviour and the body proportions to also infer sex and age.

Label	Name	Behaviour
I	Indifference	The individual shows no interest for the lure. It does not look at the picket
C	Curiosity	The individual is somehow attracted by the lure. It sniffs and remains around near the picket for a while. It does not touch the trap
FM	Facial-marking	The individual shows a typical facial marking behaviour rubbing the cheeks and the forehead on the picket. C is always included in this behaviour
SM	Spray-marking	The individual marks the picket by spraying on the picket. C is always included in this behaviour
SI	Strong interaction	The individual strongly interacts with the lure by rubbing the face and the body, sitting by the picket and scratching it with the nails. C and FM are always included in this behaviour.
D	Diffidence	The individual looks at the lure appearing suspicious and insecure. It does not get too close to the pickets. C is always included in this behaviour
F	Fear	After a D or a I behaviour the individual reacts suddenly leaving the sampling station. C is always included in this behaviour

Table 1. Ethogram of the behaviour detected by the camera-trapping survey.

To investigate the reactions toward the bait we compiled an ethogram (Wells and Egli 2004; Ellis and Wells 2010) including seven possible behaviours: indifference (I),

curiosity (C), facial marking (FM), strong interaction (SI), spray marking (SM), diffidence (D) and fear (F) (Table 1). If during the same shooting more than one behaviour occurred, we considered only the strongest one (e.g.if a cat displayed curiosity followed by facial marking and strong interaction, we considered only the "strong interaction" event).To infer any dependence relationship between the number of samples collected per session and the number of sessions elapsed we used a Poisson regression with the logarithm as the link function.

RESULTS

Genetic identifications

During the sampling period we collected a total of 63 non-invasive samples (30 hair samples and 33 scats), out of which 12 (36.7%; one scat and 11 hair samples) were assigned to non-target species according with analyses of mitochondrial CR.
Eighteen (eight hairs and ten scats) out of 51 (35.3%) wildcat samples were successfully genotyped at mitochondrial regions and/or microsatellites loci. Seventeen yielded reliable ND5 mtDNA haplotypes (eight from hairs and nine from scats) while eight were successfully genotyped with STR markers (two and six respectively). Concerning the sampling strategy, capture success rate was 0.082 genotyped hair samples per 100 trap nights and 1 scat per 42.5 km.. Wildcat samples were found only between March and June.

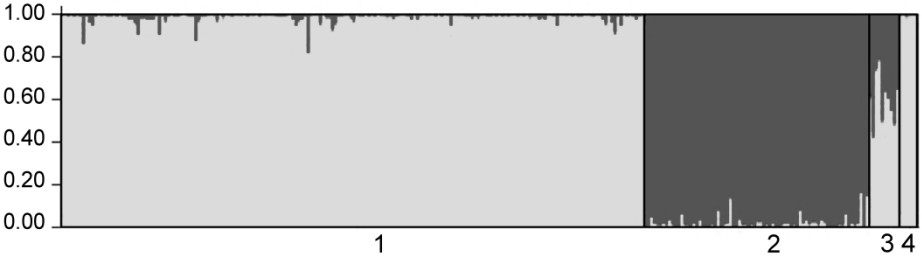

Figure 3. Proportions of posterior probability assignments as inferred by microsatellite analysis in Structure. Light grey correspond to wildcat proportion membership (Qw), dark grey correspond to domestic proportion membership (Qd). 1 = wildcat reference population; 2 = domestic cat reference population; 3 = admixed reference population; 4 = samples collected in this study.

The rate of allelic drop (ADO) was of 12.1% while the rate of false alleles (FA) was of 1.6% across all PCR reactions. The ten autosomal loci used for genotypization yielded a value of PIDsib = 0.0001. No sample showed more than two alleles, guaranteeing that no contamination occurred among samples. Microsatellite analyses allowed the detection of six individuals (three males and three females). The

test performed with STRUCTURE assigned all individuals to the F. s. silvestris subspecies with Qw values > 0.92 (Figure 3)while by mitochondrial analyses of ND5 subunit two main haplogroups (W, D, Figure 4) were identified: the wildcat haplogroup (W) and the domestic haplogroup (D). We confirmed as pure wildcats only the individuals with concordant attributions by all the three markers (STR, mtDNA and SMCY-STR [for male individuals], table 2). Only one individual (individual 1, Table 2) met these requirements Three individuals (3,4 and 6, Table 2) were attributed to the wildcat according to their nuclear genotype (Qw>90) but their mtDNA haplotypes showed all the polymorphisms peculiar of domestic cat (H1 and H5 in figure 4). Furthermore the samples belonging to one of these individuals (individual 3 in Table 2) were found about one km away from the nearest human settlement. The individual 6 was also captured by the camera-traps and showed, moreover, a wildcat phenotype (Figure 5). Two individuals (2 and 5, Table 2) assigned to the wildcat cluster by microsatellite analyses did not yielded reliable mitochondrial haplotypes.

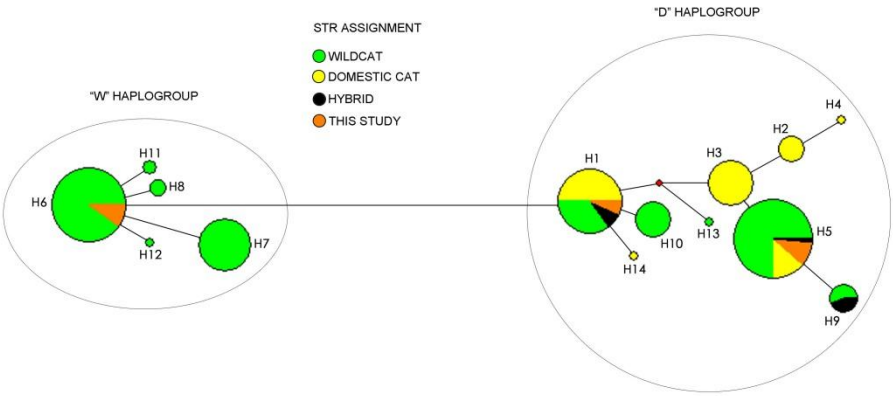

Figure 4. The network representing the phylogenetic relationships between the Italian samples reference (Mattucci et al. 2013), among which are those produced by our sampling campaign (this study). The two haplogroups (W and D) identify the samples, previously analysed with STR, through the presence of the diagnostic mutations indicated by Driscoll et al (2011)

Considering the minimum convex polygon (MCP, Figure 2), we found 2.6 captures/10km2. Taking into account the added buffer, the rate changed to 1.41 captures/10km2.

We found a slight but significant dependency between the number of sessions elapsed since the first inspection and the number of samples collected per session ($\beta k = 0,22$; $p < 0.01$)

Genetic sampling					Camera-trap sampling					
Individuals	Source	Sex	Qw (I.C.)	mtDNA	Individual	DNA	Sex	Age	Behaviour	Notes
1	scat	M(w)	0,99(0,96-1,00)	W	A	mtDNA (W)	M	AD-J	I	Pregnant
2	scat	M(w)	0,99(0,96-1,00)		B		F	AD	SI	
3	scat	F	0,98(0,91-1,00)	Di	C		F	AD-J	I	Pregnant, hybrid?
4	scat	F	0,99(0,97-1,00)	Di	D		F	AD	I	
5	scat	F	0,99(0,96-1,00)		E		M	AD-J	SM	
6	hair	M(w)	0,99(0,97-1,00)	Di	F	(6) mtDNA(W)	M	AD-J	C	
					G		M	AD-J	SI-SM	
					H		F	AD	MF	
					I		M	AD-J	C	

Table 3. Wildcat Individuals identification by genetic analyses (numbers) and camera-trapping (letters). In the mtDNA assignments column W= wildcat haplotype, Di= putative introgressed domestic haplotype. Individual 6 was the only one caught with both me

Figure 5. The individual "6" (a juvenile/adult male of wildcat), caught while depositing the hair sample that allowed its genetic identification

Camera-trapping layer

We obtained a total of 570 captures of animals, out of which 35 (6.1%) were Felis silvestris. Based on the coat colour marking patterns, size and proportion of the body and behaviour, 25 were referable to F. s. silvestris, five to F. s. catus and at least one to a putative hybrid. From 20 videos out of 35 (57.1%) we were able to detect at least nine different individuals of wildcat, five males and four females (including the putative hybrid) and five different individuals of domestic cat (three males and two females). One wildcat female and the putative hybrid appeared in an advanced state of pregnancy. The total capture rate of wildcats was 3.1 captures/ 100 trap-days. Considering the total area of 30.54 km2, we calculated 2.9 wildcats captures /10 km2 and 1.6 domestic cats capture/10 km2. Activity patterns of wildcats in the study area were mainly nocturnal (76% of capture between 9:00 pm and 5:00 am). 92.3% of the capture events occurred between May and June with a significant dependency on the number of sessions elapsed ($\beta k = 0,65$; $p < 0.01$). Considering all wildcat captures, in 51.7% of cases the individuals showed no interest for the lures while in 20.6% hairs were successfully trapped (Figure 6). Regarding the single recognized individuals six

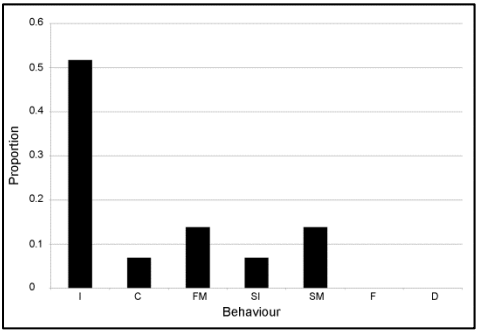

Figure 6. Proportion of single behavioural classes based on the number of discrete events in which each was displayed on the total of the recorded events (35). I: indifference; C: curiosity; FM: facial marking; SI: strong interaction; SM: spray marking; F: fear; D: diffidence

out of the nine detected individuals (four males and two females, individuals B, E, F, G, H, I) showed an interest in the lures. Four of them (two males and two females, individuals B, F, G, H) scratched on the picket leaving hair-samples (FM or SI), while one (a male individual, 5) only performed spray marking (Table 2).

DISCUSSION

The concomitant use of three census methods allowed us to detect in the study area between six and nine wildcats individuals (capture density between 1,41 ad 2,9 individuals per 10 km2) and revealing the presence of at least five domestic cats (capture density 1.6 individuals per 10 km2).

Hair-trapping proved to be the weakest method to obtain good genetic samples of wildcat compared with scats survey and camera-trapping. These results are consistent with previous studies on carnivores (Long et al. 2007; Comer et al. 2011; Monterroso et al. 2013). Hair-trapping success (0,08/100 trap-days) was similar to that reported by recent surveys for felids (0.07/100 trap-days, Steyer et al. 2012; 0.015/100 trap-days, García-Alaníz et al. 2010). The low capture efficiency of hair-traps may depend on the low interest shown by wildcats for valerian. A study by Monterroso et al. (2011) highlights that only 11.5% of the wildcats detected showed an investigative behaviour towards the bait. Anile et al (2012b) in Sicily and Kilshaw and MacDonald (2011) in Scotland found that none of the captured wildcats were interested in the valerian lures. Monterroso et al. (2013) obtained scarce results in collecting wildcat hairs in Spain. We found that 20.6% of wildcats reacted with the expected behaviour, leaving hair samples on the picket. The variability in the results arisen in the application of these technique could depend on the fact that the response to odorous baits is genetically inherited (Bradshaw 1992). Thus wildcats of different populations (especially of isolated population such as Sicilian and Scottish) could show different reactions. Furthermore only two (11%) of cat hairs provided a reliable individual genotypes, maybe due to a faster DNA degradation caused by a greater exposition of the pickets to environmental factors compared to the scats. Other studies found varying results fluctuating from 0 to 100% (Steyer et al 2013) or confirming the low genotyping rate (10%, Monterroso et al. 2013). Our results highlight the low reliability of using valerian-baited lures in genetic surveys which may lead to the

underestimation of population size in case of the exclusive use of this technique. Despite these limitations, considering the difficulty in finding non-invasive good quality samples, this method remains a good quantitative sample integration. Indeed, in our study, hair samples contributed to the characterization of the genetic profile of the individuals (especially regarding mtDNA). A strong point of this technique lies in its possible combination with the camera-trapping, that makes possible the association of the genetic data with the picture of an individual although, in our study, only one individual (6-G) was both genotyped and camera-trapped. Several hair-trapping surveys reported that in the breeding season (December- February) wildcat detection probability is the highest (Weber et al. 2008; Kéry et al. 2011; Steyer et al. 2012). However, in our own case we found a different trend. The significant dependency between the number of sessions elapsed since the first inspection and the number of captures/samples collected per session indicated that the number of samples collected increased approaching the spring. This trend may have been determined by seasonal variations in environmental conditions (food availability, snow cover...) and/or a trap-happy effect of baited traps.

Scat survey is widely used in non invasive monitoring of elusive carnivores and has proved to be a reliable source of samples for genetic analyses (Caniglia et al. 2011; Ruiz-González et al. 2013; Anile et al. 2014; DeMatteo et al. 2014). In our study it provided the majority of the biological samples that yielded reliable individual genotypes. The genotyping success rate of the faecal depositions samples (1/42.5 km) as well as the proportion of samples that yielded reliable individual genotype (25%) was lower compared with most recent Italian study, carried out in Sicily and based on a similar experimental design, (1/27.9 km, 36%, Anile et al. 2014). However, in the cited work, only samples previously selected as "fresh" were analysed and the population of wildcat was well known and stable. The general low rate of success in genetic analyses using both mtDNA and microsatellites may be due to the considerable length of the amplicons of mtDNA and/or to environmental factors (temperature, UV, etc. ..) that could have acted on the samples in the time interval between sampling sessions (Broquet et al. 2007). Nevertheless the average error rates (ADO = 12.1%, FA= 1.6%) and PIDsib for the used loci are in line with the recommended values (Waits and Paetkau 2005; Broquet et al. 2007).

Overall the genetic analyses revealed a cytonuclear discordance in the subspecies assignation in at least three individuals (3, 4, 6, Table 2) that were assigned to wildcat cluster using microsatellites (Qw > 0.9) but presented a "domestic" mitochondrial haplotype. However the presence of mitochondrial haplotypes typical of domestic population in some putative wildcats do not necessary reveals the occurrence of old hybridization or introgression, even if it may be, in several cases, a plausible explanation (Driscoll et al 2011). Indeed the presence of domestic haplotypes in wildcat individuals has been found in several populations in Europe (Randi et al. 2001; Driscoll et al. 2007; Hertwig et al. 2009). Furthermore, regarding the ND5 sub-region,

the domestic cat shares with Felis silvestris libyca (from which it derived) the same diagnostic polymorphisms that differentiate it from the European wildcat (Driscoll et al. 2007). So an alternative explanation for silvestris/catus haplotype sharing could be that some haplotypes represent a conserved ancient heritage already present in the common ancestor of F. s. silvestris and F. s. libyca (Hertwig et al. 2009). Alternatively a certain degree of gene flow between F. s. silvestris and F. s. libyca might have occurred as a result of undetected population migrations before domestication. For these reasons, further investigation are required to better understand the phenomenon and allow a correct use of mitochondrial markers in conservation genetics studies.

The results arising from camera-trapping (3.1/100 trap-days) highlight a higher capture-rate compared with similar studies carried out in comparable ecological contexts such as in Turkey and Scotland (1,8/100 trap-nights, Can et al. 2011; 2.3/100 trap-nights, Kilshaw and Macdonald 2011) while comparable results were obtained by Anile et al. (2012a) in Sicily (2,9/100 trap-nights). Camera trapping is one of the most functional methods to allow the effective monitoring of several species (Silveira et al. 2003). It can provide estimates of population parameters (abundance, density…) as well as valuable information about behaviour, circadian rhythms and species interactions (O'Connell et al. 2011). However it may suffer from overestimation errors, in particular in the determination of the abundance with capture-recapture methods, especially in studies for elusive animals with few identification marks and living in low density (Foster and Harmsen 2012) This should be taken into account considering the higher number of wildcats we detected using camera-traps compared with genetic survey. Furthermore, the images quality provided by cameras using a 940 nm flashlight allowed a reliable individual recognition only in 57.1% of the captures of Felis silvestris spp. This result is comparable with the one obtained by Can et al. (2009) (59,1%) that used cameras with similar features of ours while Anile et al. (2012a), using a white flashlight, obtained 95.2% useful pictures. However, compared with genetic survey, camera-trapping is highly efficient in contacting also the most elusive individuals. Indeed, the camera-trapping survey counted a relative high number of domestic cats while genetic survey did not show a clear presence of Felis s. catus in the study area. Has been proved that the probability of finding faecal depositions varies greatly between the domestic and wild subspecies, being lower for the domestic cat (Corbett 1979; Lozano and Urra, 2007; Lozano et al., 2013). This fact could have affected the results of our scat survey. These aspects are very important when monitoring wildcat populations, since its management should involve the knowledge of the sympatric relationship with the domestic cat (Randi et al. 2001; Pierpaoli et al. 2003; Oliveira et al. 2008a; 2008b; Hertwig et al. 2009).

This comparison allows us to state that the individual techniques result to be functional but still need to complement each other to balance the gaps.

CONCLUSIONS

In this study we evaluated the integration of three non-invasive monitoring methods to improve monitoring efficiency and obtain a good amount of data in studying the European wildcat. The use of a layered design allowed us to collect heterogeneous data using a single sampling effort and to offset the weaknesses of each method. However some precautions are necessary to improve an integrated approach. We recommend avoiding the exclusive use of hair-trapping due to the variability of response among individuals and to the possible influences of sex and season that could invalidate the results. In collecting the genetic samples, we suggest reducing as much as possible the sampling intervals to prevent DNA degradation and performing genetic analyses as soon as possible (Waits & Paetkau, 2004). We recommend the use of two camera-traps per station (Kilshaw and MacDonald 2011), one set in a high-resolution photo mode with white flashlight for a better individual identification and the second in video mode or multi-shot mode to capture their behaviours (O'Connell et al. 2011). Using a multi-method approach we could confirm the presence of the species and identify a number of wildcats individuals ranging from six (from genetic analyses) to nine (from camera-trapping) while also verifying the invasiveness of the domestic cats and the risk of hybridization. Moreover, it was possible to determine some behavioural and ecological aspects.

According to a recent study carried out in other areas of the National Park (Ragni et al. 2014), our data suggest the presence of a well-established and stable population. On the other hand, the widespread presence of domestic cats, requires further study as to implement conservation measures.

The wildcat is a solitary and secretive species whose monitoring is difficult. Knowledge about the population parameters, the genetic status and the ecology often involves many years of sampling in the same area. On the other hand, wildlife management administrations need such information for planning effective conservation measures, especially in areas where the presence of the species has been confirmed only recently. Our results suggest that integrating some non-invasive techniques, monitoring performances can be improved, allowing the collection of sound data in a relatively short time.

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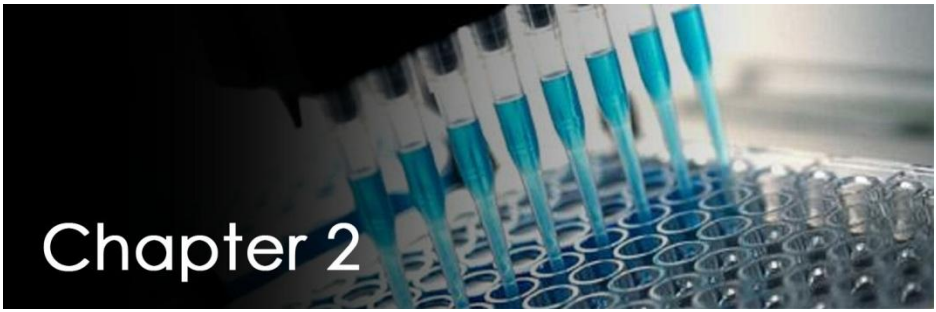

COMBINING USE OF MOST INFORMATIVE AUTOSOMAL SNPS WITH UNIPARENTAL MARKERS (MTDNA AND Y-CHROMOSOME) FOR THE ASSESSMENT OF HYBRIDIZATION IN EUROPEAN WILDCATS (*FELIS SILVESTRIS*)

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ABSTRACT

Because of a recent divergence and entirely overlapping ranges, introgressive hybridization between free-ranging domestic cats and European wildcats (*Felis silvestris silvestris*), might locally threaten the survival and conservation of indigenous wildcats populations. Identifying pure wildcats and investigating the ancestry of admixed individuals is thus crucial for supporting appropriate conservation and managing programs of European wildcat. In this study, we present a single nucleotide polymorphism (SNP) based approach combined with mitochondrial and Y-linked chromosome polymorphisms that allows the identification of introgressed individuals. First, we analysed the genetic variation of 151 highly informative SNPs ($F_{ST} > 0.8$) on 187 European cat samples, including 45 village domestic cats, 100 putative European wildcats and 42 previously known or putative wild *x* domestic hybrids. The power of all the loci to accurately identify admixture events and discriminate the different hybrid categories was evaluated. Results from Bayesian model-based computations of simulated and real genotypes show that the 151 SNPs provide successful estimates of admixture, with 100% hybrid individuals (up to second generation backcrosses) being correctly identified in STRUCTURE analyses and 100% using the NEWHYBRIDS' algorithm. None of the unclassified cats were wrongly allocated to another hybrid class. The chromosome Y-linked markers further analysed, proved to be useful for identifying wild and domestic cat males based on distinctive polymorphism. Furthermore, both mitochondrial sequenced regions (ND5 and part of the control region), clearly separated the subspecies in two well distinct haplogroups, with the exception of a few domestic shared haplotypes, suggesting the occurrence of old

introgressive events into wildcats populations or an ancient pre-domestication sharing of haplotypes with *F. s. libyca* (the most related subspecies of the domestic cat). The integration of both uniparental and nuclear markers assignments, provided a complete insight of introgression level in wildcats populations analysed. Finally, a total of 11 hybrids were identified: 8 were detected by both nuclear and uniparental markers, and 3 were identified because of the presence of nuclear genotype wild and shared domestic mitochondrial haplotype. This approach may be useful to further reconstruct both the historical and recent evolution of wildcat populations and, hopefully, to develop sound conservation guidelines for its legal protection in Europe.

Keywords: *Felis silvestris*, European wildcat, domestic cat, hybridization, introgression, single nucleotide polymorphisms, mitochondrial DNA, Y Chromosome, conservation genetics

INTRODUCTION

Anthropogenically driven changes of the spatial distribution of species are increasing the incidence of hybridization events (Reusch and Wood 2007), critically threatening the native fauna (Wayne and Brown 2001; Randi 2008). Especially in endangered taxa, hybridization (and introgression) occurring between wild species and their domestic counterparts may disintegrate the genetic integrity of the wild conspecific (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). Genetic introgression of domestic alleles into native gene pools may, in fact, introduce genes favoured under artificial selection that are maladaptive in the natural environment, disrupting locally adaptation or increasing genetic homogenization (Rhymer & Simberloff 1996). Both of these processes can reduce the fitness of wild species raising risks of genetic extinction, loss of local adaptations or outbreeding depression (Rhymer & Simberloff 1996; Allendorf *et al.* 2001; Lynch & O'Hely 2001; McGinnity *et al.* 2003; Hutchings & Fraser 2008).

Interbreeding between domesticated and wild counterparts has been observed in carnivores, ungulates, fowl, anurans and many fish species (Rhymer & Simberloff 1996; Williams *et al.* 2002). One remarkable example to underline the consequences of anthropogenic hybridization on natural populations is the current situation of the European wildcat (*Felis silvestris silvestris*). The human-mediated global dispersal of the domestic cat, together with the demographic decline of European wildcats' population and the fragmentation of suitable habitat (McOrist and Kitchener 1994, increased the risk of anthropogenic hybridization and promoted the extinction of some natural populations during the last century. In addition, the fertility of the hybrid offspring (Pierpaoli *et al.* 2003), could have facilitated the dilution of the wild genotypes over progressive generations.

Since evidence of extinction of localized wildcat populations has already been detected in central Europe (Suminski 1962), the prevention of hybridization has been identified as the greatest priority for the persistence of the subspecies (Driscoll & Nowell 2010). Hence, accurate detection of hybrid individuals and quantification of introgression rate in potentially threatened populations are the main challenges for assessing wildcat conservation's status and, subsequently, developing appropriate conservation measures.

Over the last decade, the hybridization pattern with feral domestic cats had been assessed through molecular approaches. In particular, the genotyping of several highly polymorphic molecular markers, specifically microsatellites (short tandem repeats - STR), and partial mitochondrial DNA sequences, combined with new Bayesian statistical tools, have radically improved knowledge of the genetics of European wildcat (e.g. Beaumont *et al.* 2001; Randi *et al.* 2001; Pierpaoli *et al.* 2003; Kitchener *et al.* 2005; Lecis *et al.* 2006; Oliveira *et al.* 2008; Eckert *et al.* 2010; O'Brien *et al.* 2009; Hertwig *et al.* 2009). The empirical evidence available so far suggests that

hybridization occurred sporadically in some European countries (i.e. Spain, France, Germany and Italy), but extensively in others, as in Scotland and in Hungary (Beaumont *et al.* 2001; Pierpaoli *et al.* 2003), where, most likely, the presence of forests patches and traditional agriculture with human settlements facilitates the meeting between free-ranging domestic cats and wildcats.

The recent domestication of cats from the subspecies *Felis s. libyca* in northern Africa (Driscoll et al 2007, Vigne *et al.* 2012) and the protracted coexistence of domestic and wild cats raised fear that widespread interbreeding would have led to genetic pollution and rendered uncertain any identification of 'pure' wildcat populations. Until recently, type and number of markers showed a limited power of hybrid detection after the first few generations of backcrossing (Oliveira *et al.* 2008a, b; Hertwig *et al.* 2009; Say *et al.* 2012). Thereby, a set of more powerful markers is required to assess the level of introgression in natural wildcat populations (Vaha & Primmer 2006; Randi 2008).

Over the last decades, next-generation sequencing (NGS) technologies generated a large quantity of nucleotide sequence data, promising to improve vastly the ability to study hybridization and introgression by using both molecular phylogenetic approaches and population genetic studies.

For backcross detection, single nucleotide polymorphism (SNP) markers appear promising because of: (i) reduced propensity for homoplasy due to lower mutation rates; (ii) higher density and more uniform distribution in genomes; (iii) suitability for successful high-throughput genotyping and straightforward comparability and transportability across laboratories and detection protocols; and (iv) highly successful application in fragmented DNA samples, e.g. non-invasive and historical DNA (see for reviews: Brumfield *et al.* 2003; Morin *et al.* 2004; Garvin *et al.* 2010). Nussberger *et al.* (2013), recently identified a promising set of 48 nuclear SNPs for detecting European wildcats, domestic cats and their admixed progeny. However, the reference samples used in this work were limited to Switzerland, and the SNPs power for hybrid detection has probably been overestimated by computing simulations using individuals too much differentiated than average.

In recent years, research aimed at understanding the processes of domestication and evolution of the species led to important developments in the use of uniparental markers to trace the membership of the lineages to the different populations (Driscoll *et al.* 2007; Luo *et al.* 2007; Driscoll *et al.* 2011). The linear inheritance and non-recombinant quality of these markers are making frequent their use in the identification of possible introgression events not identifiable through the use of nuclear markers (Hertwig *et al.* 2009, McEwing *et al.* 2012, Nussberger *et al.* 2013).

In this work we aimed to developed a two-steps protocol for assessing 'pure' reference cats, and for estimating introgression in conspecific wildcats by using in a first step the Illumina Infinium iSelect 63K Cat DNA Array to amplify 187 cat

samples of two *Felis silvestris* subspecies (*silvestris* and *catus*) and a number of known and putative admixed individuals previously identified with high polymorphic microsatellites panel set (Pierpaoli *et al.* 2003; Mattucci *et al.* in prep). Since this work was largely motivated by the need to routinely identify admixture events in conservation studies of European wildcat populations, we focused in extracting the minimum number of highly informative SNPs able to efficiently detect current levels of hybridization between wild (*Felis s. silvestris*) and domestic (*Felis s. catus*) cats.

