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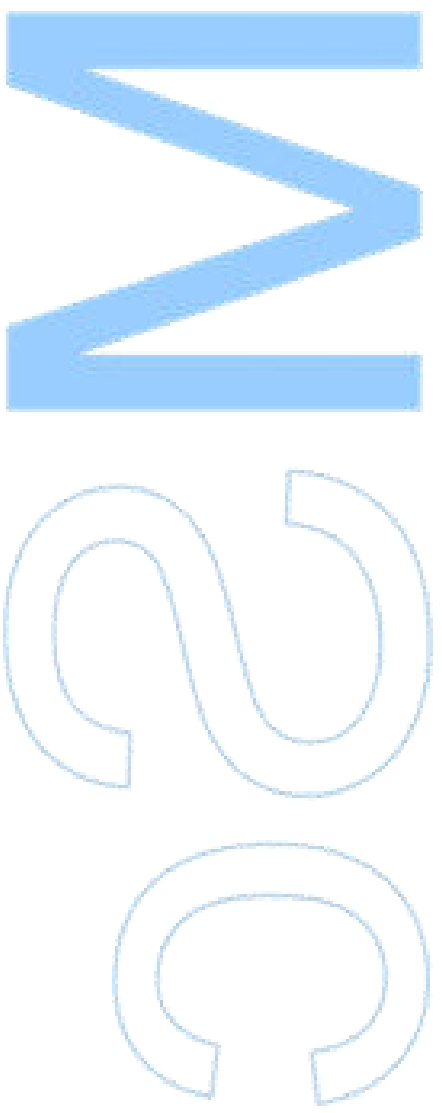UNIVERSIDADE DO PORTO

Assessing hybridization between wildcat and domestic cat: the particular case of Iberian Peninsula and some insights into North Africa

Luana Isabel Ferreira da Silva Costa Ramos
Dissertação de Mestrado apresentada à
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Assessing hybridization between wildcat and domestic cat: the particular case of Iberian Peninsula and some insights into North Africa

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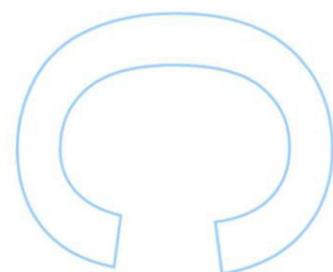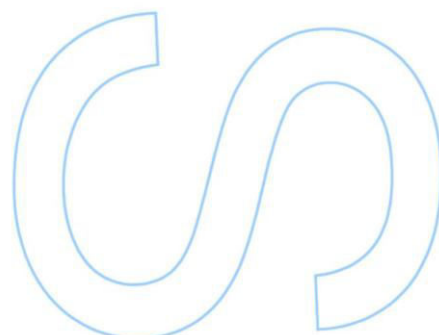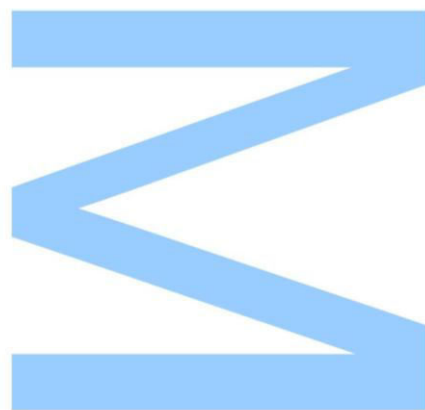
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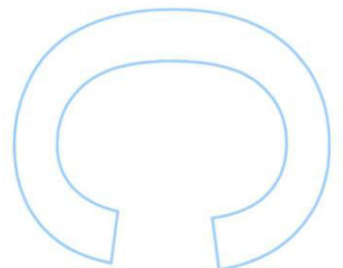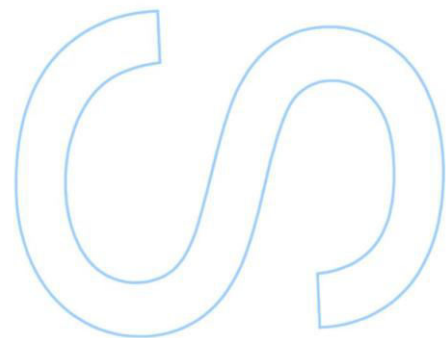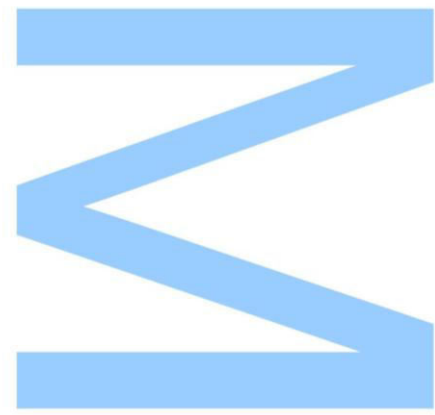
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O Presidente do Júri,

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Acknowledgements

During the development of this thesis there were many people who were essential in so many aspects that I could not finish without properly acknowledging them.

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"He will kill mice and he will be kind to Babies when he is in the house, just as long as they do not pull his tail too hard. But when he has done that, and between times, and when the moon gets up and night comes, he is the Cat that walks by himself, and all places are alike to him. Then he goes out to the Wet Wild Woods or up the Wet Wild Trees or the Wet Wild Roofs, waving his wild tail and walking by his wild lone."

The Cat that Walked by Himself, Rudyard Kipling

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Abstract

The wildcat, *Felis silvestris*, is a polytypic species that comprise six ecological and genetically different subspecies. Five subspecies occur in the wild and have a very wide distribution, occupying Europe, Africa and Asia. The domestic subspecies is nowadays one of the most iconic pets and is distributed throughout all continents. The earliest evidences of domestication were found in the Near East around 9500 years ago, and the northern African wildcat is considered its most probable ancestor.

The wild populations have been suffering extensive decline during the last decades, mainly due to anthropogenic threats like habitat destruction and direct persecution, with particularly severe consequences in Europe. The concomitant effects of these threats and increasing spread of domestic cats facilitated the contact between wild and domestic subspecies, leading to a significant growth in hybridization events. In Europe, several molecular studies regarding wildcat hybridization were developed during the last decade, identifying areas where hybridization events are rare and isolated, contrasting with other areas where introgressive hybridization is widespread. In Iberian Peninsula, previous studies revealed a clear genetic distinction between the wild and domestic subspecies, with few hybridization events restricted only to Portugal. Nevertheless, recent evidence points out to more geographically widespread hybridization. In North Africa there are also evidences of admixture, but this subspecies is still poorly studied.

Regarding this work, we address some questions regarding the population differentiation and possible hybridization among three wildcat subspecies, *F. s. silvestris* (European wildcat), *F. s. lybica* (northern African wildcat) and *F. s. catus* (domestic cat). The main goals were to obtain a significant number of samples collected across the Iberian Peninsula and North Africa, in order to determine levels of genetic diversity and differentiation between the three wildcat subspecies and perform a hybridization survey in these areas; to perform a more intense non-invasive sampling in some defined locations across Iberian Peninsula, for assessing hybridization at population level; and select and optimize a panel of microsatellite markers that allow accurate detection of wildcat hybridization.

A total of 252 samples, including 62 reference samples (42 domestic and 20 wildcats), and 99 invasive and 91 non-invasive new samples were analysed using a panel of fourteen highly polymorphic unlinked autosomal microsatellites, previously optimized for amplification of invasive and non-invasive DNA samples. Bayesian based

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clustering methods were implemented in software STRUCTURE and NEWHYBRIDS to distinguish the two wildcat and the domestic cat subspecies and their hybrids.

The three subspecies demonstrated high levels of genetic diversity. African wildcats and domestic cats revealed very high genetic similarity, while European wildcats seemed to be more differentiated from the other two subspecies. European wildcats and domestic cats showed clear distinct genepools in the Iberian Peninsula, although 12 hybrids were found widespread through the Iberian territory (20% hybridization rate). A comparison study between population level rates of hybridization was not possible given the high scat misidentification rate (78.4%) that prevented the analyses of a significant number of samples per location. Moreover, simulation results indicate that the panel of microsatellites provided accurate results in distinction between European wildcats, domestic cats and their hybrids, but did not provide accurate distinction of hybrid ancestry classes. Distinction between northern African wildcat and domestic cats was also ambiguous, due to the high genetic similarity between the two subspecies, but evidences of possible admixture were found

These results were discussed under the light of conservation and management plans for the endangered wildcat subspecies, since more strict measures should be considered. Priority should be given to the restoration and protection of large habitats, with healthy prey populations, in order to avoid spreading of wildcats into humanized areas while looking for food and shelter. Moreover, accurate identification of feral domestic cats and hybrids is essential to implement neutering programmes. More informative and diagnostic markers, such as single nucleotide polymorphisms for example, are necessary, not only for the accurate identification of hybrids but also of their ancestry class, in order to fully understand the hybridization dynamics of each population and develop appropriate conservation plans.

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Resumo

O gato-bravo, *Felis silvestris*, é uma espécie politípica que inclui seis subespécies ecológica e geneticamente diferentes. As cinco subespécies selvagens têm uma distribuição bastante vasta, ocupando os continentes Europeu, Africano e Asiático. A subespécie doméstica é um dos animais de companhia mais carismáticos, com uma extensa distribuição geográfica que ocupa quase todos os continentes. Os indícios mais antigos de domesticação foram encontrados no Médio Oriente há cerca de 9500 anos atrás, sendo o gato-bravo Africano considerado hoje o mais provável ancestral do gato doméstico.

As populações selvagens têm sofrido, durante as últimas décadas, um dramático declínio, devido maioritariamente a ameaças antropogénicas como a destruição de habitats e perseguição, com consequências especialmente severas na Europa. Os efeitos simultâneos destes perigos e o aumento da dispersão de gatos domésticos facilitam o contacto entre as subespécies selvagens e doméstica, conduzindo a um significativo aumento da hibridação. Na Europa, vários estudos moleculares direcionados para a deteção de hibridação entre gato-bravo e gato doméstico foram desenvolvidos durante a última década, possibilitando a identificação de áreas onde a hibridação é esporádica, contrastando com outras onde é possível verificar uma extensa e generalizada introgressão de genes domésticos. No caso particular da Península Ibérica, alguns estudos revelaram padrões genéticos distintos entre as duas subespécies, com alguns casos de hibridação encontrados exclusivamente em Portugal. Porém, um estudo mais recente aponta para um cenário de hibridação mais disperso pela península. Alguns estudos no Norte de África indicam também possível existência de hibridação, mas as populações desta região encontram-se ainda muito pouco estudadas.

Este trabalho aborda questões de diferenciação genética e hibridação entre três subespécies de gato-bravo, *F. s. silvestris* (gato-bravo Europeu), *F. s. lybica* (gato-bravo Africano) e *F. s. catus* (gato doméstico). Os principais objetivos propostos incluem a obtenção de um número significativo de amostras recolhidas na Península Ibérica e no Norte de África, de modo a determinar os níveis de diversidade genética e diferenciação entre as três subespécies e avaliar a incidência de hibridação nestas áreas; um intenso esforço de amostragem direcionado para algumas populações Ibéricas, com recolha de amostras não-invasivas, de forma a recolher informação sobre taxas de hibridação a nível populacional; e a seleção e otimização de um painel

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de microssatélites que permitam a correta deteção de hibridação em amostras invasivas e não-invasivas.

Um total 252 amostras, incluindo 62 amostras de referência (42 gatos domésticos e 20 selvagens), e 99 novas amostras invasivas e 91 não-invasivas foram analisadas, e um painel de catorze microssatélites autossómicos altamente polimórficos e não ligados entre si foram selecionados e otimizados para a amplificação de amostras de ADN invasivo e não-invasivo. Métodos de análise Bayesianos foram implementados nos *softwares* STRUCTURE e NEWHYBRIDS de forma a distinguir as subespécies selvagens a doméstica, e os híbridos resultados dos seus cruzamentos.

As três subespécies demonstraram um elevado nível de diversidade genética. Gatos domésticos e Africanos revelaram uma elevada similaridade genética, enquanto os gatos-bravos Europeus demonstraram maior diferenciação genética relativamente aos seus conspecíficos Africanos e domésticos. Na Península Ibérica foi encontrada uma clara distinção genética entre as subespécies Europeia e doméstica, apesar de terem sido encontrados 12 híbridos dispersos por este território (taxa de hibridação de 20%). O estudo comparativo entre taxas de hibridação a nível populacional não foi possível dada a elevada taxa de identificações erróneas nos excrementos recolhidos (78.4%) que impediu a análise um número suficiente de amostras por localização. Além disso, os resultados da análise de genótipos simulados indicaram que, apesar de o painel de microssatélites permitir uma correta identificação de gatos-bravos Europeus, domésticos e híbridos, este não possibilita uma distinção correta das classes de hibridação. A distinção entre gatos Africanos e domésticos foi também ambígua, dada a elevada semelhança genética entre as duas subespécies, embora tenham sido encontradas evidências de possível miscigenação.

Estes resultados foram discutidos à luz de planos de conservação para o gato-bravo, tendo em conta que medidas de conservação mais restritas deveriam ser consideradas. O restauro e proteção de habitats vastos e favoráveis com populações abundantes de presas é uma prioridade, de forma a impedir que o gato-bravo disperse para áreas mais humanizadas em busca de alimento. Além disso, a correta identificação de gatos domésticos ferais é essencial para implementar programas de esterilização. Marcadores moleculares mas informativos e diagnósticos, tais como polimorfismos de nucleotídeos simples (SNPs), são necessários não só para a correta identificação de híbridos, mas também das classes de hibridação, de modo a compreender com precisão a dinâmica de hibridação de cada população e desenvolver planos de conservação apropriados.

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Palavras-chave

Felis silvestris, gato-bravo Europeu, gato-bravo Africano, gato doméstico, hibridação, Península Ibérica, microssatélites, genética da conservação, amostragem genética não-invasiva.

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List of Abbreviations

AMOVA – Analysis of Molecular Variance

BLAST – Basic Local Alignment Search Tool

bp – Base Pairs

CFA – Cat Fanciers Association

CITES – Convention on International Trade in Endangered Species of Wild Fauna and Flora

CR – Control Region

DNA – Deoxyribonucleic Acid

FCA – Factorial Correspondence Analysis

FCA – *Felis silvestris catus*, domestic cat

FLY – *Felis silvestris lybica*, northern African wildcat

FSI – *Felis silvestis silvestris*, European wildcat

IRBP – Interphotoreceptor Retinoid Binding Protein

IUCN – International Union for Conservation of Nature and Natural Resources

HWE – Hardy-Weinberg Equilibrium

LE – Linkage Equilibrium

MCMC – Monte Carlo Markov Chain

mtDNA – Mitochondrial DNA

NCBI – National Center for Biotechnology Information

NUMT – Nuclear Mitochondrial DNA

PBS – Phosphate-Buffered Saline

PCR – Polymerase Chain Reaction

P_{ID} – Probability of Identity

P_{IDsib} – Probability of Identity between Siblings

SNP – Single Nucleotide Polymorphism

STR – Short Tandem Repeat

TICA – The International Cat Association

ya – Years Ago

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Of all God's creatures there is only one that cannot be made the slave of the lash. That one is the cat. If man could be crossed with the cat it would improve man, but it would deteriorate the cat.
Mark Twain

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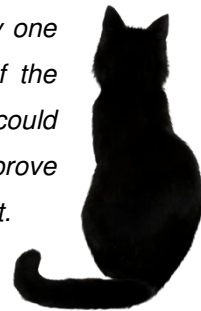
1. Introduction

1.1. The Wildcat

The wildcat (*Felis silvestris* Schreber, 1777, order Carnivora, family Felidae) is a medium sized carnivore that inhabits Europe, Asia and Africa (Lozano & Malo 2012). The first historical occurrence of the European wildcat was reported by fossil deposits of the Holsteinian Interglacial of Pleistocene in Europe (Sommer & Benecke 2006), and from here began its expansion to other continents (Nowell & Jackson 1996; Lozano & Malo 2012), having today one of the most widespread distributions among felids (Kitchener & Rees 2009).

The species conservation status is globally considered by the IUCN Red List of Threatened Species as Least Concern (Driscoll & Nowell 2010). It is also currently strictly protected under national (in most European countries) and international legislation, by the Bern Convention, the European Habitats Directive and CITES (Driscoll & Nowell 2010; CITES 2014). It is a polytypic species, and although the number of subspecies is still debatable it is usual to consider the European wildcat *F. s. silvestris* Schreber, 1775; the northern African wildcat *F. s. lybica* Forster, 1780 and the central Asian wildcat *F. s. ornata* Gray, 1830 (Randi *et al.* 2001; Pierpaoli *et al.* 2003; Yamaguchi *et al.* 2004; Kitchener & Rees 2009; Driscoll & Nowell 2010). However, recent data added to this group the southern African wildcat *F. s. cafra* Desmarest, 1822; and the Chinese desert wildcat (or Chinese Alpine Steppe cat) *F. s. bieti* Milne-Edwards, 1872 (see figure 1; Driscoll and Nowell, 2010; Driscoll *et al.*, 2007). Additionally, it also includes the domesticated form *F. s. catus* (Driscoll & Nowell 2010).

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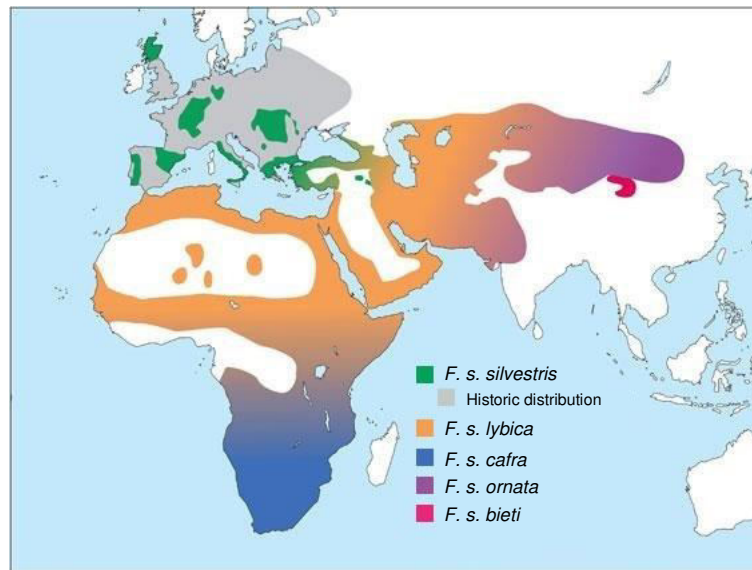
Figure 1 – Approximate distribution of *Felis silvestris* subspecies, adapted from Driscoll *et al*. (2007).

1.1.1. The wild subspecies

The wildcat species *Felis silvestris* comprises five ecologically, geographically and genetically different subspecies with natural occurrence in the wild (Driscoll *et al.* 2007). Information regarding them is not homogenous, since some are extensively studied while for others little information is known (Phelan & Sliwa 2005; Herbst & Mills 2010). Southern African wildcat was considered the same subspecies as the northern African wildcat *F. s. lybica* (Driscoll *et al.* 2007), and, therefore, some studies regarding the African wildcat did not distinguish the two (for example, Wiseman *et al.*, 2000). This subspecies occurs in southern Africa, and although the boundaries of the distribution range between *F. s. lybica* and *F. s. cafra* are not completely clear, morphological data point out to the area of Tanzania and Mozambique (Driscoll and Nowell, 2010 and references therein). In Asia, the Central Asian wildcat is distributed from east of the Caspian Sea into western India, north to Kazakhstan and into western China and southern Mongolia (Driscoll *et al.* 2007; Driscoll & Nowell 2010). It can be found near human settlements and cultivated areas, and it is mainly threatened by hunting for fur trade and hybridization with domestic cat (Nowell and Jackson, 1996 and references therein). The Chinese desert wildcat is poorly studied (Nowell & Jackson 1996; He *et al.* 2004). It was previously thought to be another species (Nowell & Jackson 1996) until 2007 when Driscoll and colleagues reclassified it as a subspecies of *F. silvestris*. It is endogenous to western China, although its distribution range is still uncertain (He *et al.* 2004), and is considered the least numerous subspecies and classified as Vulnerable by the IUCN because of its very restricted range (Driscoll & Nowell 2010)

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and intensive hunting for fur (Nowell & Jackson 1996; He *et al.* 2004). All subspecies are mainly threatened by human-caused mortality, either by habitat loss and predator control measures, or by illegal hunting for fur, especially in the case of the Chinese desert wildcat (Driscoll & Nowell 2010). Moreover, there are evidences of hybridization between domestic cats (*F. s. catus*) and all wild subspecies (Driscoll *et al.* 2007). Although the incidence of hybridization with the domestic cat is considered lower outside Europe, it is still significant (Wiseman *et al.* 2000; Driscoll & Nowell 2010), and more research focused on these subspecies should be performed to understand the real impact of hybridization.

The other two wild subspecies of *F. silvestris* coexist in Europe, the European wildcat, from Portugal to Romania and the African wildcat in some Mediterranean islands (Sardinia, Corsica and Crete). In addition to these subspecies, the domestic form is distributed through the entire continent (figure 2).
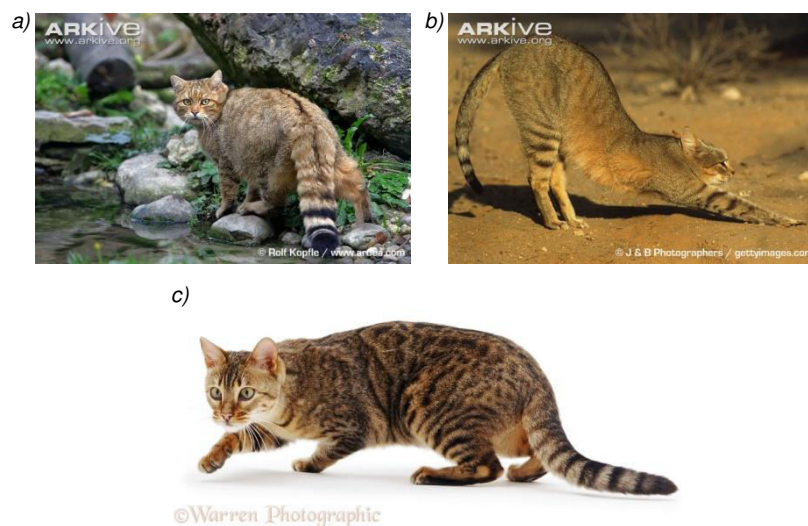
Figure 2 – The three *F. silvestris* subspecies that coexist in Europe: a) European wildcat; b) African wildcat (both from *www.arkive.org*) and c) domestic cat (from *www.warrenphotographic.co.uk).*

*The northern African wildcat (*F. s. lybica*)*

Europe is home not only to the European wildcat and the domestic cat, but also to the northern African subspecies, as they live in Sardinia for at least 3000 years according to fossil records, brought there by Neolithic navigators (Pierpaoli *et al.* 2003). This subspecies is also distributed along Africa – occurring discontinuously throughout the north from Morocco until Egypt, across the savannas of western Africa, eastwards until the Horn of Africa, Sudan and Ethiopia, and finally through south-eastern Africa were it is replaced by the southern African wildcat – and the Arabian Peninsula and part of south-western Asia (Yamaguchi *et al.* 2004; Driscoll & Nowell 2010), demonstrating an extremely wide distribution range. Moreover, this subspecies shows

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a broad habitat tolerance, including true deserts as the Sahara, but avoiding tropical rainforests (Driscoll & Nowell 2010).

African and European wildcats split recently and are, thus, closely related (Pierpaoli *et al.* 2003). Nevertheless, their general appearance is slightly different. The African subspecies have a distinct tapering tail and less visible tabby stripes, and the coat colour range from reddish brown to sandy yellow (see figure 2; Nowell and Jackson, 1996; Yamaguchi *et al.*, 2004). They are predominantly nocturnal and prey mostly on rodents (Nowell & Jackson 1996).

African wildcats are very similar, both morphologically and genetically, to domestic cats (Nowell & Jackson 1996; Driscoll *et al.* 2007) and, as mentioned, there are evidences that wildcats in Africa and Near East might be threatened by hybridization with the domestic cat (Phelan & Sliwa 2005; Driscoll *et al.* 2007). Although this is considered the primary threat to this wild feline (Nowell & Jackson 1996), the rapid development of urbanized areas (Phelan & Sliwa 2005) is also threatening their habitats and populations. Moreover, the large home ranges (51.21 km^2) of this subspecies documented by Phelan and Sliwa (2005) in the United Arab Emirates might contribute to a higher probability of encounters with highly humanized areas and consequently with the domestic cat, which might also result in disease transmission from feral domestic cats to the wild populations. This might happen in other areas of their distribution as well, where further research is needed (Nowell & Jackson 1996).

Accurate information regarding this subspecies is still lacking. Considering their interaction with the domestic cat throughout their distribution range, and with the European wildcat in Near East, thorough ecological and genetic studies are essential to understand the populations' dynamics of these subspecies and their genetic relation. Moreover, studies concerning this feline should be a priority in order to prevent further threats and population declines, and to implement accurate conservation measures.

The European wildcat (F. s. silvestris)

From the Iberian Peninsula to the Caucasus Mountains, to Scotland in the north and to the Mediterranean in the south, including the island of Sicily, the European wildcat range occupies almost all Europe and a part of south-western Asia (Yamaguchi *et al.* 2004; Lozano & Malo 2012). It is usually found in mosaic environments with areas of enclosed structure to hide, and open areas to hunt, but can be found in a variety of different habitats, as long as there is enough shelter and prey and are not excessively humanized or intensively cultivated (Nowell & Jackson 1996; Klar *et al.* 2008; Driscoll &

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Nowell 2010). In the Mediterranean areas the scrubland is especially important (Lozano *et al.* 2003; Monterroso *et al.* 2009; Lozano & Malo 2012).

This subspecies is generally brown-gray or dark gray with tabby pattern, with a black dorsal line ending at the base of a broad bushy tail with a large black tip, and is usually larger and more robust than its domestic conspecific (Nowell & Jackson 1996; Yamaguchi *et al.* 2004; Lozano & Malo 2012). According to Kitchener and colleagues (2005), some pelage characters are better for subspecific differentiation, as the length of the dorsal stripe, shape of the tail tip and its characteristic bands, presence of broken stripes or spots on flanks and number of strips on the shoulder. The European wildcat is solitary and territorial, marking its territories with faeces and other signs, and it is mostly nocturnal, moving mainly at dusk or during the night, discreetly and quietly, making it an elusive animal (Germain *et al.* 2008; Lozano & Malo 2012). Its diet is based on rodents and rabbits (Nowell & Jackson 1996; Sarmento 1996; Lozano *et al.* 2006), with a preference for the last when abundant, on which it specializes optionally (Lozano *et al.* 2006; Lozano & Malo 2012). Life expectancy is at maximum 15 years in captivity, sexual maturity is reached within the first year and, depending on the region, the mating period occurs during winter-spring, mainly from January to March, and after around two months of gestation females have a mean of three or four cubs, that disperse before the winter (Germain *et al.* 2008; Lozano & Malo 2012).

Although it is among the most common of wild felids, the wildcat faces serious threats to its long time survival (Driscoll *et al.* 2011). The historical post-Pleistocene range of the European wildcat was much wider, but suffered a massive decline during the 18th and 19th centuries (Lecis *et al.* 2006). Especially during the last two centuries, the huge increase in human population in Europe put the wildcat under severe pressure, and lead to population fragmentation and consequent isolation at regional and local levels (Pierpaoli *et al.* 2003; Randi 2008; Oliveira *et al.* 2008b; Lozano & Malo 2012). The major threats known to influence the decline of the European wildcat are the loss of habitat, mainly through deforestation, massive eucalyptus plantations and urbanization (Driscoll & Nowell 2010; Lozano & Malo 2012); non-natural anthropogenic mortality, such as the use of traps for carnivores control, hunting for their fur, poisoning and road kills (Nowell & Jackson 1996; Driscoll & Nowell 2010); reduced prey availability, mainly by hunting or diseases like myxomatosis that affect rabbits (Lozano & Malo 2012); loss of genetic integrity through hybridization and introgression of domestic cat genes (Nowell & Jackson 1996; Randi 2008); and disease transmission, being the most worrying the feline immunodeficiency virus that causes a suppression of immunity, affecting mostly domestic cats but has already appearing in some wildcat populations, in which is not usually found, probably contaminated by domestic cats

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(Račnik *et al.* 2008; Millán & Rodríguez 2009). Fortunately, the solitary behaviour of wildcats restrains the rapid spread of viruses (Lozano & Malo 2012). Another threat to the European wildcat might be competition with other species. In some European countries the competition with the European lynx is considered an important threat to wildcat populations, as well as in the Iberian Peninsula where the Iberian lynx might be partially responsible for decrease or exclusion of some populations of wildcat, as in Sierra Morena or Doñana National Park (Lozano & Malo 2012; Soto & Palomares 2014). Having this in consideration, the reintroduction plans of this critically endangered lynx species can be a potential problem for the wildcat, and thus it must be carefully studied. More thorough studies should be done in order to better understand the interactions between both species (Lozano & Malo 2012).

The conservation status of this endangered cat differs regionally, for example, from Critically Endangered in Scotland (Kitchener *et al.* 2005; Driscoll & Nowell 2010) to Vulnerable in Portugal (Cabral *et al.* 2005) and Near Threatened in Spain (Palomo *et al.* 2007). However, there are still areas in Europe that lack important information on the presence or absence of the species, patterns of dispersal, effects of natural and artificial barriers on fragmentation and isolation, demographic patterns and genetic characteristics of the populations (Oliveira *et al.* 2008a; Driscoll & Nowell 2010; Lozano & Malo 2012; Hartmann *et al.* 2013).

It is particularly worrying that the concomitant effects of all the threats listed before, like habitat destruction, population fragmentation and isolation, decrease in prey availability and increase in human density, lead the wildcat to face a more serious pressure caused by encounters with the domestic conspecific. It is arguable if hybridization is or not the most threatening problem, because, in fact, after centuries of sympatry with the domestic cat, the low frequency of hybridization described for some populations may be an evidence that some natural barriers to gene flow exist (Randi *et al.* 2001; Pierpaoli *et al.* 2003; Oliveira *et al.* 2008a; b; O'Brien *et al.* 2009; Eckert *et al.* 2010; Lozano & Malo 2012). This can be caused mainly by different activity rhythms of feral domestic cats. However, both male and female domestic cats have longer mating periods than wildcats, increasing the chances of concordance in time and space use patterns of the two subspecies (Germain *et al.* 2008). Therefore, hybridization might be increasing as a consequence of all the aforementioned natural and anthropogenic problems that cause further decline of the wild populations and a spread of domestic cats (Pierpaoli *et al.* 2003; Oliveira 2012). The closer the wildcats get to villages or other human settlements, mainly looking for food, closer they are to domestic cats, and the hybridization threat increases. Overall, hybridization occurs as a consequence of all

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other threats, making the domestic cat one of the most serious threats to wildcats' genetic integrity.

1.1.2. From a wild feline to a household pet

The process of domestication can be described as a variety of microevolutionary changes caused by natural and humanly directed selection, occurring in an anthropogenic environment during a mutualistic relationship between humans and other animals (Hu *et al.* 2013).

Domestication is one of the most successful and important processes in the evolution of human civilizations (Diamond 2002; Driscoll *et al.* 2007). It started with the evolution of human cultures from hunter-gatherers to farmers, approximately 10500 years ago (ya), and revolutionized human demography and social behaviour (Diamond 2002). Contrary to plants, few animals were domesticated (Cameron-Beaumont *et al.* 2002). One of them was the cat. Despite being one of the most emblematic and iconic domesticated animals (O'Brien *et al.* 2008), evidences of the cat domestication process are scarce and further investigation is needed to fully understand the complete process (Driscoll *et al.* 2009a; Hu *et al.* 2013). As mysterious as interesting, the process of domestication of the cat was reason to some speculation. Although it was previously thought that cats were first domesticated in Ancient Egypt (Driscoll *et al.* 2009a) due to archaeological findings of a captured *Felis silvestris lybica* (Linseele *et al.* 2007), it was latter proved that the mentioned cat remain belonged to another species, *Felis chaus* (Linseele *et al.* 2008). Nevertheless, the findings show clear evidence that Egyptians held several species in captivity, including cats, showing an ancient desire to control wild animals (Linseele *et al.* 2007, 2008). With these evidences and the large number of mummified cats and latter paintings of already domesticated individuals (see figure 3) is not difficult to understand why there was so much speculation about a possible domestication of the cat in Egypt (Malek 1993; Linseele *et al.* 2007).

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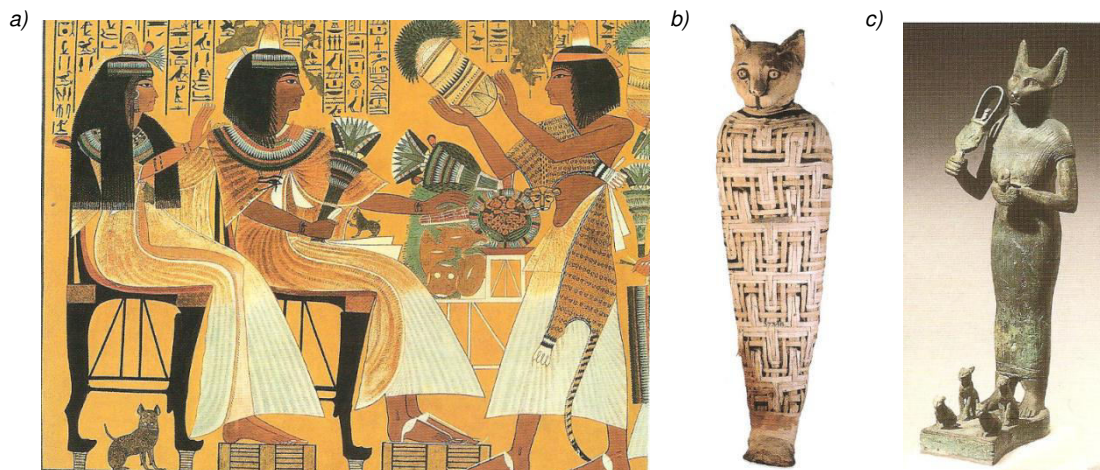
Figure 3 – Different art forms picturing cats in Ancient Egypt that evidence close relations between humans and this feline, adapted from Malek (1993). a) A wall painting portraying a kitten in the lap and its mother under the chair (page 57); b) cat mummy with elaborate pattern (page 127; British Museum); c) goddess Bastet, often represented as a cat (page 104; British Museum).

However, earlier archaeological remains suggest that the process started instead in the Mediterranean island of Cyprus, approximately 9500 ya, where an eight months old African wildcat skeleton was found intentionally buried next to a human, suggesting a spiritual link between the two (figure 4; Vigne *et al.*, 2004). Moreover, other archaeological remains of African wildcats were found in Cyprus near ancient villages, as early as 10600 years ago, evidence of very antique interaction between this feline and humans (Vigne *et al.* 2012). Also, the fact that cats were probably brought to an island where no native felines were found reinforces the evidence for this interaction (Vigne *et al.* 2004; Linseele *et al.* 2007; Driscoll *et al.* 2009a), and Cyprus is now considered the most probable location for the beginning of the process of domestication (figure 4). Genetic evidence confirmed the archaeological proofs through the use of Short Tandem Repeats (STR) and mitochondrial DNA variation by Driscoll and co-workers (2007), placing probable domestication origins in the Near East. It appears so that the taming of the cat began while humans were creating the first settlements in Middle East's Fertile Crescent (Driscoll *et al.* 2009a).

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Figure 4 – Location of the island of Cyprus in the Mediterranean Sea, highlighted by a red circle. On top, the small African wildcat remains found intentionally buried next to a human skeleton in Cyprus, adapted from Vigne *et al.* (2004).

As aforementioned, there is a lack of knowledge concerning the domestication of the cat, mainly in the period between the first evidences of domestication in Cyprus (9500 ya) and the first proofs of fully domesticated cats in Egypt (3600 ya; Driscoll *et al.*, 2009b; Hu *et al.*, 2013; Linseele *et al.*, 2007). In a recent study developed in ancient Chinese villages some cat bone remaining dating back to 5500 ya were found, and the morphometric identification suggested domesticated individuals (Hu *et al.* 2013). Moreover, evidence from isotope analysis also suggested the possibility that one of the discovered cats might had lost its hunting skills, and scavenged for discarded food or was even fed by humans, therefore showing signs of commensal relations and mechanisms of domestication (Hu *et al.* 2013). However, Bar-Oz and colleagues (2014) state that there is some ambiguity in the interpretation of Hu *et al.*'s (2013) evidences, and that the cats found were just an introduction of domesticated cats from the Fertile Crescent, or, more likely, a commensalism relation between humans and local small bodied wild cat species. They additionally state that most animals that entered commensal interactions with humans did not undergo a domestication process (Bar-Oz *et al.* 2014), which was probably the case. This studies and results reinforce the lack of information on the domestication of the cat during the aforementioned time period, and the importance of further investigation that helps understand thoroughly the cat's path to domestication.

Despite all the uncertainty there are some facts about cat's domestication that most researchers agree with. Hu and colleagues (2013) reinforce other authors' suggestion that the domestication of cats is related to the favourable service they provided for humans – control of rodents that destroyed the crops of the first farmers; and from the benefit they took from it – accessible and abundant food resources (Vigne

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et al. 2004; Driscoll *et al.* 2007; Linseele *et al.* 2007; Lipinski *et al.* 2008). This ultimately resulted in positive selection of cats with the tamest behaviours, that more easily approached the human settlements (Hu *et al.* 2013). Possibly the "large eyes and "cute" features", as stated by Driscoll and co-workers (2009a), stimulated humans to take kittens home and start taming them. Furthermore, evidence points the African wildcat *F. s. lybica* as the most probable ancestor of the domestic cat, not only because it is argued that it has a more docile behaviour (within the *F. silvestris* subspecies) that made it easier to domesticate and had a distribution more proximate to the first human settlements (Cameron-Beaumont *et al.* 2002; Linseele *et al.* 2007; Driscoll *et al.* 2009a) but also because the domestic cat is genetically more closely related to this subspecies than to any other (Driscoll *et al.* 2007, 2011; Mattucci *et al.* 2013). In fact, in Driscoll and colleagues' (2007) research all sampled domestic cats clustered together with *F. s. lybica* in a single group, distinct from the other *F. silvestris* subspecies.

There are additional evidences showing that cat's path into domestication is quite particular. They are the only domesticated species in the Felidae family, which is peculiar considering that *F. silvestris* did not fulfil important criteria for animals to be domesticated, as they are obligate carnivores and, therefore, do not have the capability of digesting every type of food; and lack strong social hierarchies, as they are solitary and defend their territory, thus not being able to follow a "leader" (Diamond 2002; Driscoll *et al.* 2009a; b). Moreover, unlike other domesticate species, cat contribution to human survival was minimal (Driscoll *et al.* 2009a). Additionally, the modern cats are still self-sufficient if they need to, exhibiting some hunting skills and behaviours ranging from untamable to highly affectionate (Lipinski *et al.* 2008; Driscoll *et al.* 2009a). There are even some authors who consider *F. s. catus* to be only partially domesticated, as the criteria of human controlled breeding and food supply is not valid to some feral cats (Bradshaw *et al.* 1999; Cameron-Beaumont *et al.* 2002; Driscoll *et al.* 2009a). However the concept of "domesticated" is by itself very difficult to define since the whole process is a continuous transition, different for each species (Driscoll *et al.* 2009b).

Once domesticated, cats spread worldwide, initially along trade routes between ancient civilizations (Lipinski *et al.* 2008; Driscoll *et al.* 2009a). Nowadays is a prolific and cosmopolitan species that occupies most habitable locations of the world, in almost total sympatry with their wild conspecifics (Randi *et al.* 2001; Lipinski *et al.* 2008), including most sea islands, and present in all continents with exception of Antarctica (Driscoll *et al.* 2011). It is one of the most popular pets worldwide (Driscoll *et al.* 2011). Menotti-Raymond and colleagues (1997) stated that in the United States, in the 90's, 65 million cats lived in approximately one third of the households, and Driscoll

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and colleagues (2011) estimated 600 million cats living in household association worldwide, with an additional 600 million living independently of humans.

The process of cat domestication did not initially undergo strong artificial selective pressures for complex traits related to behaviour, performance or production unlike most other domesticated species, since the wild characteristics were advantageous for control of pests and associated zoonotic diseases (Menotti-Raymond *et al.* 2003; Lipinski *et al.* 2008; Driscoll *et al.* 2009b). In fact, the crossbreeding between already domesticated animals and wild ones was good to preserve these traits (Lipinski *et al.* 2008). These facts contributed greatly to maintain their genetic similarity. The selection for breed creation started very late, probably within the past 150 years, mainly in Europe and America, and only based on aesthetic traits of interest, contrasting with the majority of other domesticated species for which selection for important traits started very early (Menotti-Raymond *et al.* 2003; Lipinski *et al.* 2008). The small subset of cats that were subjected to intensive artificial selection ultimately resulted in today's fifty-five breeds recognised by "The International Cat Association" or forty-two recognised by the "Cat Fanciers' Association" (Bradshaw *et al.* 1999; Lipinski *et al.* 2008; CFA 2013; TICA 2013). This process still endures, as new breeds are "created" and recognised, even by crossing domestic cats with wild species such as the Asian leopard cat, that originated the Bengal breed (Lipinski *et al.* 2008; Driscoll *et al.* 2009a). Artificial selection acted on a few *loci* related to phenotypic characteristics and has generated the different coat colours and fur types (Menotti-Raymond *et al.* 2003), but unlike dogs, for example, which demonstrate a huge variety of sizes, cat breeds do not have such variability because they were not selected for any specific task (Driscoll *et al.* 2009a).

Pure breeds have phenotypic characteristics that are highly unlikely to persist in feral or wild populations, like the shortened jaw and long fur of the Persian breeds (Bradshaw *et al.* 1999). However, the similarity between non-breed domestic cats and wildcats is widely visible. Despite the variety of coat colours in domesticates, they still retain the overall morphologic aspect of their wild ancestors with just a few differences in the size of the legs, brain and intestine, probably due to their recent domestication, and also to the low artificial selection that non-breed populations were subjected (Bradshaw *et al.* 1999; Randi *et al.* 2001; Randi 2008; Driscoll *et al.* 2009a). These non-breed cats are often feral. Bradshaw and colleagues (1999) describe the feral domestic cats as free ranging individuals with different relationships with humans, and that are able to hunt by themselves but also to scavenge food resources left accidently or deliberately by man. These are the individuals that come in contact with wild populations, and that may eventually interbreed.

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1.2. Hybridization

Hybridization is one of the most concerning subjects for conservation biologists (Allendorf *et al.* 2001; Randi 2008). It can be defined as the interbreeding between individuals from two groups or populations which are genetically distinguishable, even if not taxonomically distinct, and can be extended to crossings between domesticated species and their wild relatives and to horizontal gene transfer between different microorganisms (Rhymer & Simberloff 1996; Arnold 2004; Mallet 2005). It can be widespread or localized, both spatially and temporarily, common or rare, depending on the *taxa* involved (Abbott *et al.* 2013). In general, it is quite common on a species level, since it is estimated that 10-30% of animal and plant species hybridize regularly (Mallet 2005; Abbott *et al.* 2013). Arnold (2006) suggests that this genetic exchange is present in such a wide range of species, since virus and bacteria to plants and animals, that we might need to consider a "web-of-life" rather than a more simplistic "tree-of-life".