Second, we analysed the genetic variation occurred in the Y chromosome and in both the control region and the NADH dehydrogenase subunit 5 of the mtDNA. The integrated molecular panel set obtained combining the 151 most informative SNPs with the uniparental markers, was then used to accurately identify 'pure' reference cat and second generation backcrosses and to investigate ancient introgressive/sharing events occurred in wildcat populations, in order to assess the efficiency and reliability of these markers and promote and prioritize conservation efforts for the subspecies survival in the near future.

MATERIALS AND METHODS

Sampling

We genotyped 100 presumed European wildcat samples (*Fsi*) which were opportunistically collected from found-dead or trapped animals across seven countries in Europe (Table 1) These samples were randomly selected from a larger tissue collection maintained at ISPRA (Ozzano dell'Emilia, Bologna, Italy), with the aim to include individuals from locations spread in the entire distribution range of the European wildcat and representing the main sub-populations that were genetically identified in Europe (Mattucci *et al.* in prep.). The European wildcats were first morphologically identified according to diagnostic phenotypic traits (Schauenberg 1969, 1977; French *et al.* 1988; Ragni and Possenti 1996), and then assigned to their own subspecies through microsatellite genotyping and Bayesian analyses (Mattucci *et al.* 2013; Mattucci *et al.* in prep.). From the same tissue collection we randomly selected and genotyped 45 domestic cats (*Fca*) sampled from five European countries. Moreover, we included 42 known (captive; N = 4) or putative (natural; N = 38) hybrids (*Hy*) sampled from seven European countries, which were genetically identified in previous studies through Bayesian admixture analyses of multilocus microsatellite genotypes (Mattucci *et al.* 2013; Mattucci *et al.* in prep.). The first four hybrids were obtained in captivity from controlled *silvestris* x *catus* crosses (Ragni 1993). Six natural hybrids from eastern Italian Alps include five full-sibs extracted from the uterus of a road-killed apparently pure European wildcat female, were genetically identified in other studies (Pierpaoli *et al.* 2003; Lecis *et al.* 2006). Only in mitochondrial analyses we included also eight *Felis s. libyca* from Sardinia in order to

better clarify some phylogenetic relationship. All samples used in this study were re-analysed at 31 unlinked autosomal microsatellite loci following procedures described in Mattucci *et al.* (2013).

Subspecies	**Locations**	**Size**
Domestic cats (*F. s. catus*)	Italy	18
	Poland	4
	Portugal	10
	Spain	12
	Greece	1
		45
Putative *silvestris* x *catus* hybrids	Captivity	4
	Italy: eastern Alps	6
	Italy: central Apennines	14
	Italy: southern Apennines	6
	Luxembourg	2
	Portugal	6
	Spain	1
	Bosnia & Herzegovina	1
	Germany: southern-western	2
		42
European wildcats (*F. s. silvestris*)	Slovenia	14
	Italy: eastern Alps	20
	Italy: central Apennines	12
	Italy: Sicily	4
	Germany: central	10
	Germany: southern-western	15
	Belgium: Wallonia	5
	Luxembourg	1
	Portugal	7
	Spain	12
		100
African wildcats (*F.s libyca)* (only mitochondrial analyses)	Italy: Sardinia	8

Table 1. Locations and size of the European wild, domestic and putative hybrid cat (*Felis silvestris* ssp.) samples genotyped in this study.

SNP genotyping and quality-controls

We extracted the DNA samples using the Quiagen Blood&Tissue Kit (Quiagen®) using the manufacturer instructions. All samples were quality-controlled for DNA degradation in a 1,5% agarose minigels run for 40 minutes. We used the Illumina Infinium iSelect 63K Cat DNA Array (Illumina Inc., San Diego, CA) to genotype the cat samples at 62,897 autosomal SNPs (referred to as the 63K panel).

A total of 37 cats, belonging to the wild, domestic and admixed populations were removed from the initial dataset, because of a missing rate per individual (MIND) < 0.2. Hence, we obtained a reduced dataset of 150 fully genotyped samples that we used for all the subsequently elaborations.

The initial 63K panel set was pruned using PLINK (Purcell *et al.* 2007) for loci that were invariant, showing individual missing rates per SNPs (GENO) < 0.2, or minor allele frequency MAF < 0.05. We further pruned the panel for loci in linkage disequilibrium (LD), filtering SNPs with r^2 0.5 within 50 SNP sliding windows, shifted and recalculated every 5 SNPs. Quality-control excluded 33.833 for missing rate (i.e. no genotyping (n = 33) and GENO filter (n = 649)), allele frequencies (i.e. fixed (n = 30) and MAF filter (n = 14,513), INDEL and Linkage Disequilibrium (7 and 18,601 respectively), improving the SNP genotype call rate to 97% in the remaining analysed cat samples (n = 150). After this pruning we saved a panel of 26,361 SNPs (referred to as the 26K panel; Table 2)

Removed SNPs	**Filter type**	**Description**
7	INDEL	Insertions/deletions
30	fixed	Invariant
33	no genotyping	No genotyping results
649	GENO	Missing rate per SNP > 0.2
14513	MAF	Minor Allele Frequencies < 0.05
18601	LD	Linkage Disequilibrium r2 > 0.5
Removed samples		
37	MIND	Missing rate per sample > 0.2

Table 2. Number of SNPs and samples pruned after quality-controls of the total data set (63K SNPs tuped in 150 cat samples) in PLINK.

Screening for the most informative SNPs and statistical analyses

We used HIERFSTAT (Goudet 2005) to estimate the F-statistics and variance components, and identify the most divergent SNPs among the two cat subspecies (European wildcats and domestic cat). From the pruned 26K panel we kept the SNPs showing F_{ST} values > 0.80 (n = 151). The power of the 151 SNPs to identify individual genotypes without "shadow effects" was evaluated calculating the probability-of-

identity (PID and PIDsibs; Mills *et al.* 2000; Waits *et al.* 2001) in GENALEX 6.41 (Peakall & Smouse 2006). These SNPs were then ranked for their diagnostic hybridization power by computing their informativeness for assignment index (*In*) with INFOCALC (Rosenberg *et al.* 2003; 2005). *In* measures the expected logarithm of the likelihood ratio that an allele is assigned to one of the two parental populations, compared with a hypothetical 'average' population whose allele frequencies equal the mean allele frequency across sub-populations.

We used summary statistics to describe the extent of genetic variability within-subspecies, and divergence between European wildcats and domestic cats, excluding the 42 admixed genotypes (known and putative hybrids) that were identified in the admixture analyses (see below). These genotypes were, afterwards, used in the hybridization analyses. We computed values of observed (H_O) and unbiased expected (H_E; Nei 1978) heterozygosity for all locus-population combinations, Hardy-Weinberg and linkage disequilibrium tests (HWLE; using the Markov chain exact test with a chain length of 100,000 and 3,000 dememorization steps), AMOVA and F-statistics (testing the null hypothesis of no differentiation by permuting genotypes between populations with 10,000 replicates at $P < 0.001$) in ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010). Allelic richness (N_{AR}) was estimated in FSTAT 2.9.3.2 (Goudet *et al.* 2002)

Admixture analyses and assignment of the individual genotypes

We used STRUCTURE 2.3.3 (Pritchard *et al.* 2000; Falush *et al.* 2007; Hubisz *et al.* 2009) to identify the most likely population clusters in the sample and perform admixture analyses. Each run of STRUCTURE was replicated five times, with 10^4 burn-in followed by 10^5 simulations, combining the "admixture" model with correlated or independent (respectively "*F* and *I*") allele frequency models, both with or without prior non-genetic information (option *usepopinfo* = 1 or = 0, respectively). The optimal number of clusters (*K*) was identified by the 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: (i) the average proportion of membership (Q_i) of the sampled populations to the inferred clusters; (ii) the individual proportion of membership (q_i) to one or more than one (in case of admixed genotypes) of the inferred clusters; and (iii) the 90% credibility intervals (CI) of the q_i values STRUCTURE was run using the pruned dataset ($N = 150$), that is including the putative European wild cats, the domestic cats and hybrids, and the 151 most informative SNPs. The most likely genotypic classes of the admixed cats were estimated using NEWHYBRIDS (Anderson and Thompson, 2002). This software estimates the posterior probability of each genotype to belong to each of six genotypic classes corresponding to hybrid categories (H_i): parental subspecies (domestic – DC, or wild – WC cats), first (F1) and second (F2) crosses, and the first backcrosses BD = F1 x D; BW = F1 x W). We run ten independent replicates of

NEWHYBRIDS with the default computational parameters and with Jeffreys' priors which were chosen to down-weight the influence of an allele that might be rare in one species and absent in the other (Anderson and Thompson, 2002). The power of the 151 SNPs to correctly identify genotypes belonging to known genotypic classes was assessed by simulations. Starting from the allelic pool of those individuals who showed multi-locus genotypes (including SNPs, STR, mtDNA and chr Y markers) with the greatest Bayesian assignation values and a consistence with the uniparental markers assignment (see section below) we used the software HYBRIDLAB 1.0 (Nielsen *et al.* 2006) to generate 30 multi-locus genotypes of each parental (wildcat x wildcat; domestic cat x domestic cat), F1 (wildcat x domestic cat), F2 (F1 x F1), BX_Fca and BX_Fsi backcross categories (1,2,3,4 see Supplementary Table S2). The simulated genotypes were analysed using STRUCTURE and NEWHYBRIDS under the same settings of the admixture analyses described above. Q_i threshold values for all analyses where established by the minimum value for which all parental cats could be correctly assigned. Observed genotypes that displayed admixed genetic assignments or for which molecular assignments opposed their prior morphological identifications in the hybridization analyses of STRUCTURE and NEWHYBRIDS were also analysed together with the simulated genotypes.

Mitochondrial DNA sequencing and Y-chromosome typing

For the mtDNA analyses we included also eight samples belonging to the *F.s.libyca* subspecies, collected in Sardinia, in order to better characterize the topography of trees and networks.

We sequenced 877 bp (including the primers) of the mtDNA NADH dehydrogenase subunit 5 (ND5; nucleotides 13131 - 14007 mapped on the mitochondrial genome of the domestic cat; NCBI Reference Sequence NC001700), which, according to Driscoll *et al.* (2011), should contains seven diagnostic SNPs discriminating European wildcats and domestic cats. This sequence was amplified using the PCR primers F2B (5'-TGCCGCCCTACAAGCAAT-3') and R3B (5'-TAAGAGACGTTTAATGGAGTTGAT-3'; Driscoll *et al.* 2011). In addition, we sequenced 719 bp of the mtDNA control-region (sites 16236 - 16955) using 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). Each 10 µL PCR reaction contained 2 µL of DNA (c. 50 ng), 0.8 µL of 10X Taq Buffer advanced (Eppendorf) with self-adjusting Mg^{2+} concentration, 0.80 µL of 0.2% bovine serum albumin (Sigma-Aldrich), 0.36 µL of 2.5 mM dNTPs (Eppendorf), 0.15 µL of each 10 mM primer solution (Bionordika), 0.04 µL of 5U/µL HotStart Taq polymerase (Eppendorf) and 5.70 µL of purified water (Eppendorf). PCRs were performed in a Veriti® Thermal Cycler (Life Technologies) with the following thermal profile: initial denaturation and Taq activation at 94 °C for 15 min, followed by 50 cycles of 30 s at

94 °C, 60 s at 55 °C and 60 s at 72 °C. The PCR cycling was followed by 10 min final extension at 72 °C. PCR products were stored at 4 °C and then purified by exonuclease digestions using 1 µL of EXO-SAP-IT® (Affymetrix) per samples, incubated at 37° C for 30 min, then at 80 °C for 15 min. The purified amplicons were Sanger-sequenced using the Life technologies BigDye® Terminator v1.1 Cycle Sequencing Kit. Each 10 µL reaction contained 1 µL of amplified DNA, 1 µL of BigDye 1.1 (Life Technologies), 0.2 µL of either the forward or reverse primer and 7.8 µL of purified water. Sequencing was performed in a Veriti® Thermal Cycler with 25 cycles of 10 s at 96 °C, 5 s at 55° C, 4 min at 60 °C and a storage at 4 °C. Unincorporated nucleotides were eliminated by adding to each PCR 2 µL of 3M NaOAc and 10 µL of purified water, followed by ethanol precipitation Purified PCRs were combined with 10 µL of Hi-DI formamide (Life Technologies), denatured for 3 min at 95 °C, and finally separated on an ABI 3130 DNA Analyzer.

We genotyped two Y-linked markers: a portion of the SRY gene and microsatellite SMCY-7, which showed diagnostic polymorphisms with different fixed alleles in the two cat subspecies (Pecon-Slattery *et al.* 2004; King *et al.* 2007; Luo *et al.* 2007; Nussberger *et al.* 2013). We sequenced 376 bp of the SRY using the primers SRYF (5'–GGCCTGTGTGTCGTTTAACA –3') and SRYR (5'-GTTTTTCCACAGGAGGGATG -3') (Nussberger *et al.* 2013) and the same chemical and thermal conditions used for ND5 sequencing. We amplified the biallelic microsatellite SMCY-7 in 10 µL PCR reactions containing 1,5 µL of DNA solution (> 50 ng), 0.8 µL of 10X Taq Buffer with self-adjusting Mg^{2+} concentration (Eppendorf), 0.80 µL of bovine serum albumin 0.2% (Sigma Aldrich), 0.36 µL of dNTPs 2.5 mM (Eppendorf), 0.2 µL of each primer 10 mM (Bionordika), 0.04 of Taq polymerase 5U/µL (Eppendorf) and 5.80 µL of purified water (Eppendorf, Milano, Italy). Each reaction was amplified under the following PCR condition using a Veriti® Thermal Cycler (Life Technologies): 94°C for 2 min for initial denaturation and Taq activation, followed by 10 cycles of 40 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. Every cycle temperature decreased by 0,5 °C. The PCR cycling was followed by 25 cylces of 40 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C with a final extension for 10 min at 72 °C. PCR products were stored at 4 °C. The amplified product was combined with 10 µL of Hi-DI formamide (Life Technologies) and denatured for 3 min at 95 °C. Products were separated on an ABI 3130 DNA Analyzer.

The mtDNA sequences were aligned in SEQSCAPE 2.5 (Life Technologies) and mapped on the entire mtDNA genome of the domestic cat (NCBI Reference Sequence: NC_001700) on which the seven known variants were tagged. All the aligned sequences were in addition manually checked in BIOEDIT 7.1.11 (Hall, 1999) to verify the presence of ambiguous positions. The exported sequences were then trimmed using BIOEDIT respectively into equal sequences of 669 bp (for ND5 region, positions 13243 – 13911) and 604 bp (For the CR, 16302 – 16905) for independent

sequence analyses to maintain full-length, double-stranded, high-quality sequence data across all samples

We then translated the coding region of ND5 sequences searching for stop codon suggesting the presence of nuclear copies of mtDNA. The microsatellite fragments were analysed in GENEMAPPER® 4.0 (Applied Biosystems).

Phylogenetic analyses

The number of polymorphic sites, haplotype (h) and nucleotide diversity (π) were computed separately for each region of mtDNA (ND5 and CR) using DNASP 5.10.01 (Librando and Rozas, 2009). Genetic variability between and within populations (wildcat, domestic cat and hybrids, as defined by the SNPs admixture analyses) and haplogroups was calculated by means of an analysis of molecular variance (AMOVA) in ARLEQUIN 3.5.1.3 (Excoffier and Lischer 2010) estimating the significance of the parameters by 10 000 permutations of the distance matrix. A mismatch distribution analyses between individuals (Rogers and Harpending, 1992) was performed to test populations/groups trends using a population growth–decline model in DNASP 5.0 (Librado & Rozas 2009). Multimodal distributions were considered consistent with demographic stability, whereas sudden expansion would generate a unimodal pattern (Slatkin & Hudson 1991). Tajima's D (Tajima 1989) and FU and Li's F (Fu and Li 1993) statistics were computed to test demographic expansion in DNAsp 5.10.01. Significance of parameters was obtained by means of coalescent simulations of a panmictic population of constant size, given the number segregating sites, for a total of 1000 simulations.

Best nucleotide substitution model scheme for the sequences was computed in PARTITIONFINDER V1.1.0 (Lanfear *et al.* 2012) using Bayesian information criterion, (BIC). For the first and second codon positions in ND5 Kimura's two parameters (K80) models with invariable sites (I=0,80) was selected as best-fitting model. For third position of ND5 and control region was selected the Hasegawa, Kishino and Yano (HKY) model whit invariable sites (I=0,87)

We used NETWORK v4.6 (Fluxus Technology Ltd). to construct a network to infer relationships among haplotypes using a median-joining (MJ) algorithm (Bandelt *et al.* 1999) with $\varepsilon = 10$ and a transversions/transition weighting of 3:1 and then we cleaned up the resulting scheme using MP calculation (Polzin *et al.* 2003).

In order to verify the consistency and direction of the gene flow between the wildcat and domestic cat (excluding the *libyca* samples) we used the isolation with migration analytic approach implemented in the software IMa (Hey and Nielsen 2007) for generating relative likelihoods/posterior probabilities for complex demographic population genetic models between closed related taxa. We considered the populations as defined using nuclear molecular markers admixture analyses (SNPs and microsatellites) We derived the mutation rates of feline ND5 (0,0228/site/Myr) from

Driscoll et al (2007) and of CR (0,32/site/Myr) from Sigurđardóttir et al (2000) and used the following prior parameters values as starting point for the calculations: q1=q2=20, qa=60, m1=m2=2, t= 24,28. We performed three independent parallels runs with identical setting parameters and different starting seed values using MCMC mode. We used 30 chains per run with a geometric heating scheme setting a burning period of 1 000 000 steps and ending up with more than 30 000 000 of total steps, saving about 300 000 genealogies per run. Once assured an adequate convergence with sufficient values of effective sample size (ESS > 200) and low values of autocorrelation we combined the results of the three runs (about 900 000 genealogies) running the load-trees mode.

We reconstruct a Bayesian phylogenetic tree and estimated the divergence times in the relationships among haplotypes using BEAST v2.1.3 (Bouckaert et al 2014) using the substitution model scheme previously selected by PARTITIONFINDER. We set the rate variation among sites using a gamma distribution with four discrete categories. Due to the strong relationship between the two taxa we selected a strict molecular clock model with fix mean substitution rate calculated as the geometric mean of the mutation rates of the two regions used (8.5x10-8/site/year). Constant population size was selected as coalescent prior. Since the authors of the software discouraged the inclusion of an outgroup to root the tree (Drummond and Bouckaert 2014), we let the software to estimate the rooting point giving as prior calibration point the interval in which falls the common ancestor between *Felis s silvestris* and *F.s.libyca/catus* as illustrated in Driscoll et al (2007) using a uniform distribution with bounds values of 230 000-173 000 years BP constraining the group to be monophyletic. The Bayesian posterior probabilities (BPPs) as well as the high posterior densities for the node ages (HPDs) were extrapolated performing three independent MCMC runs of 100 000 000 steps with a burn-in period of 10 000 000 steps and picking genealogies every 2000 steps. The results of the three chains were simultaneously analyzed in TRACER v1.6 (Rambaut et al 2013). The values of ESS (always exciding 200) and the equilibrium showed by the trace plots suggested that the number of steps was more than adequate for a correct estimation of the posterior distribution of the parameters.

To support phylogenetic reconstructions two further trees were calculated using respectively neighbour-joining algorithm (NJ, Saitou and Nei 1987) and maximum-likelihood method (ML: Felsenstein 1981) and using, for ML tree reconstruction, the heuristic search by topological rearrangement of an initial tree (Near-Neighbour-Interchange). The robustness of the trees was assessed by bootstrap resampling (BS) (10 000 random replications for NJ analysis; 5000 random replications for ML analysis) (MEGA v6, Koichiro et al. 2013)

RESULTS

Variability of the 151 selected SNPs

Summary statistics and descriptive statistics for each of the 151 selected SNP are shown in Table S1 (Supplementary). All SNPs were polymorphic among the wildcats (n = 89), implying a MAF > 5%. In contrast, amongst the domestic cats , seven SNPs (about 5%) were monomorphic (n = 44) and 52 SNPs (about 34%) showed a MAF < 5%.We did not detect any significant deviations from HWE, following Bonferroni correction (P < 0.000065), in anyone of the single SNP loci. On average, the genetic variability described by the selected SNP panel was significantly partitioned between wild and domestic cats (average F_{CT} = 0,864; P <0,001), with single-locus F_{CT} pairwise values ranging between 0,797 (ChrUn13.13324872) and 0,982 (ChrC1.124364347; AMOVA P < 0,001; Table S1). In the domestic cat group the mean value of H_E was 0,101 (± 0.005 SD), ranging from H_E = 0,000 (at the seven monomorphic loci in domestic cats), to H_E = 0,325 (ChrC1.63091997). In the wildcats the average expected heterozygosity was H_E = 0,127 (± 0.004 SD), ranging between 0,011 (ChrC1.59406628) and 0,236 (ChrUn26.10046275). Average values of allelic richness proved to be significantly high (P < 0.001) both in domestic (A_R = 1.930) and wildcat group (A_R = 1.998). The average informativeness for assignment score (In) was 0,459, ranging from 0,002 (ChrB1.193634290; ChrB2.117030105) to 0,641 (ChrC1.105529441) (see Table S1).

Individuals' assignment and admixture analyses

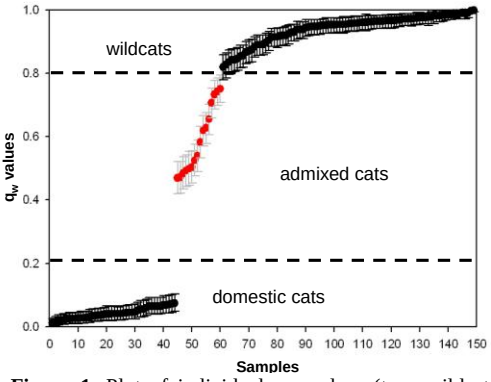

Figure 1. Plot of individual qw values (to a wildcat cluster) and their 90% credibility intervals CI. The qi thresholds = 0.20 - 0.80 (interrupted lines) define the admixed genotypes. Pure wild and domestic cats are in black, admixed individuals are in red

The admixture analysis computed on simulated genotypes (generated by HYBRIDLAB), running the "admixture" with both the allele frequencies models, and using K = 2 in STRUCTURE, was able to efficiently recognize 100% of the parental individuals at a threshold of qi = 0,80 (Supplementary Table S2) and correctly identify all the F1- F2 hybrids and the backcross genotypes of I-II generation. However, all the backcross genotypes of III- IV generation, were assigned to the backcrossed subspecies with qi > 0.80 and could not be distinguished from

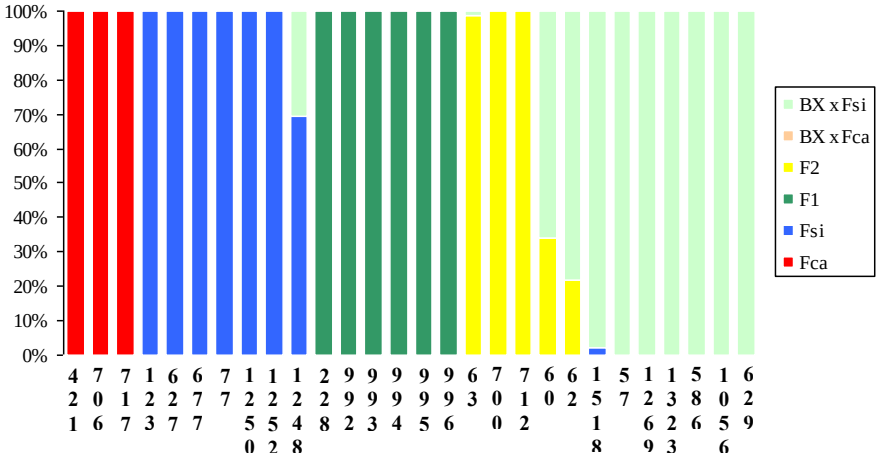

Figure 2. NEWHYBRIDS' assignment of 10 known hybrids and 19 'putative' admixed individuals to the different hybrid categories. Each individual is represented by a single vertical bar colored according to the proportion of their genome descending from each of the inferred clusters or hybrid class.

the parental populations.

At the best assignment cluster $K = 2$ (showing the highest values of ΔK and ΔF_{ST}, see Figure 1) 44 domestic cats ($Q_I = 0{,}989$ and individual q_d ranging from 0,933 to 1,000) and 89 European wildcats ($Q_{II} = 0{,}965$ and $q_W = 0{,}918 – 1{,}000$) were clearly assigned to their expected cluster with high NEWHYBRIDS' membership probabilities ($qi > 0{,}99$, see Table 3 and Figure 1). Ten previously identified admixed European wildcats (Pierpaoli *et al.* 2003) were confirmed as admixed, showing qi from 0,470 to 0,655 (90% CI = 0.420 - 0.702) to the wildcat cluster (see Table 3). Moreover, they were mostly assigned to their known hybrid category: Six individuals from the southern Apennines ($n = 1$) and the northern-eastern Alps ($n = 5$) as F1, four captive-breed individuals as BxFSI (ID 57), BxFSI/F2 (ID 60, ID 62) and F2 (ID 63), see Table 3 and Figure 2. Six of the 19 putative admixed European wildcats (Mattucci *et al.* 2013; Oliveira *et al.* in prep) analyzed in this study showed individual q_W ranging from 0,525 to 0,751: two from Italy (central Apennines and Maremma), one from Luxembourg, one from Bosnia & Herzegovina and two from Portugal. Moreover, they were clearly classified as BxFSI (ID 269, ID 1323, ID 586, ID 1056) and F2 (ID 700, ID 712) with high posterior probabilities ($qi > 0.99$). However, the remain 13 putative admixed European wildcats partially showed q_i values to the domestic cluster ranging from 0.928 to 0.978 (CI 0.898 - 0.993) for three individuals each from SW Germany, Portugal and Spain and mainly were assigned to the wildcat cluster with an individual q_i value ranging between 0,800 – 0,976 (CI 0.759 - 0.991) for ten individuals from Italy (central Apennines and Maremma, $n = 4$; eastern Alps, $n = 1$; southern

Apennines, n = 4) and SW Germany (n = 1), see Table 3. NEWHYBRIDS' clustering proved to be highly efficient to correctly allocated 10 of the 13 previously misclassified putative admixed cats to their parental category (qi > 0,99); with the only exception of ID 1518 (from Italian central Apennines) and ID 1629 (from the SW Germany) classified as BxFSI (qi > 0,99) and ID 1248 (from southern Apennines) partially classified as pure wildcat (FSI) and BxFSI (0,695 < qi < 0,979), see Figure 2.