Nevertheless, scientist's perspectives on this subject vary immensely. As an example, botanists have often regarded hybridization as any other evolutionary process while zoologists have mostly considered it as a conservation problem (Rhymer & Simberloff 1996; Mallet 2005; Genovart 2008). Moreover, hybridization is also controversial because it has set some doubts regarding species concepts, particularly to those who considered a more static concept with reproductive barriers such as the Biological Species Concept (Mallet 2005; Genovart 2008). The study of the process of hybridization has an intrinsic and mutual connexion with both the concept of species and speciation itself, and therefore, these topics present extraordinary opportunities for discussion (see Arnold, 2006).

One of the main reasons why hybridization is such a controversial topic relies on the immensity of different backgrounds that can lead species to hybridize and, consequently, the variety of consequences or "creative results" (Arnold 2004; Abbott *et al.* 2013). Trying to categorize it, globally, as beneficial or not is topic for great discussion and to some disagreement. The consequences depend not only on the rates of dispersal, gene flow between the parental species and their specific stage of divergence, and the selective pressures acting on parental and hybrids, but also on several ecological factors (Genovart 2008; Abbott *et al.* 2013). Anthropogenic hybridization, i.e. caused by human activities (introduction of exotic species, habitat destruction or release of domesticated or artificially grown species), is one particular case, and is often more worrying than natural hybridization since it can get worse with the intensification of human activities (Allendorf *et al.* 2001; Genovart 2008). Crossings

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between westslop cutthroat trout (*Oncorhynchus clarki lewisi*) and populations of rainbow trout (*Oncorhynchus mykiss*) from hatchery stocks are a well known example. When in natural sympatry, these two species show considerable reproductive segregation, but when artificially grown rainbow trout is introduced in cutthroat trout's waters they interbreed forming genetically admixed populations (Hitt *et al.* 2003).

Depending on a variety of factors, reproductive and/or behavioural, among others, the resulting hybrids can be sterile, fertile only among themselves, or between them and one or both the parental species (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). These situations have distinct effects on the populations, and require specific conservation efforts. Particularly, when hybrids cross with individuals of the parental populations some alleles of one population can introgress into the genepool of the other (Rhymer & Simberloff 1996; Allendorf *et al.* 2001; Abbott *et al.* 2013). Introgressive hybridization can, in one hand, lead to disruption of local adaptations gained by natural selection, loss of genetic diversity by homogenization of two distinct genepools (Randi 2008) and ultimately to extinction, mainly in rare species (Rhymer & Simberloff 1996). It is especially common that two hybridizing populations adapted to very different environments create hybrids with a combination of alleles that might be less suitable to survival and reproduction in their new environments, or that interbreeding reshuffles specific combinations of genes and create new ones that can be deleterious or simply less fit (Rhymer & Simberloff 1996). This phenomenon (outbreeding depression) will affect considerably those hybridizing populations that have a significant amount of genetic divergence (Allendorf *et al.* 2001).

On the other hand, in some cases, even with ongoing hybridization, the frequency of the introgressed alleles do not increase and the process can be regarded as merely a part of the evolutionary process of the species (Allendorf *et al.* 2001). Furthermore, hybridization can even bring new combinations of alleles that are favourable for the population, and this new diversity can be maintained without progress towards speciation until environmental changes lead to divergence (Abbott *et al.* 2013). However, if introgression is more frequent it can lead to the persistence of hybrid zones with widespread introgression or complete admixture, potentially acting as a powerful evolutionary force, changing the genetic identity of the populations involved and eventually leading to new populations of mixed ancestry (Allendorf *et al.* 2001; Abbott *et al.* 2013). This can take place when F1 hybrids have increased fitness compared with the parental subspecies (heterosis), and therefore the frequency of backcrosses, and consequent introgression, increases (Rhymer & Simberloff 1996).

From losing one or both the parental species, to the establishment of a stable hybrid zone where the parental species and the hybrids occur, or even to the creation

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of a new species if the hybrids are under positive selection (Genovart 2008), the outputs of the process of hybridization can be very different, and therefore, will continue to provide interesting case studies and topic for intense debates.

Planning conservation actions requires a thorough study of each particular case. Allendorf and co-workers (2001) define different categories of hybridization and suggest different conservation guidelines for each, showing how important it is to adequate conservation to the particularities of each scenario. For example, in the case of complete admixture it might be wise to preserve the hybrids, as they may fit the ecological purposes of one or both the parental species (Allendorf *et al.* 2001). On the other hand, if hybridization is extensive but the parental populations are still present, conservation actions can focus on them, depending on how endangered they are (Allendorf *et al.* 2001). Under some very specific conditions, when genetic variability is so low that the long time survival of the species is threatened, hybridization can even be seen as an important tool to manage some endangered populations, since the introduction of individuals from a close population might help introduce new alleles and increase variability (Reisenbichler & Rubin 1999; Allendorf *et al.* 2001; Arnold 2006). Although this might lead to loss of unique genetic traits of the endangered species, it is still a considerable option if carefully studied and all the potential harms understood (Reisenbichler & Rubin 1999; Allendorf *et al.* 2001), as otherwise can lead to terrible consequences, as pointed out by Rhymer and Simberloff (1996).

As Allendorf and colleagues (2001) mention, the conservation policies for hybridization have been, over time, as controversial and unstable as the topic itself, and the development of one flexible enough to apply to the majority of cases seem very complex. As aforementioned, for each case intensive research is needed in order to understand the hybridization process and to be able to provide accurate conservation measures for each particular scenario (Genovart 2008). However, some cases raise more delicate questions than others.

One particularly controversial example occurs between domestic and wild species. Hybridization between domesticated animals and plants and their wild relatives had an important role in the evolution of the first and its genetic enrichment, ultimately leading to the development of highly efficient breeds by artificial selection (Arnold 2004). For instance, the high level of diversity of maize (*Zea mays* ssp. *mays*) was often explained by multiple origins of domestication from its wild ancestor, teosinte, until Matsuoka and colleagues (2002) found evidence of a single domestication event and subsequent hybridization with the wild ancestor that increased the genetic diversity of the domesticate. It is possible that these hybridization events, that occur mostly in higher altitudes, had allowed some races of maize to survive and

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mature in such environments (Matsuoka *et al.* 2002; Arnold 2004). The process of domestication of the dog is also an example of the importance of introgression of wild alleles, as it is argued that repeated hybridization between dog and grey wolf was an important source of genetic variability on which artificial selection then acted (Vilà *et al.* 1997). This increase in genetic diversity is especially important when domestication creates an accentuated bottleneck with consequent decrease in variability (Arnold 2004). Nevertheless, the consequences are not always good, especially for wild species. The introgression of alleles from the domesticated population can decrease fitness in the wild by disrupting important adaptations created by natural selection, threatening the genetic integrity of the wild species (Randi 2008).

A different controversial issue is intraspecific hybridization. It can occur at subspecies, races or population levels, and is often not considered a conservation concern since populations of the same species naturally share alleles and, thus, the introduction of some genetic variation can be beneficial (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). Nevertheless, sometimes the majority of genetic diversity of a species is among those infraspecific levels (Rhymer & Simberloff 1996), and hybridization can result in decrease of diversity by homogenization. Moreover, the introgression of some alleles might cause disruption of important local adaptations present in different populations (Allendorf *et al.* 2001). For example, the escape of some individuals from salmon hatcheries/aquaculture facilities may cause decrease in fitness of local wild populations through intraspecific hybridization (Reisenbichler & Rubin 1999; Allendorf *et al.* 2001). Similarly, the spread of domestic cats into wildcat territories might represent a threat to the endangered wild subspecies.

1.2.1. Wildcat/domestic cat hybridization

Closely related species are likely to hybridize more often (Abbott *et al.* 2013). As aforementioned, domestic cats and their wild relatives are genetically very similar so it is predictable that, when in sympatry, hybridization can occur. As the divergence of the domestic cat lineage happened in sympatry with the wild ancestor, they were probably in constant crossbreeding, increasing their genetic proximity (Driscoll *et al.* 2009b).

When hybrids are fertile not only among themselves but also with the parental species hybridization tends to increase progressively (Allendorf *et al.* 2001). For the particular case of the cat, this is a noticeable problem since it is known that hybrids generated from the crossbreeding of the two subspecies (F1) are fertile and can

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reproduce with other hybrids and with the parental subspecies (Pierpaoli *et al.* 2003). Hybrid individuals might be less fit because they were never exposed to natural selection, and therefore wild populations with admixture might be pushed to habitats more favoured by domesticates (urban areas), which can lead to greater and greater admixture (Driscoll *et al.* 2011). Moreover, the modification of habitats (mainly caused by human interference) can lead to fragmentation and isolation, which can cause wild individuals from isolated populations to hybridize with domesticates, given that it is more difficult for them to find conspecific mates (Rhymer & Simberloff 1996). In addition, domestic cats often spread and are able to live in wild territories (Sarmento *et al.* 2009), contributing to increase the range overlap, and therefore, making hybridization a persistent problem for the wildcat (Driscoll *et al.* 2011).

The introduction of domestic genes in the wild populations' gene pool might lead to the prejudicial disruption of locally adaptive gene complexes (Driscoll *et al.* 2011). On the other hand, the introduction of domestic genes can be favoured by natural selection, as they can somehow have a more tamed behaviour and access resources related to human activities (Driscoll *et al.* 2011). Either way, genetic integrity of the wild populations is potentially compromised by hybridization (Pierpaoli *et al.* 2003; Driscoll *et al.* 2011), and might result in extinction of the wild subspecies by homogenization of the genetic diversity.

The available studies demonstrate that domestic/wildcat hybridization rates are very diverse throughout Europe, with huge contrast between some areas where only sporadic events occur and others where extensive hybridization persists (Randi 2008). Several reasons can be related with these differences. Oliveira and colleagues (2008a) propose some, as the higher impact of habitat changes on original forest landscapes of central Europe than in mosaic Mediterranean landscapes of south Europe, the different habits towards domestic cats as the practice of feeding feral cats, and different past demographic declines that might have allowed feral domesticates to cross-breed in different ways.

The first studies using sets of molecular *loci* combined with specialized software confirmed that rates of hybridization could not be generalized (Beaumont *et al.* 2001; Randi *et al.* 2001; Pierpaoli *et al.* 2003). In one of the first hybridization studies, Randi and colleagues (2001) found in Italy one recent (based on 12 microsatellite *loci*) and three putative old generation hybrids (based on discordant nuclear/mitochondrial identification) out of 48 putative European wildcats, suggesting a negligible impact of hybridization on this country (2.1%). In contrast, Beaumont and colleagues (2001) found in Scotland that the analysed free living cats contained a mixture of wild and domestic genes probably influenced by past introgression, thus showing widespread

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hybridization, based both on 9 microsatellites and various pelage characteristics. Pierpaoli and co-workers (2003) did an extensive study around Europe, sampling Portugal, Belgium, Switzerland, Italy, Germany, United Kingdom, Slovenia, Hungary and Bulgaria, and also on the Mediterranean island of Sardinia (*F. s. lybica*), based on morphological traits and 12 microsatellite markers. These authors found no hybrid individuals in the Sardinian cats and confirmed the negligible rate of hybridization in Italy. The widespread hybridization scenario in Scotland was also confirmed. Furthermore, Hungary also showed considerable signs of hybridization since the Hungarian wildcats were partially assigned to the domestic cat group, with 12 identified hybrids out of 46 sampled individuals (26.1%). Hybrid individuals were also found in Bulgaria (1 in 35 sampled individuals, 2.9%) and in Portugal (1 in 15 sampled individuals, 6.7%). Later, the results for Italy – low rate of hybridization – and Hungary – extensive admixture – were also confirmed with the use of 27 microsatellites, including 21 linked markers, by Lecis and colleagues (2006). In France, O'Brien and co-workers (2009) found distinct genepools for the two subspecies despite clear evidence of admixed genotypes. The authors conclude that hybridization is rare in this country and that there is a high frequency of genetically pure wildcats. Hertwig and colleagues in 2009 and Eckert and colleagues in 2010 studied hybridization in Germany using 11 and 8 microsatellite *loci* together with alloenzyme *loci*, respectively. Although Eckert and colleagues found some traces of past introgression with no recent evidence of extensive hybridization, Hertwig and co-workers found a hybridization rate of 18.4% in the country, with higher impact on the western population. Still, domestic and wildcats genepools in this country are clearly differentiated, which demonstrates that hybridization is not extensive as in Scotland or Hungary. Later on in 2013, Oliveira did an extensive study of European populations covering almost all the species distribution, with a set of 38 unlinked microsatellites that once more confirmed the highly admixed nature of cat populations in Scotland and Hungary, contrasting with other generally non-admixed European countries where some hybrids can be found. Moreover, the author studied *F. s. lybica* sampled in the islands of Sardinia and Corsica, but also on North Africa, although no hybrids were found within these locations.

Oliveira and colleagues (2008b) did the first genetic study focused in Portuguese wildcat populations and found 4 hybrids, which corresponded to approximately 14% of the sampled individuals. Hybrid individuals were spread through the sampling area, one in the north, one in the centre and two in the south of Portugal (Oliveira *et al.* 2008a). Afterwards, a more extended analysis was done in Portugal and Spain, improving sample size and geographical range, which confirmed the presence

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of hybrids in Portugal with no evidence for hybridization in Spain (Oliveira *et al.* 2008a). Later, on the behalf of a thorough study of hybridization in Europe, Oliveira (2012) found the first evidences of hybridization in Spain, and confirmed previous evidences of hybridization in Portugal. The wildcat was formerly widespread through Portugal and Spain, but the human influence on habitats and population dynamics presented several threats that lead to population decline and higher proximity between domestic and wildcat ranges, eventually leading to increasing hybridization (Oliveira *et al.* 2008b). According to Lozano and Malo (2012), Iberian Peninsula is of particular importance because it is the larger population centre, contributing to a quarter of all European populations, but they also state an important deficiency in genetic studies regarding hybridization, mainly in Spain. Also, Driscoll and colleagues (2007) point out the possible role of the Iberian Peninsula as a glacial refugium (Kitchener & Rees 2009), highlighting the importance of this area for European wildcat genetic diversity.

These studies based on advanced molecular markers and appropriate software allowed a more accurate and thorough study of cat hybridization throughout Europe. Nevertheless, *F. s. lybica*'s populations of North Africa are still poorly studied, and although no evidence of hybridization was found in recent studies (Oliveira 2012), hybridization might still be an important threat for this subspecies (Nowell & Jackson 1996), for which further studies with larger sample sizes are needed.

Setting a threshold for the proportion of admixture for a population to be considered in danger is complex (Allendorf *et al.* 2001) and this ultimately leads to discordant opinions about conservation measures to be applied. Nevertheless, all research regarding hybridizing *taxa* is important mainly to detect non introgressed populations for conservation purposes (Randi 2008). Detecting the amount of pure populations is also important because the less pure populations exist more important the hybrid populations become (Allendorf *et al.* 2001). For instance, in the most affected wildcat populations, mainly Scottish and Hungarian as previously stated, if there are not enough pure wild individuals, protection of hybrids might be the only way to maintain the ecologic function of the species in the ecosystems. In contrast, in other European populations that seem to experience low frequencies of hybridization, conservation measures should focus on pure wild individuals and on identification and neutering of hybrids to preserve the subspecies genetic purity, as Pierpaoli and colleagues (2003) defend. Neutering is an important method to control hybrids, especially because they have similar home ranges to those of wildcats, sometimes overlapping, and are therefore responsible for maintaining or increasing hybridization (Germain *et al.* 2008; Oliveira 2012). Either way, it is important to understand the ecological factors influencing hybridization in each different population. For example,

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according to Germain and colleagues (2008), in their study area in France hybridization might be lower in the winter because of the confinement of the domestic cats in buildings. This is plausible to occur in other locations around Europe.

In general, it is common that the majority of backcross hybrids are almost undistinguishable morphologically from the parental species, and therefore the frequency of admixture might be largely underestimated if only phenotypic characteristics are considered (Mallet 2005), particularly when the parental species are morphologically very identical. Molecular tools enable more accurate identification of hybrids and of pure populations for conservation purposes. Nevertheless, hybrids are often genetically very similar to the parental species, especially backcrosses, and even microsatellites might not be powerful enough to identify all admixed individuals (Oliveira 2012; Nussberger *et al.* 2013). Thus, improving the molecular toolbox for detecting hybridization is demanding.

1.3. Molecular tools

Every study requires techniques that enable researchers to reach the outlined objectives. Molecular techniques have been evolving for a few decades. Today, they facilitate thorough analyses that allow more comprehensive knowledge on several species, and ultimately lead to more complete and accurate conservation plans. These techniques are in constant update.

Particularly, the study of hybridization started with the use of several morphologic characteristics, with subsequent use of molecular tools and software that are continuously advancing into new and more informative ones. In the case of cat studies, some authors described several pelage characteristics that were used to classify individuals as wild, domestic or hybrid (some of those characteristics are shown in figure 5; Beaumont *et al.*, 2001; Daniels *et al.*, 1998; Kitchener *et al.*, 2005). Morphological characteristics, as skull measurements or cranial volume (which are highly correlated with pelage characters; Beaumont *et al.*, 2001) and intestinal indexes (Pierpaoli *et al.* 2003) were traditionally used to distinguish wildcats from the domestic form (Yamaguchi *et al.* 2004; Kitchener *et al.* 2005).

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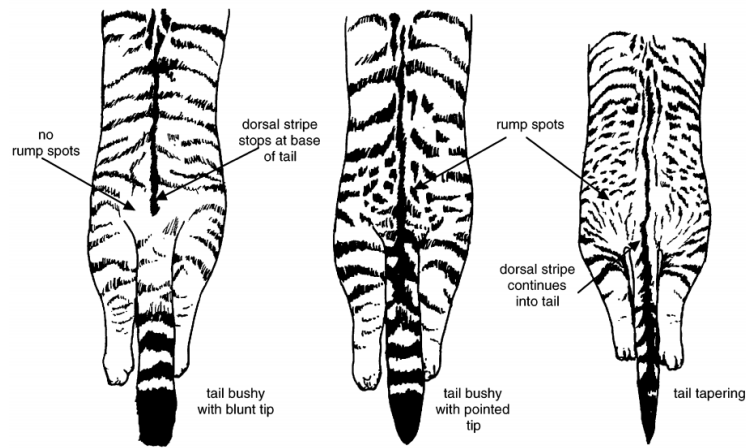
Figure 5 – Some pelage characteristics related with tail shape, dorsal stripe and rump spots, used in morphologic identification of wildcats (left), hybrids (middle) and domestic cats (right). Adapted from Beaumont *et al.* (2001).

It is plausible that in wild living domestic cats and hybrids selection acts against coat colours different from the wild phenotype, which reduces morphological divergence between the wild and domestic subspecies, making distinction based on these traits more difficult (Randi *et al.* 2001). Although Randi and colleagues (2001) show that it is possible to identify African wildcats, European wildcats and domestic cats combining morphological and behavioural traits with the geographical origin, hybrid individuals proved to be much more difficult to identify. Morphological and morphometric traits are not diagnostic to accurately distinguish subspecies, and even less for the identification of hybrid individuals (Beaumont *et al.* 2001; Randi *et al.* 2001; Lecis *et al.* 2006; Driscoll *et al.* 2007; O'Brien *et al.* 2009; Devillard *et al.* 2014), especially if only a rapid examination in the field is possible (Ballesteros-Duperón *et al.* 2014) or if samples are collected from individuals found dead and often deteriorated (Oliveira *et al.* 2008b; O'Brien *et al.* 2009). This lack of accuracy in morphologic identification also happens in other close hybridizing *taxa* as wolf and dog (Verardi *et al.* 2006). Particularly, after some generation of backcrossing, identification of hybrids based on morphologic traits becomes nearly impossible, and thus the real impact of hybridization might be underestimated and the real dynamic of the hybridization process in some populations misunderstood (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). However, until the mid-1960s the detection of hybrids was based on morphological characteristics alone, with the assumption that hybrid individuals should have an intermediate phenotype between the two parental, which is not always true (Allendorf *et al.* 2001). By the same time, the development of protein electrophoresis (alloenzymes) revolutionized the identification of hybrids, and later on, the development of more advanced techniques allowed the study of more *loci* with sophisticated

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software (Allendorf *et al.* 2001).The possibility of identifying admixed individuals and quantifying introgression in closely related hybridizing populations has extraordinary potential for the development of conservation and management action plans.

1.3.1. Molecular markers

The introduction of molecular markers allowed a thorough and more comprehensive study of natural populations and, in particular, of the process of hybridization. Rhymer and Simberloff (1996) refer some markers that were usually used for this purpose, as alloenzymes, random amplified polymorphic DNA (RAPDs), mitochondrial DNA, microsatellites, among others. In particular, the use of highly polymorphic microsatellites combined with Bayesian clustering methods provided an accurate methodology to domestic cat/wildcat individual assignment and identification of hybrids (Oliveira *et al.* 2008b). Nevertheless, other molecular markers are also used, mainly in combination with microsatellites, and others are becoming more and more popular, mainly when considering some limitations of microsatellites.

1.3.1.1. Mitochondrial DNA

Mitochondrial DNA is often a first approach to the study of hybridization, for identification of haplotypes that are specific from each parental population (Wayne & Jenks 1991; Rhymer & Simberloff 1996), and to detect past maternal introgression when the mitochondrial haplotype does not match the nuclear DNA or morphologic results (Driscoll *et al.* 2007; Randi 2008; Hertwig *et al.* 2009). Since it is maternally inherited it can also provide evidence about the direction of hybridization, i.e. if it is more frequent that males of one population are breeding with females of another, or the reciprocal (Rhymer & Simberloff 1996; Hertwig *et al.* 2009). Nevertheless, for more detailed study and correct identification of hybrids, mtDNA should be used along with biparentally inherited nuclear markers (Väli *et al.* 2010).

Insertions of mitochondrial DNA into the nuclear genome are a problem to population genetic studies and phylogenies, since the inserted fragments, *numts*, are paralogs of the authentic sequence but have different evolution rates (Lopez *et al.* 1996; Antunes *et al.* 2007). The majority of cat mitochondrial DNA is inserted in the nuclear genome, and since the domestic cat mitochondrial and nuclear genomes' release it has been easier to assess these *numts* in the cat, providing evidence of multiple independent insertions and duplications widespread across most cat

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chromosomes, and that the proportions of these insertions are comparable to those of man, the highest among mammals (Lopez *et al.* 1996; Antunes *et al.* 2007). Randi and colleagues (2001) amplified mtDNA and stated that in some cases putative *numt* sequences were amplified, which were divergent and phylogenetically basal to the true mtDNA sequences. Nevertheless, several authors amplify some regions of the mtDNA supposedly without nuclear copies, since these can provide important information on past introgression of mitochondrial sequences due to hybridization events (Randi *et al.* 2001; Driscoll *et al.* 2007; Hertwig *et al.* 2009; Eckert *et al.* 2010). However, the portion of mtDNA genome to be amplified and studied has to be chosen carefully, because the heterogeneous mutation rates between true mtDNA and *numts* can lead to significantly biased information.

1.3.1.2.   Microsatellites

Microsatellites, or Short Tandem Repeats (STRs), are tandemly repetitive DNA sequences, usually evolutionary neutral and occurring randomly throughout the genome (Bennett 2000; Li *et al.* 2002; Guichoux *et al.* 2011). The repeat motifs are usually short, with one to six base pairs (Bennett 2000; Li *et al.* 2002; Guichoux *et al.* 2011). Microsatellites have been the marker of choice for many genetic studies, mainly due to their abundance in the genome and high polymorphism (a consequence of their high mutation rate; Bennett, 2000; Guichoux *et al.*, 2011; Li *et al.*, 2002; Väli *et al.*, 2010). These markers provide remarkable information for infering population structure, due to their high allelic richness, considerably higher than SNP markers (Guichoux *et al.* 2011). However, this characteristic along with homoplasy might reduce the power for discriminating sister species, as there are more chances of allele sharing, therefore diminishing their power for hybrid detection, especially beyond the first generation (Morin *et al.* 2004; Nussberger *et al.* 2013). In fact, a large number of makers are necessary to detect introgressed alleles, especially when these markers are highly polymorphic and not diagnostic, as happens in the case of microsatellites (Nussberger *et al.* 2013). Moreover, successful amplification and analysis of microsatellites rely on multiple technical methodologies that should be considered during the whole genotyping process, since choosing the most adequate *loci*, designing the appropriate primers, optimizing multiplex reactions and selecting of the most suitable software for data analyses, among many others (Guichoux *et al.* 2011).

A vast set of STR markers was developed for the domestic cat by Menotti-Raymond and colleagues (1997) for forensic reasons, as domestic cat hairs can sometimes appear in crime scenes and can be used as evidence. The possibility of

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using biological material that yields DNA in low quality or quantity by amplifying small tandem repeats in multiplex reactions was outstanding for the forensic sciences (Menotti-Raymond *et al.* 1997). Shortly after the development of these markers, smaller sets started to be used for conservation studies regarding European wildcat/domestic cat hybridization (Beaumont *et al.* 2001; Randi *et al.* 2001), and have been the preferred option for these studies ever since (Oliveira 2012). However, the distinction of individuals, either parental or hybrid, of intraspecific *taxa* is difficult given the aforementioned limitations of microsatellites. Consequently, in order to improve detection of hybrids, hybridization analyses were improved with the use of Bayesian based clustering methods that provide a probabilistic assessment of individuals to a cluster (Oliveira *et al.* 2008a). These methods are powerful to assess population differentiation, even when reference genotypes are not accessible and/or the hybridization rates are variable, since they are not highly influenced by the proportion of hybrids (Anderson & Thompson 2002; Vähä & Primmer 2006; Oliveira *et al.* 2008a).

The use of linked *loci* might also be beneficial for the study of admixture in natural populations, when linkage groups are known (Falush *et al.* 2003; Lecis *et al.* 2006; Vähä & Primmer 2006), as modelling the "admixture linkage disequilibrium" might enhance the detection of older generation hybrids (Verardi *et al.* 2006; Randi 2008). Nevertheless, closely linked markers are not independent, thus, are less informative than the same number of independent markers and a considerable number of linkage groups is recommended (Lecis *et al.* 2006), requiring increased laboratory effort. Also, the combined use of linked and unlinked microsatellite *loci* can bring more advantages than the use of either alone as shown by Lecis and colleagues (2006). However, Nussberger and colleagues (2013) state that unlinked markers are best for detection of hybrids, which supports that the use of linked *loci* to study admixture is still somewhat controversial (Hertwig *et al.* 2009).

It is important to consider that even with the use of advanced software and a carefully selected set of microsatellites, some hybrid individuals, especially backcrosses, might not be identified (Oliveira 2012). Therefore, the real impact of hybridization might be underestimated. Consequently, more powerful and diagnostic markers are required to accurately detect admixture in natural wild populations of wildcats (Nussberger *et al.* 2013), and single nucleotide polymorphisms are becoming increasingly popular (Oliveira 2012; Nussberger *et al.* 2013).

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1.3.1.3.   Single Nucleotide Polymorphisms

The popularity of single nucleotide polymorphisms (SNPs) for ecology and conservation genetic studies, in particular for the study of hybridization, has been increasing (Morin *et al.* 2004; Seddon *et al.* 2005; Väli *et al.* 2010). SNPs seem a promising tool in these cases for their characteristics. They are usually biallelic, at most tetrallelic, have low degree of homoplasy and are more likely diagnostic than microsatellites (Nussberger *et al.* 2013). Besides, SNPs have several technical advantages over microsatellites, like result's compatibility between laboratories with no need for calibration, they are easier to multiplex because do not rely on detection of fragment length, and most importantly, PCR amplification products can be very short which allows to work better with low quality, fragmented samples (Seddon *et al.* 2005; Guichoux *et al.* 2011; Nussberger *et al.* 2013). Additionally, SNPs might be genotyped with several techniques, in contrast to microsatellites that are usually genotyped using capillary gel electrophoresis coupled with fluorescent based detection (Guichoux *et al.* 2011). Furthermore, SNPs are even more abundant in the genome than microsatellites, in coding and non-coding regions, providing broader genome coverage (Morin *et al.* 2004; Guichoux *et al.* 2011).

The power of SNPs for admixture analyses is based mostly in their highly differentiated allele frequencies between the hybridizing *taxa* (Nussberger *et al.* 2013). Nevertheless, their lower mutation rate might not detect very recent population expansions or structure (Guichoux *et al.* 2011). Also, SNPs have higher ascertainment bias than microsatellites, which makes the population from which SNPs were selected appear more variable and, therefore, influence estimates of population diversity and structure (Morin *et al.* 2004; Seddon *et al.* 2005; Guichoux *et al.* 2011).

Oliveira (2012) selected a set of SNPs including some randomly dispersed through the domestic cat genome, others in morphologic and disease candidate genes with presumed phenotype/genotype correlation in domestic cats and others in candidate genomic regions that revealed polymorphic positions between European wildcat and domestic cat or for which high variability was known among domestic cat. Although she found no diagnostic SNPs, these markers can help identify differential rates of introgression across different genomic regions. Nussberger and colleagues (2013) also adopted this type of genetic marker and developed a set of SNPs for wildcat and domestic cat using a small portion of the genome through high-throughput sequencing of reduced representation libraries and selecting unlinked SNPs with different fixed alleles in the two subspecies. As the wildcat/domestic cat hybridization study move forward to the use of these markers (Mullikin *et al.* 2010; Oliveira 2012;

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Nussberger *et al.* 2013), it is essential to understand the different advantages and efficiency of each type of marker, and which provide the best combination of informative results vs cost of development/genotyping. It should be considered that the combination of two or more types of molecular markers might also be a suitable option for hybridization studies (Väli *et al.* 2010), since different types of markers from the entire genome, preferably representing both neutral and non-neutral variation can provide the most unbiased view of introgression dynamics (Oliveira 2012).

Although the development of advanced molecular methodologies allowed a more accurate study of several *taxa* and, particularly, the detection of hybridization, it is still difficult to have access to a large quantity of samples, mainly at a population level. The majority of samples are collected opportunistically, and animal captures involve high costs and have a low efficiency. The development of more sophisticated molecular techniques also provided an opportunity to increase sample sizes with non-invasive genetic sampling (Beja-Pereira *et al.* 2009), which was previously not feasible due to the low quantity and quality of the extracted DNA.

1.3.2. Non-invasive genetic sampling

Non-invasive population genetics is a combination of techniques to be applied in the field, laboratory and during analytical work that allow the collection, genotyping and analyses of elusive and/or rare animals without disturbing, trapping or even seeing them (Taberlet *et al.* 1999; Broquet *et al.* 2007). Limitations concerning invasive sampling are mostly critical for carnivores, especially for endangered ones whose population densities have decreased largely (Mills *et al.* 2000). The possibility of collecting samples non-invasively had a huge impact on population and conservation genetic studies. The possibilities range from collection of faeces, urine, saliva, hair snares, regurgitated pellets or shed feathers (Taberlet *et al.* 1999; Mills *et al.* 2000), among other remnants or droppings left by animals during their normal activities. Non-invasive genetic sampling is more time effective and allows the collection of a larger number of samples in populations of elusive and rare species, also reducing the anthropogenic pressures related to wildlife trapping and handling (Oliveira *et al.* 2008a; Beja-Pereira *et al.* 2009). In theory, it is possible to perform the same kind of population genetic studies that are usually done with good quality invasive samples (Beja-Pereira *et al.* 2009). However, non-invasive genetics deal with some limitations, especially during laboratory procedures, due to low quantity of target DNA, low quality (degraded)

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DNA, contaminations by alien DNA and/or PCR inhibitors that can lead to genotyping errors and reduced amplification success (Taberlet *et al.* 1999; Broquet *et al.* 2007).

There are some laboratory concerns when dealing with non-invasive samples to improve the analyses of this kind of samples. For instance, it is recommended that samples are correctly and carefully stored, the extraction should be performed in a separate room with sterile conditions to prevent contaminations, performing independent amplification replicas to confirm the genotype, using negative controls to detect contaminations, using specific primers and carefully chosen molecular markers, among many others (Bonin *et al.* 2004; Broquet *et al.* 2007; Beja-Pereira *et al.* 2009; Kolodziej *et al.* 2013). These are extremely important for reducing the chances of contamination and to reduce genotyping error rates. All samples are prone to genotyping errors that occur when the genotype identified by molecular analyses does not match the real genotype of the individual, and these can bias the final results (Bonin *et al.* 2004). These errors are associated with the amplification of DNA. In the case of microsatellite amplification two types of genotyping errors are more frequently considered – allelic dropouts that occur when one allele is not amplified and produce false homozygotes, and false alleles that occur during the initial steps of the PCR reaction and result in the amplification of artefacts often misidentified as true alleles, producing false heterozygotes (Taberlet *et al.* 1999; Valière 2002; Broquet & Petit 2004). These errors can influence allele frequencies, and consequently interfere with analyses of Hardy-Weinberg equilibrium, inbreeding, population structure, individual identification, population size, among other, and can happen for many different unpredictable reasons during all the genotyping procedure (Bonin *et al.*, 2004 and references therein). Genotyping error rates are mainly considered when dealing with non-invasive samples, due to the low quality and quantity of the extracted DNA, and should be assessed to understand how reliable the resulting genotypes are. Different methods are used for calculating error rates, for example, by comparison between a reference genotype (obtained from a good quality, invasive sample) and non-invasive genotype, among several independent replicas (provided by independent amplification of DNA or, when possible, independent extractions), between independent replicas and the consensus genotype (Bonin *et al.* 2004; Kolodziej *et al.* 2013).

European wildcats are extremely elusive and have low population densities in Iberian Peninsula. These characteristic difficult the collection of large sample sizes by invasive sampling procedures that imply long, extensive and persistent efforts (Oliveira *et al.* 2008a; b). Therefore, collection of non-invasive samples seems a promising tool for the study of this endangered feline.

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1.4. Objectives

The Iberian Peninsula is a particularly interesting area to study the European wildcat. Although some ecological and genetic studies have been done lately (Lozano *et al.* 2003; Sarmento *et al.* 2006; Oliveira *et al.* 2008a; b; Millán & Rodríguez 2009; Monterroso *et al.* 2009), more recent and widespread studies are needed to fully understand its distribution and abundance, current threats and ecological factors that influence fitness, to produce clear and efficient conservation plans. Particularly, it is crucial to understand population dynamics and threats at local levels, especially regarding interbreeding with domestic cats and consequent pollution of the wildcat genepool. Genetic studies are still necessary to thoroughly understand the hybridization dynamics of these endangered populations, namely by enlarging the information across the whole Iberian range, but also by assessing the real levels of hybridization within some populations. Furthermore, given the extreme lack of information concerning northern African wildcats and taking into account that hybridization might also be threatening this endangered subspecies (Driscoll *et al.* 2007), it is of major importance to investigate these populations and raise awareness for this poorly studied feline.

Considering this, two major objectives to this work were outlined.

i) Evaluate the occurrence of hybridization in the Iberian Peninsula, and in order to achieve this objective we aim to:
   a. Optimize a panel of microsatellites to detect hybridization using invasive and non-invasive samples;
   b. Determine levels of genetic variability and differentiation in European wildcats and in domestic cats;
   c. Study the hybridization process at a population scale.

ii) Test the optimized microsatellite panel in the detection of hybridization between the northern African wildcat and the domestic cat in North Africa. In order to achieve this objective we aim to:
   a. Determine levels of genetic variability and differentiation in both subspecies;
   b. Access the occurrence of hybridization between *F. s. lybica* and *F. s. catus*.

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2. Methodologies

2.1. Sample collection

In order to achieve an extensive sample set of Iberian cats, several public and private institutions were contacted to collect and provide us samples from across the Iberian Peninsula's wildcat range. A total of 99 invasive samples (tissue from dead animals, blood, hair or saliva) were collected from animals found dead or captured on the scope of ongoing projects, the Life Lynx program and a Valladolid wildcat association or opportunistically by other researchers and veterinarians across the Iberian Peninsula (putative domestic and European wildcats; n=77) and north Africa (putative African wildcats; n=22). Whenever possible, samples were identified by the collectors as putative wildcats (European and African) or domestic cats, based in morphologic characteristics (size, coat colours, skin and tail patterns). Also, a total of 91 scat samples were collected either on the behalf of other research projects such as the study of Iberian Peninsula mesocarnivores (Monterroso 2013), or by field biologists specifically for this study, including one scat from North Africa. Although non-invasive sampling procedures varied slightly among collectors, in general they were performed by surveying designed transects on foot and scats were collected taking all precautions to prevent contaminations from the collector or cross-contaminations from other samples. The main reason for the collection of non-invasive samples was to increase the total number of samples per population in Iberian Peninsula.

Overall, a total of 190 new samples were extracted and analysed, from across the Iberian Peninsula and North Africa (figure 6 and table S 1, Supplementary Material I). Additional 62 reference samples from the Iberian Peninsula were already genotyped in previous works developed in CIBIO/InBIO-UP (Portugal), and were chosen based on their high probability of assignment to the pure wild (n=20) and pure domestic (n=42) subspecies, based on morphology and genetic analyses. These samples were amplified along other invasive samples for the new set of microsatellites.

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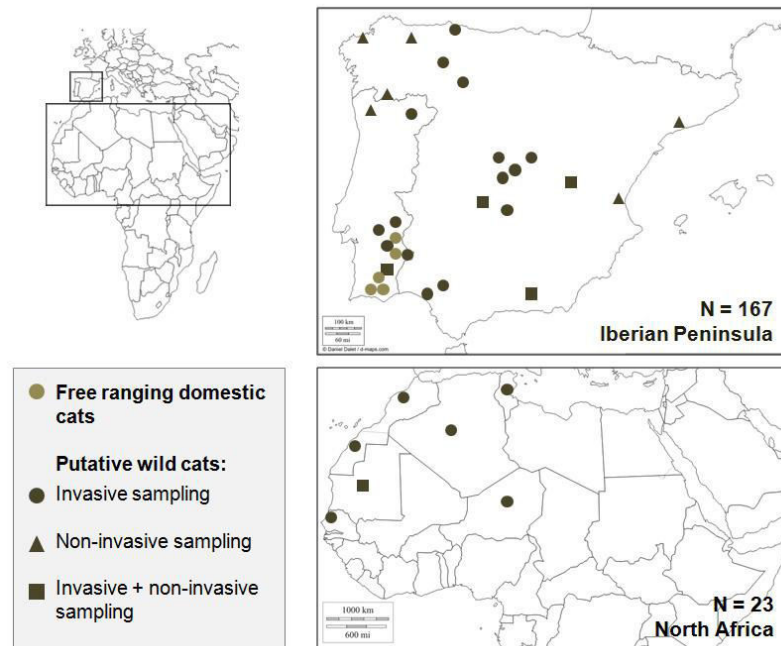
Figure 6 – Approximate location of cat samples collected in this study across the Iberian Peninsula and North Africa.

Moreover, 9 random bred house cats were sampled for both scats and saliva by their owners, in order to assess the genotyping efficiency of non-invasive genetic procedures.

2.2. DNA extraction and quantification

Invasive samples

Tissue, hair and blood samples were stores frozen or in 96% ethanol, and DNA was extracted with EasySpin Genomic DNA Tissue Kit (Citomed), following manufacturer's protocol, and DNA from saliva was extracted using the Buccal Swab Spin Protocol (in QIAamp® DNA Mini and Blood Mini Handbook, pages 36-38, Quiagen). DNA from clotted blood samples was extracted using the same protocol as used for blood samples, but with previous wash in PBS solution (Citomed) to clean the samples from possible PCR inhibitors.

The approximate quantity and quality of extracted DNA was tested by electrophoresis in 0.8% (w/v) agarose gel containing GelRed (DNA fluorescent dye; BioTarget). Three μl of bromophenol blue were added to two μl of extracted DNA and then loaded in the gel. Gels were run at 300V and the extracted DNA was visualized in a UV transilluminator device (Bio-Rad). DNA samples were then diluted accordingly.

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Non-invasive samples

Non-invasive scat samples were stored at room temperature in 96% ethanol until extraction, and dried at 60ºC for approximately 2 days before extraction. DNA was extracted following Frantz *et al.* (2003) protocol after the GuSCN/silica method (Boom *et al.* 1990), with an additional final step for further removal of potential PCR inhibitors using pre-rinsed Microcon® YM-30 centrifugal Filter Units (Millipore, Billerica, MA). Negative controls were included to monitor potential DNA contaminations. The procedures were performed in a dedicated low quality DNA laboratory, under sterile conditions and positive air pressure in order to prevent contaminations.

To assess the concentration of DNA, some samples were quantified with Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen) method in VICTOR[3] Multilabel Plate Reader (PerkinElmer).

All DNA samples were stored at -20ºC until later use.

2.3. DNA amplification

Selection of microsatellite markers

A set of microsatellites was chosen among the 38 microsatellites amplified by Oliveira (2012), which were developed for the domestic cat by Menotti-Raymond and colleagues (1997, 1999, 2003) and chosen according to the assortment made by Lipinski *et al.* (2008) following criteria of high heterozygosity, high polymorphism and wide chromosomal distribution. Microsatellites with higher values of F_{ST} and R_{ST} per *locus* between domestic cats from Europe and wildcats from the Iberian Peninsula were selected, since it is expected that those are the best to discriminate between Iberian wildcats and domestic cats. These genetic parameters were calculated using FSTAT v.2.9.3.2 (Goudet 2001). Using domestic cats from across Europe does not influence the calculations since this subspecies does not present genetic structure in this continent (Pierpaoli *et al.* 2003; Oliveira *et al.* 2008b). Also, the probability of identity (P_{ID}) and the probability of identity between siblings (P_{IDsib}; Mills *et al.*, 2000; Waits *et al.*, 2001) were calculated for the Iberian wild individuals to identify the microsatellites with higher power of individual identification, using software GIMLET v.1.3.3 (Valière 2002). P_{ID} can be defined as the probability of two randomly sampled individuals from the same population having the same genotype at multiple *loci* (Waits *et al.* 2001). Microsatellites with the lowest P_{ID} values will be the ones that perform the more precise individual identification. Considering these parameters, 15 autosomal

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unlinked microsatellites (see table 1) were chosen for the development of this work, although one (FCA262) was subsequently removed from analyses.

Table 1 – Description of 15 microsatellites used to genotype all *Felis silvestris* samples. Locus name, chromosomal location (Chr), number of repetitions (NR; *locus* marked with * show intermediate alleles) and primer sequences, according to Menotti-Raymond *et al.* (1999). Allele range was obtained after genotyping of all samples. FCA262 (marked with **) was removed from analyses and therefore, the allele range is shown according to Oliveira (2012).