Dataset	ID Code	Populations	STRUCTURE		NEWHYBRIDS					
			Q_d	Q_w	Domestic	Wild	F1	F2	Bx I	Bx II
Domestic cats			0.957	0.043	1.000					
Felis silvestris catus n = 44			(0.933-0.977)	(0.023-0.066)						
European wildcats			0.015	0.985		1.000				
Felis silvestris silvestris n = 89			(0.035-0.083)	(0.916-0.964)						
Known admixed cats	57	Captivity (Italy)	0.345	0.655						1.000
Felis silvestris x catus n = 10			(0.298-0.394)	(0.606-0.702)						
	60	Captivity (Italy)	0.381	0.619				0.341		0.659
			(0.334-0.430)	(0.570-0.666)						
	62	Captivity (Italy)	0.373	0.627				0.220		0.780
			(0.325-0.423)	(0.577-0.675)						
	63	Captivity (Italy)	0.417	0.583				0.989		0.011
			(0.368-0.467)	(0.533-0.632)						
	228	Italy:Southern Apennines	0.501	0.499			1.000			
			(0.447-0.554)	(0.446-0.553)						
	992	Italy: eastern Alps	0.496	0.504			1.000			
			(0.445-0.547)	(0.453-0.555)						
	993	Italy: eastern Alps	0.529	0.471			1.000			
			(0.479-0.579)	(0.421-0.521)						
	994	Italy: eastern Alps	0.515	0.485			1.000			
			(0.464-0.566)	(0.434-0.536)						
	995	Italy: eastern Alps	0.530	0.470			1.000			
			(0.479-0.580)	(0.420-0.521)						
	996	Italy: eastern Alps	0.507	0.493			1.000			
			(0.457-0.558)	(0.442-0.543)						

Putative admixed cats *Felis silvestris x catus n* = 19	67	Italy: Central Apennines and Maremma	0.144 (0.111-0.181)	0.856 (0.819-0.889)		0.994			0.006
	123	Italy: Central Apennines and Maremma	0.110 (0.080-0.143)	0.890 (0.857-0.920)		1.000			
	627	Italy: Central Apennines and Maremma	0.126 (0.092-0.164)	0.874 (0.836-0.908)		1.000			
	677	Italy: Central Apennines and Maremma	0.147 (0.112-0.184)	0.853 (0.816-0.888)		1.000			
	1269	Italy: Central Apennines and Maremma	0.249 (0.206-0.293)	0.751 (0.707-0.794)					1.000
	1323	Italy: Eastern Alps	0.258 (0.215-0.302)	0.742 (0.698-0.785)					1.000
	1518	Italy: Central Apennines and Maremma	0.180 (0.141-0.221)	0.820 (0.779-0.859)		0.021			0.979
	77	Italy:Southern Apennines	0.024 (0.009-0.043)	0.976 (0.957-0.991)		1.000			
	1248	Italy:Southern Apennines	0.164 (0.127-0.203)	0.836 (0.797-0.873)		0.695			0.305
	1250	Italy:Southern Apennines	0.129 (0.095-0.166)	0.871 (0.834-0.905)		1.000			
	1252	Italy:Southern Apennines	0.116 (0.085-0.150)	0.884 (0.850-0.915)		1.000			
	586	Luxembourg	0.293 (0.247-0.341)	0.707 (0.659-0.753)					1.000
	1056	Bosnia & Herzegovina	0.267 (0.223-0.313)	0.733 (0.687-0.777)					1.000
	421	Germany: Southern-western	0.978 (0.959-0.993)	0.022 (0.007-0.041)	1.000				
	629	Germany: Southern-western	0.200 (0.161-0.241)	0.800 (0.759-0.839)					1.000
	700	Portugal	0.475 (0.425-0.524)	0.525 (0.476-0.575)			1.000		

706	Portugal	0.971	0.029	1.000		
		(0.951-0.987)	(0.013-0.049)			
712	Portugal	0.460	0.540			1.000
		(0.411-0.510)	(0.490-0.589)			
717	Spain	0.928	0.072	1.000		
		(0.898-0.955)	(0.045-0.102)			

Table 3. Individual membership proportions (qi) and inferred ancestry of 16 putatively admixed cats according to the Bayesian analyses performed in STRUCTURE and NEWHYBRIDS. In STRUCTURE, individuals were assigned into two cluster corresponding to the domestic (Qd) and wild (Qw) groups excluding prior population information. In NEWHYBRIDS, individuals were assigned into six different genotypes classes: pure domestic and wild cat, F1, F2, Bx I (backcross with domestic cat) and Bx II (backcross with wildcat). STRUCTURE 90% credibility intervals (CI) are shown in brackets.

Mitochondrial DNA sequences

We aligned 669 bp (positions 13243 – 13911) of the mtDNA ND5 gene in 144 samples. The alignment did not showed indels or stop codons and the aminoacid sequence was concordant with the domestic cat ND5 protein (NCBI Reference Sequence NC001700). These findings suggest that we did not amplify nuclear copies (numts) of mtDNA ND5. The alignment showed 18 different haplotypes with 23 polymorphic sites and 20 parsimony informative sites. The CR analyses yielded 122 reliable sequences 604 bp (16302 – 16905) that included 48 haplotypes, characterized by 49 polymorphic sites, 40 parsimony informative sites and five indels. In 121 samples was possible to carry out a multi-fragment alignment of the two regions showing 54 haplotypes with a total of 60 polymorphic sites, 59 parsimony informative sites and five indels. Table 4 shows an overview on the key statistics of genetic variability.

The multimodal mismatch distribution (Supplementary Figure S1) of the three groups (wildcat, domestic cat and hybrid) as defined by SNPs analyses (cf. SNPs section) as well as the not significant Tajima's D and Fu and Li's F statistics approaching zero suggested a long term stability in the groups size history (Table 4).

The network (Figure 3) representing the relationships among the multi-alignment haplotypes (Supplementary Table S3), including two haplotypes (DL.1 and DL.2) carried exclusively by the eight *libyca* samples, showed two major groups clearly separated by 14 mutations, among which are included the seven diagnostic positions on ND5. The first group (hence called W) counted 24 haplotypes for a total of 55 individuals. The haplogroup appeared quite compact with one main haplotype represented by 15 samples and a mean of about two individuals for the remaining haplotypes. The second group (hence called D) showed a more complex structure comprehending three haplogroups (D1, D2, D3) for a total of 32 haplotypes and 73

	N	N° of haplotypes	Polimorphic sites	Parsimony informative sites	InDel	Nucleotide diversity π (± SD), percentage	Haplotype diversity h (± SD)	Tajima's D	FU & Li's F
ND5	144	18	23	20		0,909 (± 0,019)	0,867 (± 0,014)	-	-
CR	122	48	49	40	5	1,939 (± 0,064)	0,955 (± 0,008)	-	-
ND5-CR	121	54	60	59	5	1,400 (± 0,028)	0,965 (± 0,007)	-	-
Wildcat	79	34				1,150 (± 0,074)	0,940 (± 0,015)	0,663 (p>0,10)	0,609 (p>0,10)
Domestic	28	21				0,781 (± 0,056)	0,958 (± 0,014)	0,371 (p>0,10)	0,039 (p>0,10)
Admixed	14	9				1,245 (± 0,129)	0,859 (± 0,089)	0,852 (p>0,10)	0,481 (p>0,10)
W	55	24				0,255 (±0,148)	0,910 (± 0,029)	-1,401 (p=0,06)	-1,120 (p>0,10)
D1	18	11				0,278 (±0,165)	0,928 (± 0,040)	-1,113 (p>0,10)	-0798 (p>0,10)
D2	30	6				0,255 (± 0,150)	0,827 (± 0,026)	-0,293 (p>0,10)	-0,893 (p>0,10)
D3	18	13				0,169 (± 0,110)	0,928 (± 0,051)	-1,780 (p<0,05)	-1,647 (p=0,06)

Table 4. Genetic variability of multi-fragment alignments (ND5 and CR) observed within the populations as defined by SNPs analyses (wildcat, domestic, admixed) and within the haplogroups (W, D1, D2, D3) extrapolated from the phylogenetic analyses (excluding lybica samples).

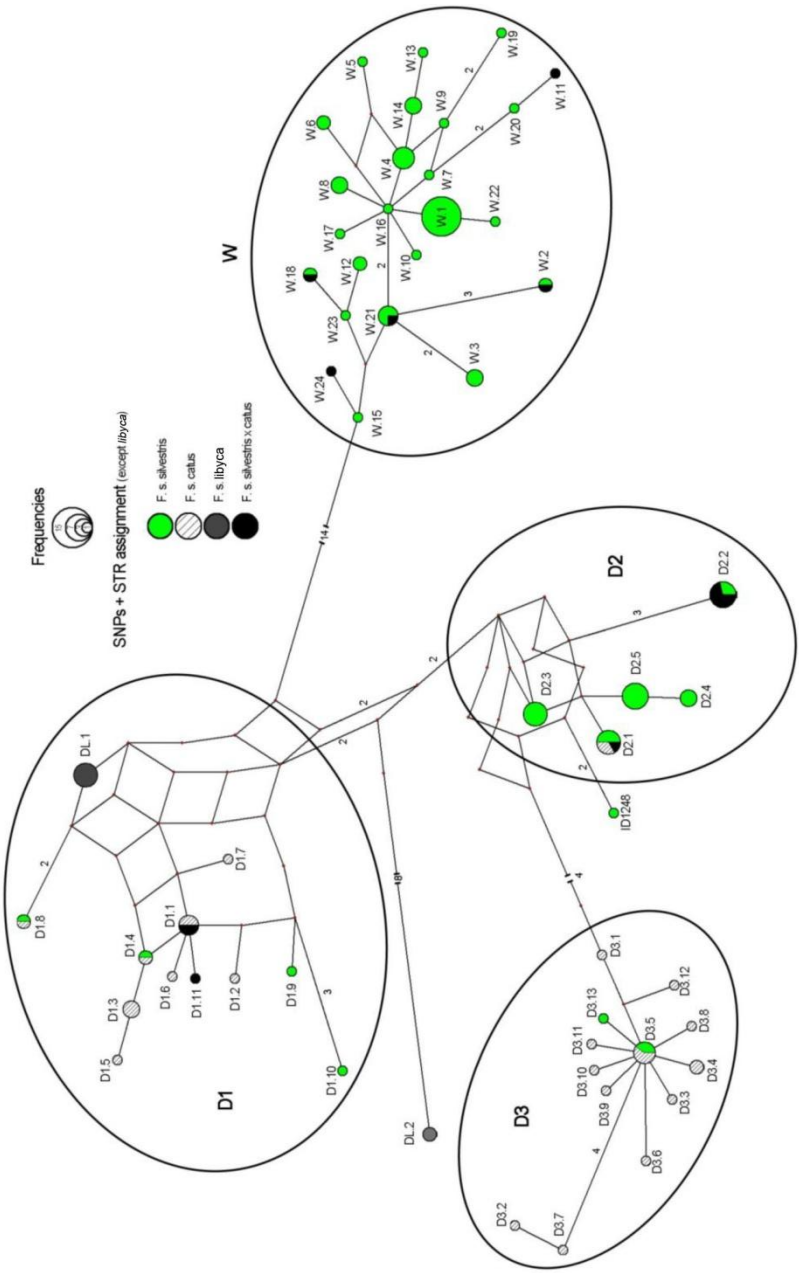

Figure 3. Median-joining network of the European wildcat (F.s.silvestris), Domestic cat (F.s.catus) the putative admixed individuals and the African wildcat (F.s.lybica) mtDNA haplotypes. The haplotypes are coloured according assignation based of nuclear markers (SNPs and STRs). The size of the circles is proportional to the haplotypes frequency.

individuals. Comparing this information with the data from nuclear markers, we found that 50 of the 55 individuals of group W were classified as pure wildcat and 5 as wild-domestic hybrids according to the STRUCTURE results (cf. SNPs section). None of the 28 domestic cat clustered within this group whereas they all fell in the group D where we found also a relatively high number of putative wildcat individuals. Specifically in the haplogroup D1, composed by 12 haplotypes, clustered 11 domestic cats, four wildcats, three hybrids and six *libyca*. The haplogorup D2 (five haplotypes) was characterized by a massive presence of wildcat (21), six hybrids and only two domestic cats. One more haplotype, carried only by one individual (ID 1248, from Italian southern Apennines), resulted quite isolated from this group. The haplogroup D3 presented a more homogeneous star-like structure with 13 haplotypes including 15

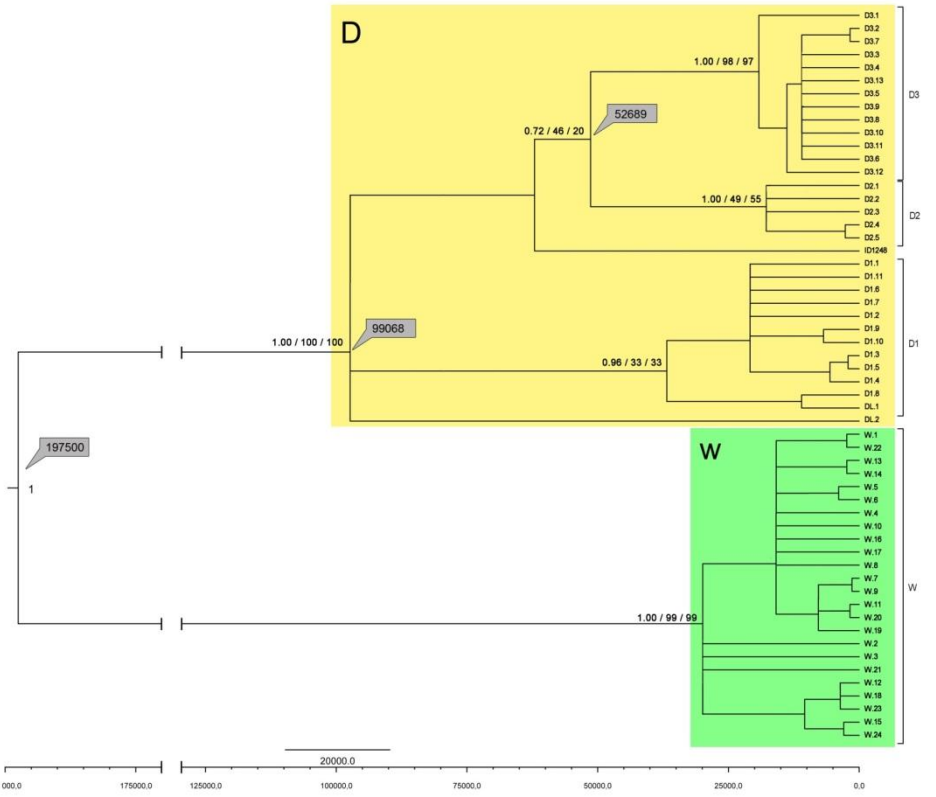

Figure 4. Phylogenetic tree of the of the European wildcat (F.s.silvestris), Domestic cat (F.s.catus) the putative admixed individuals and the African wildcat (F.s.libyca) mtDNA haplotypes. For the principal nodes are provided respectively the high posterior densities of the Bayesian trees, and the bootstrap values of neighbour joining and maximum likelihood trees (HPD/NJ/ML). In the dark grey boxes are given the ages of the nodes.

domestic cat and three wildcats (Table S3). Lastly the haplotype DL.2, represented by the last two *libyca* samples, resulted well separated from all three described haplogroups. A network resulting only from the ND5 sequences (Supplementary Figure S2) showed an identical assignation for the principal two haplogorups (W and D). Considering the multi-alignment haplogroups the mismatch distributions and the Tajima's D and the Fu and Li's F did not reveal any relevant population dynamic except for haplogroups W and D3 which showed near significant negative values of parameters (Table 4) and bell-shaped curves suggesting slight positive trends (Figure Sl). The network structure was concordant with the topology of the phylogenetic tree (Figure 4) where the principal groups were supported using the three clustering method (NJ, ML and Bayesian). The tree presented a clear bisection between the W haplogroups and the more heterogeneous D. Furthermore, all the subsequent clades corresponded with the network reconstruction.

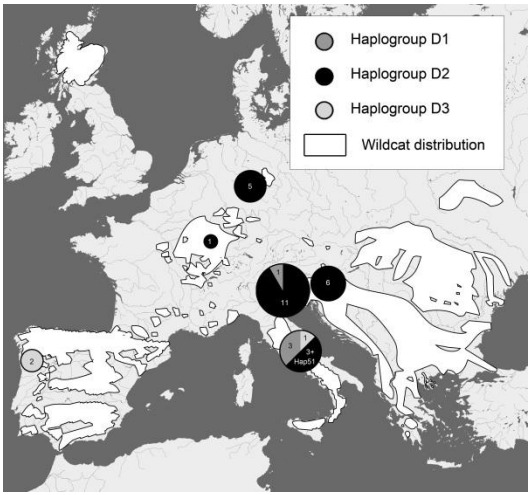

Figure 5. Geographical distribution for the principal macro-areas of the haplogroups that presented haplotypes shared between wildcat and domestic cat. The size of the circles is proportional to the number of samples. The numbers inside the slices show the frequency for each haplogroup.

Regarding the geographic distribution of the shared haplotypes (Figure 5) we note in particular how the haplogroup D3 presented a high frequency in eastern Italy/Slovenia area progressively decreasing in central Europe and Italy. Assuming as the calibration point the interval between 173 000 and 230 000 years BP in which fell the divergence node between the *F. s. silvestris* and *F.s.catus/libyca* clades (Driscoll et al 2007) we estimated different periods of differentiation especially in the group D where resulted particularly useful to clarify the presence of haplotypes belonging to wildcat individuals. The differentiation of three principal clades of haplogroup D (D1, D2-D3, DL.2) began 0,099 Mya (95% high posterior density, HPD, 0,056 – 0,153 Mya). This datum is in accordance with the hypothesis of a multi-matrilineal coalescence of today domestic clades deriving from at least five different *Felis s. libyca* lineages whose common ancestor was dated back more than 100 000 years ago (Driscoll et al 2007). The other important node was the one separating groups D2 and D3 that fell about 0,053 Mya (95% HPD 0,026 – 0,088 Mya), much before the age suggested by any archaeological evidence for cat

domestication (Vigne et al 2004; Hu *et al.* 2014). This could suggests a first earliest important transmission/sharing of haplotypes between *libyca* and *silvestris* populations rather than a massive introgression after domestication process. Nevertheless the presence of several less frequent haplotypes featured by wildcat individuals originated nearby domestication period (D1.8, D1.9; D1.3; D1.7; D3.13; D3.15) seems to confirm a certain degree of introgression. This was also supported by IMa analyses of migration that reported very low global migration rates in wildcat – domestic cat direction (0,013; 90% HPD 0,001 – 0,255) and a domestic cat – wildcat migration rate an order of magnitude greater (0,213; 90% HPD 0,07 – 0,521) (Figure 6).

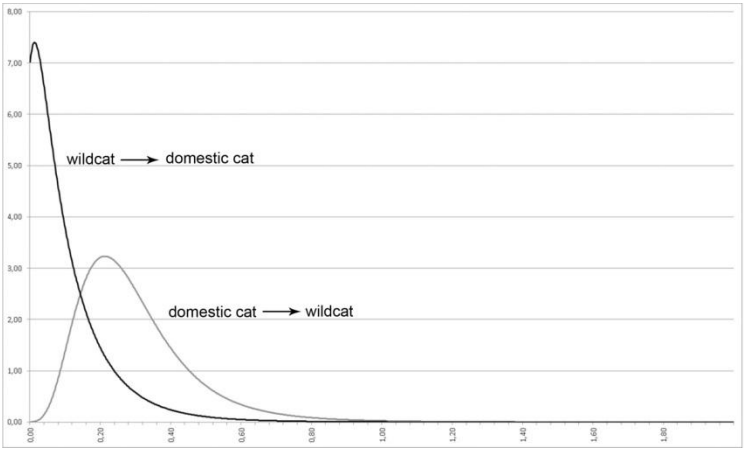

Figure 6. Probability distribution for the total migration rates between wildcat (F.s.silvestris) and domestic cat (F.s.catus) extrapolated by the sofwtare IMa using the total individuals for each population.

The comparison between AMOVA analyses among the groups defined using the SNPs (wildcat, domestic cat and hybrids) and among mtDNA haplogroups (W, D1, D2, D3) showed sharp differences. In the first analysis most of the variation was explained within populations (62,65%) while considering the haplogroups the variation among populations was 87,42% (Table 5).

Source of variation	d.f	Variance components	Percentage of variance	P
Among populations	2	4,163	37,35	<0.001
Within populations	118	6.984	62,65	<0.001
Among haplogroups	3	12,640	87,68	<0.001
Within haplogroups	117	1,776	12,32	<0.001

Table 5. Analysis of molecular variance among/within the three putative populations (wildcat, domestic and admixed) and among/within the four principal haplogroups inferred from the phylogenetic analyses

Y chromosome genotyping

We successfully genotyped 86 males that yielded reliable genotypes for both the SRY and microsatellite Y-linked markers. Sequences of the SRY gene showed the expected polymorphic site with two alleles (A and G) at position 1956. The microsatellite showed the expected two allele respectively of 271bp and 273 bp. In all the amplified samples the two markers showed coherent genotypes. 1) allele A of the SRY gene was always associated to the microsatellite allele 271; this haplotype was found in all the 43 samples except two: 123 and 495 (henceforth named "haplotype WY"). Sample 123, from central Italy, clustered in the haplogroup D3 and was previously identified as an admixed individual in the analysis of microsatellites markers (qw = 0,904 C.I. = 0,728-1,000) and afterwards was re-assigned as wildcat by using SNPs (qw = 0,922; C.I. = 0,886-0,954). The sample 495, from eastern Italy, was identified as wildcat consistently by all markers ($qw_{str+snps}$ = 0,979 C.I. = 0,925-0,983). 2) The allele G of the SRY gene was always associated to the microsatellite allele 273. This haplotype (henceforth named "haplotype DY") was found in 26 over 28 of the genotypes that were genetically identified as domestic cats. Two presumed domestic cats (1011 and 1331) showed haplotype WY. Assignation of these samples to the domestic subspecies was concordant in all autosomal markers (1011 $qw_{str+snps}$ = 0,006 C.I. = 0,000-0,038; 1331 $qw_{str+snps}$ = 0,067 C.I. = 0,067-0,140). Among the 15 hybrid males there were eight WY and seven DY (Table 6).

		Haplotype DY	Haplotype WY
SNPs assignment	**Domesitc**	26	2
	Wildcat	2	41
	Admixed	7	8
	Tot	35	51

Table 6. Chromosome Y haplotypes based on SMCY-7 STR and SRY gene SNP and relative frequencies in the putative populations

DISCUSSION

We described here a multi-locus protocol for admixture detection, to improve the reliability of hybrids identification and deeply investigate ancient introgressive events. Thus, 151 most informative SNPs (with average F_{ST} varying between 0,797 and 0,982 in European subpopulations) were selected from the Illumina Infinium iSelect 63K Cat DNA Array, and combined with two pairs of uniparental markers, showing distinctive diagnostic mutations for wild and domestic cats. In particular, the ND5 region and part of the control region (Tiedemann *et al.* 1996; Freeman *et al.* 2001; Driscoll *et al.* 2007) for maternal lineage and Y-linked markers (Pecon-Slattery *et al.* 2004; King *et al.* 2007; Luo *et al.* 2007; Nussberger *et al.* 2013) for paternal lineage, helped to provide a complete overview of the hybridization in European wildcat populations.

To assess the diagnostic power of markers, it appeared crucial choose the right reference, avoiding any hybrid or introgressed individuals, and ensuring the representativeness of the genetic diversity of the parental populations. Although this last factor might influence the correct allocation of backcrossed individuals through Bayesian algorithms (Falush *et al.* 2003), markers power has been assessed on a reduced sample set (n = 187), compared to recent STRs study across Europe (n = 1114; Mattucci *et al.* in prep), that however provided a representation of the five macro populations of wildcat detected with 38 STRs in Mattucci *et al.* (2014).

Comparison in the assignment efficiency (SNPs and STRs)

SNPs were ascertained from a 63K cat array, which has been designed on ~9.55 million SNPs from the three combined genome sequencing efforts and subsequently has been tested on 12 different breeds, 10 wildcats, 10 western and 10 eastern random bred cats, in addiction to five trios, the Abyssinian (Cinnamon) and the 6 cats from the Hill's SNP discovery project. Thus, the selected SNPs would be expected to be generally diverse amongst random bred cats compared to wildcats. But genetic diversity, including *Ar* and H_E, proved to be significantly high both in domestic and wildcat group, showing similar mean values, most likely as a result of the heterogeneous sampling planned by taking into account the genetic-geographic repartition of wildcats in Europe. Generally, genetic variability is expected to be lower in domesticated forms relatively to their wild counterparts, due to the domestication bottleneck caused by the low number of founder individuals and the restricted gene flow imposed by human constrains (Doebley *et al.* 2006). However, the progenitor of the domestic cat is considered the *Felis s. libyca* subspecies (Driscoll *et al.* 2007; Lipinski *et al.* 2008), thus a direct comparison between the proposed wild progenitor and domesticate cannot be evaluated in this study.

The selected panel of 151 most informative SNPs proved to be efficient in identifying 100% of parental genotypes and first-second generation hybrids with a posterior probability > 0,80, but none of third-fourth generation hybrids. Increasing the posterior probability to > 0,95, according to Nussemberg *et al.* (2013), whose informative SNPs panel identified over 86% of all hybrids, we correctly identified 100% of all simulated individuals (including both parental and different generation hybrids) but only 90% of empiric domestic cats and 66% of empiric wildcats. Thus, we chose an assignment threshold of 0,80 less stringent but more discriminant than previous studies (Nussemberg *et al.* 2013; Oliveira *et al.* in prep), allowing the correct identification of all sampled parental cats and all firs-second generation introgressed cats.

Dataset	Individual Code	Populations	151 SNPs		31 STRs	
			Q_d	Q_w	Q_d	Q_w
Known admixed cats	ID 57	Captivity (Italy)	0.345	0.655	0.454	0.546
Felis silvestris x catus n = 10			(0.298-0.394)	(0.606-0.702)	(0.303-0.609)	(0.391-0.697)
	ID 60	Captivity (Italy)	0.381	0.619	0.479	0.521
			(0.334-0.430)	(0.570-0.666)	(0.320-0.644)	(0.356-0.680)
	ID 62	Captivity (Italy)	0.373	0.627	0.485	0.515
			(0.325-0.423)	(0.577-0.675)	(0.324-0.649)	(0.351-0.676)
	ID 63	Captivity (Italy)	0.417	0.583	0.490	0.510
			(0.368-0.467)	(0.533-0.632)	(0.326-0.657)	(0.343-0.674)
	ID 228	Italy:Southern Apennines	0.501	0.499	0.679	0.321
			(0.447-0.554)	(0.446-0.553)	(0.533-0.815)	(0.185-0.467)
	ID 992	Italy: eastern Alps	0.496	0.504	0.493	0.507
			(0.445-0.547)	(0.453-0.555)	(0.342-0.647)	(0.353-0.658)
	ID 993	Italy: eastern Alps	0.529	0.471	0.575	0.425
			(0.479-0.579)	(0.421-0.521)	(0.416-0.730)	(0.270-0.584)
	ID 994	Italy: eastern Alps	0.515	0.485	0.575	0.425
			(0.464-0.566)	(0.434-0.536)	(0.416-0.729)	(0.271-0.584)
	ID 995	Italy: eastern Alps	0.530	0.470	0.546	0.454
			(0.479-0.580)	(0.420-0.521)	(0.388-0.701)	(0.299-0.612)
	ID 996	Italy: eastern Alps	0.507	0.493	0.500	0.500
			(0.457-0.558)	(0.442-0.543)	(0.345-0.656)	(0.344-0.655)

Putative admixed cats *Felis silvestris x catus* n = 19	ID 67	Italy: Central Apennines and Maremma	**0.144**	**0.856**	**0.238**	**0.762**
			(0.111-0.181)	(0.819-0.889)	(0.111-0.380)	(0.620-0.889)
	ID 123	Italy: Central Apennines and Maremma	0.110	0.890	0.127	0.873
			(0.080-0.143)	(0.857-0.920)	(0.000-0.281)	(0.719-1.000)
	ID 627	Italy: Central Apennines and Maremma	**0.126**	**0.874**	**0.344**	**0.656**
			(0.092-0.164)	(0.836-0.908)	(0.171-0.522)	(0.478-0.829)
	ID 677	Italy: Central Apennines and Maremma	**0.147**	**0.853**	**0.214**	**0.786**
			(0.112-0.184)	(0.816-0.888)	(0.078-0.370)	(0.630-0.922)
	ID 1269	Italy: Central Apennines and Maremma	0.249	0.751	0.342	0.658
			(0.206-0.293)	0.707-0.794)	(0.200-0.495)	(0.505-0.800)
	ID 1323	Italy: eastern Alps	0.258	0.742	0.246	0.754
			(0.215-0.302)	(0.698-0.785)	(0.123-0.385)	(0.615-0.877)
	ID 1518	Italy: Central Apennines and Maremma	0.180	0.820	0.533	0.467
			(0.141-0.221)	(0.779-0.859)	(0.351-0.714)	(0.286-0.649)
	ID 77	Italy:Southern Apennines	0.024	0.976	0.156	0.844
			(0.009-0.043)	(0.957-0.991)	(0.036-0.297)	(0.703-0.964)
	ID 1248	Italy:Southern Apennines	0.164	0.836	0.285	0.715
			(0.127-0.203)	(0.797-0.873)	(0.144-0.436)	(0.564-0.856)
	ID 1250	Italy:Southern Apennines	**0.129**	**0.871**	**0.255**	**0.745**
			(0.095-0.166)	(0.834-0.905)	(0.120-0.408)	(0.592-0.880)
	ID 1252	Italy:Southern Apennines	**0.116**	**0.884**	**0.361**	**0.639**
			(0.085-0.150)	(0.850-0.915)	(0.217-0.517)	(0.483-0.783)
	ID 586	Luxembourg	**0.293**	**0.707**	**0.154**	**0.846**
			(0.247-0.341)	(0.659-0.753)	(0.003-0.306)	(0.694-0.997)
	ID 1056	Bosnia & Herzegovina	0.267	0.733	0.399	0.601
			(0.223-0.313)	(0.687-0.777)	(0.232-0.571)	(0.429-0.768)
	ID 421	Germany: Southern-western	0.978	0.022	0.829	0.171
			(0.959-0.993)	(0.007-0.041)	(0.639-1.000)	(0.000-0.361)
	ID 629	Germany: Southern-western	0.200	0.800	0.417	0.583
			(0.161-0.241)	(0.759-0.839)	(0.263-0.573)	(0.427-0.737)
	ID 700	Portugal	0.475	0.525	0.404	0.596
			(0.425-0.524)	(0.476-0.575)	(0.243-0.570)	(0.430-0.757)

ID 706	Portugal	0.971	0.029	0.593	0.407
		(0.951-0.987)	(0.013-0.049)	(0.430-0.750)	(0.250-0.570)
ID 712	Portugal	0.460	0.540	0.643	0.357
		(0.411-0.510)	(0.490-0.589)	(0.467-0.813)	(0.187-0.533)
ID 717	Spain	0.928	0.072	0.675	0.325
		(0.898-0.955)	(0.045-0.102)	(0.515-0.822)	(0.178-0.485)

Table 7 Individual membership proportions (qi) of known and putatively admixed cats according to the Bayesian analyses performed in STRUCTURE with admixture and correlated allele frequencies model, excluding prior population information (POP = 0), and by using 151 most informative SNPs and 31 STRs (Mattucci *et al.* 2013; Mattucci *et al.* in prep). STRUCTURE qi values correspond to allocations with K=2 to the domestic (Qd) and wild (Qw) inferred clusters. with their 90% credibility intervals (CI). Cats resulting with both markers admixed are shaded in light grey.