Locus	Chr	NR	Primer sequences (5' – 3')	Allele range
FCA023	B1	2	F:CAGTTCCTTTTTCTCAAGATTGC R:GCAACTCTTAATCAAGATTCCATT	155-179
FCA035	D2	2	F:CTTGCCTCTGAAAAATGTAAAATG R:AAACGTAGGTGGGGTTAGTGG	159-181
FCA043	C2	2	F:GAGCCACCCTAGCACATATACC R:AGACGGGATTGCATGAAAAG	141-161
FCA096	A2	2	F:CACGCCAAACTCTATGCTGA R:CAATGTGCCGTCCAAGAAC	207-257
FCA097	B1	2*	F:TAATGTTCAACTTGAATTGCTTCC R:GAACAGTAGTTTGCCCATACAGG	152-175
FCA126	B1	2	F:GCCCCTGATACCCTGAATG R:CTATCCTTGCTGGCTGAAGG	145-177
FCA132	D3	2	F:ATCAAGGCCAACTGTCCG R:GATGCCTCATTAGAAAAATGGC	161-185
FCA149	B1	2	F:CCTATCAAAGTTCTCACCAAATCA R:GTCTCACCATGTGTGGGATG	143-159
FCA220	F2	2*	F:CGATGGAAATTGTATCCATGG R:GAATGAAGGCAGTCACAAACTG	226-242
FCA223	B3	2	F:CTGGGCACTAGGTGTGCAC R:GGTCTTGGATTAGAACCGAGG	218-256
FCA229	A1	2	F:CAAACTGACAAGCTTAGAGGGC R:GCAGAAGTCCAATCTCAAAGTC	171-195
FCA262**	D2	2	F:ATCTCTTCCATGGTGTGTGATG R:TACAGAATACTCCCCCCGC	163-195
FCA310	C2	2	F:TTAATTGTATCCCAAGTGGTCA R:TAATGCTGCAATGTAGGGCA	132-158
FCA391	B3	4	F:GCCTTCTAACTTCCTTGCAGA R:TTTAGGTAGCCCATTTTCATCA	238-282
FCA698	D1	2	F:GGGAAATAACAGGCTAGCAGG R:TCAGGCTTCACACTCACAGTG	226-282

Invasive samples

Initially, the 15 microsatellites were distributed in two multiplexes (table S 2), Supplementary Material II) according to their allele range and the possible interactions between primers, checked using AUTODIMER v.1.0 (Vallone & Butler 2004). Multiplex reaction MixII was later subdivided, since some samples with small DNA quantity/quality were not amplified properly at all *loci* (see table S 2). All PCR reactions were performed using the M13-tailed primer method (Oetting *et al.* 1995; Neilan *et al.* 1997), modifying all forward primers with universal tails fluorescently labelled with 6-FAM, VIC, NED and PET dyes (Applied Biosystems; see Beja-Pereira *et al.*, 2009) on a T100 Thermo Cycler (Bio Rad). Primer multiplexes included the forward primers 10x

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diluted, the respective tail primers and the reverse primers. A final PCR volume of 10 µl was used, including 5 µl of Multiplex PCR Master Mix (Quiagen), 1 µl of Primer Multiplex and 1 or 2 µl of DNA according to its estimated concentration (corresponding to approximately 5-10 ng of DNA), completed with destilled H_2O. For testing possible contaminations, all PCR reactions included a negative control. PCR conditions included an initial denaturation step of 15 min at 95ºC, followed by a touchdown programme with a total of 7 cycles of 30s at 95ºC, 45s at 59-56ºC and 30s at 72ºC, decreasing 0.5ºC per cycle. Following these, 25 cycles were performed with similar conditions but with annealing temperature of 56ºC, and 8 cycles at 53ºC. A final extension step of 30min at 60ºC was also performed.

The amplification success was tested by electrophoresis in 2% (w/v) agarose gel, with the use of a 100-1000bp DNA ladder Marker V (NZYtech). The amplified DNA fragments were separated by size in an automatic sequencer ABI3130xl Genetic Analyzer (Applied Biosystems) with the use of an internal marker GeneScan™ 500 LIZ (Life Technologies, Applied Biosystems).

Non invasive samples

Non invasive samples were submitted to similar procedures with slight modifications due to its particularities (Beja-Pereira *et al.* 2009). Scat samples of sympatric species are often difficult to distinguish and, although collected by experienced field biologists, should always be genetically identified to the species level. Thus, extracted DNA was initially used to identify the species and distinguish cat samples by amplifying a fragment (600 bp) of the mitochondrial DNA Control Region, using primers CR1 and CR2 (Palomares *et al.* 2002). PCR conditions included an initial denaturation step of 15min at 95ºC, followed by 40 cycles of 20s at 95ºC, 20s at 58ºC and 20s at 72ºC, with a final extension of 10min at 60ºC. PCR results were treated with two enzymes, ExoI and FastAP, to remove single stranded DNA. Sequencing reaction was performed using the forward primer, with PCR conditions that included an initial denaturation step of 3min at 94ºC, followed by 24 cycles of 10s at 96ºC, 5s at 55ºC and 4min at 60ºC. Sequence results were finally cleaned with Sephadex G-50 Medium (GE Healthcare Bio-Sciences AB) and separated in the automatic sequencer ABI3130xl. Species identification was performed using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990) on the National Center for Biotechnology Information (NCBI) database (Benson *et al.* 2012; Acland *et al.* 2014).

A fragment of the Interphotoreceptor Retinoid Binding Protein (IRBP) nuclear gene, known for its capacity to distinguish mesocarnivore species (Oliveira *et al.* 2010), was additionally used for species identification. PCR conditions for this reaction were

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slightly different, with 30 seconds of denaturation time during the 40 cycles and a final extension of 5min at 72ºC. Subsequent procedures were performed equally. All reactions were performed in a T100 Thermo Cycler (Bio Rad).

The 14 microsatellite *loci* were rearranged in three smaller multiplexes in order to facilitate the amplification in low quantity and low quality DNA (see table S 2). In order to increase the quantity of DNA template for the amplification, a combination of two reactions was performed. A first pre-amplification PCR reaction using 1µl of primer multiplexes containing the forward and reverse primers for each microsatellite, 5µl of Multiplex PCR Master Mix (Quiagen) and 2µl of template DNA, following PCR conditions with initial denaturation of 15min at 95ºC, 20 cycles with 30s at 95ºC, 60s at 57ºC and 30s at 72ºC, with final extension of 30min at 60ºC; and a second PCR reaction using as template 1µl of pre amplified solution, and primer multiplex containing only the tail primer and reverse primer, following the same PCR conditions as used for DNA extracted from invasive samples. Multiplex MixNI3 required further optimization and thus was later portioned into two smaller multiplexes (see table S 2).

For all second PCR reactions, four replicas were amplified in order to accurately identify the genotypes for each *locus*. The same sequencing procedure as for invasive samples was applied and the four replicas were sequenced independently.

2.4. Data analysis

Microsatellite sequencing results were visualized using the software GENEMAPPER 4.0 (Applied Biosystems) and resulting genotypes were determined by comparison with size standard fragments of the internal marker.

For the non-invasive samples, resulting genotypes for the four replicas were compared and the correct alleles were inferred by the consensus between the four genotypes. For a heterozygous genotype to be considered it had to be present in at least two replicas. On the other hand, for a homozygous genotype to be considered it had to be present in at least three replicas. This is crucial to avoid errors related to genotyping low quality DNA, like allelic dropout and false alleles. These error rates were calculated using software PEDANT v.1.0 (Johnson & Haydon 2007a; b), and were then used to obtain the consensus threshold in software GEMINI v.1.3.0 (Valière *et al.* 2002). Lastly, the consensus threshold was used to run the "consensus genotypes" option in software GIMLET v.1.3.3 (Valière 2002) in order to obtain a consensus for the four replicates taken into account the error rate, and to compare this with the previous manually done one.

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Given the fact that different scat samples might belong to the same individual, the presence of repeated samples was checked running the "regroup genotypes" option on software GIMLET v.1.3.3. This procedure was not necessary for invasive samples since there is no risk of repeated individuals.

Error rates were also calculated using the test samples for comparison, since it is expected that genotyping of good quality invasive samples is more accurate and the resulting genotypes can be used as references, and therefore this comparison will provide realistic rates of allelic dropout and false alleles for the genotyping of non-invasive samples (Kolodziej *et al.* 2013). These calculations were performed by comparison between the consensus genotypes for the non-invasive test samples and the invasive genotypes of the same samples (used as reference), using GIMLET v.1.3.3.

Samples with 30% or more missing data were excluded from analysis. Finally, for all samples the potential presence of null alleles, after Bonferroni correction, and scoring errors were assessed using MICRO-CHECKER v.2.2.3 (Van Oosterhout *et al.* 2004). With the complete database, comprising invasive and non-invasive sample genotypes, the Probability of Identity (P_{ID}) and Probability of Identity between Siblings (P_{IDSib}) were calculated using GIMLET v.1.3.3, in order to assess the power of individual identification of the set of microsatellites.

2.4.1. Genetic diversity analysis

Genetic diversity was analysed for each of the three subspecies separately and excluding all putative admixed individuals found in hybridization analysis using a more conservative threshold of $q>0.90$ for STRUCTURE results (see below), in order to assure that only pure individuals were used.

Deviations from Hardy-Weinberg equilibrium (HWE; Markov chain length of 1000000 and 10000 dememorization steps) and from pairwise linkage equilibrium (LE; 10 initial conditions and 10000 permutations) for all *locus*-population combinations were assessed using software ARLEQUIN v.3.5.1.3 (Excoffier & Lischer 2010). For both, the significance level for p-values was adjusted using Bonferroni correction. The same software was used to compute allele frequencies, mean number of alleles (N_A) and observed and expected heterozygosities (H_O and H_E). F_{IS} over all *loci* for each subspecies was estimated using FSTAT v.2.9.3.2 (Goudet 2001). Allelic richness (Ar) and private allelic richness (PAr) for each subspecies were computed using HP-RARE v.1.1 (Kalinowski 2005), following a rarefaction procedure that compensates for different sample sizes (Kalinowski 2004). Therefore, the number of genes was set to 22

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given the low African wildcat sample size of 11 individuals. Pairwise F_{ST} (Weir & Cockerham 1984) and R_{ST} (Slatkin 1995) statistics were calculated to infer genetic differentiation among the three subspecies, using software ARLEQUIN. An analysis of molecular variance (AMOVA) was conducted among and within groups for the three subspecies and for each pairwise combination (*F. s. silvestris* vs *F. s. catus*; *F. s. catus* vs *F. s. lybica* and *F. s. silvestris* vs *F. s. lybica*) in software ARLEQUIN, with 10000 permutations using number of different alleles (F_{ST}-like) to calculate molecular distances.

2.4.2. Individuals' assignment and admixture analysis

In order to assess the capacity of the selected microsatellites to differentiate domestic and wildcats, and to have a preliminary analysis of populations' structure, a Factorial Correspondence Analysis (FCA) was performed on software GENETIX v.4.0 (Belkhir *et al.* 2004) with a database comprising all European wild and domestic individuals. For a more detailed study and accurate distinction of the subspecific origin of the sampled individuals (individuals' assignment) a Bayesian analysis was performed on software STRUCTURE v.2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2007; Hubisz *et al.* 2009) with individuals of domestic and European wild subspecies. Prior information was used for reference individuals of both *taxa*. The analysis was performed using the admixture model and assuming correlated allele frequencies (which is often more efficient for analyses of closely related groups; Falush *et al.*, 2003), with 250000 burn-in followed by 1000000 Monte Carlo Markov Chain (MCMC) iterations, each run repeated independently 5 times to check the consistency of the results. The number of populations (K) was set to 2. The threshold of $q>0.85$ to assign an individual to a cluster was established by posterior analyses (see below). Subsequently, software NEWHYBRIDS v.1.1 Beta (Anderson & Thompson 2002) was used to thoroughly study the hybridization class of the putative hybrids found previously. Six hybrid classes were defined: i) pure wildcat (FSI), ii) pure domestic cat (FCA), iii) F1 hybrids (F1), iv) F2 hybrids (F2), v) first generation backcross with wildcat (BxFSI), vi) first generation backcross with domestic cat (BxFCA). The burn-in period of 100000 was performed, followed by 500000 MCMC runs and "Uniform" priors were used for mixing proportions and allele frequencies.

Taking into account that Bayesian analysis lacks a statistical validation of the assumed distribution of priors, simulations are required to evaluate the power of the set of microsatellite for assigning each individual to a parental or hybrid class (Nielsen *et*

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al. 2006). Accordingly, simulated genotypes of parental and hybrid classes were generated using software HYBRIDLAB v.1.0 (Nielsen *et al.* 2006). Twenty randomly selected individuals from the reference database, of both European wildcats and domestic cats, were used to create forty simulated genotypes of each parental subspecies. These were then used to simulate forty genotypes of each hybrid class, including second generation backcrosses with domestic and wildcats. All resulting simulated genotypes were analysed in STRUCTURE using the same conditions as previous analyses. NEWHYBRIDS was also performed using same conditions as preceding analyses but varying the number of classes to test, either assuming six aforementioned classes or assuming eight classes that include second generation backcross with wild (Bx_2FSI) and domestic cats (Bx_2FCA).

2.4.3. Population structure analysis

In order to investigate the existence of structure in the wildcat populations of Iberian Peninsula, a dataset consisting of only pure wild individuals was used to compute a Factorial Correspondence Analysis in GENETIX. Individuals were identified as Portuguese or Spanish samples to simplify posterior visualization of results. The same database was used subsequently on software STRUCTURE with the same conditions as previous analyses, but with no prior information. The number of populations (K) was tested from 1 to 10, and the optimal number of clusters was identified according to the Evanno method (Evanno *et al.* 2005) implemented on the web version of STRUCTURE HARVESTER (Earl & VonHoldt 2012).

2.4.4. African wildcats' individual assignment

To infer the power of the set of microsatellites in discriminating African wildcats from European wildcats and domestic cats, the full dataset comprising all samples of the three subspecies was used first on GENETIX software for a preliminary graphic view of the distinction among the three subspecies. For more detailed analysis STRUCTURE software was used with the same conditions as previously. Prior information was used only for European wild and domestic cats, since there were no reference samples for African wildcats. The number of clusters was tested from K=2 to K=5 and optimal number of clusters identified as aforementioned in the previous analysis. Then, the same software was used to understand how accurately African wildcats, domestic cats and putative hybrids between the two were identified, using a dataset with only

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reference domestic individuals and African wildcat samples. Same conditions were used and number of clusters was forced to 2. No simulations were performed for African wildcat samples because no reference samples were available, but the same threshold value was used as for previous analyses.

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3. Results

From the initial 99 invasive samples, 84 were analysed, 69 from the Iberian Peninsula and 15 from North Africa, resulting in 84.8% success for extraction and amplification of invasive samples.

From the total 91 scat samples, 45 were already extracted and identified as *F. silvestris* on the behalf of other research projects. From the remaining 46 scat samples extracted during the course of this project, 38 were successfully extracted (extraction success of 82.6%). Within these 38 samples, 7 were identified by the fragment of mitochondrial DNA Control Region or IRBP nuclear gene as wolfs/dogs (*Canis lupus*), 20 as red foxes (*Vulpes vulpes*) and 3 as other mammals or contaminated by prey DNA. Therefore, only the remaining 8 samples were identified as cats (*Felis silvestris*; 21.1% accurate morphological identification of scats) and, together with the 45 previously identified, were used in further analysis. From these 53 samples 22 were eliminated from analysis due to excessively fragmented DNA that was not possible to amplify (58.5% amplification success). Samples with the same genotype or with only one allele difference, sampled in the same region, were considered the same individual and therefore, three samples were eliminated from further analysis. Mean concentration of DNA for non-invasive samples was 3.09 ng/μl, ranging from 1.18 to 17.21 ng/μl.

The complete database was checked for missing data and four samples with more than 30% missing data were eliminated. European wildcats and domestic cats showed evidences of null alleles in 6 and 4 *loci*, respectively.

The selected microsatellites showed overall high values of F_{ST} and R_{ST} (table 2). FCA096 showed the highest F_{ST} value (0.257) and FCA229 showed the highest R_{ST} (0.665). FCA132 and FCA043 showed the lowest F_{ST} (0.059) and R_{ST} (0.197), respectively. Allelic richness and expected heterozygosity were, overall, high for the three analysed cat subspecies (table 2).

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Table 2 – Information regarding microsatellite *loci* used to genotype all *Felis silvestris* samples. F_{ST} and R_{ST} values were calculated with reference samples (European wildcats and domestic cats) for the selection of markers. For each subspecies the values for number of samples (N, including reference individuals and pure individuals identified in STRUCTURE), number of alleles (N_A), allelic richness (Ar) and observed and expected heterozygosity (H_O and H_E) are shown.

Locus	F_{ST}	R_{ST}	*F. s. silvestris* (N=68)				*F. s. catus* (N=93)				*F. s. lybica* (N=11)			
			N_A	Ar	H_O	H_E	N_A	Ar	H_O	H_E	N_A	Ar	H_O	H_E
FCA023	0.21	0.47	5	3.81	0.62	0.68	12	6.75	0.66	0.67	9	9.00	0.73	0.89
FCA035	0.11	0.25	12	8.06	0.35	0.84	6	3.00	0.37	0.54	6	6.00	0.46	0.41
FCA043	0.20	0.20	11	5.46	0.53	0.65	9	5.58	0.63	0.70	9	9.00	0.91	0.86
FCA096	0.26	0.59	11	8.37	0.91	0.88	12	5.45	0.50	0.53	14	14.00	0.91	0.95
FCA097	0.12	0.30	9	5.93	0.61	0.69	9	6.35	0.74	0.83	8	8.00	0.91	0.83
FCA126	0.08	0.43	10	6.08	0.74	0.78	10	6.08	0.71	0.78	10	10.00	0.82	0.91
FCA132	0.06	0.31	12	7.17	0.73	0.78	10	7.40	0.84	0.85	9	9.00	0.82	0.87
FCA149	0.11	0.30	6	3.38	0.43	0.52	7	5.30	0.62	0.76	6	6.00	0.82	0.81
FCA220	0.17	0.57	9	6.30	0.54	0.77	6	4.60	0.61	0.62	8	8.00	0.73	0.90
FCA223	0.10	0.46	7	4.52	0.66	0.66	14	7.32	0.66	0.81	10	10.00	0.82	0.91
FCA229	0.22	0.67	8	5.01	0.51	0.66	11	5.72	0.61	0.72	8	8.00	0.82	0.87
FCA310	0.15	0.22	2	1.16	0.02	0.02	10	5.23	0.75	0.76	9	9.00	0.73	0.84
FCA391	0.09	0.54	10	7.75	0.46	0.86	7	4.64	0.62	0.64	11	11.00	0.91	0.93
FCA698	0.11	0.41	10	5.64	0.61	0.74	15	9.37	0.85	0.88	11	11.00	0.91	0.93

Probability of Identity (P_{ID}) and Probability of Identity between Siblings (P_{IDSib}) were calculated to assess the power of individual identification of the set of microsatellites. FCA698 is the most informative *locus* and the overall values for the set of 14 microsatellites were 3.06 e^{-18} and 7.33 e^{-7} for P_{ID} and P_{IDSib}, respectively (table 3). P_{ID} value is considered low below 0.01 and as the overall value decreases, the statistical confidence in the individual identification increases (Waits *et al.* 2001).

Table 3 – Probability of Identity (P_{ID}) and Probability of Identity between Siblings (P_{IDSib}) in increasing order of single *locus* values for the 14 microsatellites. The first *locus* is the most informative one and subsequent values are cumulative.

	P_{ID}	P_{IDSib}
FCA698	2.16E-02	3.10E-01
FCA132	6.43E-04	9.97E-02
FCA096	1.82E-05	3.24E-02
FCA229	6.57E-07	1.07E-02
FCA126	2.62E-08	3.59E-03
FCA223	1.03E-09	1.21E-03
FCA097	4.21E-11	4.07E-04
FCA391	2.52E-12	1.48E-04
FCA023	1.70E-13	5.47E-05
FCA220	1.28E-14	2.08E-05
FCA043	9.58E-16	7.90E-06
FCA035	1.07E-16	3.30E-06
FCA149	1.47E-17	1.43E-06
FCA310	3.06E-18	7.33E-07

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Genotyping error rates were calculated for non-invasive samples. Overall, the rates of allele dropout and false alleles calculated among replicas were very low. The mean values for allele dropout and false alleles were 0.005 and 0.000, respectively (table 4).

Table 4 – Values of allelic dropout and false alleles per *locus*, for non-invasive samples.

	Allelic dropout	False alleles
FCA023	0.000	0.000
FCA035	0.000	0.000
FCA043	0.000	0.000
FCA096	0.000	0.000
FCA097	0.000	0.000
FCA126	0.000	0.000
FCA132	0.000	0.000
FCA149	0.016	0.000
FCA220	0.011	0.000
FCA223	0.000	0.000
FCA229	0.000	0.000
FCA310	0.000	0.000
FCA391	0.040	0.000
FCA698	0.000	0.000

Error rates per *locus* were also calculated with test samples (invasive and non-invasive) and are presented below. Mean values are 0.016 and 0.050 for allelic dropout and false alleles, respectively (table 5).

Table 5 – Error rates per *locus* (allelic dropout and false alleles) for non-invasive samples, based on genotyping of invasive and non-invasive samples.

	Allelic dropout	False alleles
FCA023	0.000	0.000
FCA035	0.000	0.000
FCA043	0.000	0.000
FCA096	0.000	0.250
FCA097	0.000	0.000
FCA126	0.000	0.000
FCA132	0.000	0.000
FCA149	0.000	0.200
FCA220	0.000	0.000
FCA223	0.143	0.000
FCA229	0.000	0.000
FCA310	0.000	0.000
FCA391	0.000	0.000
FCA698	0.000	0.000

3.1. Genetic diversity

All microsatellites were polymorphic for the three analysed cat subspecies. Both European wildcats and domestic cats exhibited some *loci* significantly deviating from HW equilibrium and some combinations of *loci* in linkage disequilibrium (table 6).

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There was no evidence of deviations to HWE or LE in all African wildcat samples (table 6). Number of alleles per *locus* ranged from 2 to 12, from 6 to 15 and from 6 to 14 in European wild, domestic and African wildcats, respectively (table 6). The lowest number of alleles (N_A=2) occurred in the *locus* FCA310 and the highest (N_A=15) in the *locus* FCA698. The observed heterozygosity varied between the lowest value of 0.02 for *locus* FCA310 and the highest value of 0.91 for *locus* FCA096, both in the European wildcat subspecies. Expected heterozygosity ranged between 0.02 for *locus* FCA310 in European wildcats and 0.95 for *locus* FCA096 in African wildcats. Mean observed heterozygosity values were always lower than expected heterozygosity with F_{IS} values greater than zero, especially for European wildcats. African wildcats showed the highest allelic richness (9.14) and private allelic richness (3.27), while European wildcats exhibit the lowest allelic richness (5.62) and domestic cats the lowest private allelic richness (0.81, table 6).

Table 6 – Genetic diversity parameters using 14 microsatellites for the three analysed cat subspecies, excluding putative hybrids. N – number of samples; N_A – mean number of alleles *per locus*; Ar – allele richness; PAr – private allele richness; H_o – observed heterozygosity; H_e – expected heterozygosity; F_{IS} – inbreeding coefficient; HWE – number of *loci* with significant deviations of HW equilibrium (significance level α=0.001, Bonferroni corrected) and LE – number of *loci* pairs in linkage disequilibrium for 91 pairwise comparisons (significance level α=0.0005, Bonferroni corrected). Standard deviation for N_A, H_o and H_e are shown in brackets.