By comparing Bayesian admixture outcomes for known and putative hybrids, using highly informative SNPs and STRs (see Table 7), it has been possible evaluate the performance of both markers in hybrid's detection. Ten known hybrids were clearly identified as admixed by both markers with *qi* to the wildcat cluster ranging from 0,470 – 0,655 (90% CI = 0,420 – 0,702) for SNPs to 0,321 – 0,546 (90% CI = 0,185 – 0,697) for STRs (see Table 7). Although the evidence of comparable *qi* assignment, the 90% CI proved to be wider for STRs than for SNPs.

On the contrary, differences in markers performance were shown in putative admixed identification. The admixture ancestry of the 19 putative hybrids, has been, in fact, confirmed by both markers, in six individuals, namely: two individuals from Italy (respectively ID 1269 from central Apennines; ID 1323 from *Eastern* Alps), two from Portugal (ID 700; ID 712), one from Bosnia & Herzegovina (ID 1056), and one individual from Luxembourg (ID 586), whose previously assignment with STRs (Mattucci *et al.* in prep) showed the lower value of Credibility Intervals to the wildcats cluster of 0,694 (namely under the assignment threshold). However, the range of their membership probabilities to the wildcat cluster and related CI resulted to be extremely narrow with SNPs when compared to STRs values (respectively 0,525 – 0,742 with 90% CI = 0,476 – 0,794, and 0,357 – 0,846 with 90% CI = 0,187 – 0,997). three further individuals might be considered as admixed because, even if their assignment probabilities with SNPs are ranging from 0,800 to 0,836, the lower value of Credibility Intervals resulted to be under the assignment threshold (ranging from 0.,759 to 0,797). On the contrary, 10 individuals previously misclassified as putative hybrids based on STRs assignment, using the panel of most informative SNPs were identified as 'pure wildcat' from central-southern Apennines and Maremma (ID 67, ID 627, ID 677, ID 77, ID 123, ID 1250, ID 1252), and as 'pure domestic cats' from southern-western Germany (ID 421), Portugal (ID 706), and Spain (ID 717), see Table 7.

The uncertain assignment of admixture individuals revealed with 31 STRs has been overcame with SNPs, that proved to be more reliable than STRs, providing

narrow IC ranges and individual membership probabilities clearly partitioned between wild and domestic clusters, even if the reference cats and the putative hybrids belonging to different geographic (and possibly genetic) populations. Most likely, the high allelic richness in combination with homoplasy might have reduced the microsatellites diagnostic power for hybrid recognition, since there are more possibilities of allele sharing between two hybridizing taxa. Thus, microsatellites appeared to be more suited to recognize genetic population structure (Guichoux *et al.* 2011) than identify introgression events. Recent studies have already demonstrated the SNPs potentiality to equal or even outperform microsatellites not only for individual ancestry (Lao *et al.* 2008), but also for population assignment (e.g. Seddon *et al.* 2005; Narum *et al.* 2008; Smith and Seeb 2008; Coates *et al.* 2009) and pedigree studies (Santure *et al.* 2010, Hauser *et al.* 2011), and proved to have large allele frequency differences among populations (Freamo *et al.* 2011).

Moreover, contrary to Nussemberg *et al.* (2013) recent outcomes, that limit the power of their SNPs panel on a regional level (specially beyond the Swiss borders) while encouraging their use in a wider sample, our set of 151 highly informative SNPs proved to be surely efficient and applicable with all genetic populations of cats disclosed in Europe (Mattucci *et al.* in prep), since different genetic-geographic reference cats has already been tested for ancestry detection analyses.

The repeatedly crossbreeding occurred in the past with both parental groups and admixed individuals (Bewick 1807; Driscoll *et al.* 2009; Driscoll *et al.* 2007; Suminski 1962) and the fertility of hybrids, might have diluted the proportion of domestic alleles through the generations into the wildcat gene pool, leaving traces only in non-recombining mtDNA or Y Chromosome regions. Thus, the uniparental markers might allow a further detection of hybridization by crossing maternal and paternal diagnostic polymorphism between domestic and wild cats with SNPs Bayesian assignments (Table 8). Driscoll *et al.* (2011) recently proposed a set of mitochondrial markers present on a portion of 2604 bp including ND5, ND6 and the Cytb that should ensure an effective distinction between domestic and wildcats. In this study we selected the coding portion of the gene ND5 which presented the highest density of such polymorphisms (seven) to which we added a portion of the control region (Freeman *et al.* 2001).

Uniparental markers

Both mitochondrial sequenced regions (ND5 and part of the control region), clearly split the subspecies in two well distinct haplogroups (D and W) separated by the seven diagnostic mutation of ND5 plus further seven mutations present on the control region. While the haplogroup W was pretty compact and composed only by previously assigned wildcat and admixed individuals, fully confirming the reliability of these polymorphisms in the mitochondrial characterization of the wildcat

populations, the haplogroup D, that hence was expected to contain haplotypes only present in domestic and admixed individuals, was much more differentiated and showed at least three main sub-haplogroups (D1, D2 and D3) in which were detected several wildcat individuals. In particular, the haplogroup D2 showed to be present with a high frequency in wildcat individuals and with low frequency in domestic cats. The presence of these sharing haplotypes opened the way to two different hypotheses. In one hand it might be possible that, as a consequence of introgressive events occurred in the past, most likely between a domestic female and a wild male, a domestic signature entered in the wild mitochondrial region through these common haplotypes as there is no evidence for the translocation and release to the European wild of wild-type *F. s. libyca* (Driscoll et al 2011). On the other hand, it also might be possible that these haplotypes were already present in a common ancestor of the two subspecies, much earlier domestication, and we now found them shared between the two subspecies, meaning that the markers selected in this work are not reliable and diagnostic to distinguish wild from domestic cats and to investigate on introgressive episodes.

Bayesian phylogenetic reconstructions, along with distance-based and maximum-likelihood methods, confirmed the network arrangement providing information about the ancestry of the haplogroup of interest. Besides confirming a multiple matrilineage ancestry dating back to about 100,000 years ago (Driscoll et al 2007), it gave a time estimate of the separation between haplogroups D2 and D3. This node goes back to about 53,000 years ago, long time before any documented domestication process took place (Vigne et al 2004, Hu *et al.* 2014). The fact that after this date haplogroup D2 has started a process of isolation the result of which is visible today (considering our sample) mostly in wild individuals (see Table S3) suggests that before this event might be occurred one or more events of genetic transmission/sharing among ancient populations of African (*libyca*) and European (*silvestris*) wildcat. This hypothesis is consistent with the considerations of Macdonald *et al.* (2010) assuming different step migration from European regions towards areas of the Near East during the late Pleistocene, probably affected by glacial-interglacial cycles (Kurtén 1965a,b, Macdonald 2010). The geographic distribution of haplogroup D2 (Figure 5) appears to show a core in the south-central region of Europe. Whereas in our sample are not present an adequate number of individuals coming from the eastern part of the distribution area, such data are not however in contradiction with the possible return from the south-eastern regions of a hypothetical population carrier of these haplotypes. It is therefore crucial to expand the dataset including a representative sample of this conjunction area. The migration rates resulted from the IMa analyses and the presence of several less frequent haplotypes featured by wildcat individuals originated nearby domestication period (D1.8, D1.9; D1.3; D1.7; D3.13; D3.15) seem to confirm, however, what might actually be the remnant of a most-recent introgression event. Although not in a highly significant (Table 4, Figure S1) values Tajima'sD and FU &

LI's F, along with the mismatch distributions, suggest a stable trend of haplogorup D2 and the presence of expansion for the other groups (including W). This datum is likely, given the historical and recent spreading of domestic cats population.

Dataset	ID Code	Populations	STRUCTURE assignemnt with 155 SNPs		mtDNA	Y Chr
			Q_d	Q_w	ND5+CR	
Known admixed cats	ID 57	Captivity (Italy)	0.345	0.655	D2.1	W
Felis silvestris x catus n = 10			(0.298-0.394)	(0.606-0.702)		
	ID 60	Captivity (Italy)	0.381	0.619	D1.1	W
			(0.334-0.430)	(0.570-0.666)		
	ID 62	Captivity (Italy)	0.373	0.627	D1.11	W
			(0.325-0.423)	(0.577-0.675)		
	ID 63	Captivity (Italy)	0.417	0.583	D1.1	D
			(0.368-0.467)	(0.533-0.632)		
	ID 228	Italy:Southern Apennines	0.501	0.499	W.2	D
			(0.447-0.554)	(0.446-0.553)		
	ID 992	Italy: eastern Alps	0.496	0.504	D2.2	D
			(0.445-0.547)	(0.453-0.555)		
	ID 993	Italy: eastern Alps	0.529	0.471	D2.2	D
			(0.479-0.579)	(0.421-0.521)		
	ID 994	Italy: eastern Alps	0.515	0.485	D2.2	D
			(0.464-0.566)	(0.434-0.536)		
	ID 995	Italy: eastern Alps	0.530	0.470	D2.2	D
			(0.479-0.580)	(0.420-0.521)		
	ID 996	Italy: eastern Alps	0.507	0.493	D2.2	D
			(0.457-0.558)	(0.442-0.543)		
Putative admixed cats	ID 67	Italy: Central Apennines and Maremma	0.144	0.856	W.21	F
Felis silvestris x catus n = 19			(0.111-0.181)	(0.819-0.889)		
	ID 123	Italy: Central Apennines and Maremma	0.110	0.890	D3.13	D
			(0.080-0.143)	(0.857-0.920)		
	ID 627	Italy: Central Apennines and Maremma	0.126	0.874	W.23	F
			(0.092-0.164)	(0.836-0.908)		
	ID 677	Italy: Central Apennines and Maremma	0.147	0.853	W.21	F
			(0.112-0.184)	(0.816-0.888)		
	ID 1269	Italy: Central Apennines and Maremma	0.249	0.751	W.18	W
			(0.206-0.293)	0.707-0.794)		
	ID	Italy: *Eastern* Alps	0.258	0.742	W-ND5	-

1323		(0.215-0.302)	(0.698-0.785)		
ID 1518	Italy: Central Apennines and Maremma	0.180 (0.141-0.221)	0.820 (0.779-0.859)	W.21	W
ID 77	Italy:Southern Apennines	0.024 (0.009-0.043)	0.976 (0.957-0.991)	W-ND5	-
ID 1248	Italy:Southern Apennines	0.164 (0.127-0.203)	0.836 (0.797-0.873)	D.4	W
ID 1250	Italy:Southern Apennines	0.129 (0.095-0.166)	0.871 (0.834-0.905)	D1.9	W
ID 1252	Italy:Southern Apennines	0.116 (0.085-0.150)	0.884 (0.850-0.915)	D1.10	W
ID 586	Luxembourg	0.293 (0.247-0.341)	0.707 (0.659-0.753)	W.11	W
ID 1056	Bosnia & Herzegovina	0.267 (0.223-0.313)	0.733 (0.687-0.777)	D-ND5	F
ID 421	Germany: Southern-western	0.978 (0.959-0.993)	0.022 (0.007-0.041)	D-ND5	F
ID 629	Germany: Southern-western	0.200 (0.161-0.241)	0.800 (0.759-0.839)	D-ND5	-
ID 700	Portugal	0.475 (0.425-0.524)	0.525 (0.476-0.575)	-	-
ID 706	Portugal	0.971 (0.951-0.987)	0.029 (0.013-0.049)	W-ND5	-
ID 712	Portugal	0.460 (0.411-0.510)	0.540 (0.490-0.589)	W.24	W
ID 717	Spain	0.928 (0.898-0.955)	0.072 (0.045-0.102)	-	-

Table 8. Power to detect wild x domestic cat hybrids combining individual Bayesian assignment performed in STRUCTURE by using 151 most informative SNPs. and the haplotypes classification detected by using maternal (mtDNA) and paternal (Y Chromosome) diagnostic polymorphism between domestic (D) and wild (W) haplogroups.Individuals presenting only Nd5 reliable sequence are indicated with the homonymous suffix. Individuals needing further analyses to overcome the current lacking of uniparental informations despite the clear SNPs classification. are underlined.

Crossing nuclear and uniparental information

Both uniparental markers confirmed the admixture assignment, previously detected with nuclear SNPs and STRs, of all know hybrids. Four of them showed, in fact, a complementary wild and domestic uniparental assignment, while five of the

remains six reported the domestic patrilineal mutation and one of the suggested shared domestic haplotypes (D2.2). At last one individual (ID63) reported the haplotype D1, most likely resulting by a past introgressive event. Among the nine putative hybrids detected with high informative SNPs (see above), six individuals, whose admixture ancestry has been assessed with both nuclear markers (SNPs and STRs), evidenced wild haplotypes in both mitochondrial and chromosome Y, with the exception of samples lacking uniparental information (for Y ID 1323 and ID 1056 and for both markers ID 700), and ID 1056 from Bosnia and Herzegovina that showed the shared domestic mitochondrial haplotype. The remains 3 individuals, recognized as 'putative hybrids' with SNPs because of their wide CI range, presented wild haplotype for both markers (for ID 1518) and complementary haplotypes (for ID 1248), while the individual ID 629 showed only the shared domestic mitochondrial haplotype.

For what concern the individuals re-classified as 'pure parental' with SNPs (n = 10), we found congruencies between SNPs and mitochondrial identification only in 5 cats but completely lacking the paternal information, one individual missed both uniparental assignment (ID 717), while 3 showed incongruences between SNPs and mitochondrial identification (namely nuclear genotype wild and shared domestic mitochondrial haplotype), see Table 8.

The integration of both uniparental and nuclear markers assignments, provided a complete insight of introgression level in wildcats populations analyzed (see Table 8). Finally, a total of 11 hybrids were identified among the 19 'putative' hybrids of which: 8 were detected by both nuclear (STRs and SNPs) and uniparental (mtDNA and Y) markers, and 3 were identified because of the presence of nuclear genotype wild and shared domestic mitochondrial haplotype (ID 123, ID 1250, ID 1252). Because of the history of domestic/wildcat introgression (Bewick 1807; Driscoll *et al.* 2009; Driscoll *et al.* 2007; Suminski 1962), individuals showing only STR and SNP data have been considered as incomplete for admixture analysis. Thus, the individual ID 700 from Portugal, clearly identified with both SNPs and STRs markers as admixed, has not been considered in the final hybrids count (n = 11), being lacking of uniparental information, and should be further analyzed improving the markers performance.

CONCLUSIONS

Hence, the uniparental markers analyzed proved to be extremely efficient in subspecies identification, providing matrilineal and patrilineal signature of potentially distant admixture events, once crossed with nuclear (SNPs or STRs) assignment information. The insights provided by the time estimates on the possible origin of shared haplotypes gave an important contribution to the correct use of uniparental mitochondrial markers. It has been demonstrated that one should be very careful in defining introgressed an individual who bring a domestic mitochondrial haplotype, as

we shown how this can be not only a trace of a distant hybridization event, but also a testimony of the evolutionary history of the species. To clarify this aspect is undoubtedly necessary further research on a larger and more representative sample and try to define reliable haplogroups references for a proper assignment.

To ensure a finely detection of cryptic hybrids in wildcat populations and assess the introgression degree of highly compromised populations of Scotland and Hungary (Beaumont *et al.* 2001), it should be needed extend the admixture analyses, by using this combined and useful multi locus panel, and increase the representativeness of cats genetic diversity in Europe, by implementing samples from each macro and sub populations detected by Mattucci *et al.* (2014).

The new throughput technologies under development for domestic cats will soon allow the evaluation of the entire genome of *F. silvestris* species, supporting the identification of more diagnostic loci and potentially indicating areas of the genome involved with domestication. Other phenotypic polymorphisms, such as melanism at the *Agouti* locus (*ASIP*) (Eizirik *et al.* 2003), would likely be an important diagnostic for domestic cat introgression into wildcats might be equally highly informative. Thus, combined repertoires of highly informative autosomal SNPs, X and Y-linked markers and mtDNA variants promise to involve a deep investigation of cat domestication effects on wildcats populations.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Description of 151 most informative SNP loci used in this study, and values of single locus genetic diversity in the genotyped domestic cats (Fca; N = 44) and European wildcats (Fsi; N = 89) : SNP: access number of each SNP. Chr: chromosome number in the domestic cat karyotype (Pontius *et al.* 2007); absolute position bp (Mullikin *et al.* 2010) AR: allelic richness; HE: expected heterozygosity; FIS: Inbreeding coefficient; pw-FCT : pairwise estimate of genetic differentiation between European wild and domestic cats (computed as Fct in AMOVA; Arlequin.); IN: informativeness for assignment index (computed in INFOCALC) .

SNP	Chr	Position	A_R		H_E		F_{IS}		pw F_{CT}	I_N
			Fca	Fsi	Fca	Fsi	Fca	Fsi		
chrC1.124364347	C1	124364347	1,814	1,972	0.023	0.014	mono	mono	0.982	0.544
chrD2.28093358	D2	28093358	1,967	2,000	0.045	0.028	-0.012	-0.007	0.964	0.544
chrC1.120057704	C1	120057704	1,000	2,000	0.000	0.061	mono	0.386	0.959	0.436
chrD1.156500121	D1	156500121	2,000	1,992	0.082	0.034	-0.030	-0.012	0.949	0.484
chrUn.34671542	UN	34671542	1,000	2,000	0.000	0.078	mono	0.311	0.947	0.459
chrA1.154897381	A1	154897381	1,795	2,000	0.022	0.066	mono	0.315	0.946	0.457
chrC1.243444286	C1	243444286	1,795	2,000	0.022	0.087	mono	0.481	0.931	0.407
chrA1.67830943	A1	67830943	2,000	2,000	0.069	0.064	-0.025	0.385	0.929	0.587
chrD3.66450925	D3	66450925	1,995	2,000	0.067	0.066	-0.024	-0.030	0.928	0.455
chrB4.142126995	B4	142126995	2,000	1,992	0.133	0.034	-0.065	-0.012	0.926	0.465
chrUn1.10713841	UN1	10713841	1,000	2,000	0.000	0.107	mono	-0.055	0.925	0.440
chrB1.162220276	B1	162220276	1,993	2,000	0.066	0.072	-0.024	-0.033	0.924	0.439
chrE2.44868665	E2	44868665	1,974	2,000	0.046	0.086	-0.012	0.220	0.922	0.393
chrD2.101514624	D2	101514624	1,967	2,000	0.045	0.088	-0.012	0.219	0.921	0.543
chrC1.59406628	C1	59406628	2,000	1,787	0.187	0.011	-0.105	mono	0.920	0.510
chrB2.117030105	B2	117030105	1,999	2,000	0.089	0.064	0.485	0.385	0.920	0.002
chrUn31.884121	UN31	884121	1,999	2,000	0.087	0.067	-0.036	-0.030	0.920	0.414
chrC2.71556573	C2	71556573	1,999	2,000	0.091	0.065	-0.038	-0.029	0.919	0.423
chrA3.123183917	A3	123183917	1,999	2,000	0.087	0.068	0.485	-0.031	0.918	0.400
chrF1.24859656	F1	24859656	1,999	2,000	0.091	0.071	-0.038	-0.032	0.914	0.446
chrUn38.11625325	UN38	11625325	1,993	2,000	0.066	0.087	-0.024	-0.042	0.913	0.411
chrA1.166331725	A1	166331725	1,814	2,000	0.023	0.112	mono	-0.057	0.913	0.416
chrA1.31688322	A1	31688322	1,993	2,000	0.066	0.090	-0.024	-0.044	0.911	0.544
chrD4.36674221	D4	36674221	2,000	2,000	0.110	0.066	0.373	-0.030	0.911	0.477
chrUn5.2974540	UN5	2974540	1,000	2,000	0.000	0.127	mono	0.111	0.910	0.409
chrC1.236293313	C1	236293313	1,996	2,000	0.069	0.092	-0.025	0.479	0.908	0.418
chrA3.40321217	A3	40321217	1,960	2,000	0.044	0.107	-0.012	0.157	0.907	0.402
chrD4.41337045	D4	41337045	1,967	2,000	0.045	0.110	-0.012	0.156	0.905	0.426
chrF2.34671501	F2	34671501	1,995	2,000	0.067	0.100	-0.024	0.185	0.902	0.398
chrUn.59973692	UN	59973692	1,854	2,000	0.024	0.128	mono	0.128	0.900	0.582
chrB1.90775428	B1	90775428	1,999	2,000	0.089	0.096	0.485	0.186	0.896	0.488
chrD4.115991773	D4	115991773	1,999	2,000	0.089	0.098	-0.037	-0.049	0.895	0.460

chrA1.186398902	A1	186398902	2,000	1,991	0.201	0.033	0.333	-0.012	0.894	0.426
chrD1.132172994	D1	132172994	1,000	2,000	0.000	0.151	mono	0.072	0.893	0.548
chrB3.1681903	B3	1681903	1,960	2,000	0.044	0.126	-0.012	0.290	0.893	0.481
chrF1.58410096	F1	58410096	1,999	2,000	0.089	0.101	-0.037	0.651	0.892	0.514
chrE1.127826462	E1	127826462	2,000	2,000	0.130	0.078	-0.063	-0.037	0.892	0.392
chrB3.74631411	B3	74631411	2,000	2,000	0.110	0.092	-0.050	0.218	0.890	0.476
chrE2.69893420	E2	69893420	1,995	2,000	0.067	0.117	-0.024	0.366	0.890	0.443
chrC1.92123224	C1	92123224	1,974	2,000	0.046	0.128	-0.012	-0.068	0.889	0.440
chrD4.32906349	D4	32906349	1,996	2,000	0.069	0.116	-0.025	0.327	0.889	0.526
chrC1.216357902	C1	216357902	1,000	2,000	0.000	0.156	mono	0.204	0.889	0.569
chrA3.5987880	A3	5987880	2,000	2,000	0.107	0.097	-0.049	0.186	0.887	0.529
chrA2.194725092	A2	194725092	1,795	2,000	0.022	0.146	mono	-0.081	0.886	0.427
chrB4.119147	B4	119147	1,960	2,000	0.044	0.135	-0.012	-0.073	0.885	0.428
chrC1.26196706	C1	26196706	2,000	2,000	0.165	0.066	0.186	-0.030	0.885	0.380
chrB4.123591019	B4	123591019	1,960	2,000	0.044	0.142	-0.012	0.643	0.883	0.397
chrA3.2785813	A3	2785813	2,000	2,000	0.110	0.104	-0.050	0.417	0.881	0.467
chrB2.146764150	B2	146764150	1,999	2,000	0.089	0.116	-0.037	0.327	0.880	0.514
chrE3.69030840	E3	69030840	2,000	2,000	0.110	0.106	0.373	0.417	0.879	0.451
chrC2.170223552	C2	170223552	1,999	2,000	0.089	0.118	-0.037	0.132	0.879	0.450
chrUn.9699383	UN	9699383	2,000	2,000	0.110	0.107	-0.050	-0.054	0.878	0.582
chrE2.20288683	E2	20288683	2,000	2,000	0.130	0.096	0.649	-0.048	0.878	0.472
chrA1.169461878	A1	169461878	2,000	2,000	0.165	0.076	-0.089	-0.035	0.877	0.469
chrUn12.17303165	UN12	17303165	1,999	2,000	0.087	0.126	-0.036	-0.067	0.874	0.426
chrB3.114518607	B3	114518607	1,999	2,000	0.087	0.127	-0.036	0.646	0.873	0.445
chrUn12.8261513	UN12	8261513	1,999	2,000	0.089	0.126	-0.037	-0.067	0.873	0.433
chrD3.124519058	D3	124519058	2,000	2,000	0.127	0.106	-0.062	-0.054	0.871	0.508
chrA1.257652083	A1	257652083	2,000	2,000	0.107	0.118	-0.049	0.519	0.870	0.390
chrUn.77050150	UN	77050150	1,999	2,000	0.087	0.133	-0.036	0.125	0.869	0.526
chrD4.110867181	D4	110867181	2,000	2,000	0.112	0.117	-0.051	-0.061	0.869	0.481
chrC1.33827163	C1	33827163	1,967	2,000	0.045	0.158	-0.012	0.224	0.868	0.484
chrF1.10508463	F1	10508463	2,000	2,000	0.150	0.097	-0.077	0.419	0.867	0.495
chrD3.121660315	D3	121660315	2,000	2,000	0.133	0.107	0.293	-0.055	0.867	0.500
chrA2.74350148	A2	74350148	1,993	2,000	0.066	0.146	-0.024	-0.081	0.867	0.445
chrF1.1700092	F1	1700092	2,000	2,000	0.172	0.086	-0.093	0.481	0.866	0.518
chrD2.98140067	D2	98140067	1,960	2,000	0.044	0.165	-0.012	0.377	0.865	0.420
chrB1.95582849	B1	95582849	1,795	2,000	0.022	0.175	mono	0.159	0.864	0.465
chrA3.17994107	A3	17994107	1,999	2,000	0.087	0.138	-0.036	-0.075	0.864	0.413
chrB2.94207958	B2	94207958	1,999	2,000	0.087	0.140	-0.036	-0.076	0.863	0.526
chrA1.271452674	A1	271452674	1,999	2,000	0.087	0.140	-0.036	0.257	0.863	0.447
chrC1.50317920	C1	50317920	1,960	2,000	0.044	0.165	-0.012	0.043	0.862	0.503
chrA2.143540215	A2	143540215	2,000	2,000	0.165	0.093	0.186	-0.045	0.861	0.391
chrE2.61902026	E2	61902026	1,993	2,000	0.066	0.156	-0.024	0.536	0.860	0.500

chrA2.123045290	A2	123045290	2,000	2,000	0.146	0.107	-0.075	-0.055	0.860	0.442
chrC1.105529441	C1	105529441	2,000	2,000	0.165	0.096	-0.089	0.186	0.860	0.641
chrA1.245760324	A1	245760324	1,993	2,000	0.066	0.156	-0.024	-0.088	0.859	0.466
chrB1.178805063	B1	178805063	1,993	2,000	0.066	0.156	0.661	0.204	0.859	0.397
chrB3.70368504	B3	70368504	2,000	2,000	0.112	0.130	-0.051	0.110	0.858	0.452
chrA2.171627840	A2	171627840	1,999	2,000	0.087	0.146	0.485	0.075	0.857	0.521
chrUn15.2682639	UN15	2682639	1,999	2,000	0.087	0.146	-0.036	0.384	0.857	0.462
chrA2.132974752	A2	132974752	1,967	2,000	0.045	0.172	-0.012	-0.099	0.856	0.461
chrE1.132017960	E1	132017960	1,999	2,000	0.087	0.148	-0.036	0.384	0.856	0.466
chrA3.120458264	A3	120458264	2,000	2,000	0.107	0.137	-0.049	0.258	0.855	0.436
chrA1.210108596	A1	210108596	2,000	2,000	0.206	0.077	0.106	0.261	0.855	0.466
chrF2.42999512	F2	42999512	1,814	2,000	0.023	0.185	mono	0.014	0.855	0.526
chrUn.45668217	UN	45668217	1,999	2,000	0.087	0.150	-0.036	0.383	0.855	0.472
chrB3.150053764	B3	150053764	2,000	2,000	0.127	0.126	-0.062	-0.067	0.854	0.390
chrB3.51419880	B3	51419880	2,000	2,000	0.127	0.127	-0.062	0.111	0.853	0.486
chrA2.222548225	A2	222548225	2,000	2,000	0.201	0.080	0.109	0.260	0.853	0.483
chrB3.127289249	B3	127289249	1,993	2,000	0.066	0.164	-0.024	0.181	0.853	0.507
chrD3.78037429	D3	78037429	1,967	2,000	0.045	0.178	-0.012	-0.103	0.853	0.460
chrB1.118680910	B1	118680910	1,995	2,000	0.067	0.164	-0.024	0.044	0.852	0.539
chrB1.100367105	B1	100367105	1,795	2,000	0.022	0.191	mono	0.122	0.851	0.392
chrUn13.12209356	UN13	12209356	2,000	2,000	0.127	0.133	-0.062	-0.071	0.849	0.437
chrB3.39630826	B3	39630826	1,795	2,000	0.022	0.200	mono	0.005	0.847	0.454
chrB3.86869224	B3	86869224	1,999	2,000	0.087	0.159	-0.036	0.056	0.847	0.466
chrB4.68540749	B4	68540749	1,960	2,000	0.044	0.184	-0.012	0.016	0.847	0.458
chrA1.180113591	A1	180113591	2,000	2,000	0.206	0.090	0.332	0.219	0.844	0.432
chrUn.41472022	UN	41472022	2,000	2,000	0.165	0.115	0.186	-0.059	0.843	0.533
chrA2.62766160	A2	62766160	1,999	2,000	0.087	0.165	-0.036	0.181	0.842	0.479
chrC2.132017434	C2	132017434	2,000	2,000	0.165	0.118	0.186	-0.062	0.841	0.421
chrC2.262161	C2	262161	2,000	2,000	0.184	0.107	-0.103	0.369	0.841	0.524
chrA2.205830088	A2	205830088	2,000	2,000	0.107	0.154	0.374	0.059	0.840	0.476
chrF1.75274841	F1	75274841	1,999	2,000	0.089	0.165	-0.037	0.318	0.840	0.491
chrD4.55661288	D4	55661288	1,795	2,000	0.022	0.206	mono	0.326	0.840	0.463
chrC1.201204339	C1	201204339	2,000	2,000	0.110	0.156	-0.050	0.058	0.838	0.519
chrB1.71995132	B1	71995132	2,000	2,000	0.268	0.056	0.162	-0.024	0.837	0.499
chrD1.91944678	D1	91944678	1,993	2,000	0.066	0.185	-0.024	0.014	0.835	0.526
chrA3.101023230	A3	101023230	2,000	2,000	0.228	0.088	0.071	-0.042	0.834	0.524
chrB4.80902801	B4	80902801	1,960	2,000	0.044	0.201	-0.012	0.234	0.834	0.415
chrB4.109683320	B4	109683320	2,000	2,000	0.169	0.126	-0.091	-0.067	0.833	0.520
chrA2.66162743	A2	66162743	1,795	2,000	0.022	0.214	mono	0.192	0.833	0.365
chrB1.193634290	B1	193634290	1,999	2,000	0.089	0.175	-0.037	0.289	0.833	0.002
chrUn12.6652777	UN12	6652777	2,000	2,000	0.187	0.116	0.143	-0.060	0.832	0.473
chrC1.52927181	C1	52927181	2,000	2,000	0.172	0.126	-0.093	0.290	0.832	0.360

chrA1.50468195	A1	50468195	1,996	2,000	0.110	0.164	0.373	0.044	0.831	0.446
chrA1.275002460	A1	275002460	2,000	1,998	0.298	0.044	0.095	-0.017	0.831	0.470
chrB3.26272231	B3	26272231	1,000	2,000	0.000	0.229	mono	0.354	0.830	0.444
chrA3.164199478	A3	164199478	1,999	2,000	0.089	0.178	-0.037	0.547	0.830	0.457
chrB2.8692400	B2	8692400	2,000	2,000	0.236	0.087	0.047	-0.042	0.829	0.003
chrUn15.2234009	UN15	2234009	2,000	2,000	0.150	0.145	-0.077	0.230	0.827	0.461
chrD3.10653740	D3	10653740	2,000	2,000	0.130	0.158	0.294	0.349	0.826	0.474
chrUn7.2511376	UN7	2511376	2,000	2,000	0.127	0.161	0.295	0.201	0.825	0.566
chrA3.97197194	A3	97197194	2,000	2,000	0.146	0.150	-0.075	0.073	0.824	0.452
chrUn5.4430690	UN5	4430690	1,795	2,000	0.022	0.225	mono	-0.038	0.824	0.475
chrUn8.1386135	UN8	1386135	2,000	2,000	0.107	0.176	0.374	0.288	0.822	0.419
chrA2.179540281	A2	179540281	2,000	2,000	0.223	0.106	0.074	-0.054	0.821	0.439
chrC2.105768020	C2	105768020	2,000	2,000	0.236	0.097	0.239	-0.048	0.820	0.481
chrUn13.14266848	UN13	14266848	1,993	2,000	0.066	0.203	0.661	0.215	0.820	0.607
chrB4.30520254	B4	30520254	2,000	2,000	0.223	0.107	0.074	0.369	0.819	0.471
chrB1.158491011	B1	158491011	2,000	2,000	0.219	0.110	-0.132	-0.056	0.819	0.472
chrA1.122465543	A1	122465543	1,967	2,000	0.045	0.217	-0.012	-0.032	0.818	0.547
chrC2.34543191	C2	34543191	2,000	2,000	0.146	0.158	-0.075	-0.089	0.818	0.389
chrD4.78705483	D4	78705483	1,999	2,000	0.087	0.195	-0.036	0.355	0.817	0.497
chrC1.15780754	C1	15780754	2,000	2,000	0.184	0.135	0.391	0.093	0.817	0.489
chrB4.95831693	B4	95831693	2,000	2,000	0.165	0.148	-0.089	0.229	0.816	0.502
chrB1.81168124	B1	81168124	1,795	2,000	0.022	0.233	mono	0.427	0.815	0.500
chrE2.3933982	E2	3933982	2,000	2,000	0.201	0.126	-0.117	0.112	0.815	0.545
chrA1.194996231	A1	194996231	1,993	2,000	0.066	0.210	-0.024	0.087	0.814	0.472
chrE2.65383456	E2	65383456	2,000	2,000	0.187	0.137	-0.105	0.092	0.813	0.474
chrB2.112444547	B2	112444547	2,000	2,000	0.127	0.176	0.295	0.288	0.812	0.483
chrD1.4140039	D1	4140039	1,999	2,000	0.087	0.201	-0.036	0.328	0.811	0.582
chrB4.43191231	B4	43191231	1,993	2,000	0.066	0.217	-0.024	0.279	0.808	0.006
chrC1.46354234	C1	46354234	2,000	2,000	0.219	0.126	0.076	0.468	0.805	0.412
chrC1.95227611	C1	95227611	1,999	2,000	0.087	0.208	-0.036	0.196	0.805	0.623
chrUn26.10046275	UN26	10046275	1,960	2,000	0.044	0.236	-0.012	0.041	0.803	0.526
chrC1.63091997	C1	63091997	2,000	2,000	0.325	0.055	0.173	0.388	0.802	0.417
chrA2.116555292	A2	116555292	2,000	2,000	0.283	0.086	0.127	0.220	0.801	0.478
chrUn30.18116	UN30	18116	2,000	2,000	0.127	0.191	0.295	0.357	0.799	0.423
chrUn13.13324872	UN13	13324872	1,999	2,000	0.087	0.217	-0.036	0.176	0.797	0.477

Supplemetary Table S2. Average proportions membership (Qi) and inferred ancestry of sampled domestic cats (Fca) and wildcats (Fsi) combining with 10 different hybrid classes. All populations are running with "admixture" and correlated allele frequencies model in STRUCTURE (K = 2). On the left are reported the average proportion membership of each populations to wild and domestic clusters (Qw= wildcats cluster; Qd = domestic cluster). On the right are numbered the genotypes assigned at six different threshold values to their own genetic cluster: simulated domestic and wild cats (Fca sim and Fsi sim) are correctly assigned at a threshold of 0.95. First and second generation hybrids (F1 and F2) are assigned equally to both two clusters. I and II generation backcrosses with wild and domestic cats are assigned at a threshold of 0.75 while III and IV generation backcrosses with wild and domestic cats are chiefly assigned at a threshold of 0.85. The observed wild and domestic cat genotypes are correctly identified at a threshold of 0.80 and 0.90 respectively.

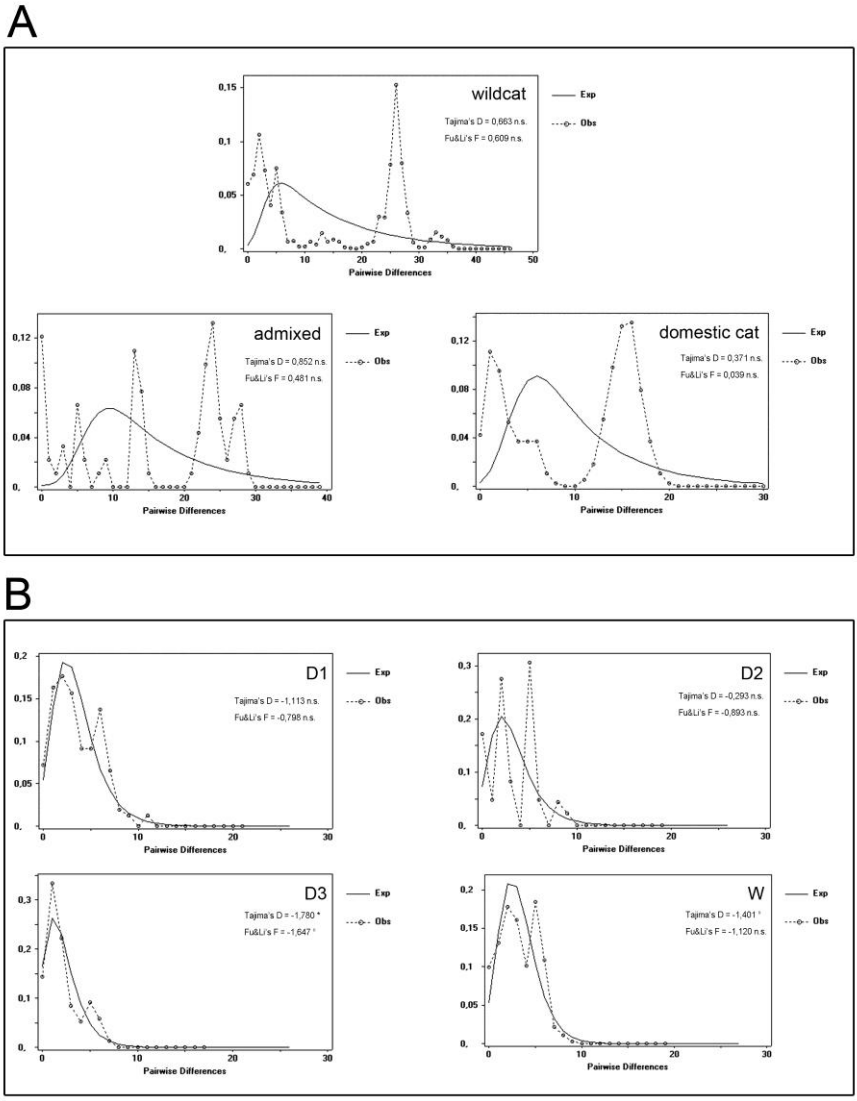
	Q_w	Q_d	70%	75%	80%	85%	90%	95%	N
Fca	0.031	0.969	10	10	10	10	10	9	10
Fsi	0.938	0.062	53	53	53	49	40	35	53
Fca sim	0.022	0.978	30	30	30	30	30	30	30
Fsi sim	0.970	0.030	30	30	30	30	30	30	30
F1	0.490	0.510							
F2	0.492	0.508							
BX1_Fca	0.256	0.744	29	7					30
BX1_Fsi	0.730	0.270	29	14					30
BX2_Fca	0.247	0.753	27	8					30
BX2_Fsi	0.730	0.270	30	20					30
BX3_Fca	0.135	0.865	30	30	30	25			30
BX3_Fsi	0.852	0.148	30	30	30	20			30
BX4_Fca	0.138	0.862	30	30	30	25	2		30
BX4_Fsi	0.851	0.149	30	30	30	19			30

Supplementart Table S3. Frequency in each population (wildcat, domestic, admixed) of the 54 haplotypes found in the 121 multi-fragment alignments. Haplotypes are grouped in the haplogroups (W, D1, D2, D3) defined in the phylogenetic analyses

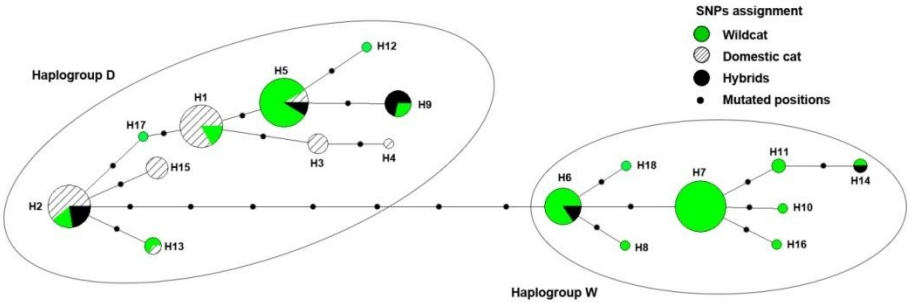
	ID	wildcat	domestic	admixed	n° samples
Haplogroup W	W.1	15			15
	W.2	1		1	2
	W.3	3			3
	W.4	5			5
	W.5	1			1
	W.6	2			2
	W.7	1			1
	W.8	3			3
	W.9	1			1
	W.10	1			1
	W.11			1	1
	W.12	2			2
	W.13	1			1
	W.14	3			3
	W.15	1			1
	W.16	1			1
	W.17	1			1
	W.18	1		1	2
	W.19	1			1
	W.20	1			1
	W.21	3		1	4
	W.22	1			1
	W.23	1			1
	W.24			1	1
Haplogroup D1	D1.1		2	2	4
	D1.2		1		1
	D1.3		3		3
	D1.4	1	1		2
	D1.5		1		1
	D1.6		1		1

	D1.7		1		1
	D1.8	1	1		2
	D1.9	1			1
	D1.10	1			1
	D1.11			1	1
Haplogroup D2	D2.1	3	2	1	6
	D2.2	2		5	7
	D2.3	6			6
	D2.4	3			3
	D2.5	7			7
	ID1248	1			1
Haplogroup D3	D3.1		1		1
	D3.2		1		1
	D3.3		1		1
	D3.4		2		2
	D3.5	2	3		5
	D3.6		1		1
	D3.7		1		1
	D3.8		1		1
	D3.9		1		1
	D3.10		1		1
	D3.11		1		1
	D3.12		1		1
	D3.13	1			1
Total	54	79	28	14	121

Supplementary Figure S1. Mismatch distributions for the different samples Arrangements. A organized by populations; B organized by haplogroups. Inside each plot are reported the values of Tajima'sD and Fu & Li's F. *: p< 0;05; ° : p ≈ 0,05.



Supplementary Figure S2. Median-joining network of the European wildcat (*F.s.silvestris*), Domestic cat (*F.s.catus*) and putative admixed haplotypes only considering ND5 region of mtDNA. The haplotypes are coloured according to the assignation based on nuclear markers (SNPs and STRs). The size of the circles is proportional to the haplotypes frequency



PHYLOGEOGRAPHY OF THE EUROPEAN WILDCAT (*FELIS SILVESTRIS SILVESTRIS*) IN EUROPE: TODAY STRUCTURE AND HISTORICAL INFERENCES ON SPECIES' BIOGEOGRAPHY.

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ABSTRACT

The European wildcat (*Felis silvestris silvestris*) is characterized by a fragmented and genetically differentiated population. However, the phylogenetic relationships and the biogeographic history of the species is still unclear. Furthermore the long sympatry with the domestic cat that has characterized the last 10 000 years makes particularly interesting the understanding of possible past introgression events. In this work we sequenced a portion of ND5 gene of mitochondrial DNA that has proved to contain polymorphisms able to distinguish wild from domestic subspecies and to provide good phylogeografic information. We performed a phylogenetic and phylogeographic analysis on a sample of 707 individuals (212 domestic cats, 423 wildcats and 72 putative hybrids from Scotland and Hungary) collected across Europe and previously typed at 31 microsatellite loci and assigned to their subspecies by means of Bayesian clustering. We included 10 more wildcats individuals whose sequences were deposited in GeneBank. The results showed two main differentiated clades (W and D) whose haplotypes featured respectively the wild and domestic polymorphisms. While all the 212 domestic cats presented the expected domestic haplotypes we found that also 174 (40,1%) of wildcats shared some D group haplotypes. Also 60 putative hybrids (83,3%) carried a haplotype of group D. The 74,7% of wildcats featuring domestic haplotypes were grouped in a separate clade (including two haplotypes Dw4 and Dw6) that diverged from the rest of group about 37 700 years ago and that was present across all Europe. Group W showed a clear geographic structure in which to a first Mediterranean/continental main differentiation began about 64 200 years ago followed a separation among central Europe (and part of the Iberian peninsula), Italy (that presented a certain degree of haplotype sharing with Iberian peninsula and Eastern Europe) and Scotland regions. This work provided a

comprehensive phylogenetic and phylogeographic analysis of European wildcat that tried also to clarify the origin of shared haplotypes advancing new and complementary hypothesis about the biogeographic history of the species.

Keywords: *Felis silvestris*, phylogenetics, phylogeography, biogeography, introgression, wildcat.

INTRODUCTION

The wildcat *Felis silvestris* is a polytypic species comprising six ecologically, 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), in the Mediterranean islands of Corsica, Sardinia, and Crete (Randi and Ragni 1991, Driscoll *et al.* 2007); and the domestic descendant of *libyca* North African cats, the domestic cat (*Felis silvestris catus*) that has been spread throughout the entire continent, as well as in the entire World. Archaeological remains suggest that the European subspecies probably appeared in the continent around 450 000-200 000 y BP (Kitchener 1991, Sommer and Benecke 2006), descending from the Martelli's cat (*Felis lunensis*, Martelli 1906), which was found in Europe during the early Pleistocene (Kitchener 1991; Nowell and Jackson 1996) and differentiated into the late African subspecies between 230 000 and 173 000 yars ago (Driscoll et al 2007). The presence of African wildcats in Mediterranean islands is a consequence of human translocations at very early stages of domestication, probably less than 11 000 years ago by Neolithic navigators (Vigne *et al.* 2012).

Current patterns of European wildcats distribution and genetic structure are likely a reflection of both natural and anthropogenic events. In one hand, the range shifts during the climatic oscillations of the Pleistocene contributed to shape wildcat's demographic history and genetic diversity (Kitchener and Rees 2009). On the other hand, intensive human persecution in the 18th and 19th centuries (Stahl and Artois 1994), led to a strong demographic declines for most of the species range (Nowell and Jackson 1996) due to deforestation, habitat modifications and local decline of major prey (e.g. Lozano *et al.* 2007, Monterroso *et al.* 2009).

In the last years, several studies have focused on the analysis of genetic diversity and hybridization patterns among European wildcats. Some populations across Europe have now been investigated using new genetic and statistical tools. Examples of detailed analyses can be found for Iberian peninsula (Oliveira *et al.* 2008a, Oliveira *et al.* 2008b), France (O'Brien *et al.* 2009), Italy (Randi (Randi *et al.* 2001), Germany (Hertwig *et al.* 2009, (Eckert *et al.* 2010), Hungary (Lecis *et al.* 2006) and Scotland (Beaumont *et al.* 2001), where the analysis of mitochondrial variation and/or microsatellites diversity suggested varying degrees of hybridization between wild and domestic cats. Recently Mattucci et al (2014) carried out an investigation of the genetic structure of 1114 wildcats in Europe using a panel of 38 microsatellite loci, analyzing the effects of the recent population decline and fragmentation faced by the species. However, a comprehensive phylogeographic history of wildcats in Europe is still missing, and the structure of population subdivision is known only in a recent time-scale. Therefore, reconstructing evolutionary patterns of population structuring of

wildcats in Europe, and estimating within and between population genetic diversity, is needed to identify evolutionary or conservation units and forecast their conservation perspectives.

In the last few years a genetic study on the origin of domestic cats (Driscoll et al 2007) shed light on some aspects of phylogenetic and phylogeographic history of *Felis silvestris*, in particular focusing on mtDNA sequence variation and identifying a panel of genetic markers useful in the discrimination between subspecies and consequently between European wildcat and domestic cats (Driscoll et al 2011). The application of this panel (or part of it) in several studies revealed some interesting discordance between nuclear and mitochondrial subspecies' identifications (Hertwig *et al.* 2009, Nussberger *et al.* 2014, Witzenberger and Hochkirch 2014) generally associated with signals of domestic cat introgression (Driscoll *et al.* 2011). However these studies included a small number of individuals, often with a low geographical coverage. Moreover, the paleo-biogeographic history of the species (Kurtén 1965, 1968, Macdonald *et al.* 2010) suggested a deeper investigation on larger and more representative sample.

The aims of this study are: 1) to reconstruct a phylogeographic status and history of wildcat in Europe and estimate the main biogeographic and evolutionary steps in relation to the last climatic fluctuations (late Pleistocene glacial cycles); 2) to deepen the causes of recent found discordance between nuclear and mitochondrial markers in several putative wildcat individuals in order to make more reliable and safe the use of these markers in the identification of reference wild individuals and cryptic introgression.

MATERIALS AND METHODS

Sampling

A total of 707 biological samples (tissue, blood, buccal swabs, hair and skin samples), belonging to 423 putative European wildcats, 212 domestic cats, and 72 putative admixed cats from Hungary (n = 58) and Scotland (n = 14) were collected from six macro-regions of Europe (divided in a total of 12 sub-regions, see Table 1) in twelve years (from 1998 to 2010). All samples were stored in 5 volumes of 95% ethanol (tissues, skins and hairs) or Tris/SDS buffer (blood, buccal swabs; Longmire *et al.* 1997), and kept at -20°C at the ISPRA genetic laboratories (Ozzano dell'Emilia, Bologna, Italy)

The European wildcat sample derives from opportunistic collections of found-dead or trapped animals, covering the majority of species range in Europe (Table 1 and Figure 1 for details). All putative wildcats were previously morphologically identified by collectors according to wildcat phenotype, life history,

cranial and intestinal indexes, stomach content and/or biometric indices (Schauenberg 1969, 1977, French *et al.* 1988, Ragni and Possenti 1996).

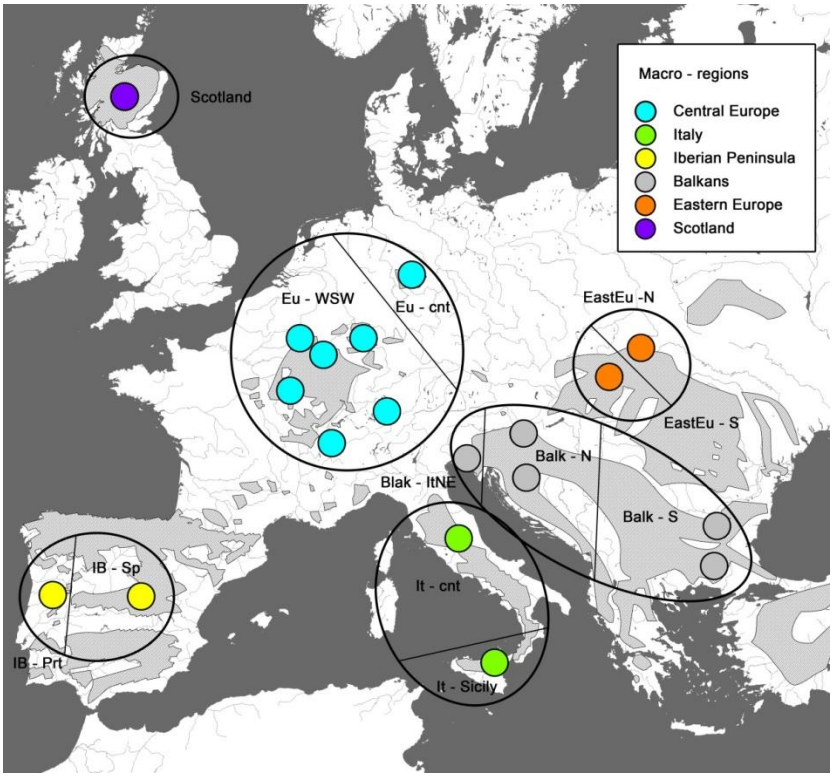

Figure 1. Map with the indicative location of the sampling points (one for country). Macro-regions are in different colours (see legenda). Each macro-region is divided in the respective sub-regions

The domestic cat sample comes from free-ranging cats that had no regular feeding or housing connection to humans as well as from random bred cats having some connection to humans or cats owned and cared for by humans but not belonging to any specific breed.

Total DNA was isolated using standard phenol-chloroform (Sambrook and Russell 2006) or the QIAGEN DNeasy tissue and blood extraction kits (Qiagen Inc, Hilden, Germany), depending on the available quality and quantity of each sample. Each extraction run included two negative controls.

All samples were previously typed at 31 loci (Mattucci Phd Thesis) and were assigned to the domestic (*Felis silvestris catus*), wild (*Felis silvestris silvestris*) or

admixed populations by means of Bayesian clustering using the software STRUCTURE 2.3.3. Following the criterion suggested by Mattucci et al (2013) we considered an assignment threshold of qi = 0.90. By this time the individuals in this study will be considered as “wildcat”, “domestic cat” or “admixed” in according to this analysis.