Subspecies	N	N_A	Ar	PAr	H_o	H_e	F_{IS}	HWE	LE
F. s. silvestris	68	8.71 (±2.87)	5.62	1.34	0.55 (±0.21)	0.68 (±0.215)	0.19	6	11
F. s. catus	93	9.86 (±2.80)	5.91	0.81	0.65 (±0.13)	0.72 (±0.11)	0.09	2	6
F. s. lybica	11	9.14 (±2.07)	9.14	3.27	0.81 (±0.12)	0.85 (±0.13)	0.06	0	0

Genetic differentiation among subspecies inferred by pairwise F_{ST} and R_{ST} statistics varied from 0.090 and 0.222, and 0.091 and 0.690, respectively (table 7). All values revealed moderate to great divergence between each pair of subspecies, and for the majority of combinations of R_{ST} values were considerably higher than F_{ST}. For both parameters, the lowest values were observed between African wildcats and domestic cats.

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Table 7 – Pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) statistics for European wildcats, domestic cats and African wildcats, with exception of putative hybrids. All values are statistically significant (p<0.05).

	F. s. silvestris	*F. s. catus*	*F. s. lybica*
F. s. silvestris	-	0.621	0.690
F. s. catus	0.222	-	0.091
F. s. lybica	0.190	0.090	-

Analyses of Molecular Variance (AMOVA) for the three subspecies *F. s. silvestris*, *F. s. catus* and *F. s. lybica*, and for each pairwise combination (table 8) were performed excluding individual level. When considering the three subspecies, highest percentage of variation is found within these, although a considerable proportion of variation is attributed to differentiation among subspecies, supported by F_{ST} values that indicate considerable genetic variability among the three *taxa* (0.188). This pattern of higher percentage of variation within subspecies was also found in all other comparisons. Nevertheless, for European wildcat/domestic cat comparison the percentage of variation found among subspecies was higher, confirmed by a higher F_{ST} value, while for domestic cat/African wildcat comparison the variation within subspecies was considerably lower, also confirmed by a lower F_{ST} value.

Table 8 – Analyses of Molecular Variance (AMOVA) for the three cat subspecies (FSI/FCA/FLY) and three pariwise combinations. All fixation indexes' values are significant (p<0.05). FSI – European wildcat; FCA – domestic cat; FLY – African wildcat.

		Variance	% variation	F_{ST}
FSI/FCA/FLY	Among subspecies	1.146	18.76	0.19
	Within subspecies	4.960	81.23	
FSI/FCA	Among subspecies	1.227	20.56	0.21
	Within subspecies	4.894	79.44	
FCA/FLY	Among subspecies	0.505	8.96	0.09
	Within subspecies	5.129	91.04	
FSI/FLY	Among subspecies	1.031	17.34	0.17
	Within subspecies	4.911	82.66	

3.2. Individuals' assignment and admixture analysis

Factorial Correspondence Analysis provided a preliminary examination of individual's partition into different clusters. The graphical result for Iberian individuals (figure 7) showed a distinction in axis 1 (horizontal, 7.51%) for the reference samples of both European wildcats (right) and domestic cats (left). Sampled individuals across

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Iberian Peninsula clustered either with the reference wildcats or the reference domestic cats, with a few exceptions that were positioned between the two defined groups, evidence for possible hybrids present in these populations (figure 7). Axis 2 suggests that domestic cats are more homogeneous than wildcats, which are more spread through this axis.
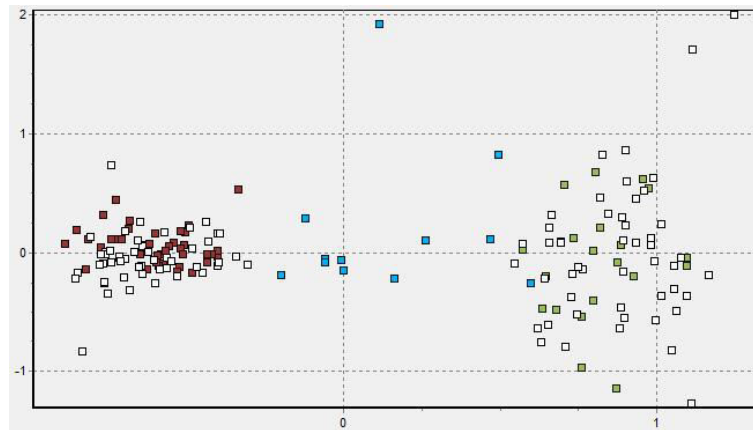
Figure 7 – Factorial Correspondence Analysis (FCA) with European wildcat references (green squares) and domestic cat references (red squares). New sampled individuals from all the Iberian Peninsula are represented by white squares. Blue squares represent 12 putative hybrids (see also STRUCTURE analysis below). Axis 1 and 2, horizontal and vertical respectively, are the two principal correspondence factors.

Bayesian analyses were performed in order to have a proportion of allocation of each individual to a given cluster. First, simulated genotypes were analysed using two Bayesian softwares (STRUCTURE AND NEWHYBRIDS) to infer the threshold to consider an individual as "pure". Results from STRUCTURE reveal that all simulated pure European wildcats and domestic cats were assigned to their correct cluster with an average proportion of membership $Q_{FSI} = 0.965$ and $Q_{FCA} = 0.961$ (see table 9). The lower limits of the 90% confidence intervals were always higher than 0.85 and therefore, considering these results and previous studies (Oliveira 2012), a threshold of qi>0.85 was defined to allocate an individual to one of the parental clusters defined in STRUCTURE. All hybrids showed a much wider confidence interval range than parentals, with F1 and F2 hybrids showing the widest range. First and second generation hybrids were never misinterpreted as pure individuals and first generation backcrosses were incorrectly identified as parentals less often than second generation backcrosses. Results from second generation backcrosses demonstrate that these individuals are very often misinterpreted as pure individuals (backcrosses with wildcat misidentified as wildcats and backcrosses with domestic cat misidentified as domestic cats) and have a low percentage of correct assignments which is also verified by the average proportion

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of assignment of $Q_{Bx2FSI} = 0.899$ and $Q_{Bx2FCA} = 0.884$ that is higher than the defined threshold for identification of pure individuals, which can also be observed in the bar plot in figure 8.

Analysis performed in NEWHYBRIDS provided 100% correct assignment of pure individuals when considering just six classes of hybridization, with a lower threshold of qi>0.75 (table 9). Nevertheless, the analysis using this software showed very low percentage of correctly identified individuals for second generation hybrids and for first generation backcrosses. Also, second generation backcross hybrids remained unclassified when these classes were included in the tests, and in both cases were mainly identified as pure individuals or backcrosses of first generation. If a lower threshold is considered, more individuals of all classes are correctly identified. However, this decrease in the threshold also implies an increase of individuals incorrectly assigned to other classes. Still, this threshold was used in subsequent analysis of NEWHYBRIDS to identify the hybridization class of the samples individuals, and only six classes were tested.

Table 9 – Assignment of simulated genotypes. Forty individuals of each class were simulated, including pure European wildcats (FSI); pure domestic cats (FCA); first (F1) and second (F2) generation hybrids, backcrosses of first (BxFSI, BxFCA) and second generation (Bx$_2$FSI, Bx$_2$FCA). Simulated individuals were analysed using two Bayesian softwares: a) STRUCTURE, showing average proportion of membership for wildcat (Q$_{FSI}$) and domestic cat cluster (Q$_{FCA}$) with respective 90% confidence intervals in brackets, percentage of correctly assigned individuals (%N) and number of individuals incorrectly assigned to one of the pure clusters (n); b) NEWHYBRIDS, showing percentage of individuals assigned to their correct class at different thresholds (%N qi>0.85; %N qi>0.75) and respective number of individuals assigned to an incorrect class (n).

a)	Q$_{FSI}$	Q$_{FCA}$	%N	N
FSI *qi>0.85*	0.965 (0.867,1.000)	0.035 (0.000,0.133)	100	-
FCA *qi>0.85*	0.039 (0.000,0.143)	0.961 (0.857,1.000)	100	-
F1 *0.40<qi<0.60*	0.484 (0.256,0.706)	0.516 (0.294,0.736)	70	0
F2 *0.40<qi<0.60*	0.483 (0.264,0.706)	0.517 (0.294,0.736)	42.5	0
BxFSI *0.60<qi<0.85*	0.801 (0.604,0.953)	0.199 (0.047,0.396)	70	12 FSI
BxFCA *0.60<qi<0.85*	0.234 (0.070,0.438)	0.766 (0.562,0.930)	75	9 FCA
Bx$_2$FSI *0.60<qi<0.85*	0.899 (0.740,0.993)	0.101 (0.007,0.260)	22.5	31 FSI
Bx$_2$FCA *0.60<qi<0.85*	0.116 (0.018,0.276)	0.884 (0.724,0.982)	37.5	25 FCA

b)	8 Classes				6 Classes				
	%N *qi>0.85*	n	%N *qi>0.75*	n	%N *qi>0.85*	n	%N *qi>0.75*	N	
FSI	95		100		100		100		

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FCA	87.5		97.5		97.5		100
F1	45		62.5		42.5		52.5
F2	0	2 F1	5	3 F1 2 BxFCA	0	1 F1 2 BxFSI 2 BxFCA	2.5	3 F1 3 BxFSI 4 BxFCA
BxFSI	0	3 FSI 1 F1	2.5	7 FSI 1 F2	17.5	8 FSI 1 F1	32.5	9 FSI 1F1
BxFCA	2.5	2 FCA	32.5	3 FCA 1 F1	27.5	2 FCA	42.5	3 FCA 1 F1
Bx_2FSI	0	10 FSI	0	21 FSI	-	22 FSI 1 BxFSI	-	25 FSI 1 BxFSI
Bx_2FCA	0	13 FCA 4 BxFCA	0	19 FCA 8 BxFCA	-	18 FCA 8 BxFCA	-	20 FCA 15 BxFCA
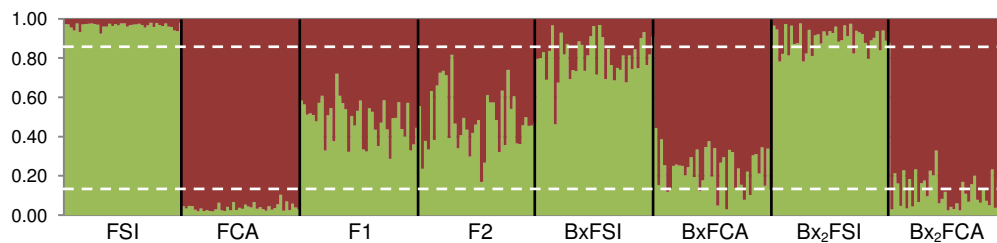
Figure 8 – Structure analysis of simulated microsatellites genotypes of European wildcats (green) and domestic cats (red), for K=2. Dashed lines indicate the threshold at q = 0.85. FSI – pure European wildcats; FCA – pure domestic cats; F1 – first generation hybrids; F2 – second generation hybrids; BxFSI – first generation backcrosses with wildcat; BxFCA – first generation backcrosses with domestic cat; Bx_2FSI – second generation backcrosses with wildcat; Bx_2FCA – second generation backcrosses with domestic cat.

Assignment analysis performed in STRUCTURE provided a more accurate allocation of individuals to one of the parental clusters (K=2), as well as the identification of hybrid individuals. All reference European wildcats were assigned to cluster 1 (FSI) with mean proportion of $Q_{FSI}=0.986$. On the other hand, all reference domestic cats were allocated to cluster 2 (FCA) with average proportion of $Q_{FCA}=0.984$. From the 97 analysed individuals from the Iberian Peninsula with unknown ancestry (including invasive and non-invasive sampling), 48 were allocated to the wildcat cluster with an average proportion of membership of $Q_{FSI}=0.987$, and 37 were identified as domestic cats with equal average proportion of $Q_{FCA}=0.987$. The remaining 12 individuals were not assigned to any of the two clusters, and therefore were considered hybrids. Their proportions of assignment to cluster 1 ranged from 0.260 to 0.833 (see table 10). A subsequent analysis of these individuals was implemented in NEWHYBRIDS to infer the class of hybridization (see figure 9). Five individuals (2EM, 87NS, FG31, CNI1322 and CNI1432) revealed high proportion of assignment to the F2 hybrid class which is congruent with STRUCTURE results. Sample 79EM was also identified with

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q>0.75 to the pure wild population, although there was also a considerable proportion of assignment to backcross with wildcat (q=0.188). This is compatible with results from STRUCTURE that also indicate high similarity with the wildcat cluster (0.812), although not above the threshold. These results seem to indicate that this individual might be an older generation backcross with European wildcats, which explains the high genetic similarity with this subspecies. Remaining individuals have proportions of assignment divided across several classes and, thus, are not assigned to any particular class, particularly considering difficult correct identification of hybrid classes demonstrated by simulation analyses. Individual G12-9 had considerable proportion of assignment to the pure wild population, backcrosses with pure wildcat class or F2 class, most probably being a backcross hybrid with higher similarity with European wildcats. On the other hand, individual 1EM demonstrated higher similarity with the domestic population, with considerable proportion of assignment to the F2 hybrid class. Individuals FG12 and FG15 demonstrated some proportion of assignment to F2 and backcross with domestic cat hybrid classes, which is congruent with their higher proportion of assignment to the domestic cat population in STRUCTURE. Sample FG46 showed high proportion of assignment to F2 hybrid class, although not above the threshold, showing also some proportion of assignment to backcross with domestic cat hybrid class. Sample CNI1403 demonstrated high proportion of assignment to the wildcat cluster in both analyses, also demonstrating some proportion of assignment to F2 and backcross with wildcat hybrid classes. This individual is possibly an old generation hybrid, genetically more similar to European wildcats.

These results are concordant with the Factorial Correspondence Analysis, since all hybrid individuals identified with Bayesian analysis are graphically located between the domestic and wildcat groups (figure 7).

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Table 10 – Assignment of admixed individuals using STRUCTURE and NEWHYBRIDS. For each sample results from both analyses are represented, including both parental classes and respective 90% Confidence Intervals for STRUCTURE and all six hybridization classes tested with NEWHYBRIDS. In these last, bold values are above the threshold of qi>0.75 defined using simulation analyses, and other high values are underlined.

Sample code	STRUCTURE				NEWHYBRIDS					
	FSI	FCA	90% CI	90% CI	FSI	FCA	F1	F2	BxFSI	BxFCA
G12-9	0.796	0.204	(0.560,0.997)	(0.003,0.440)	<u>0.390</u>	0.000	0.025	<u>0.229</u>	<u>0.355</u>	0.001
1EM	0.260	0.740	(0.055,0.488)	(0.512,0.945)	0.000	<u>0.396</u>	0.001	<u>0.491</u>	0.000	0.111
2EM	0.504	0.496	(0.286,0.721)	(0.279,0.714)	0.000	0.000	0.051	**0.882**	0.043	0.024
87NS	0.358	0.642	(0.151,0.580)	(0.420,0.849)	0.000	0.017	0.000	**0.941**	0.001	0.040
79EM	0.812	0.188	(0.611,1.000)	(0.000,0.389)	**0.770**	0.000	0.001	0.041	<u>0.188</u>	0.000
FG12	0.319	0.681	(0.095,0.561)	(0.439,0.905)	0.000	0.103	0.094	<u>0.520</u>	0.004	<u>0.280</u>
FG15	0.370	0.630	(0.155,0.601)	(0.399,0.845)	0.000	0.021	0.077	<u>0.657</u>	0.005	<u>0.240</u>
FG31	0.417	0.583	(0.195,0.645)	(0.355,0.805)	0.000	0.020	0.043	**0.839**	0.014	0.084
FG46	0.407	0.593	(0.183,0.638)	(0.362,0.817)	0.000	0.022	0.100	<u>0.719</u>	0.013	<u>0.146</u>
CNI1322	0.567	0.433	(0.307,0.811)	(0.189,0.693)	0.030	0.001	0.000	**0.945**	0.021	0.004
CNI1403	0.833	0.167	(0.580,1.000)	(0.000,0.420)	<u>0.671</u>	0.000	0.004	<u>0.140</u>	<u>0.184</u>	0.000
CNI1432	0.475	0.525	(0.212,0.728)	(0.272,0.788)	0.003	0.056	0.000	**0.919**	0.006	0.016
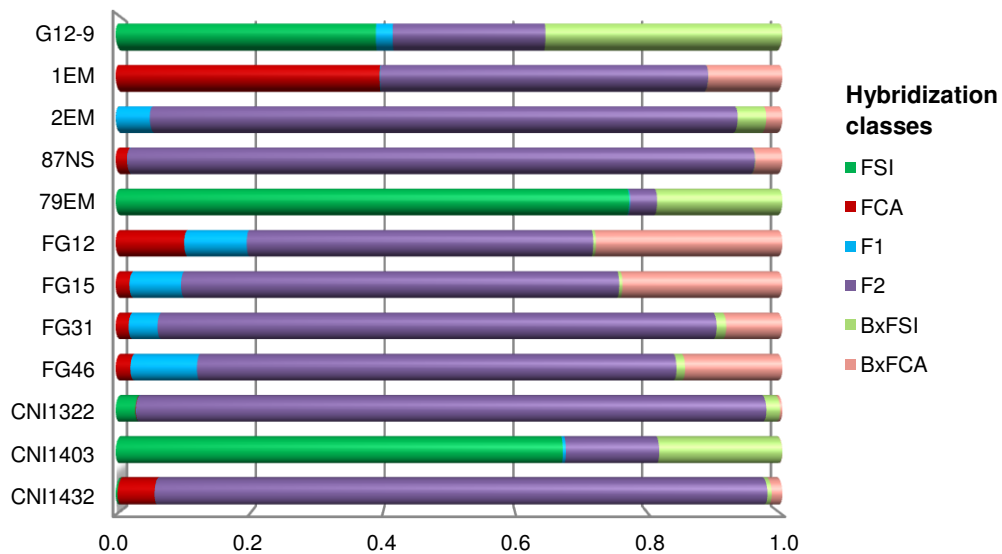
Figure 9 – Proportion of admixed individuals' assignment to each of the six hybrid classes, using NEWHYBRIDS. FSI – Pure European wildcat; FCA – pure domestic cat; F1 – first generation hybrids; F2 – second generation hybrids; BxFSI – first generation backcrosses with European wildcat; BxFCA – first generation backcrosses with domestic cat.

Eight hybrid cat samples were collected in the Spanish locations of Valladolid, Toledo, Ciudad Real, Cabañeros National Park and Muniellos Natural Reserve in Spain, and four in the Portuguese locations of Mértola (Guadiana Valley National Park) and Barrancos (figure 10). This scenario shows that hybridization is spread through Iberian Peninsula, mainly in south Portugal and north and central Spain. Seven

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individuals were previously identified morphologically as European wildcats and two as putative hybrids. The genotypes of the remaining three individuals were retrieved from scat samples, therefore without morphological information. These samples were collected between 2010 and 2014, which demonstrates that hybridization events occurred over the past years and continue to occur in the present.
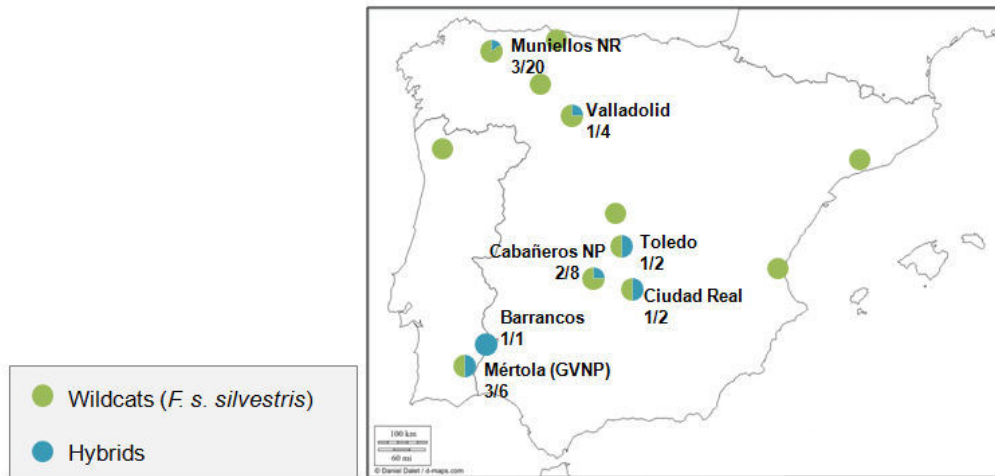
Figure 10 – Location of the populations where pure European wildcats were identified (green) and proportion of hybrid individuals (blue) throughout the Iberian Peninsula, according to genetic analyses. The number of hybrid cats in comparison with the total number of samples is shown.

Moreover, 11 samples previously identified as wildcats or with dubious morphology (putative hybrids), collected in wildcat territories near Madrid, Segovia, Sevilla, Granada and in Muniellos Natural Reserve in Spain, and near Estremoz, Montemor-o-Novo and Guadiana Valley National Park in Portugal, were genetically identified as domestic cats. Although these individuals might be old generation backcrosses with domestic cat that retained wildcat phenotypic traits, they were considered wrong morphological identifications.

3.3. Population structure analysis

Analysis of population structure for pure wild individuals of Iberian Peninsula was initially assessed with a Factorial Correspondence Analysis (figure 11). This analysis did not show any clear distinction among populations or regions. Thus, these results showed no evident genetic substructure within the Iberian Peninsula wildcat populations.