Subspecies	Macro-region	Sub-region		Country		
Domestic cat						**212**
	Italy					59
		It - cnt	59			
				Italy	59	
	Central Europe					25
		Eu-WSW	25			
				Germany	24	
				Switzerland	1	
	Iberian peninsula					111
		IB - Prt	53			
				Portugal	53	
		IB - Sp	58			
				Spain	58	
	Balkans					2
		Balk - N	2			
				Slovenia	2	
	Eastern Europe					15
		EastEU - N	15			
				Poland	15	
Wildcat						**433**
	Italy					109
		It - cnt	101			
		It - Sicily	8			
				Italy	109	
	Central Europe					158
		Eu - cnt	34			
				Germany	34	
		Eu-WSW	124			
				Germany	94	
				Belgium	15	
				Luxemburg	9	
				Austria	2	
				Switzerland	1	
				France	0(3)	

	Iberian peninsula					48
		IB - Prt	24			
				Potugal	21(3)	
		IB - Sp	24			
				Spain	20(4)	
	Balkans					104
		Balk - N	30			
				Slovenia	29	
				Croatia	1	
		Balk - ItNE	70			
				Italy (NE)	70	
		Balk - S	4			
				Romania	2	
				Bulgary	2	
	Eastern Europe					14
		EastEu - N	10			
				Poland	10	
		EastEu - S	4			
				Hungary	4	
Hybrid						**72**
	Eastern Europe					58
		EastEu - S	58			
				Hungary	58	
	Scotland					14
		Scot	14			
				Scotland	14	

Table 1. Origin of the 717 samples used in the study. The samples are listed by subspecies, according to microsatellite identifications, macro-regions (Italy, Central Europe, Iberian Peninsula, Balkans, Eastern Europe, Scotland) , sub-regions (It – cnt: central Italy; It – Sicily: Sicily; Eu – cnt: center-north Germany; Eur – WSW: southern and west region of central Europe; IB – Prt: Portugal; IB – Sp: Spain; Balk – N: northern Balkans; Balk – S: Southern Balkans; Balk – ItNE eastern Italian Alps; EastEU – N: Poland; EastEU – S: Hungary) and country. Samples from eastern Italian Alps were considered part of the Balkans group in according to the recent phylogeographic findings (Mattucci et al 2013). Numbers in parentheses indicate the sequences downloaded from the NCBI database (Driscoll et al 2007).

Sequencing procedure and data analysis

We sequenced 877 bp (including the primers) of the mtDNA NADH dehydrogenase subunit 5 (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 7 diagnostic SNPs discriminating European wildcats (*Felis silvestris silvestris*) and domestic cats (*Felis silvestris catus*). We selected this region also due to its informativess on wildcats phylogeny (Driscoll

et al 2007). This sequence was amplified using PCR primers F2B (5'-TGCCGCCCTACAAGCAAT-3') and R3B (5'-TAAGAGACGTTTAATGGAGTTGAT-3') (Driscoll *et al.* 2011). Each 10 µL PCR reaction contained 2 µL of DNA (c. 50 ng), 0.8 µL of 10X Taq Buffer advanced (Eppendorf) with self-adjusting Mg^{2+} (Eppendorf), 0.80 µL of 0.2% bovine serum albumin (Sigma-Aldrich), 0.36 µL of 2.5 mM dNTPs (Eppendorf), 0.15 µL of each 10 mM primer solution (Bionordika), 0.04 µL of 5U/µL HotStart Taq polymerase (Eppendorf) and 5.70 µL of purified water (Eppendorf, Milano, Italy). PCRs were performed in a Veriti Thermal Cycler (Life Technologies) with the following thermal profile: 94°C for 15 min for initial denaturation and Taq activation, followed by 50 cycles of 30 s at 94°C, 60 s at 55°C and 60 s at 72°C. The PCR cycling was followed by a final extension for 10 min at 72°C. PCR products were stored at 4°C and then purified by exonuclease digestions (1 µL of EXO-SAP per samples, incubated at 37° C for 30 min, then at 80° C for 15 min). The purified amplicons were Sanger-sequenced. Each 10 µL reaction contained 1 µL of amplified DNA, 1 µL of BigDye 1.1 (Life Technologies), 0.2 µL of either the forward or reverse primer and 7.8 µL of purified water. Sequencing was performed in a Veriti Thermal Cycler with 25 cycles of 10 s at 96° C, 5 s at 55° C, 4 min at 60° C and a storage at 4° C. Sequences were cleaned from unincorporated label nucleotide by precipitation adding to each PCR product a 12 µL mix composed by 2 µL of NaOAc 3M and 10 µL of purified water. Then were added 50 µL of 100% EtOH. The mix was then centrifuged at 12500 rpm for 10 min. The supernatant was extracted and eliminated manually using a transfer pipette. The precipitate was washed with 70 µL of 70% EtOH and centrifuged at 12500 rpm for 7 min. The supernatant was eliminated and the precipitate was left to dry in the dark. The purified product was combined with 10 µL of Hi-DI formamide (Life Technologies) and denatured for 3 min at 95°C. Products were separated on an ABI 3130 DNA Analyzer.

We used 10 additional wildcat sequences downloaded from the NCBI database (accession numbers: EF587158, EF587164, EF587168, EF587166, EF587170, EF587169, EF587171, EF587156, EF587162, EF587159; Driscoll *et al.* (2007), and used as positive controls in alignment and analyses procedures. All sequences were aligned using SEQSCAPE software v2.5 (Life Technologies). The sequence of the entire mtDNA genome of the domestic cat (NCBI Reference Sequence: NC_001700), trimmed at the above-mentioned positions, was used as reference sequence over which we tagged the seven known variants. All sequences were then checked by eye and further trimmed using BIOEDIT v7.1.11 (Hall, 1999) into equal sequences of 669 bp (positions 13243 – 13911) for subsequent sequence analyses to maintain full-length, double-stranded, high-quality sequence data across all samples. Haplotypes (h) and nucleotide diversity (π) were computed using DNAsp v5.10.01 (Librando and Rozas, 2009). We used NETWORK v4.6 (Fluxus Technology Ltd). to construct networks to infer relationships among haplotypes using a median-

joining (MJ) algorithm (Bandelt *et al.* 1999) with ε = 10 and a transversions/transition weighting of 3:1 and then we cleaned up the resulting scheme using MP calculation (Polzin *et al.* 2003). An analysis of molecular variance (AMOVA) was performed in ARLEQUIN v3.5.1.3 (Excoffier and Lischer 2010) on Euclidean pairwise genetic distances estimating the significance of the parameters by 10 000 permutations of the distance matrix. The analysis was conducted at a level of subspecies clustering inferred by microsatellites (wildcats, domestic cats and hybrids), macro-regions and sub-regions. We used the same software to compute Tajima's D (Tajima, 1989) and FU and Li's F (Fu and Li, 1993) statistics to test demographic expansion. We performed a spatial analysis of molecular variance (SAMOVA) using the software SAMOVA 2.0 (Dupanloup et al 2002) that implements an approach to define groups of populations that are geographically homogeneous and maximally differentiated from each other using a simulated annealing procedure that aims at maximizing the proportion of total genetic variance due to differences between groups of populations. Best nucleotide substitution model scheme for the sequences was computed in PARTITIONFINDER V1.1.0 (Lanfear *et al.* 2012) using Bayesian information criterion, (BIC). For the first and second codon positions in ND5 Kimura's two parameters (K80) models with invariable sites (I=0,80) was selected as best-fitting model. For third position of ND5 was selected the Hasegawa, Kishino and Yano (HKY) model whit invariable sites (I=0,87).

We reconstructed a Bayesian phylogenetic tree and estimated the divergence times in the relationships among haplotypes using BEAST v2.1.3 (Bouckaert et al 2014) using the substitution model scheme previously selected by PARTITIONFINDER. We set the rate variation among sites using a gamma distribution with four discrete categories. Due to the strong relationship between the taxa we selected a strict molecular clock model with fix mean substitution rate (2,28x10-8/site/year). Constant population size was selected as coalescent prior. Since the authors of the software discouraged the inclusion of an outgroup to root the tree (Drummond and Bouckaert, 2014), we let the software to estimate the rooting point giving as prior calibration point the interval in which falls the common ancestor between *Felis s silvestris* and *F.s.libyca/catus* as illustrated in Driscoll et al (2007) using a uniform distribution with bounds values of 230 000-173 000 years BP constraining the group to be monophyletic. The Bayesian posterior probabilities (BPPs) as well as the high posterior densities for the node ages (HPDs) were extrapolated performing three independent MCMC runs of 100 000 000 steps with a burn-in period of 10 000 000 steps and picking genealogies every 2000 steps . The results of the three chains were simultaneously analysed in TRACER v1.6 (Rambaut et al 2013). The values of ESS (always exciding 200) and the equilibrium showed by the trace plots suggested that the number of steps was more than adequate for a correct estimation of the posterior distribution of the parameters. To support phylogenetic reconstructions two further trees were calculated using respectively neighbour-joining

algorithm (NJ, Saitou and Nei, 1987) and maximum-likelihood method (ML, Felsenstein, 1981) and using, for ML tree reconstruction, the heuristic search by topological rearrangement of an initial tree (Near-Neighbor-Interchange). The robustness of the trees was assessed by bootstrap resampling (BS) (10 000 random replications for NJ analysis; 5000 random replications for ML analysis) using the software MEGA 6.06 (Koichiro et al 2013)

RESULTS

The mtDNA sequence alignment did not show indels or stop codons, and the aminoacid sequence was concordant with the domestic cat ND5 protein (NCBI Reference Sequence NC001700). Thus, we excluded the amplification of munts or pseudogenes. We identified a total of 28 haplotypes among the 717 sequences of *F. silvestris* spp (including the GeneBank sequences), counting 32 polymorphic sites, 23 of which were parsimony informative sites. One GeneBank sample yielded a private haplotype (W14 from Fsi113).

Phylogenetic analyses in wildcats, domestic cats and admixed groups

We performed a first phylogenetic analysis using all the 717 individual mtDNA sequences. The overall haplotype diversity was h = 0,8620 ± 0,0060 (SD) and the nucleotide diversity π = 0,8700 ± 0,0130 (SD) (Table 2). The phylogenetic tree reported in Figure 2 resulted in a concordant topology for the main clades by using the three different clustering methods (Neighbour-Joining, NJ; Maximum likelihood, ML and Bayesian). A first main node 1 (100/100/1) divided the samples in two principal haplogroups (hence called D and W) each one including 14 haplotypes. Haplogroup D included a total of 447 (62,25%) individuals. This haplogroup presented an haplotype diversity h = 0,7457 ± 0,0116 (SD) and a nucleotide diversity π = 0,2467 ± 0,1610 (SD) (Table 2). It showed a further supported subdivision at the node 2 (100/88/0,99) in Group 1 (including four haplotypes and 134 individuals) and Group 2 (10 haplotypes and 313 individuals). Haplogroup W counted a total of 270 individuals (37,7%) featured by 14 haplotypes. The haplotype diversity was h = 0,7196 ± 0,0214 (SD) and a nucleotide diversity π = 0,2554 ± 0,1656 (SD) (Table 2). The node 4 (96/100/1) split this clade in Group 3 and Group 4 including respectively 9 haplotypes (88 samples) and 5 haplotypes (182 samples). We calculated a median-joining network (Figure 3, A) in which the two main haplogroups (D and W), separated by seven mutations, were clearly identifiable. All haplotypes in haplogroup D presented the seven DNA polymorphisms described in Driscoll et al (2011) for the *F. s. libyca/F. s. catus* lineage, while haplogroup W was featured exclusively by haplotypes carrying typical *F. s. silvestris* polymorphisms in the same seven corresponding positions of haplogroup D.

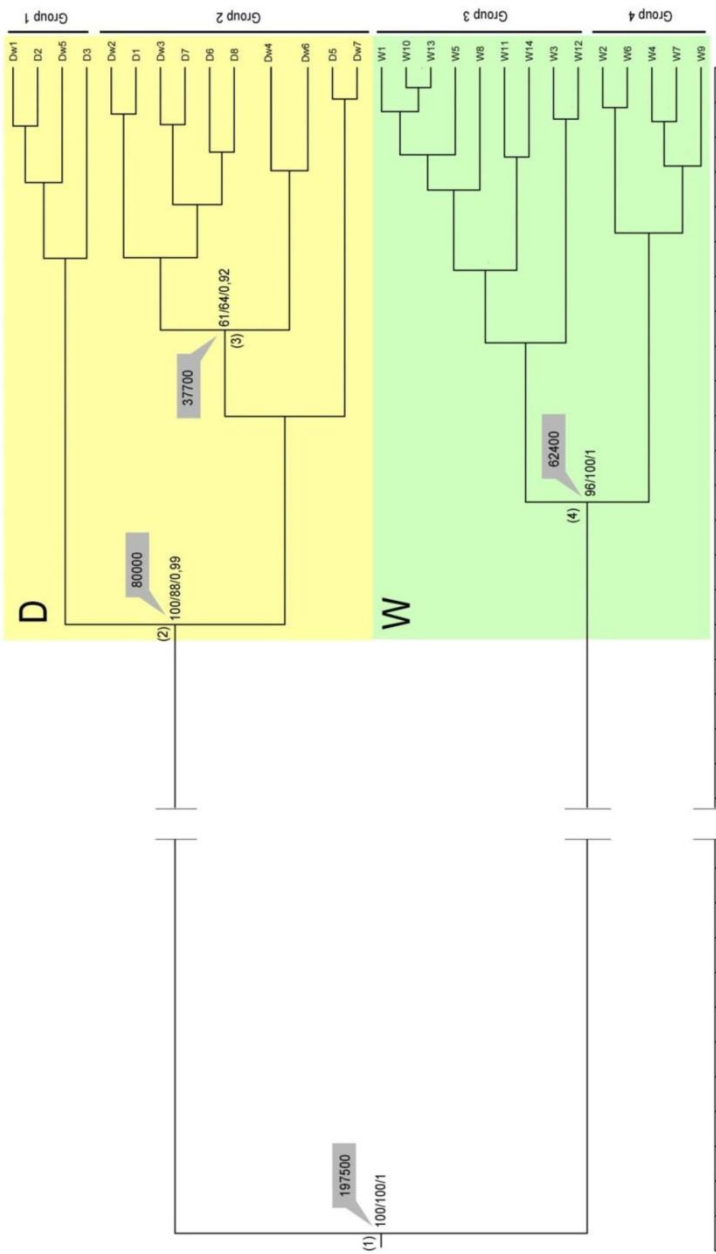

Figure 2. Bayesian tree with the phylogenetic relationship of the ND5 haplotypes from all the 718 samples. Grey cartoons indicate the age estimated for nodes. For the numbered nodes are provided respectively the bootstrap values for Neighbour-joining and Maximum-likelihood trees and the higher posterior density for Bayesian tree.

	N	Number of haplotypes	Nucleotide diversity ($\pi \pm SD$)	Haplotype diversity ($h \pm SD$)	Tajima's D	p-value	Fu & Li's F	p-value
Overall	717	28	0,8700 ± 0,0130	0,8620 ± 0,0060				
Domestic	212	12	0,2300 ± 0,1500	0,7350 ± 0,0180	**-0,4133**	**0,078**	**-2,3367**	**0,026**
Hybrids	72	8	0,6864 ± 0,3780	0,7977 ± 0,0268	1,1447	0,173	4,1293	0,442
Wild	433	20	1,0625 ± 0,5524	0,8130 ± 0,0108	2,2972	0,405	4,5339	0,280
Haplogroup D	447	14	0,2467 ± 0,1610	0,7457 ± 0,0116	-0,3468	0.067	-2,2705	0,046
Haplogroup W	270	14	0,2554 ± 0,1656	0,7196 ± 0,0214	**-0,4516**	**0,059**	**-2,7893**	**0,030**
Italy	55	4	0,0483 ± 0,0551	0,1731 ± 0,0675	**-1,0254**	**0,043**	**-1,7795**	**0,009**
Central Europe	117	7	0,1653 ± 0,1207	0,6760 ± 0,0336	-0,3593	0,098	-0,7058	0,103
Iberian Peninsula	40	5	0,2451 ± 0,1640	0,6372 ± 0,0631	0,4335	0,135	1,0906	0,260
Balkans	43	2	0,0139 ± 0,0278	0,0465 ± 0,0439	**-1,4799**	**0,001**	**-0,7225**	**0,042**
Eastern Europe	8	4	0,2296 ± 0,1743	0,7500 ± 0,1391	1,3467	0,234	-0,3747	0,030
Scotland	7	1						
Haplogroup DW	235	7	0,2079 ± 0,1419	0,5813 ± 0,0336	0,4150	0,223	0,7415	0,202
Italy	54	4	0,2069 ± 0,1435	0,4612 ± 0,0578	1,2878	0,359	1,9418	0,388
Central Europe	41	3	0,0365 ± 0,0471	0,0963 ± 0,0624	**-2,0023**	**0,000**	**-1,1067**	**0,047**
Iberian Peninsula	8	2	0,0641 ± 0,0730	0,4286 ± 0,1687	0,3335	0,479	0,5363	0,132
Balkans	61	5	0,2599 ± 0,1700	0,5710 ± 0,0553	1,4606	0,366	1,8278	0,341
Eastern Europe	64	4	0,1989 ± 0,1389	0,6562 ± 0,0346	1,2350	0,384	1,9931	0,406
Scotland	7	1						

Table 2. Genetic variability observed overall the entire sample, within the populations and for the main genetic phylogroups found for wildcats. Tajima's D and Fu and Li's F statistics test are provided.

Referring to the subspecies assignation based on the 31 microsatellites loci performed on the samples by Mattucci et al (2014) we were able to characterize each haplotype on the basis of the population belonging (wildcats = 433, domestic cats = 212 or putative hybrids = 72) of individuals. We divided the haplotypes in three main categories described by prefixes: "d" for the haplotypes assigned to the haplogroup D and detected exclusively in domestic cat genotypes; "w" haplotypes assigned to the haplogroup W and detected exclusively in wildcat cat genotypes and "Dw" haplotypes assigned to the haplogroup D, but detected in presumed wildcat or admixed domestic cat genotypes. Haplotype frequencies for each population are summarized in Table S1. The majority of domestic cats (n = 175, 82,5%) fell in Dw haplotypes that shared with wildcats and hybrids. Also 174 wildcats (40,1%) were featured by domestic mtDNA variants

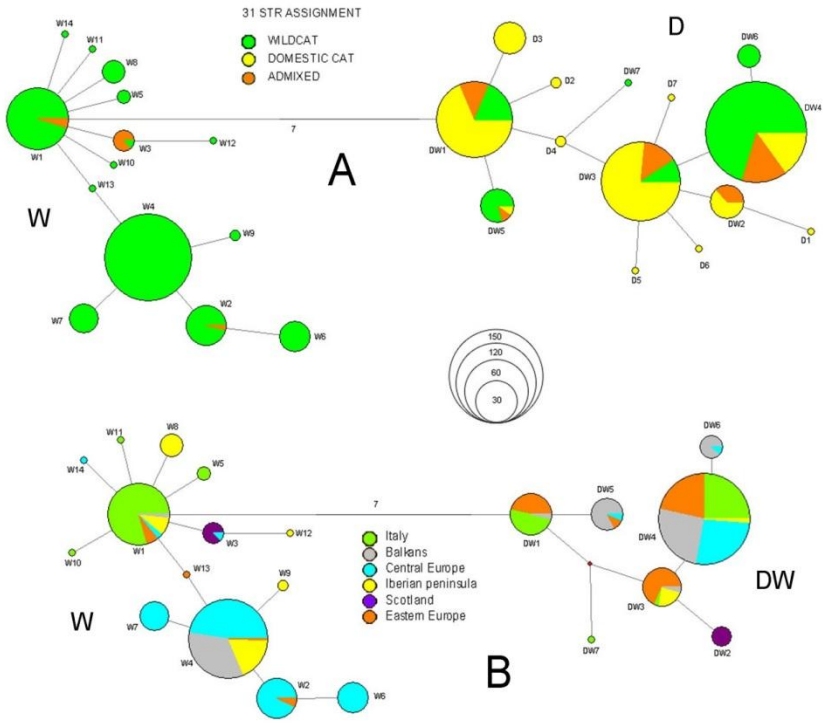

Figure 3. Median-joining networks of mtDNA haplotypes. Network A includes all 718 samples and each haplotype was divided and coloured according to the frequency for each population as assigned using. Network B includes only the 505 wildcats and admixed individuals and each haplotype was divided and coloured according to the macro-region membership. The numbers of mutations (greater than one) between haplotypes are indicated. The size of the circle is proportional to the frequency of each haplotype.

(Table S3) and 130 of these (74,7%) were found showing haplotypes Dw4 and Dw6.These same haplotypes were present in few domestic cats frequency (12%) and represented a separate clade in Group 2 (node 3, 61/64/0,92, Figure 2). Sixty out of the 72 admixed individuals form Scotland (7) and Hungary (53) were featured by "Dw" haplotypes and the 41,7% of these presented haplotype Dw4. Haplogroup W included 249 wildcats, 11 admixed individuals (seven from the Scottish population and four from Hungary) and the 10 GeneBank wildcat individuals. Haplotypes with highest relative frequency were W1 (n = 63, 23,3%) and W4 (n = 124, 45,9%). No domestic cat was found carrying any haplotype of haplogroup W (see table S1 for further information). Considering the three taxonomic groups (wildcat, domestic cats and

hybrids) as different populations we performed a first AMOVA analysis (Table 3) detecting an higher percentage of variation within population (63,3 %) than among (36,7 %) and a FST = 0,37 (P < 0,01).

Source of variation	Variance Components	Percantage of variation	Differentiation indexes
1			
Among populations (Domestic, Wildcats, Hybrids)	1,51	36,66	ϕST =0,37
Within populations	2,60	63,34	
2			
Among groups W/WD	5,68	86,68	ϕCT = 0,87
Among macro-regions /within groups	0,37	5,60	ϕSC = 0,42
Within macro-regions	0,51	7,72	ϕST = 0,92
3			
Among macro-regions (within group W)	0,59	58,24	ϕST = 0,58
Within macro-regions	0,42	41,76	
4			
Among macro-regions (within group DW)	0,12	17,21	ϕST = 0,17
Within macro-regions	0,60	82,79	
5			
Among macro-regions (no Scotland)	0,43	0,46	ϕCT = 0,47
Among sub-regions/within macro-regions	0,08	8,65	ϕSC = 0,16
Within sub-regions	0,41	44,68	ϕST = 0,55
6			
Among macro-regions (no Scotland)	0,06	8,63	ϕCT = 0,09
Among sub-regions/within macro-regions	0,07	9,44	ϕSC = 0,10
Within sub-regions	0,57	81,93	ϕST = 0,18

Table 3. Analyses of molecular variance based on mtDNA data. ϕST: differences among all the populations of an analysis (1: subspecies; 2,3,4: macro-regions; 5,6: sub-regions); ϕCT differences: among groups (2: haplogroups W/DW; 5,6: macro-regions); ϕSC: differences among populations within a groups (2: macro-regions; 5,6: sub-regions). Scotland included only one sub-region so was excluded from sub-regions analyses (5,6). All values were highly significant (p < 0,05). 1

Phylogeographic analyses of European wildcats and admixed individuas

We focused the phylogeographic analyses on wildcat and putative admixed populations pruning the initial database from the domestic cats and leaving a total of 433 wildcats and 72 admixed individuals. including 21 distinct haplotypes, defined by 26 polymorphic sites, 21 of which were parsimony informative. Hence we will refer to the D haplogroup pruned from the domestic cat (therefore including 174 wildcats and 61 hybrids) as the haplogroup DW whose parameters of genetic diversity are

consultable in Table 2. The geographic distribution of haplotypes for each haplogroup (W and DW) is showed in Figure 4 in which the haplotypic proportion for each sub-region (see Table 1 for their definition) is represented by the different pies subdivisions. The Group W showed a clear separation among peninsular Italy/south-eastern Europe, continental Europe/Iberian Peninsula and Scotland with a certain degree of haplotype sharing (especially for haplotype W1) between Italy and Iberian peninsula. Group DW showed a pervasive presence of haplotype Dw4 and a generic lack of geographic structure except for Scotland and Iberian peninsula (in particular Portugal). Domestic cats were removed from the initial network and the circles representing the different haplotypes were partitioned according to the six macro-regions from which the individuals were sampled (Figure 3, B). The two haplogroups W and DW showed a differentiation index FCT = 0,87 ($p < 0,01$, Table 3) and a quite different geographic partitioning: haplogroup W was roughly divided into a first star-like haplogroup corresponding to Group 3 (n = 88, including haplotypes W1, W3, W5, W8, W10, W11, W12, W13, W14) with a dominance of Italian individuals (n = 55, 63,2%) followed by the Iberian peninsula (n = 16, 18,2%) and a second one, corresponding to Group 4 (n = 182, haplotypes W2, W4, W6, W7, W9), including mostly central European (n = 114, 62,6%) and Balkans (n = 42, 23%) individuals. Counter wise haplogorup DW, including a total of 235 samples, did not presented any evident geographic structure. The population of Scotland (n = 14, composed by putative admixed/feral cats) is equally present in both the haplogroup W and DW and was represented by one haplotype for each haplogroup (respectively W3, n = 7 and Dw2, n = 7) and in particular haplotype W3 is shared only with one German individual (ID 760) while Dw2 appear to be a Scottish private haplotype. In the Hungarian admixed population (n= 58) most of the individuals (n = 54, 93,1%) carried haplotypes of DW group and only four individuals showed wild mitochondrial haplotypes (see Table S1). We performed a SAMOVA to discover statistically significant spatial subdivision. Geographic partitioning on group W led to the detection of four main geographic groups: Scotland, Italy (center and Sicily) / Hungary, Portugal and rest of Europe with a high significant FCT = 0,63 ($p < 0,01$) and a percentage of variation among groups of 63,21% versus a low value of FSC = 0,14 ($p<0,01$) with a variation among population within groups of 5,29%. This datum is concordant with the phylogenetic clustering of haplogroup W divided in Group 3 and Group 4 (Figure 2). On the contrary haplogroup DW only showed a separation of Scotland and Portugal regions, while the rest of Europe seemed devoid of a clear geographic structure with an overall FCT = 0,28 ($p < 0.05$) and a the majority of variation explained within populations (63,06%). To further substantiate this description we performed independent AMOVA analyses based on the described macro-regions and sub-regions from which samples came (Table 1). At a macro-regions level of group W (Table 3) was detected a FST = 0,58 ($p < 0,01$) among populations (58,24% of variance explained among populations). The FST pairwise matrix between population (Table

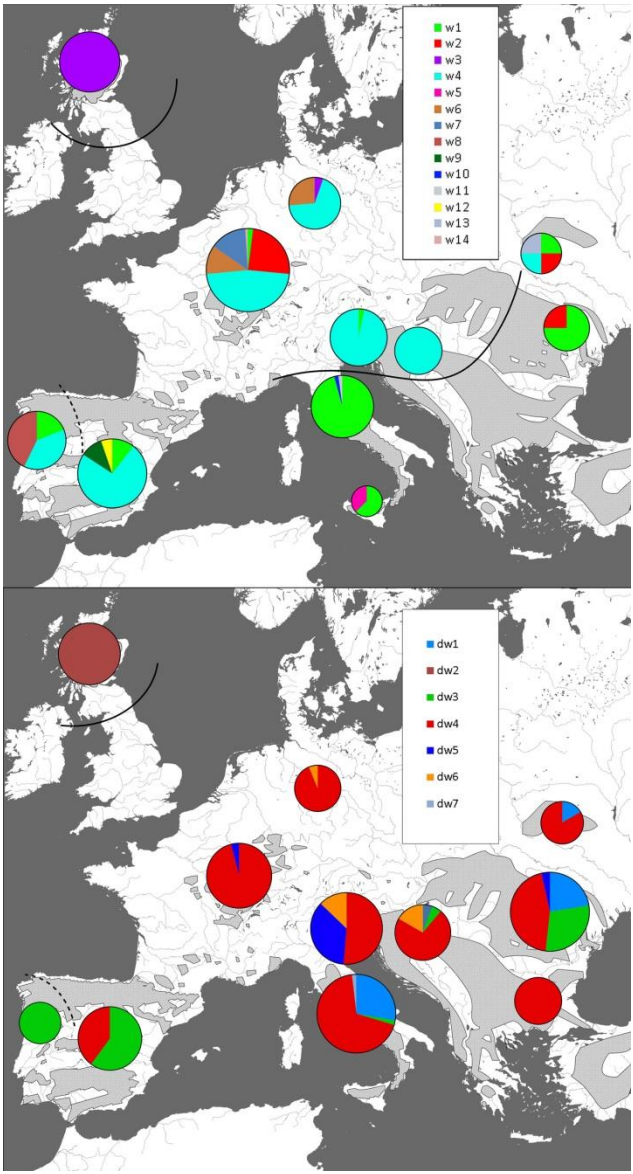

Figure 4. Map of haplotypes distribution. The map above describes the distribution of the haplotypes of group W. The map below shows the distribution of haplotypes of group DW. Circles are approximately proportional to the haplotype frequency. Black curved and broken lines indicate the geographical separation suggested by SAMOVA analyses (see results for details**)**

S2) highlighted how Scotland and Italy presented the highest value respect to all others regions. Regarding the same analysis on group DW we found a lower value of FST = 0,17 ($p < 0,01$) with the 82,8% of variation explained within populations. Here the FST pairwise matrix (Table S2) showed relative high values only for Scotland region and between Iberian peninsula and central Europe, probably because of the Portugal contribution, as previously detected by SAMOVA. Deepening the analyses at a sub-regions level (Table 3) we found in W group low FSC values (0,16; $p < 0,05$) and again central Italy, Sicily and Portuguese populations showed relative higher values of pairwise FST (Table S2). The DW group presented a lower value of FSC (0,10; $p < 0,05$) confirming the lack of structure, exception for the Portuguese population that presented relative higher values of FST (Table S2).