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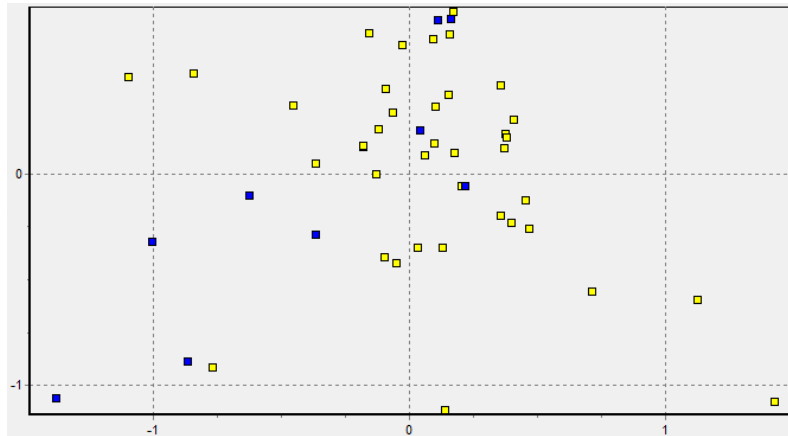
Figure 11 – Factorial Correspondence Analysis (FCA) of pure European wildcats. Individuals sampled in Spain are represented by yellow squares and individuals sampled in Portugal are represented by blue squares. Axis 1 and 2, horizontal and vertical respectively, are the two principal correspondence factors.

The existence of substructure in Iberian Peninsula populations was also studied with the use of Bayesian analysis in STRUCTURE software. The optimal number of clusters was four. Cluster 2 (represented in green, see figure 12 and 13) contained all six individuals from Cabañeros National Park in central Spain, with average proportion of membership of $Q_{CLUSTER2}=0.975$. However, the other three clusters did not provide evidence of clear geographical substructure in Iberian Peninsula, since they contain samples spread over the sampling area (see figure 13). It is possible that the number of pure European wildcats (n=48; 9 from Portugal, 39 from Spain) analysed was too low for the inference of genetic substructure.
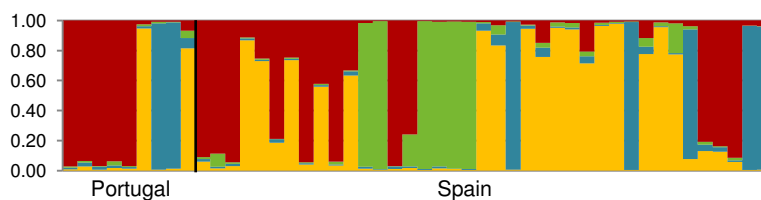
Figure 12 – Results for wildcats substructure analysis in Iberian Peninsula, with the optimal number of clusters K=4. Dataset was divided in "Portuguese wildcats" and "Spanish wildcats" for convenience.

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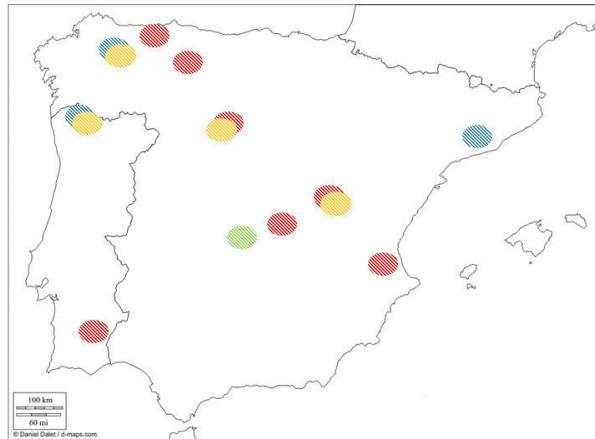
Figure 13 – Approximate distribution of the four clusters obtained with STRUCTURE. Colours correspond to the ones in STRUCTURE barplot (figure 12).

3.4.   African wildcats' individual assignment

In order to assess the power of the microsatellite panel for distinguishing the three subspecies of cats and to investigate the differentiation between African wildcats and domestic and European wildcats, a Factorial Correspondence Analysis was initially performed (figure 14). The graphical distribution of the sampled individuals showed clear distinction in axis 1 (horizontal, 6.37%) between domestic and European wildcats and the existence of hybrid individuals between the two, as seen in previous analyses. African wildcats clustered together with domestic cats, with clear distinction from European wildcats but demonstrating high similarity with the domestic cluster. When considering axis 2 (vertical, 3.52%) one African wildcat individual showed a high distinction (FG21) from the rest of the African and domestic cluster.
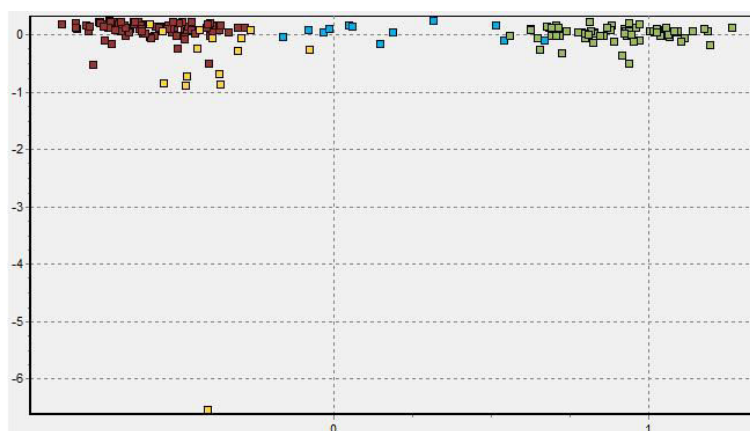
Figure 14 – Factorial Correspondence Analysis (FCA) performed with the complete dataset comprising European wildcat (green squares), domestic cats (red squares), African wildcats (yellow squares) and individuals identified as European wildcat/domestic cat hybrids in previous analyses (blue squares). Axis 1 and 2, horizontal and vertical respectively, are the two principal correspondence factors.

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STRUCTURE analyses were performed to infer more accurately the capacity of the set of microsatellites in assigning the individuals to one of the three subspecies, and to investigate the existence of African wildcat/domestic cat hybrids. The optimal number of clusters was K=3 but results with two clusters are also shown in figure 15. When two clusters were considered, all African wildcats were mainly identified as domestic cats, which reinforces the genetic proximity between these two subspecies as showed by previous results (figure 15 a). The Iberian Peninsula samples are identified as on previous analyses of individual's assignment and admixture for European wild and domestic cats only. On the other hand, considering the optimal number of clusters, K=3, overall the three subspecies are well distinguished. Among the 15 cats collected in North Africa none was misidentified as a European wildcat, 12 were identified as pure African wildcats with average proportion of membership of $Q_{FLY}=0.894$, two were identified as pure domestic cats with average proportion of membership to the domestic cluster of Q=0.938, and one showed admixed ancestry between African and domestic cats (Fli781, collected in Western Sahara, see figure 16). Sample FG21, which demonstrated high differentiation from the African wildcats' cluster in axis 2 of the FCA plot, was assigned to the African wildcat population with $q_{FG21}=0.982$. The results are clear both on the barplot in figure 15 b) and on the triangular plot in figure 15 c). In this last graphical representation the three subspecies are clearly distinguished. The African cat Fli781 that shows potential admixed genotype is represented between the African wildcat cluster and the domestic cluster, and the two African cats identified as domestics are represented within the reference domestic cats. Individuals sampled throughout the Iberian Peninsula cluster either with the European wildcats or the domestic cats, and hybrids are represented between the two clusters. There are no individuals represented between the African and European wildcat clusters, which demonstrated that these two subspecies are clearly distinguished, as aforementioned.

One individual (CNI1432) was significantly allocated to the African wildcat cluster ($q_{CNI1432}=0.838$) and is, thus, represented within the African wildcat group in figure 14 c). This individual was previously identified as a European wildcat/domestic cat hybrid, possibly of second generation (F2), and was probably misidentified in this analysis due to its similarity with the domestic cats. Other irregular results with some proportion of assignment to the African wildcat cluster occur in four previously identified hybrids (between European wildcat and domestic cat), all with no class of hybridization identified using NEWHYBRIDS, and in one previously identified domestic cat.

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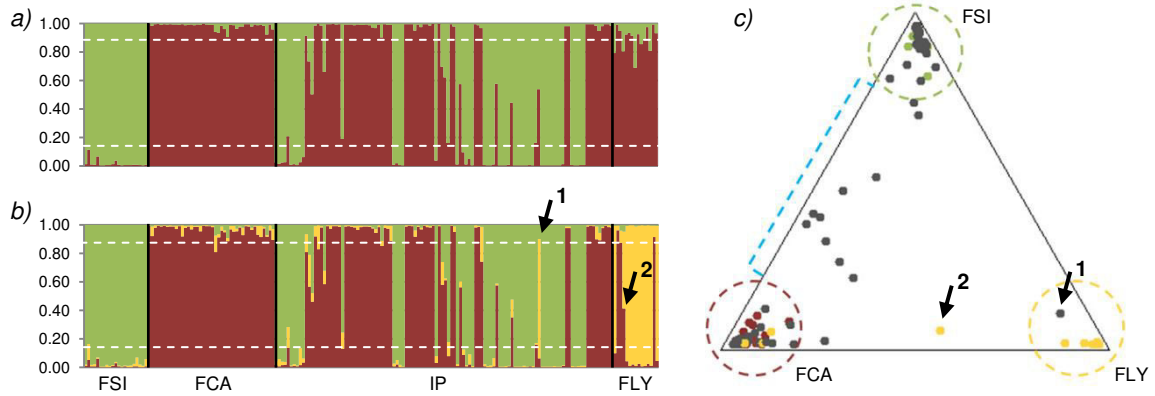
Figure 15 – Individual assignment for the three wildcat subspecies. a) Allocation of individuals in two clusters; b) allocation of individuals in three clusters (optimal K=3). Each subspecies is represented by FSI – European wildcat (green); FCA – domestic cat (red); FLY – northern African wildcat (yellow). IP are all individuals sampled in Iberian Peninsula. c) Triangular plot of Structure results for three clusters. Top corner represents the European wildcat cluster (FSI), bottom left corner represents the domestic cat cluster (FCA), bottom right corner represents the African wildcat cluster (FLY); European wildcat references and domestic cat references are represented in green and red dots, respectively, samples collected in Africa are represented by yellow dots and the new individuals sampled in Iberian Peninsula are represented by grey dots. Black arrows identify 1- individual CNI1432, 2- individual Fli781.
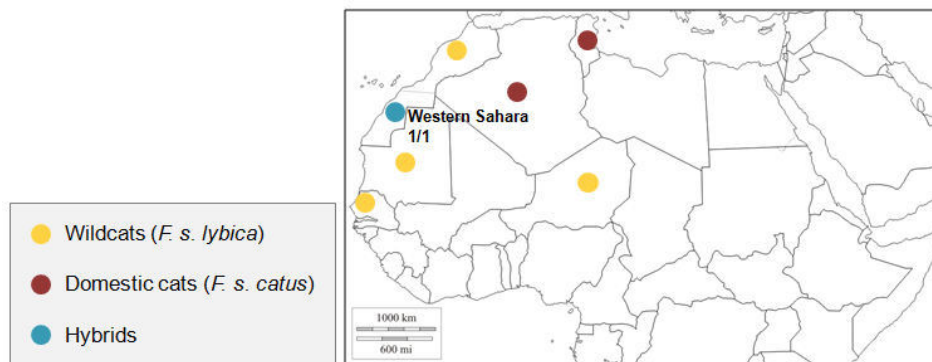
Figure 16 – Location of the individuals identified as northern African wildcats, domestic cats and hybrids throughout North Africa, according to genetic analyses.

The STRUCTURE results for reference domestic cats and African wildcats (barplot result not shown) demonstrate that some reference domestic cats were misinterpreted, showing a proportion of membership to the domestic cluster below the threshold of 0.85 (misidentified individuals with proportion of membership to the domestic cluster ranging from 0.463 to 0.844). These results explain the peculiar results found in the analysis with three subspecies, and reveal that the set of microsatellites is not informative enough to accurately discriminate these two subspecies (domestic and African wildcats), probably because of their high genetic similarity. Having this in consideration, the results of admixture between these two subspecies should be considered carefully. The sample identified as admixed might be a real hybrid or just an artefact of imprecise identification.

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4. Discussion

Conservation and management of wild populations is an increasing concern for conservation biologists. The increase in human population is threatening wildlife with growing occupation, modification and destruction of important habitats, and with the enormous pressure of human densities on the ecosystems. Planning conservation measures in order to diminish the decline of wild populations is a complex task that requires intense study of the populations' dynamics and a clear comprehension of the most threatening pressures acting on them. Anthropogenic hybridization is one of the most underlining concerns, mainly because it is often a consequence of the concomitant effects of many other threats. The wildcat *Felis silvestris*, already threatened in most of its distribution range (Nowell & Jackson 1996; Driscoll & Nowell 2010), is a clear example of the alarming indirect consequences of human pressure. This elusive species is very affected by the pervasive spread of human populations through their native habitats, which influenced drastically their population densities. Since humans usually bring along their pets, the consequent massive spread of domestic cats carried a dangerous opportunity for extensive artificial crossings between wild and domestic cat subspecies. Understanding the processes influencing hybridization, and its effects on the involved populations is essential for the construction of proper management plans. During the last decade, several researchers have performed genetic studies throughout Europe in order to understand the dynamics of European wildcat and domestic cat interactions, and the consequent hybridization scenarios (Beaumont *et al.* 2001; Randi *et al.* 2001; Pierpaoli *et al.* 2003; Lecis *et al.* 2006; Oliveira *et al.* 2008a; b; O'Brien *et al.* 2009; Hertwig *et al.* 2009; Eckert *et al.* 2010). These studies demonstrated that the rates of hybridization among European populations differ considerably from widespread admixture to sporadic events, reinforcing the idea that each situation should be carefully studied and only then considered in a comparative overview. For the wildcat, it is particularly important to understand how past and present events continuously shaped each subspecies and their populations, particularly how their interactions influence their genetic identities.

4.1. Genetic diversity among three subspecies of *Felis silvestris*

The European wildcat is the most studied among the five wild subspecies of *F. silvestris* (Driscoll & Nowell 2010). Its geographic range overlaps, in its most eastern

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part, with the distribution range of the African wildcat (Kitchener & Rees 2009). Although this African subspecies is the most probable ancestor of the domestic cat (Vigne *et al.* 2004; Driscoll *et al.* 2007) it is not yet thoroughly studied. Both wild subspecies interact with the domestic cat through almost their entire distribution range. Nevertheless, for an accurate study of their interactions it is essential first to understand their genetic patterns, and how they were shaped by their evolutionary history and recent events.

The European wildcat population of Iberian Peninsula might comprise several subpopulations, some probably geographically isolated and, thus, not breeding randomly throughout all their Iberian distribution range. A similar situation is described by O'Brien and colleagues (2009) in their study area in France. Also, positive F_{IS}, as found for the Iberian wildcats in this study (0.19), can evidence that the populations might be suffering from some inbreeding. These factors possibly explain the deviations from HWE and LE found in the Iberian wildcat population. Moreover, domestic cat populations are usually under artificial selection and non-random breeding, which explains why this subspecies also present deviations from HWE and LE (Oliveira 2012). These deviations also explain the evidence of null alleles in these subspecies, that probably represent excess of homozygote genotypes rather than actual null alleles (Hertwig *et al.* 2009).

The highest genetic diversity was observed in the African wildcats, except for mean number of alleles that were higher in domestic cats, while European wildcats showed overall lower genetic diversity. The overall high genetic diversity observed in domestic cats is in accordance with previous studies (Oliveira *et al.* 2008a; Eckert *et al.* 2010; Oliveira 2012) and might indicate that the process of domestication did not result in drastic genetic variability decrease in this subspecies (Pierpaoli *et al.* 2003), which is probably related with the fact that the domestication process did not occur under severe artificial selection (Lipinski *et al.* 2008), and eventually in more than one place (Driscoll *et al.* 2007). Also, the continuous movement of domestic cats by humans assured gene flow, which also increased the genetic diversity of this subspecies (Eckert *et al.* 2010). Nevertheless, domestic cats showed the lowest private allelic richness which might be explained by introgression of alleles from European wildcats (Beaumont *et al.* 2001) and to high genetic similarity with African wildcats (the most probable ancestor). African wildcats had the highest values of allelic richness and heterozygosity, which is in accordance with previous study by Oliveira (2012). This author point out two possible explanations related to the evolutionary history of the *taxon*: the extremely wide distribution range and habitat tolerance, which might have promoted gene flow in the past and resistance to population declines; and/or the

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crossbreeding with the domestic subspecies during the process of domestication that could have lead to the preservation of high genetic diversity. On the other hand, the lowest genetic diversity observed in the European wildcat might be a result of repeated bottlenecks during glaciations, habitat destruction, persecution and severe declines in the last centuries (Wiseman *et al.* 2000; Lecis *et al.* 2006; Lozano & Malo 2012), with consequent fragmentation and isolation of populations, that lead to its classification as Vulnerable and Near Threatened in Portugal and Spain, respectively (Cabral *et al.* 2005; Palomo *et al.* 2007). Despite demonstrating the lowest genetic diversity among the three analysed subspecies, the obtained values for expected heterozygosity and allelic richness are still high, which indicates that inbreeding depression is still not strongly affecting the populations.

When using microsatellites, different allele sizes between populations can reflect different mutation rates, and this might influence the values of population differentiation. R_{ST} statistics have in consideration the allelic size differences, and therefore, higher values of R_{ST} than F_{ST} indicate that new mutations might be responsible for a substantial proportion of variation (Pierpaoli *et al.* 2003; Oliveira *et al.* 2008b). High differentiation was observed between domestic cats and European wildcats as indicated by the high F_{ST} and R_{ST} values, showing clearly distinct gene pools in the Iberian Peninsula. These results suggest that introgression is not widespread in the Iberian populations, and that hybridization is not yet strongly affecting the genetic identity of the European wildcat. This is in accordance with previous studies in the Iberian Peninsula (Oliveira *et al.* 2008a; b; Oliveira 2012), and similar to what happens in other locations across Europe like Italy (Randi *et al.* 2001), France (O'Brien *et al.* 2009) and Germany (Hertwig *et al.* 2009; Eckert *et al.* 2010), but contrasting with areas with widespread introgression as in Scotland (Beaumont *et al.* 2001) and in Hungary (Pierpaoli *et al.* 2003; Lecis *et al.* 2006). On the other hand, high genetic similarity between African wildcats and domestic cats was demonstrated by the low F_{ST} and R_{ST} values between these two subspecies. This is congruent with previous studies (Randi *et al.* 2001; Oliveira 2012) and in accordance with the probable African ancestry of the domestic cat (Driscoll *et al.* 2007). The domestication of the cat started around 9500 ya (Vigne *et al.* 2004) and probably in constant cross-breeding between the domesticated form and the wild ancestor (Lipinski *et al.* 2008). Moreover, cats were not intensively selected for specific traits during domestication, which contributed to maintaining a high genetic similarity with their ancestor (Lipinski *et al.* 2008; Driscoll *et al.* 2009b). Finally, high F_{ST} and R_{ST} values between European wildcats and African wildcats demonstrate high differentiation that might be explained by different selective pressures and geographic isolation. There is evidence that European wildcats

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divergence occurred first, and that the other wild subspecies diverged later from each other (Kitchener & Rees 2009), which is congruent with Driscoll and colleagues' (2007) results on the mtDNA phylogenetic tree and the date of splitting events within the *Felis silvestris* species that show that the European wildcat has been a clearly different lineage for a long time (Hertwig *et al.* 2009). Moreover, the African and the European wildcats are mostly geographically separated with the exception of the region of Near East and Caucasus where their ranges overlap (Kitchener & Rees 2009). Studies focused on these areas where these two cat subspecies cohabit could provide interesting information about their interactions and genetic and ecological features under similar habitat conditions.

AMOVA results revealed higher percentage of variation within subspecies, which is in accordance with their high diversity and might indicate some substructure within subspecies. Moreover, AMOVA results between pairs of subspecies confirmed the results of pairwise F_{ST} and R_{ST}. Comparison between African wildcats and domestic cats showed the lowest percentage of variation among subspecies, which is in accordance with the low genetic differentiation demonstrated in previous analysis. Domestic cats/European wildcats and African wildcats/European wildcats showed higher percentage of variation among subspecies which is also congruent with high F_{ST} and R_{ST} values.

Overall, all three subspecies demonstrated high levels of genetic diversity, evidence that decrease in genetic variability is not yet a major concern for the conservation of the wild subspecies. Nevertheless, there are some evidences of inbreeding demonstrated by positive F_{IS} values in the three subspecies, particularly in European wildcats (0.19), and, thus, decrease in genetic diversity might be a concern in the future.

The high genetic differentiation between domestic and European wildcats found here reveals a clear distinction between these subspecies in Iberian Peninsula, without extensive introgression. Nevertheless, these populations are still possibly affected by hybridization.

4.2. Iberian wildcat survey

The European wildcat and the domestic cat coexist in Iberian Peninsula, and the increase in human density is bringing the two subspecies closer together, influencing each other both ecologically and genetically (Oliveira *et al.* 2008a;

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Sarmento *et al.* 2009). Therefore, it is crucial to infer if hybridization is threatening the genetic integrity of wildcats and monitor the interaction between the two subspecies.

4.2.1. Individuals' assignment and admixture analyses

Previous studies focused in Iberian Peninsula found clear genetic distinction between European wildcats and domestic cats, but also confirmed the existence of admixed individuals among the two subspecies (Oliveira *et al.* 2008a; b). Given that anthropogenic threats seem to be contributing significantly to the increasing contact between the two subspecies, it is of major importance to continue monitoring the Iberian populations, in order to define accurate management plans.

Individuals' assignment provided further evidence of European wildcat and domestic cat differentiation in Iberian Peninsula. Both graphical and Bayesian analyses showed accurate distinction between these two subspecies, which was further supported by clear differentiation of pure simulated genotypes that were allocated to their correct cluster with high probability of assignment. These results are congruent with the high differentiation demonstrated by high F_{ST} and R_{ST} values found in previous analyses, and confirm that Iberian wildcats preserve their genetic identity as a distinct subspecies.