Demographic analyses

Subspecies populations, haplogroups D, W and DW and populations of the macro-regions of each group were analysed by means of mismatch distributions (Figure 5) and the results were crossed with relative values of Tajima's D and Fu and Li's F statistics (Table 4). We founded a weak significant sign of population expansion in Domestic cat population with a near bell-shaped curve in mismatch plot (Figure 5) and Tajima's D = -0,4133 ($p = 0,078$) and a Fu and Li's F = -2,3367 ($p < 0,05$) (Table 4) Mismatch distribution curve (Figure 5) and slightly significant negative values Tajima's and Fu and Li's estimators suggested a low degree of population expansion of group W in contrast with the stability of group DW (Table 4). In the W group only Italian and Balkan macro-regions presented an increasing trend in the mismatch plot consistent with significant negative values of Tajima and Fu and Li's statistics (see Table 4 and Figure 5), although these values were lower than two, suggesting caution in considering a hypothesis of actual expansion. In the group DW only central Europe population seem to present an actual expansion trend with significant negative values of the statistics (Tajima's D = -2,002; $p < 0,01$ and FU and Li's F = -1,106; $p < 0,05$; Table 4) and a sharp peak in the mismatch plot (Figure 5).

Divergence time estimates

Assuming the interval inside which fall the divergence point between the *Felis silvestris silvestris* and African wildcats clade (that includes *Felis silvestris libyca* from which derived *Felis silvestris catus*) as calibration point and the mutation rate inferred for the sequenced region (from Driscoll et al 2007; 2011) we detected different periods of differentiation for the European wildcat population. The Bayesian tree of Figure 2 including all the 28 haplotypes provided dating estimates for the best supported nodes of interest. The subtree W showed a main differentiation event at node 4 about 62 400 years BP (95 % HPD 21 860 – 118 910 years BP) between Group

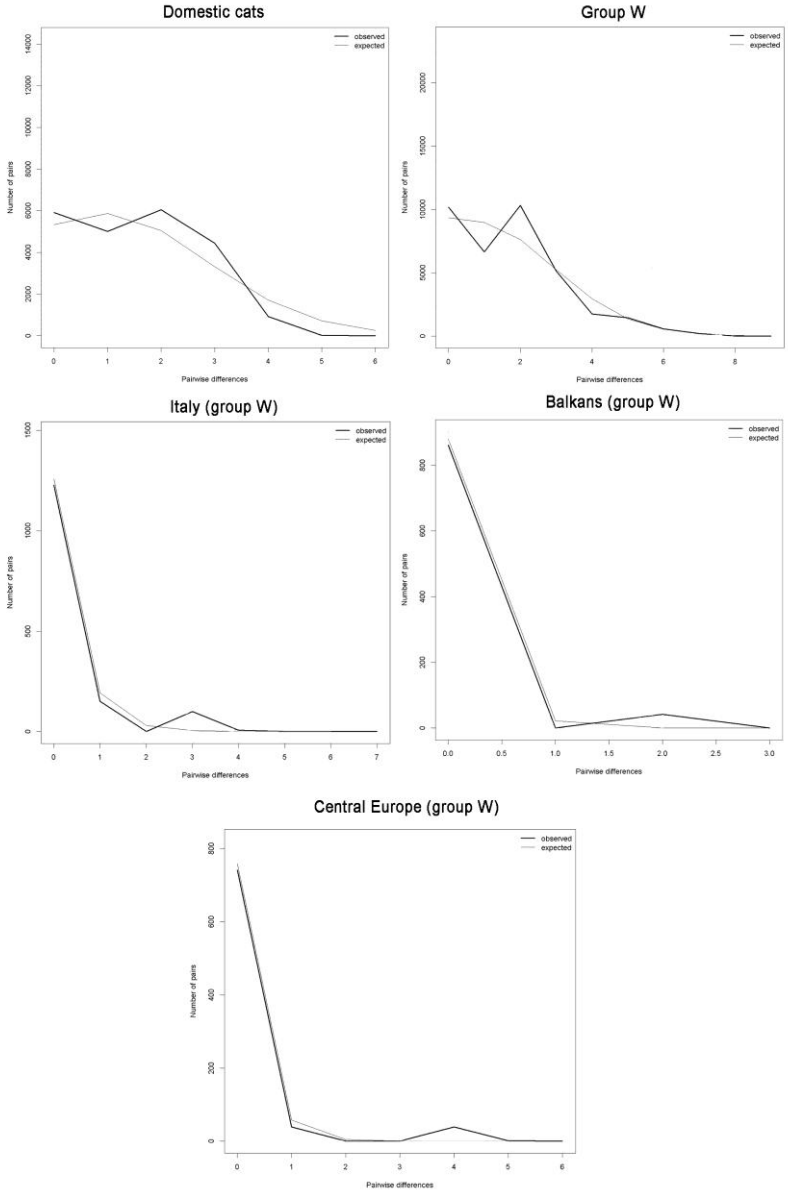

Figure 5. Mismatch distribution for the group for haplotypes that presented significant values of Tajima's D and Fu and Li's F indexes (see Table 4).

4 (mainly present in continental Europe and Iberian peninsula) and the mostly Mediterranean/Scottish haplotypes of Group 3. The clade D presented two main points of differentiation. The first one (node 2) dating back 80 000 y BP (95% HPD 31 561 – 145 850 y BP) and separating the group in the two major Group 1 and Group 2. A second important divergent point (node 3) occurred in Group 2 about 37 700 y BP (95% HPD 11 992 – 76 931 y BP) in which began the differentiation between the two shared haplotypes with the highest frequency of wildcat with respect to domestic cats (Dw4 and Dw6) from the rest of domestic / shared haplotypes (Figure 2).

DISCUSSION

We performed a phylogenetic analysis on 718 samples collected across Europe sequencing a 669 bp portion of ND5 gene of mtDNA. All samples were previously typed at 31 microsatellite loci and assigned to its putative population (wildcat, domestic cat or hybrid) by means of Bayesian clustering. We found two main phylogenetic groups (W and D) whose haplotypes showed respectively the seven characteristic polymorphisms of the two analysed subspecies *Felis s. silvestris* and *Felis s. catus* (which share the same polymorphisms with the *F. s. libyca*). Assuming that the microsatellite assignment analyses correctly identified the population of origin of wild and domestic cats, and the admixed genotypes of putative hybrids, we found 249 wildcat (58,9% of the total), 11 hybrids (15,3%) and no domestic cat carrying wild mitochondrial haplotypes (haplogroup W), while all 212 domestic cats, 174 wildcat (40,1% of the total) and 60 hybrids (83,3%) presented haplotypes of group D. A first hurdle in the interpretation of data derived from mtDNA (specifically from the ND5 region) to explain the phylogenetic history of the European wildcat is the presence of some degree of haplotypes sharing between domestic cat and wildcat. Other authors identified a number of cases of mtDNA/nuclear discordances, which were attributed to past *Felis s. catus* mtDNA introgression events since there are no evidence of recent important translocations of Felis s. libyca (the subspecies from which derived the domestic form) in Europe (Nussberger, Weber *et al.* 2007, Kery, Gardner *et al.* 2011, Nussberger, Greminger *et al.* 2013, Nussberger, Wandeler *et al.* 2014), Drisoll et al 2011, McEwing *et al.* 2012). Chromosomal recombinations during reiterated backcrossing events could, and the more rapidly evolving microsatellites, may have erased the admixture signals at the nuclear loci after a few generations (Va¨ha¨ and Primmer 2006). An alternative hypothesis arose was that ancestrally shared haplotypes, which originated in the common ancestor of *F. s. silvestris* and *F. s. libyca* (Hertwig, Schweizer *et al.* 2009) before subspecies splitting and before domestication, have been retained in some non-hybridizing European wildcat populations. Our domestic cat sample presented a quite high level of haplotype diversity (0,7350 ± 0,0180) and seemed to originate from at least two different matrilineage dating back about 80 000 Y BP (Figure 2) reflecting the multiple lineage origin of the taxon

according to Driscoll et al (2007). Following the past domestic introgression hypothesis, we expected to find the putative introgressed wild individuals to show a pretty balanced frequency of domestic haplotypes or at least to present few frequent haplotypes (for a "founder-effect" of few cross-mating domestic individuals) but, however, relatively abundant in the domestic cat populations since it is less probable that the supposed hybridization events occurred only between few haplotypes so poorly represented in domestic cats. But, according to our data, these haplotypes were not equally distributed across the wildcat individuals and they were most frequent (74,7%, Table S3) in haplotypes Dw4 and Dw6 included in a separate clade (Figure 2). Furthermore haplotype Dw4 was present only in 12,3% of our domestic cats and none of them presented the haplotype Dw6. The geographic distribution of these discordant haplotypes (group DW, Figure 4) and the scarce genetic differentiation among macro-regions of this group (Table 3) seemed to reflect a wide distribution of these haplotype (especially Dw4) in Europe while we could expect regionally localized hybridization events because of the relative low vagility of the species (Bizzarri et al 2010, Horn et al 2011, Anile personal communications) and the recent habitat fragmentation. Only Iberian peninsula and Scotland appeared to reflect this scenario (Figure 4). Trying to explain the phylogenetic history of these haplotypes applying the hypothesis of ancient haplotype sharing we might assume that the seven polymorphisms that today seem to be fixed in the domestic subspecies (as well as in *Felis s. libyca* from which it derived) were already present in a recent common ancestor of the two clades and that a wildcat lineage inherited them evolving independently until today. But the clade of haplotype Dw4 and Dw6 seemed have experienced an isolation and a differentiation from about 37 700 y BP (95% HPD 11 992 – 76 931 y BP). If this estimate long predates any evidence for cat domestication according to the last archaeological and genetic findings (Vigne *et al.* 2004, Driscoll, *et al.* 2007, Vigne 2011, Hu*et al.* 2014) it appeared too recent to support a pre-(sub)speciation origin. Indeed it is quite improbable that starting the divergence process between European wildcat and the African wildcats lineages before 173 000 years ago (Driscoll et al 2007) a hypothetical European wildcat lineage carrying polymorphism of group D evolved in the very same way to those that led to the today clade of Group 2 and started to diverge only in the last tens of thousands years. Kurtén data (1965, 1968), updated by Macdonald *et al.* (2010), introduced the hypothesis of a late Pleistocene (about 50 000 years ago) southernward waves of European wildcats towards the Levant (being the two subspecies still almost in contact between the Syrian coasts and the Turkish Taurus). This event could have led to a syntopic condition of *Felis s. silvestris* with the *Felis s. libyca* featured by the future domestic-like mtDNA polymorphisms. In this scenario the two subspecies might have experienced a degree of genetic flow, part of which could have later (37 700 years ago?) returned in Europe. However other shared haplotypes (Dw1, Dw2, Dw3, Dw5, Dw7) seem to be recently differentiated and the high frequency of domestic cats let suppose a more recent by-introgression origin. The

hypothesis introduced above could be an alternative or, more likely, a supplementary explanation for the haplotypic sharing between wildcats and domestic cats introducing a more complex evolutionary history for the European wildcat population. This hypothesis would also influence the understanding of hybridization direction since we detected domestic haplotypes in the 90,2 % of admixed individuals. If we assume the more recent introgression hypothesis it would come up that the most probable direction could be between females domestic cats and males wildcats. But since the highest frequency of admixed individuals fell in Dw4 haplotype, if we assume an ancient haplotype exchange between *F. s. silvestris* and *F. s. libyca* we could not reject the inverse hypothesis (male domestic cats and female wildcat).

The phylogenetic analyses of European wildcat population characterized by wild uniparental haplotypes (haplogroup W) separated this clade in two main subtrees (Groups 3 and 4, Figure 2) that partly reflected the geographic structure showed in Figure 4 and emerged in the SAMOVA analysis. So we found three main geographic repartitions: 1) a wide continental European zone (that however included also part of the Iberian peninsula) featuring a greater genetic variability (see Table 2) dominated by haplotype W4 (with Portugal that has a certain degree of isolation with the private haplotype W8) 2) the Italian peninsula with a low genetic variability and the dominant haplotype W1, with the private haplotype W5 in Sicily as the consequence of its isolation (Pierpaoli, Biro *et al.* 2003, Mattucci *et al.* 2013); 3) the monomorphic free-ranging Scottish cats that showed only one differentiated haplotype that we found only in another one German individual (ID 760). In general there was a certain degree of haplotype sharing between the Mediterranean and Easter Europe populations. Taking into account the number of individuals carrying haplogroup W haplotypes the 90,9% of Italian samples (n = 50) and 15% individual from Iberian peninsula (n = 6) presented the haplotype W1 as well as half (n = 4) of the samples from Eastern Europe (three admixed individual from Hungary and one from Poland). In particular the pairwise FST values between Italy and Iberian peninsula was lower than the average (0.465). This macro-regions distribution reflects that of previous studies carried out on microsatellites describing a clear differentiation between central and southern Europe (Pierpaoli *et al.* 2003), and fits in the paleo-geographic framework that sees in the last glacial cycle the main driver of isolation and subsequent differentiation (Taberlet et al 1998, Hewitt 2001, Randi 2007). Indeed the estimated time of differentiation between the central and southern group of haplotypes (Group 3 and 4, Figure 2) was about 62 400 years BP (95 % HPD 21 860 – 118 910 years BP), during the Pleniglacial period, and three of the macro-region of haplogroup W (Italy, central Europe and Balkans, Figure 4) seemed to have faced a population expansion. The lack of archaeological findings attributable to the presence of wildcat during Pleniglacial in central Europe in contrast with the relative abundance in Mediterranean regions (Sommer and Benecke 2006) and the supposed extra-Mediterranean *refugia* (Stewart *et al.* 2001; Schmitt and Varga 2012) suggested a survival of wildcat population in central/southeast Europe

during last glaciations and a consequent recolonization after a bottleneck preferably by this group. This is supported also by the pervasive presence in continental Europe one haplotype (W4). In this light, the Italian macro-region could be considered an area of genetic isolation, closed by Alpine hinge (Sommer and Benecke 2006), which maintained a certain degree of genetic flow with other southern areas of Europe (Iberian peninsula and south-eastern Europe). This pattern of continuous gene flow across southern Europe has been also reported in European pine marten (*Martes martes*, Ruiz-Gonzalez *et al.* 2013) or brown bear (*Ursus arctos*) populations (Valdiosera *et al.*, 2007). The habitat fragmentation did not seem to have affected the genetic structure, since there was not a clear sub-region differentiation among haplotypes (Table 3). But this result can be easily explained considering the type of uniparental marker used for this study and its slow mutation rate that do not allow reliable differentiation estimates for recent isolation events. Indeed studies involving more quick-evolving microsatellites detected a significant fragmentation effect in the genetic structure, in particular in Germany (Pierpaoli *et al.* 2003). Nevertheless this study detected at least three different main evolutionary significant units (ESUs) for Scotland, Italian and continental Europe (including Iberian peninsula, central Europe and Balkans macro-regions) with further signs of differentiation in the Portugal sub-region.

CONCLUSIONS

This study analysed some aspects of phylogeography of wildcat using a mtDNA marker (ND5 region). The results revealed a complex evolutionary history that includes hypothesis of relatively past domestic introgressions in the wildcat population and the possibility of a more ancient migration phase with a cohabitation and subsequent gene flow with populations of *Felis s libyca*, the ancestor of domestic cats, during late Pleistocene that today has left traces in the uniparental heritage. It is of crucial importance to understand this phenomena since they could affect the correct way to use this promising uniparental mitochondrial marker in order to help knowing the actual genetic status of the species. To better investigate this hypothesis and assess their likelihood the sample size should be expanded including specimens of European and African wildcats from the border areas of respective ranges (from Turkey to the Near East). Furthermore phylogenetic time estimates should also be applied to more fast-evolving nuclear markers (microsatellites or SNPs) harvesting the last genomic tools to enlarge the set of markers and compare the results. A migration by isolation analysis could assess the degree of gene flow among populations and shed light on the processes of post-glacial recolonization. This information, especially those regarding the haplotype sharing between domestic cats and wildcats, is going to be crucial to assess the level of introgression of domestic alleles in the wild populations and to

outline regional genetic peculiarities to allow the development of proper conservation projects and tools.

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	Haplogroup D													Haplogroup W												
	d1	d2	d3	d4	d5	d6	d7	dw1	dw2	dw3	dw4	dw5	dw6	dw7	w1	w2	w3	w4	w5	w6	w7	w8	w9	w10	w11	w12	w13	w14
Italy																												
It - cnt	1							21, **15**	7	22, **1**	8, **37**			**1**	**45**									**1**	**1**			
It - Sicily															**5**				**3**									
Balkans																												
Balk - N								1, **1**		1, **1**	**13**		**3**					**12**										
Balk - ItNE		1									**20**	**14**	**5**		**1**			**30**										
Balk - S											**4**																	
Iberian Pen																												
IB - Sp			4	2				18		29, **3**	5, **2**				**2**			**14**					**2**			**1**		
IB - Prt			12		1	1	1	7	2	21, **3**	10				**4**			**8**				**9**						
Central Eur																												
Eu - cnt											**15**		**1**				**1**	**13**		**5**								
Eu - WSW		1	1					7	3	7	3, **24**	**1**			**2**	**24**		**46**		**11**	**14**							**1**
Eastern Eur																												
EastEu - S								*12*, **1**		*15*, **2**	*25*, **1**	*2*			*3*	*1*												
EastEu - N								12, **1**		1	**5**	2			**1**	**1**		**1**									**1**	
Scotland									*7*								*7*											

Supplementary Table S1. Number of individuals of each haplotypes for the macro-regions and sub-regions as defined in Table 1. The haplotypes from the 717 individuals are divided into the two main groups D and W (see Results for details). Number of domestic cats are in a normal font, wildcat are in bold and hybrids are in underlined italics.

Supplementary Table S2. Pairwise FST tests between pairs of macro-regions and sub-regions. See Table 1 for macro and sub-regions definitions.

Macro - W	Italy	Bakans	Iberian Pen	Central Eur	Eastern Eur	Scotland
Italy	0,000					
Bakans	0,896	0,000				
Iberian Pen	0,465	0,276	0,000			
Central Eur	0,701	0,141	0,267	0,000		
Eastern Eur	0,479	0,678	0,034	0,320	0,000	
Scotland	0,911	0,984	0,712	0,823	0,802	0,000

Macro - DW						
Italy	0,000					
Bakans	0,031	0,000				
Iberian Pen	0,172	0,165	0,000			
Central Eur	0,187	0,140	0,652	0,000		
Eastern Eur	0,016	0,059	0,070	0,264	0,000	
Scotland	0,552	0,504	0,817	0,901	0,510	0,000

Sub W	It cnt	It Sicily	Balk N	Balk ItNE	IB Sp	IB Prt	Eu cnt	Eu WSW	EastEu S	EastEu - N
It - cnt	0,000									
It - Sicily	0,294	0,000								
Balk - N	0,906	0,910	0,000							
Balk - ItNE	0,901	0,907	0,037	0,000						
IB - Sp	0,734	0,599	0,029	0,057	0,000					
IB - Prt	0,418	0,288	0,477	0,554	0,294	0,000				
Eu - cnt	0,774	0,639	0,103	0,174	0,079	0,387	0,000			
Eu - WSW	0,718	0,675	0,111	0,146	0,145	0,470	0,024	0,000		
EastEu - S	0,305	0,174	0,779	0,806	0,329	0,036	0,382	0,469	0,000	
EastEu - N	0,709	0,493	0,469	0,520	0,016	0,100	0,071	0,139	0,056	0.00000

Sub DW										
Balk - N	0									
Balk - ItNE	0,154	0,000								
Balk - S	0,084	0,117	0,000							
IB - Sp	0,207	0,108	0,452	0,000						
IB - Prt	0,518	0,184	1,000	0,118	0,000					
Eu - cnt	0,007	0,225	0,135	0,598	0,899	0,000				
Eu - WSW	0,023	0,209	0,140	0,433	0,747	0,009	0,000			
EastEu - S	0,178	0,080	0,173	0,003	0,076	0,267	0,238	0,000		
EastEu - N	0,042	0,032	0,081	0,101	0,444	0,122	0,010	0,033	0,000	

ID	Origin	qw	qw C.I.	H
70	Italy	0,968	(0,840-1,000)	Dw1
73	Italy	0,943	(0,808-1,000)	Dw1
285	Italy	0,985	(0,911-1,000)	Dw1
511	Italy	0,987	(0,922-1,000)	Dw1
574	Italy	0,985	(0,914-1,000)	Dw1
659	Italy	0,992	(0,951-1,000)	Dw1
662	Italy	0,955	(0,808-1,000)	Dw1
894	Italy	0,988	(0,925-1,000)	Dw1
902	Italy	0,991	(0,948-1,000)	Dw1
906	Italy	0,94	(0,812-1,000)	Dw1
914	Italy	0,949	(0,788-1,000)	Dw1
1061	Slovenia	0,995	(0,972-1,000)	Dw1
1217	Poland	0,993	(0,959-1,000)	Dw1
1251	Italy	0,961	(0,853-1,000)	Dw1
1253	Italy	0,971	(0,875-1,000)	Dw1
1284	Italy	0,99	(0,937-1,000)	Dw1
1299	Italy	0,992	(0,952-1,000)	Dw1
1346	Hungary	0,926	(0,750-1,000)	Dw1
124	Italy	0,95	(0,808-1,000)	Dw3
296	Portugal	0,992	(0,954-1,000)	Dw3
317	Portugal	0,994	(0,967-1,000)	Dw3
616	Hungary	0,994	(0,962-1,000)	Dw3
708	Portugal	0,994	(0,966-1,000)	Dw3
1065	Slovenia	0,979	(0,881-1,000)	Dw3
1093	Spain	0,987	(0,921-1,000)	Dw3
1133	Spain	0,959	(0,792-1,000)	Dw3
1194	Spain	0,983	(0,896-1,000)	Dw3
1349	Hungary	0,936	(0,766-1,000)	Dw3
71	**Italy**	**0,996**	**(0,974-1,000)**	**Dw4**
72	**Italy**	**0,996**	**(0,976-1,000)**	**Dw4**
84	**Italy: eastern Alps**	**0,995**	**(0,970-1,000)**	**Dw4**
92	**Italy**	**0,975**	**(0,874-1,000)**	**Dw4**
94	**Italy**	**0,987**	**(0,921-1,000)**	**Dw4**
95	**Italy**	**0,983**	**(0,906-1,000)**	**Dw4**
97	**Italy**	**0,993**	**(0,956-1,000)**	**Dw4**
171	**Germany: central**	**0,979**	**(0,891-1,000)**	**Dw4**
188	**Belgium**	**0,996**	**(0,976-1,000)**	**Dw4**
197	**Belgium**	**0,997**	**(0,981-1,000)**	**Dw4**
200	**Belgium**	**0,996**	**(0,978-1,000)**	**Dw4**
201	**Belgium**	**0,995**	**(0,968-1,000)**	**Dw4**
204	**Bulgaria**	**0,953**	**(0,806-1,000)**	**Dw4**
206	**Bulgaria**	**0,989**	**(0,933-1,000)**	**Dw4**
216	**Hungary**	**0,988**	**(0,931-1,000)**	**Dw4**
368	**Germany: central**	**0,994**	**(0,964-1,000)**	**Dw4**
372	**Germany: central**	**0,995**	**(0,971-1,000)**	**Dw4**
377	**Germany: central**	**0,988**	**(0,926-1,000)**	**Dw4**
384	**Germany: central**	**0,994**	**(0,964-1,000)**	**Dw4**
385	**Germany: central**	**0,988**	**(0,930-1,000)**	**Dw4**
388	**Germany: central**	**0,996**	**(0,977-1,000)**	**Dw4**
389	**Germany: central**	**0,996**	**(0,977-1,000)**	**Dw4**
397	**Germany: south western**	**0,986**	**(0,921-1,000)**	**Dw4**
403	**Germany: south western**	**0,983**	**(0,900-1,000)**	**Dw4**
407	**Germany: south western**	**0,995**	**(0,969-1,000)**	**Dw4**
489	**Italy**	**0,994**	**(0,964-1,000)**	**Dw4**
491	**Italy**	**0,996**	**(0,979-1,000)**	**Dw4**
493	**Italy: eastern Alps**	**0,99**	**(0,938-1,000)**	**Dw4**
507	**Italy**	**0,995**	**(0,968-1,000)**	**Dw4**
512	**Italy**	**0,963**	**(0,830-1,000)**	**Dw4**
513	**Italy**	**0,994**	**(0,961-1,000)**	**Dw4**
514	**Italy**	**0,976**	**(0,869-1,000)**	**Dw4**
519	**Italy**	**0,973**	**(0,872-1,000)**	**Dw4**
523	**Italy: eastern Alps**	**0,982**	**(0,907-1,000)**	**Dw4**
524	**Italy: eastern Alps**	**0,984**	**(0,904-1,000)**	**Dw4**
526	**Italy: eastern Alps**	**0,987**	**(0,922-1,000)**	**Dw4**
527	**Italy: eastern Alps**	**0,995**	**(0,972-1,000)**	**Dw4**
528	**Italy: eastern Alps**	**0,997**	**(0,980-1,000)**	**Dw4**
531	**Italy: eastern Alps**	**0,992**	**(0,953-1,000)**	**Dw4**
532	**Italy: eastern Alps**	**0,991**	**(0,944-1,000)**	**Dw4**
540	**Italy: eastern Alps**	**0,996**	**(0,974-1,000)**	**Dw4**
544	**Italy: eastern Alps**	**0,992**	**(0,954-1,000)**	**Dw4**
545	**Italy: eastern Alps**	**0,991**	**(0,943-1,000)**	**Dw4**
573	**Italy**	**0,995**	**(0,972-1,000)**	**Dw4**
591	**Luxembourg**	**0,985**	**(0,911-1,000)**	**Dw4**
631	**Italy**	**0,985**	**(0,913-1,000)**	**Dw4**
657	**Italy**	**0,995**	**(0,972-1,000)**	**Dw4**
658	**Italy**	**0,975**	**(0,869-1,000)**	**Dw4**
660	**Italy**	**0,972**	**(0,865-1,000)**	**Dw4**
673	**Italy**	**0,924**	**(0,769-1,000)**	**Dw4**
742	**Germany: central**	**0,995**	**(0,971-1,000)**	**Dw4**
743	**Germany: central**	**0,991**	**(0,942-1,000)**	**Dw4**
744	**Germany: central**	**0,993**	**(0,956-1,000)**	**Dw4**
748	**Germany: central**	**0,993**	**(0,956-1,000)**	**Dw4**
879	**Romania**	**0,932**	**(0,759-1,000)**	**Dw4**

880	**Romania**	**0,987**	**(0,922-1,000)**	**Dw4**	**1290**	**Italy**	**0,993**	**(0,958-1,000)**	**Dw4**
886	**Italy**	**0,979**	**(0,882-1,000)**	**Dw4**	**1291**	**Italy**	**0,994**	**(0,961-1,000)**	**Dw4**
888	**Italy**	**0,991**	**(0,945-1,000)**	**Dw4**	**1292**	**Italy**	**0,981**	**(0,890-1,000)**	**Dw4**
889	**Italy**	**0,983**	**(0,904-1,000)**	**Dw4**	**1298**	**Italy**	**0,965**	**(0,852-1,000)**	**Dw4**
890	**Italy**	**0,98**	**(0,889-1,000)**	**Dw4**	**1300**	**Italy**	**0,928**	**(0,774-1,000)**	**Dw4**
899	**Italy**	**0,964**	**(0,862-1,000)**	**Dw4**	**1305**	**Italy**	**0,993**	**(0,959-1,000)**	**Dw4**
910	**Italy**	**0,996**	**(0,979-1,000)**	**Dw4**	**1306**	**Italy**	**0,932**	**(0,807-1,000)**	**Dw4**
916	**Italy**	**0,996**	**(0,977-1,000)**	**Dw4**	**1308**	**Croatia**	**0,996**	**(0,976-1,000)**	**Dw4**
920	**Italy**	**0,995**	**(0,967-1,000)**	**Dw4**	**1325**	**Italy: eastern Alps**	**0,994**	**(0,967-1,000)**	**Dw4**
926	**Germany: south western**	**0,995**	**(0,969-1,000)**	**Dw4**	**1334**	**Italy: eastern Alps**	**0,993**	**(0,958-1,000)**	**Dw4**
938	**Germany: south western**	**0,996**	**(0,976-1,000)**	**Dw4**	**1337**	**Italy: eastern Alps**	**0,994**	**(0,961-1,000)**	**Dw4**
941	**Germany: south western**	**0,991**	**(0,948-1,000)**	**Dw4**	**1339**	**Italy: eastern Alps**	**0,994**	**(0,964-1,000)**	**Dw4**
944	**Germany: south western**	**0,995**	**(0,969-1,000)**	**Dw4**	**1385**	**Germany: south western**	**0,993**	**(0,961-1,000)**	**Dw4**
946	**Germany: south western**	**0,996**	**(0,978-1,000)**	**Dw4**	**1397**	**Germany: central**	**0,989**	**(0,936-1,000)**	**Dw4**
947	**Germany: south western**	**0,997**	**(0,979-1,000)**	**Dw4**	**1399**	**Germany: south western**	**0,996**	**(0,975-1,000)**	**Dw4**
951	**Germany: south western**	**0,992**	**(0,954-1,000)**	**Dw4**	**1410**	**Germany: south western**	**0,99**	**(0,941-1,000)**	**Dw4**
989	**Italy: eastern Alps**	**0,995**	**(0,971-1,000)**	**Dw4**	**1413**	**Germany: south western**	**0,996**	**(0,975-1,000)**	**Dw4**
1000	**Italy: eastern Alps**	**0,992**	**(0,951-1,000)**	**Dw4**	**1424**	**Germany: south western**	**0,996**	**(0,973-1,000)**	**Dw4**
1001	**Italy: eastern Alps**	**0,991**	**(0,948-1,000)**	**Dw4**	**1429**	**Germany: south western**	**0,983**	**(0,902-1,000)**	**Dw4**
1009	**Italy**	**0,974**	**(0,877-1,000)**	**Dw4**	**1436**	**Germany: south western**	**0,995**	**(0,973-1,000)**	**Dw4**
1025	**Spain**	**0,975**	**(0,861-1,000)**	**Dw4**	**1438**	**Germany: central**	**0,996**	**(0,975-1,000)**	**Dw4**
1029	**Slovenia**	**0,977**	**(0,877-1,000)**	**Dw4**	**1442**	**Germany: south western**	**0,996**	**(0,978-1,000)**	**Dw4**
1032	**Slovenia**	**0,994**	**(0,964-1,000)**	**Dw4**	**1444**	**Germany: south western**	**0,996**	**(0,974-1,000)**	**Dw4**
1035	**Slovenia**	**0,993**	**(0,956-1,000)**	**Dw4**	**1446**	**Germany: central**	**0,991**	**(0,944-1,000)**	**Dw4**
1038	**Slovenia**	**0,995**	**(0,970-1,000)**	**Dw4**	492	Italy: eastern Alps	0,991	(0,946-1,000)	Dw5
1041	**Slovenia**	**0,996**	**(0,976-1,000)**	**Dw4**	525	Italy: eastern Alps	0,996	(0,977-1,000)	Dw5
1042	**Slovenia**	**0,994**	**(0,967-1,000)**	**Dw4**	533	Italy: eastern Alps	0,995	(0,969-1,000)	Dw5
1044	**Slovenia**	**0,996**	**(0,974-1,000)**	**Dw4**	538	Italy: eastern Alps	0,955	(0,829-1,000)	Dw5
1047	**Slovenia**	**0,996**	**(0,977-1,000)**	**Dw4**	547	Italy: eastern Alps	0,994	(0,962-1,000)	Dw5
1048	**Slovenia**	**0,996**	**(0,976-1,000)**	**Dw4**	572	Italy: eastern Alps	0,97	(0,860-1,000)	Dw5
1051	**Slovenia**	**0,994**	**(0,962-1,000)**	**Dw4**	674	Austria	0,967	(0,852-1,000)	Dw5
1057	**Slovenia**	**0,996**	**(0,979-1,000)**	**Dw4**	999	Italy: eastern Alps	0,991	(0,946-1,000)	Dw5
1073	**Slovenia**	**0,996**	**(0,974-1,000)**	**Dw4**	1004	Italy: eastern Alps	0,992	(0,950-1,000)	Dw5
1094	**Spain**	**0,996**	**(0,977-1,000)**	**Dw4**	1315	Italy: eastern Alps	0,995	(0,967-1,000)	Dw5
1221	**Poland**	**0,994**	**(0,964-1,000)**	**Dw4**	1316	Italy: eastern Alps	0,996	(0,973-1,000)	Dw5
1222	**Poland**	**0,995**	**(0,969-1,000)**	**Dw4**	1317	Italy: eastern Alps	0,995	(0,971-1,000)	Dw5
1223	**Poland**	**0,991**	**(0,947-1,000)**	**Dw4**	1324	Italy: eastern Alps	0,952	(0,831-1,000)	Dw5
1226	**Poland**	**0,99**	**(0,941-1,000)**	**Dw4**	1327	Italy: eastern Alps	0,996	(0,976-1,000)	Dw5
1230	**Poland**	**0,995**	**(0,971-1,000)**	**Dw4**	1333	Italy: eastern Alps	0,974	(0,869-1,000)	Dw5
1267	**Italy: eastern Alps**	**0,977**	**(0,887-1,000)**	**Dw4**	**82**	**Italy: eastern Alps**	**0,995**	**(0,969-1,000)**	**Dw6**
1287	**Italy**	**0,932**	**(0,789-1,000)**	**Dw4**	**985**	**Italy: eastern Alps**	**0,996**	**(0,975-1,000)**	**Dw6**
1288	**Italy**	**0,996**	**(0,976-1,000)**	**Dw4**	**988**	**Italy: eastern Alps**	**0,986**	**(0,917-1,000)**	**Dw6**

991	**Italy: eastern Alps**	**0,995**	**(0,972-1,000)**	**Dw6**	**1312**	**Italy: eastern Alps**	**0,996**	**(0,977-1,000)**	**Dw6**
1040	**Slovenia**	**0,994**	**(0,965-1,000)**	**Dw6**	**1340**	**Austria**	**0,987**	**(0,922-1,000)**	**Dw6**
1067	**Slovenia**	**0,988**	**(0,926-1,000)**	**Dw6**	1302	Italy	0,979	(0,881-1,000)	Dw7
1068	**Slovenia**	**0,993**	**(0,957-1,000)**	**Dw6**					

Supplementary Table S3. Llist of the 174 Wildcat samples presenting haplotypes of group *D.*Individuals showing haplotype Dw4 and Dw6 are in bold. ID: sample identification number; Origin: sampling country; qw: wildcat membership proportion from 31 STR Bayeisian analyses; qw C.I : 90%: confidential interval.

GENERAL DISCUSSION

The general aim of this thesis was the development of a multidisciplinary approach to the study of the European wildcat (*Felis silvestris silvestris*), and the assessment of its conservation status. I used a number of different disciplinary fields, including non-invasive monitoring, conservation genetics, phylogenetics and phylogeography. The research project consisted of three fundamental steps: 1) to carry out an effective and reliable non-invasive sampling strategy for wildcat monitoring; 2) to deepen and improve the use of the most recently-discovered genetic markers to discriminate among subspecies and assess the levels of hybridization and introgression; 3) to clarify the phylogeography of the species and the patterns of population diversification in its biogeographical history.

The first part concerned the development of a non-invasive sampling strategy aimed at maximizing the quantity and quality of the data collected while minimizing the sampling effort In our research we integrated the main non-invasive monitoring techniques used for wildcat monitoring, including camera-trapping (Mulder 2007; Bryce 2011; Can *et al.* 2011; Anile *et al.* 2012a), scented lures for hair-trapping (Kery *et al.* 2011; Monterroso *et al.* 2011; Steyer *et al.* 2013) and scat survey (Anile *et al.* 2014; Lozano *et al.* 2013). These three techniques were stratified using an experimental design based on systematic sampling and the opportunity to combine information resulting from two different sources (genetic and photographic), thus balance the drawbacks of each one. The biological samples were genetically analysed to identify individual genotypes and assign each sample to its putative subspecies (*Felis s. silvestris*, *Felis s. catus* or their hybrids) using 10 microsatellite loci (Menotti-Raymond and O'Brien 1995; Menotti-Raymond *et al.* 1997) and the ND5 region of mtDNA (Driscoll *et al.* 2011) for subspecies identification and introgression detection.

This protocol was applied in a portion of the Foreste Casentinesi National Park, in the northern Apennines, as this region was known in recent bibliography (Ragni et al 2014) as the northern limit of ascertained wildcat distribution in peninsular Italy. The results confirmed the presence of the species with a number of wildcat individuals ranging from six (from genetic analyses) to nine (from camera-

trapping), finding a balanced sex proportion. Furthermore, using camera-trapping results, we revealed the presence of domestic cats (at least 5) living in sympatry with wildcats and a putative pregnant hybrid whose presence was not detected by genetic sampling. Whenever possible, we integrated genetic and photographic information. Some individuals with clear wild-type phenotype showed traces of introgression at mtDNA and/or high qi (> 0,80) values of membership to the wildcat cluster after Bayesian analyses on microsatellite loci, but carried a mitochondrial haplotype with polymorphisms that are typical of domestic subspecies *Felis s. catus* or of its most recent progenitor, the *Felis s. lybica* (Driscoll *et al.* 2007; Vigne *et al.* 2011). This discordance was possibly due to traces of past genetic introgression (Driscoll *et al.* 2011; Hertwig *et al.* 2009; Nussberger *et al.* 2013).

Moreover, it was possible to determine some behavioural and ecological aspects of the species. This method has proved to be effective and allowed to evaluate the reliability of each approach. When taken separately, the results were sensibly different from those found in other studies, in particular with respect to the season in which the method was applied (Steyer *et al.* 2013) and the effects on the individuals of the attractor used (tincture of *Valeriana officinalis*) (Anile *et al.* 2012b; Kery *et al.* 2011; Steyer *et al.* 2013). These comparisons allowed us also to highlight the pros and cons of the sampling techniques, outlining further directions for their improvement.

Thus, in the second part of this work we tried to improve the latest molecular tools for a more reliable use in subspecies and hybridization assessment. Although non-invasive genetics greatly improved in the last 20 years the study and conservation of elusive species presents several issues related to the quality of the samples, the variables in genotyping error rates (Taberlet *et al.* 1999; Broquet *et al.* 2007; Knapp *et al.* 2009; Marucco *et al.* 2011; Uno *et al.* 2012; Lampa *et al.* 2013; Monterroso *et al.* 2013) and to the cost-benefit ratio in type and quantity of molecular markers needed to identify different degrees of hybridization between wildcat and domestic cat (Driscoll *et al.* 2011; Nussberger *et al.* 2013; Nussberger *et al.* 2014). Over the last decades, next-generation sequencing (NGS) technologies generated a large quantity of nucleotide sequence data providing an increasing number of molecular markers. Single nucleotide polymorphisms (SNPs) appeared promising molecular markers because of their reduced propensity for homoplasy due to (i) lower mutation rates, higher density and more uniform distribution across the genomes; (ii) suitability for successful high-throughput genotyping, straightforward comparability and transportability across laboratories and detection protocols, and highly successful application in fragmented DNA samples, e.g. non-invasive and historical DNA (Brumfield *et al.* 2003; Morin *et al.* 2004; Garvin *et al.* 2010).

Thus, in collaboration with Dr. Federica Mattucci (Laboratory of genetics, ISPRA, Bologna), we developed, on a final sample of 150 individuals collected from 13 different countries of European wildcat range, a two-step protocol for assessing ‘pure’ reference cats (wild and domestic), and for estimating introgression in

conspecific wildcats. Starting from the analysis of an initial SNP pool coming from the Illumina Infinium iSelect 63K Cat DNA Array we extracted the minimum number of highly informative SNPs (n = 151) that were able to efficiently detect the subspecies and the current levels of hybridization between wild (*Felis s. silvestris*) and domestic (*Felis s. catus*) cats. We first tested them on simulated pure and admixed genotypes, then on known captive admixed individuals and finally on the 150 samples, previously analysed with 31 STR markers. The results were then crossed with the data from uniparental haplotypes resulting from sequencing two regions of mtDNA (portions of the control region, from Freeman *et al.* 2001 and the ND5 gene, from Driscoll *et al.* 2011 for a total of 1273 bp) and two markers of the Y chromosome (SMCY STR-7, Luo *et al.* 2007 and a portion of the SRY gene Nussberger *et al.* 2013) which were analysed independently. The panel of 151 SNPs proved to be extremely effective in hybrids identification up to the second generation backcross. Furthermore, a remarkable narrowing of the confidence intervals in individual clustering makes these markers much more reliable than traditional microsatellite loci, especially when assigning individuals with wild/domestic genetic proportions (*qi*) near to the detection threshold of 0,80. The uniparental markers proved to be efficient in subspecies identification and once crossed with nuclear information (SNPs or STRs) showed some matrilineal and patrilineal discordances, thus providing the signature and direction of potentially distant admixture events. However, time estimates, haplotypes frequency and the geographic distribution of some of the discordant haplotypes suggested different possible origins for the shared haplotypes.

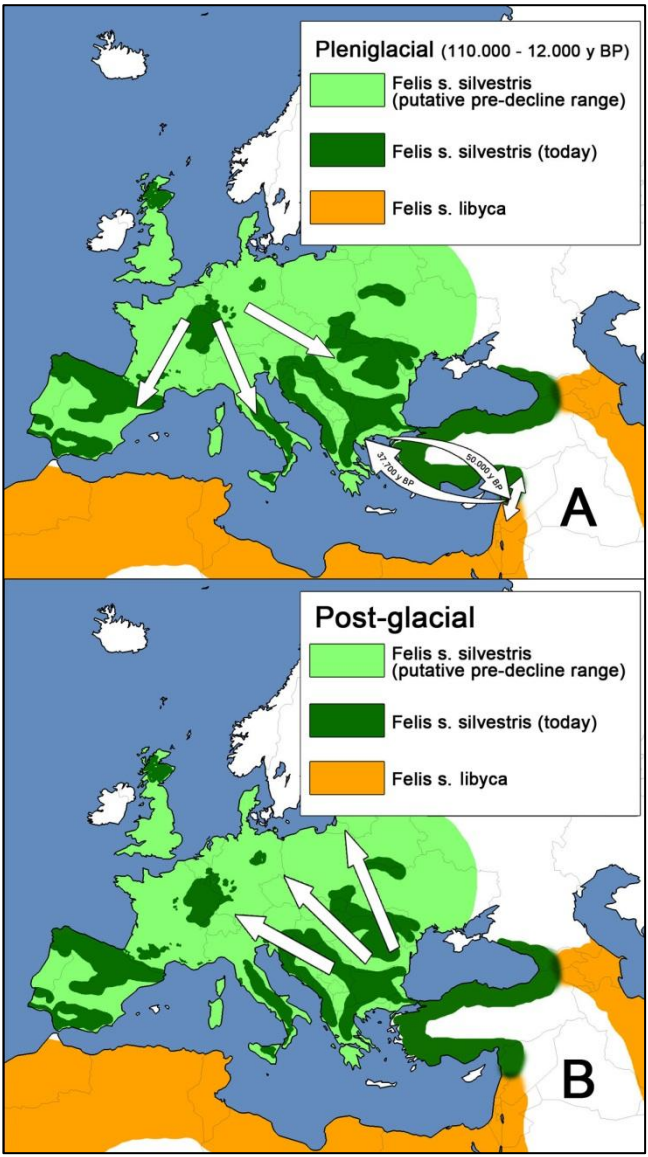
Based on the the available evidences (Driscoll *et al.* 2007) the evolutionary divergence between European wildcat (*Felis silvestris silvestris*) and the *Felis silvestris libyca/catus* clade led to several mitochondrial differentiations that should discriminate the two main groups. However in this work as well as in other studies (Driscoll *et al.* 2007; Hertwig *et al.* 2009) a relatively abundant number of wildcat individuals showed mitochondrial haplotypes typical of the domestic/*lybica* clade. Three main non-exclusive hypotheses could be proposed in order to explain this cytonuclear discordance (Moran and Kornfield 1995; Verheyen *et al.* 2003; Driscoll *et al.* 2007; Driscoll *et al.* 2009; Driscoll *et al.* 2011; Hertwig *et al.* 2009; Macdonald *et al.* 2010):

1. Since there are no evidences of *lybica* anthropogenic translocation in Europe in historical times, the cytonuclear discordance could represent the trace of an old domestic introgression derived from centuries of sympatry between the *silvestris/catus* subspecies. Such introgression could have not been detected by nuclear markers (in particular microsatellites) because chromosomal recombinations during reiterated backcrossing events may have erased the admixture signals at the nuclear loci after a few generations (Va¨ha¨ and Primmer 2006).

2. Some haplotypes are highly conserved in comparison to others and, therefore, could represent the ancient heritage from the common ancestor of *F. s. silvestris* and *F. s. lybica.*
3. As a result of migratory events in late Pleistocene, a certain degree of gene flow occurred between *Felis s. silvestris* and *Felis s. libyca* before any domestication process and left traces in part of the current wildcat population..

These last considerations extended the research perspectives of the third part of the work. Thus, in addition to analysing some aspects of the phylogeography of the species in Europe, we took advantage of the larger sample size (718 samples, including domestic cats, wildcats and admixed individuals, from 14 different countries) to investigate the hypotheses left open by the previous works on the origin of haplotypes shared between domestic cat and European wildcat. Starting from the information derived from the previous genotyping performed on the samples at 31 microsatellite loci, which provided a preliminary subspecies identification (Mattucci et al 2014), we further characterized a 669 bp portion of the ND5 region on the mtDNA that could to be useful in defining the subspecies and in describing the evolutionary history if the taxon (Driscoll *et al.* 2007). We found that the 40,1% of the wildcat carried a "domestic" haplotype and the 74,7% of these presented haplotypes (Dw4 or Dw6) which were present only in the 12% of domestic individuals. The calculations dated the time of divergence of this group back about to 37 700 y BP, clearly before any ascertained domestication process, and were consistent with the estimates performed in chapter 2. The Phylogeographic analyses found that this group is uniformly distributed across Europe.

Since our domestic cat sample presented a quite high level of haplotype diversity (0,7350 ± 0,0180, see chapter 3) and seemed to originate from at least two ancient matrilineages, if we assumed that the cytonuclear discordance of some wildcat individuals originated only from old domestic introgression we would expect to find a balanced distribution of the putative introgressed haplotypes in the introgressed wildcat population and a relatively clear geographic structure (e.g. in a localized hybridization scenario) Alternatively few common haplotypes should be present (for a "founder-effect" of few cross-mating domestic individuals), however with a consistent frequency in domestic cats, since it would be unlikely that the supposed hybridization events occurred only between few haplotypes poorly represented in the domestic cat population.



The maps resume the biogeographic hypothesis emerged from this work. Map A shows some of the possible distribution shifts and gene flows during pleniglacial period. Map B show the direction of a possible recolonization flow from extra-Mediterranean *refugia*. The arrows indicate possible migration flows. Bidirectional arrow indicates putative gene flow.

Although we found this kind of patterns in other shared (Dw) haplotypes (Dw1, Dw2, Dw3, Dw5, Dw7), this is not the case for the clade Dw4-Dw6. On the other hand, if we assume the hypothesis of a highly conserved ancient haplotype shared between the subspecies (hypothesis 2) we would expect to found much more differentiation in this group, since the estimated split between the two main clades (*F. s. silvestris* vs. *F. s. libyca*) fell much earlier than the time estimated considering the mutation rates of the region used. This finding was also confirmed using the faster-evolving control region (see chapter 2 and Hertwig et *al.* 2009). The divergence process between European wildcat and the other lineages took place before 173 000 years ago (Driscoll et al 2007). So a hypothetical wildcat lineage carrying the polymorphisms of group D, shared with *F. s. libyca* (and later with the derived *catus*), should have begun to diverge more than 100 000 years ago. Indeed it is quite improbable that this wildcat lineage might have evolved in the very same way to the ones that led to the today clade of Group 2 (Figure 2, Chapter 3) diverging only in the last tens of thousands years (according to our estimates). If we consider the paleo-biogeographic reconstruction made by Kurtén (1965, 1968) and Macdonald *et al.* (2010), we could move forward the migration hypothesis that, according to their estimates, might be occurred about 50 000 years ago toward the today Palestine regions (inhabited by *libyca* wildcats in that time) and that would fit our estimates of a supposed later "coming back" near 40 000 years ago. Anyway this hypothesis should be supported by a larger sample size also including a good representation of regions at the borders of the respective subspecies ranges *(F. s. silvestris* and *libyca*).

Phylogeographic analyses on wild haplotypes revealed a clearer geographic structure that divided the current distribution into three main regions including Scotland, continental Europe with part of Iberian peninsula (excepted Portugal that showed a certain degree of differentiation) and Mediterranean/south-eastern Europe. In particular, the Italian population was clearly isolated, maybe as a consequence of the last glacial cycles, although one of its main haplotype (W1) was also found in Iberian peninsula and south-eastern Europe.The persistence of one or more extra-mediterranean glacial *refugia* in Balkans regions (Stewart and Lister 2001; Schmitt and Varga 2012) was suggested also by the presence of one pervasive haplotype (W4) in Central Europe, probably resulted from a post-glacial recolonization. Indeed the split between the two main groups of clade W (Group 3 and 4, Figure 2 in Chapter 3) fell about 62 400 years BP (95 % HPD 21 860 – 118 910 years BP), a date that is compatible with the Pleniglacial period. These results confirm the findings by Pierpaoli et *al* (2003) using nuclear markers on the genetic structure in Europe and outline the evolutionary significant units that represent essential information for planning conservation actions. Furthermore they confirm the importance of a wide geographic coverage in phylogeographic studies based on mitochondrial DNA, providing a first reference framework for future works.

CONCLUSIONS AND FUTURE PROSPECTIVES

In this work we tried to apply an integrated approach to the study of this species, whose complex evolutionary history has crossed with that of its domestic relative. Several steps need to be taken to gain the necessary information to ensure the conservation of this species. The knowledge of its ecology, biology and ethology is a fundamental aspect that has been investigated by several authors (Lopes Fernandes 1993; Ragni and Possenti 1996; Tryjanowski *et al.* 2002; Sarmento 1996; Sarmento *et al.* 2006; Monterroso *et al.* 2009; Jerosch *et al.* 2010; Jerosch and Goetz 2011), but several aspects, especially those regarding the species behaviour, still remain unclear, although would be of great help in understanding the interactions of the wildcat with the domestic cat and shed light on behavioural processes that underlie hybridization

This work has provided few but important pieces of information about wildcat's habits, especially regarding its circadian and seasonal behaviour, and highlighted a certain degree of home range overlap between wild and domestic cats (see chapter 1), in particular through the use of camera-trapping. In the last decades, the significant reduction in the size of video recording devices allowed to obtain animal-borne videos through the application of cameras (e.g. Crittercam ®) directly on the animal. This technique allowed a better understanding of the behaviour and the use of space of several species (Marshall 1998; Heithaus *et al.* 2001; Adimey *et al.* 2007; Gad 2008; Fuentes *et al.* 2014) including the domestic cat (Loyd *et al.* 2013a; Loyd *et al.* 2013b). This technique was never applied on wildcat and could represent an interesting application that could provide valuable insights on the issues mentioned above.

Another important aspect for the species management is the knowledge of its distribution and population parameters, for which a good monitoring efficiency is essential. Wildcat monitoring is largely based on sightings, camera- and live-trapping, radio tracking, scat surveys and, opportunistically, road-kills. Because of the elusiveness of the species and its solitary behaviour it is quite difficult to contact the animal in its natural environment. Some studies attempted to monitor the populations of the European wildcat using different non-invasive techniques (Kery *et al.* 2011; Anile *et al.* 2012a; Anile *et al.* 2014; Bryce 2011; Can *et al.* 2011; Lozano *et al.* 2013; Steyer *et al.* 2013) (Kery et al. 2011; Anile et al. 2012a; Anile et al. 2014; Bryce 2011; Can et al. 2011; Lozano et al. 2013; Steyer et al. 2013) with limitations specific to each method (camera-trapping, scat survey, hair-trapping, etc.). In this study, we applied an integrated approach that aimed to compensate the drawbacks of the single techniques and allowed their contextual comparison.

In recent years, the use of drones has become very popular in various fields of research and some attempts were also made on wildlife, especially on large African animals of savannah (Schiffman 2014). In more complex environment such as the

Italian Apennines or the European woodlands it would be interesting to associate this technique with the use of thermal imaging (Long and Locher 2011; Steen *et al.* 2012; Christiansen *et al.* 2014) to try and detect wild animals and recognize a species-specific signature.

However, so far only non-invasive genetic sampling seems to provide a relatively abundant source of samples to assess the genetic status of natural populations. From this point of view one of the biggest challenges is to identify markers that join a good ability to assign the sample to an individual, its subspecies or its class of hybridization to a reliable genotyping even for highly degraded non-invasive samples. We provided a protocol that using the last resources of NGS techniques (using final pool of 151 nuclear SNPs) and the recent findings in uniparental markers (mtDNA and Y chromosome) greatly improved the ability to identify hybrids. Our analyses of mitochondrial DNA deepened the species' recent evolutionary and biogeographic history, opening new perspectives in the relationship between wild and domestic subspecies. A future attempt to reduce the number of loci could lead to the development of a SNP-chip that could be also applied to non-invasive samples and greatly speed up conservation genetic studies (Nussberger *et al.* 2014). Until now, the use of NGS tools in phylogenetics and phylogeography has been limited by the low cost-effectiveness of sequencing orthologous DNA fragments for many individuals, and by the difficulty to estimate demographic parameters by using SNPs with insufficient variability for modeling gene coalescence (Kuhner 2009; Pinho and Hey 2010). However, promising protocols have been recently proposed and developed to overcome previous drawbacks. For example, sequencing a subset of the genome, i.e., reduced-representation genomic libraries (Barbazuk *et al.* 2005) and combining individuals into a single run (Glenn 2011), might allow an economically and computationally feasible alternative (McCormack *et al.* 2011). Moreover, producing longer reads with the advent of third generation sequencing platforms (e.g., PacBio, Ion Torrent, Starlight) coupled to the latest computational tools (eg. SNAPP, Bryant et al 2012; ***∂a∂i***, Gutenkunst et al 2009) might facilitate gene tree analysis and allow the possibility to use SNPs for testing demographic hypotheses by involving gene flow (Durand *et al.* 2011; McCormack *et al.* 2011)

In conclusion, in my thesis I dealt with some of the main issues related to the conservation of the European wildcat, with a focus on genetic research. I then attempted to integrate results from different research fields (ecology, genetics and genomics), aiming at providing a more complete framework to develop future research projects and conservation policies.

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