Nevertheless, hybridization analysis provided evidence of 12 admixed individuals. Considering these among all individuals genetically identified as non domestic cats (n=60), these admixed individuals represent a hybridization rate of 20% in the whole Iberian Peninsula. This rate is higher than found in previous admixture studies in Portugal (14%) by Oliveira *et al.* (2008a) and much higher than the hybridization rate of 6.9% calculated in a previous study for Iberian Peninsula (Oliveira *et al.* 2008a). However, proper sampling of wildcat territories should be done by collecting samples at population level, including feral cat populations, which might include backcrossed individuals with domestic cat (Oliveira 2012). Non-invasive sampling can help overcome this limitation (see section 4.2.2).

Admixed individuals were distributed throughout the sampling area, evidencing a geographically widespread hybridization scenario, mainly in south Portugal and north and central Spain. These results show a considerably larger distribution of hybrids in comparison with previous studies in Iberian Peninsula, in which hybrids were exclusively identified in Portugal (Oliveira *et al.* 2008a; b), but are congruent with more recent studies that identified hybrids across the Iberian territory (Oliveira 2012). This might be a result of increase in sampling effort throughout Spain or evidence of

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increase in hybridizing populations since previous studies. These results transmit a higher concern for Spanish wildcat populations, since the identification of eight hybrid individuals in this country demonstrate that wildcats might be subject of increasing threats and, therefore, be more susceptible to artificial crosses with domestic cats. Nevertheless, a more intense sampling would be necessary to confirm the absence of hybridization in some areas that were less sampled as southern and north-eastern Spain and particularly in northern and central Portugal where Oliveira and co-workers (2008a) had previously found hybrids. Further studies in southern Spain would also be important since no hybrids were found within the 22 samples from Granada in Oliveira and colleagues' (2008a) study, but three domestic cats were identified. In the present study, two domestic cats (previously identified as wildcats by morphologic features) were also found in wild territories near Granada and Sevilla. The presence of domestic cats in wild territories can potentiate the hybridization and, thus, a more intense sampling effort should be directed towards these regions to understand if these populations are genetically pure (and, therefore, interesting for conservation purposes, mainly if reproductive programs are needed), or if hybridization events exist but were not yet found. Additionally, other 9 domestic individuals (previously morphologically identified as wildcats) were also found within other wild territories in Spain (near Madrid, Segovia and Muniellos Natural Reserve) and Portugal (Estremoz, Montemor-o-Novo and Guadiana Valley Natural Park). The identification of these individuals illustrates how feral domestic cats are capable of spreading and living in habitats of wildcats, in sympatry with their wild conspecifics. Free ranging domestic cats in wild territories are a conservation concern not only due to the risk of interbreeding, but also due to competition, and disease transmission (Ferreira *et al.* 2011).

Additional concern comes from the fact that 8 out of the 12 hybrid individuals were collected in protected areas, both in Portugal (Guadiana Valley National Park) and Spain (Cabañeros National Park and Muniellos Natural Reserve). Furthermore, domestic individuals were also found in Muniellos Natural Reserve and Guadiana Valley National Park. Previous studies also identified hybrid and domestic individuals within natural parks in Iberian Peninsula (Oliveira *et al.* 2008a; b; Sarmento *et al.* 2009). This raises important questions regarding the protection of species inside protected areas. In Iberian Peninsula it is quite common that some villages and other human settlements are located near or within protected areas, which imply the existence and spread of domestic cats through wild territories. It is of primary importance to focus conservation actions on these cases, disclosing campaigns to raise awareness for the conservation of wildcat populations and to neuter feral domestic cats.

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Hybrid samples' collection dates range from 2010 to 2014. Older hybridization events in the Iberian Peninsula are evidenced in previous studies (Oliveira *et al.* 2008a; b) which reinforces that hybridization events have happened continuously through the last years. Moreover, the identification of putative second generation hybrids (F2) and backcrosses might suggest that hybrids are not only breeding among themselves but also with both parental subspecies. However, some hybrids' ancestry class remained unknown, since the genotypes were not significantly allocated to a singular hybridization class in NEWHYBRIDS' analyses and, therefore, these results should be analysed carefully, especially taking into account the results of simulation analysis. Simulated genotypes based on reference samples were used to set a unambiguous threshold to identify hybrid genotypes and estimate the range of variation of the confidence interval of individual qi values (Randi 2008), but also to understand how well the set of microsatellites distinguish parental individuals and different hybrid classes (Vähä & Primmer 2006). The analysis of our simulated genotypes demonstrated that all parental individuals can be correctly identified using both STRUCTURE and NEWHYBRIDS, but the accuracy power decreases for hybrids, especially for second generation hybrids (F2) and backcrosses of second generation. Particularly, simulated F2 hybrids were generally not allocated to a single hybridization class or misidentified as other hybrids, and in one case a backcross hybrid was identified as a F2. Having this in consideration, assignment to any hybrid class other than F1 by NEWHYBRIDS should not be assumed as certain. The relative low number of microsatellites used might be the reason for this difficulty in correctly identifying the hybrid class of all individuals, but other similar studies also found this difficulty, namely in defining the backcrosses (e.g. Oliveira *et al.* 2008a). According to Vähä and Primmer (2006), with a high F_{ST} value such as the obtained for European wildcats and domestic cats (0.22), a set of 14 microsatellites is enough to properly distinguish pure individuals and hybrids using STRUCTURE and NEWHYBRIDS. Nevertheless, their results also suggest that for the correct identification of different hybrid classes, a higher number of microsatellites is necessary (around 48 markers), especially for backcrosses. Using a larger marker set comprising 38 microsatellites, Oliveira (2012) achieved better results for assignment of simulated genotypes to their correct category using NEWHYBRIDS, although some were still misidentified. Hybrids beyond the first generation (F2 or backcrosses) have a huge variety of possible combinations of alleles (Rhymer & Simberloff 1996), and, consequently, the proportion of contribution from the parental populations is difficult to estimate. Also, there are several admixture classes that are not considered in analyses, as F1xF2, F2xF2, F2xbackcross, F2xparental, among others, which can be present in the populations but are extremely difficult to

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identify. Regarding this difficulty in identification of some hybrid classes, some admixed individuals might not be detected, and the hybridization rates are possibly underestimated. Also, the real hybridization dynamics of the admixed population might not be completely understood due to this limitation. The accurate identification of hybridization classes provide important information for understanding the impact of this process in the population, and what conservation measures are better for each situation.

4.2.1. Population level study based on non-invasive sampling

Non-invasive sampling is an important tool to increase the number of samples and to study large territories of elusive or rare species (Broquet *et al.* 2007). It allows the performance of a uniform sampling with no morphological pre classification. Collection of invasive samples for cat hybridization studies are often biased, since there is a tendency to sample individuals with the wild type tabby phenotype or that reveal hybrid characteristics, and this can eventually lead to an underestimation of the hybridization rates (Pierpaoli *et al.* 2003; Oliveira 2012). Nevertheless, non-invasive sampling entails some disadvantages. One of the major problems is that non-invasive samples from sympatric species might be difficult to distinguish. Particularly for scat samples, identification based only on morphological characteristics is prone to errors that can bias the final results and, therefore, it is extremely important to perform species identification based on molecular markers (Oliveira *et al.* 2010; Monterroso *et al.* 2013a). From the 37 scats analysed in this study only 8 were correctly identified as *F. silvestris*, thus providing a misidentification rate of 78.4%. The majority of misidentified scats were genetically identified as red foxes (54.1%). These results are consistent with a previous study that also obtained a high misinterpretation rate of wildcats as red foxes (84.6%; Monterroso *et al.*, 2013a). The abundance of the target species influences the accurate morphological identification of its scats and, therefore, the declines in wildcat populations through Iberian Peninsula might explain the difficult collection and correct identification of scats from this endangered and rare feline (Driscoll & Nowell 2010; Monterroso *et al.* 2013a). Furthermore, the high abundance of red foxes and its marking behaviour also contributes to high detection of red fox's scats, while the dietary overlap between the two species (mainly when European rabbits are available) increase the similarity of their scats' morphology (Monclús *et al.* 2008; Monterroso *et al.* 2013a). Although the fragment of the mitochondrial Control Region used in this work usually provides more successful species identification of

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mesocarnivores' scats than the fragment of IRBP nuclear gene (Monterroso *et al.* 2013a), the size of that fragment in the cat genome (600 bp) can be difficult to amplify in DNA samples that are especially degraded or fragmented. All these particularities have to be taken into account and, therefore, the IRBP marker (Oliveira *et al.* 2010) was used in combination with the fragment of the mtDNA CR because it provides a smaller amplification fragment of 221 bp. Moreover, not all correctly identified samples are in optimal conditions to be used for further genetic analyses, since the extracted DNA might not have enough quantity or quality for subsequent amplification of other markers, for example, to obtain information on subspecific origin or hybridization. The relatively low value of microsatellite amplification success of the extracted non-invasive samples (41.5%) might be due to fragmented DNA molecules or because the concentration is frequently lower than for extractions of invasive samples. Moreover, non-invasive samples are prone to more genotyping errors during amplification than conventional invasive samples (Taberlet *et al.* 1999; Broquet *et al.* 2007; Beja-Pereira *et al.* 2009). The test samples collected in this work, specifically for the calculation of genotyping error rates, provide more realistic rates of allelic dropout and false alleles, since it is expected that using good quality invasive samples as references for comparison presents more accurate results than comparison between independent replicas (Bonin *et al.* 2004; Kolodziej *et al.* 2013). This is particularly important because the two step amplification procedure applied in this work to non-invasive samples is considerably recent and, although it allows a remarkable decrease in the required quantity of template DNA per replica (Beja-Pereira *et al.* 2009), there is still no clear information about its influence on genotyping errors. A comparative analysis of results from amplification of non-invasive samples using this two step PCR procedure and conventional amplification of good quality invasive samples might provide important information regarding genotyping errors. However, the 9 non-invasive test samples (scats) collected for this study were collected exclusively from house cats and were mainly fresh, not degraded by climate or other environmental conditions. Scats collected in the field are often exposed to several different environmental conditions for several days, weeks or even months before collection, which can deteriorate the sample quality (Taberlet *et al.* 1999). Different temperatures, moisture, precipitation, presence of fungi or parasites and sample age have an important influence on the extraction and amplifying procedures and on genotyping errors (Piggott 2004; Monterroso *et al.* 2013a). Furthermore, although to a lesser extent, different diets contain several different PCR inhibitors that also influence amplification success, thus possibly affecting genotyping errors (Murphy *et al.* 2003; Broquet *et al.* 2007). The nine domestic cats sampled for this test purpose were all fed with pet food. The different

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compositions of these diets might have an effect on DNA amplification and genotyping error rates. Piggot (2004) states that it is important that test samples are representative of the samples collected in the field. Although the collected test samples provide an insight on the real genotyping errors associated with the amplification of our non-invasive samples (0.016 and 0.050 for allelic dropout and false alleles, respectively), more accurate tests would be necessary to have more reliable results. This could be achieved by sampling a higher number of cats, preferably captive bred wildcats, fed with a diet similar to what is found in their natural habitats, and exposing scats to the diverse environmental conditions found in the field, if possible.

Furthermore, even after successful amplification of DNA, it is necessary to check for repeated genotypes, as the same individual can be sampled more than once (Beja-Pereira *et al.* 2009). Having all aforementioned restrictions in consideration, conducting population level studies using non-invasive genetic sampling requires a considerable sampling and laboratory effort and still improvements in the protocols.

The lack of population-level studies of hybridization in the Iberian Peninsula (as well as in most of the European wildcat range) prevents a more comprehensive analysis of the dynamics of hybridization at the regional scale. Studies focused on determination of population level rates of hybridization would allow comparisons between highly hybridized populations and others where hybridization is more sporadic, which could provide important information about the influence of several ecological factors on hybridization dynamics, as discussed by Hertwig and colleagues (2009) for the wildcat populations of Germany. However, due to the considerable limitations of non-invasive sampling, particularly to the great misidentification rate, the lack of sufficient quantity of samples for the Iberian areas that were non-invasively sampled (Guadiana Valley National Park, Peneda-Gerês National Park, L'Olleria (Valencia) and Paüls (Tarragona), Sierra Morena and Sierra Arana (Granada), Serra do Xurês and Coruña) it was not possible to perform the comparative study of population level hybridization rates. Only one population in Muniellos Natural Reserve had a sufficient number of correctly identified and good quality samples (n=20) and a hybridization rate of 15% was determined. Domestic cats (morphologically identified as wildcats) were also discovered in this population, which indicate their presence in this protected area. Regarding this and the high hybridization rate, further admixture studies and more intense conservation actions for the wildcat populations of this natural reserve should be considered.

The high misidentification rate and low amplification success demonstrated that an increased effort is needed to survey populations based in non-invasive sampling. The high sampling and DNA extraction effort required for such low number of usable

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samples reveal that non-invasive genetic sampling might not be cost-effective for wildcat studies in Iberian Peninsula. Nevertheless, considering its numerous advantages, it is important to test if a more careful sampling, mainly focused in areas with lower density of red foxes and with lower abundance of European rabbit (which would decrease the dietary overlap of red foxes and wildcats and, therefore, decrease the similarity in scat morphology) could be more effective (Monterroso *et al.* 2013a). Moreover, although scats are simpler to collect and one of the most common type of non-invasive sample used, there are other non-invasive sampling procedures that can be considered, such as the use hair trapping combined with appropriate attractants and camera traps (Zielinski *et al.* 2006; Monterroso *et al.* 2011, 2013b), that are already being used in wildcat studies in Europe (Steyer *et al.* 2013).

4.2.2. Substructure analysis

The dramatic population declines in the past centuries (Lecis *et al.* 2006) caused mainly by habitat destruction and human persecution (Nowell & Jackson 1996; Lozano & Malo 2012) had severe consequences in fragmentation and isolation of wildcats' populations throughout their distribution (Nowell & Jackson 1996; Pierpaoli *et al.* 2003). These factors may lead to genetic differentiation, and ultimately to decrease in genetic diversity, inbreeding or even extinction (Dixon *et al.* 2007). Oliveira (2012) identified patterns of genetic structure in European wildcats' distribution concordant with a previous study by Pierpaoli and colleagues (2003), detecting five distinct geographical macroareas. Within these geographical macroareas there are evidences of additional substructure, with several subpopulations. In the particular case of Iberian Peninsula, three subpopulations were identified by the author – northern, south-western and south-eastern Iberia. Similar substructure was found before by Oliveira and colleagues (2008b), distinguishing northern (north and centre) Iberia from southern Iberia. In contrast, our results do not resemble this genetic substructure. These results might indicate that gene flow was maintained in the past among separated populations, essentially due to wildcat's high dispersal rate, but that the recent increasing fragmentation of habitats is causing a considerable decrease in gene flow (Oliveira 2012). Fragmentation of the original habitats and destruction of ecological corridors might lead to disruption of the patterns of gene flow and, consequently, to differentiation among local isolated populations by genetic drift (Pierpaoli *et al.* 2003; Martínez-Cruz *et al.* 2007; Oliveira 2012). The low number of samples per location and the low number of markers used in this study might not be enough to detect this recent

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substructure and, therefore, our results have to be analysed with some restrictions. It is possible that a more uniform sampling effort would be necessary to have a more homogeneous number of pure wildcat samples throughout the Iberian Peninsula, in order to accurately study the possible patterns of substructure. A higher number of markers would probably also contribute to a more accurate analysis, which might explain the different results observed by Oliveira (2012) with 38 microsatellite *loci*. This author also point out that although a considerable proportion of current fragmentation might result from extensive habitat destruction and direct persecution, many other geographical, ecological and historical factors may as well contribute to explain genetic differentiation. This might explain the apparent genetic differentiation of the population of Cabañeros National Park, although further interdisciplinary studies combining ecology and genetics, comprising a higher number of samples, are necessary to thoroughly understand if this population is truly genetically differentiated or if this result is just an artefact of low sampling size.

Nevertheless, although genetic diversity is still high and does not seem to be decreasing due to population fragmentation (Oliveira 2012), continuous monitoring and management plans should be considered in order to identify possible changes in this pattern.

4.3.   A few insights into northern African wildcats

African wildcats diverged from the European subspecies recently (Driscoll *et al.* 2007), and today they share some parts of their distribution range (Kitchener & Rees 2009). On the other hand, the African wildcat is considered the most probable ancestor of the domestic cat (Vigne *et al.* 2004; Driscoll *et al.* 2007), and this widely distributed domestic pet is currently one the major threats to the wild subspecies due to hybridization (Driscoll & Nowell 2010). Regarding this, it is important to understand the genetic and ecological interactions between these three subspecies.

Bayesian analyses confirmed the clear distinction observed with high F_{ST} and R_{ST} values between European wildcats and African wildcats (see section 4.1). High similarity between domestic cats and their most probable ancestor, the northern African wildcat, was also confirmed with Bayesian analyses, particularly considering that the African subspecies is allocated in the domestic cluster when only two populations (K=2) are tested. This high genetic similarity is in accordance with previous studies (Driscoll *et al.* 2007; Oliveira 2012). Bayesian analyses separated the three subspecies in three distinct clusters, which is accordance with the high percentage of variation

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between these subspecies observed in the AMOVA results. However, the high genetic proximity among domestic and African wildcats complicates their distinction, particularly with a small set of microsatellites. In fact, considering the low F_{ST} (0.09) between these two populations, Vähä and Primmer (2006) recommend a much higher number of microsatellite *loci*, around 48 or more, for the accurate identification of parental species. Based on simulations, the authors also infer that identification of hybrids between populations with such low F_{ST} is not accurate when a low number of *loci* are used. In our species' assignment analyses some reference domestic cats were not correctly distinguished from African wildcats. Moreover, when the analysis was performed using the three cat subspecies there were some domestic cats and hybrids between European wildcats and domestic cats that were not accurately identified, which might be explained by the aforementioned high genetic similarity between African and domestic cats and consequent difficult distinction with our set of markers. In order to achieve a more accurate analysis, it is crucial to choose a higher set of markers specifically for the distinction between European, African and domestic cats. Such set of microsatellites should be chosen among the most informative for distinction of these subspecies and using adequate reference samples, as these should not contain any hybrids and need to be representative of the genetic diversity in the parental populations. This was taken in consideration when choosing the markers for distinction of European wildcats and domestic cats. However, the lack of reference samples for the African population prevented the application of this methodology for choosing markers that were also informative for the distinction between African and domestic cats. This lack of reference African wildcat genotypes also prevented simulation analyses, to infer an adequate threshold value for individual's assignment. Oliveira (2012) stated the same limitation, arguing that although clear distinction of putative African wildcats and domestic cats was possible with the set of 38 microsatellites, no admixture inferences were made for *F. s. lybica* subspecies due to lack of an accurate threshold. Regarding this, it should be a priority to construct an appropriate reference database for the African subspecies, to facilitate the selection of adequate and informative markers and for its use in simulation analyses.

Nevertheless, even considering all limitations regarding domestic/African wildcat differentiation, our results provide interesting insights into northern African wildcat population genetics, especially considering the lack of studies regarding this subspecies. Our results provide further evidence that hybridization between domestic cats and African wildcats might be occurring in North Africa, and therefore, it would be important to study North African cats thoroughly, particularly since this is considered a major threat to *F. s. lybica* populations (Phelan & Sliwa 2005; Driscoll *et al.* 2007). As

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mentioned for European wildcats, opportunistic sampling of African wildcats, as done in this study, did not provide a representative sampling of African cats. All individuals were identified morphologically as African wildcats, and no domestic cats or putative hybrids from North Africa were collected. Studying these might provide a more comprehensive insight of this subspecies' hybridization dynamics. Moreover, a more widespread and complete sampling in the North African wildcat's territories, particularly in areas where the three analysed subspecies occur (Near East; Kitchener and Rees, 2009) could provide interesting information on the genetic structure of this species and on the origin of the domestic subspecies (Pierpaoli *et al.* 2003).

4.4.   Implications for Conservation

Regarding the conservation status of Iberian wildcat populations – Vulnerable in Portugal and Near Threatened in Spain (Cabral *et al.* 2005; Palomo *et al.* 2007) – it is essential to interpret genetic and ecological studies in the light of conservation and management plans.

It is documented that the extreme declines in European wildcat populations throughout their entire range were mainly caused by human related threats like habitat destruction and persecution (Nowell & Jackson 1996). With increased protection in most European countries and international legislation, wildcat population densities had a slight recover. However, wildcats are still suffering from other threats, and anthropogenic hybridization with the domestic conspecific is currently considered the major risk to this endangered feline (Driscoll & Nowell 2010). The fact that wild and domestic genepools are still clearly distinguishable and "pure" wild individuals are found in most European countries where these two subspecies have been living in sympatry for a long time, can lead to the conclusion that some selective pressures and/or reproductive barriers are still preventing extensive hybridization. Germain and colleagues (2008) state that competition can be a behavioural barrier to hybridization in genetically close species that live in sympatry, but human activities may diminish this effect (Germain *et al.* 2008). Moreover, Hertwig and colleagues (2009) discuss that some morphotypes and genotypes must be more privileged in certain habitats. These authors state that domestic cats are better adapted to cultivated landscapes and proximity to human settlements, while wildcats are more adapted to forest environments and are more vulnerable to pathogens and viruses that affect the domestic form. Therefore, it is extremely worrying that introgression may destroy these specific advantages, possibly causing an increase in hybridization (Hertwig *et al.* 2009).

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Although low hybridized populations are still the majority of cases, the identification of extensively hybridized areas where distinction between wild and domestic cats is not clear and cat populations appear to be a hybrid swarm, such as in Scotland and Hungary (Beaumont *et al.* 2001; Pierpaoli *et al.* 2003; Lecis *et al.* 2006; Oliveira 2012) should be regarded as a warning and a motivation for the development of adequate monitoring and management plans to avoid other situations to develop in the same way. Conservation measures must be applied after careful and thorough study of the hybridizing populations, to assure that management plans fit the requirements of each particular scenario. For instance, if only first generation hybrids are identified in a population, hybrids might be sterile, or ecological, genetic or behavioural reproductive barriers are possibly preventing further hybridization. On the other hand, if backcrossed individuals are identified, introgression might be threatening the genetic integrity of the wild populations and more aggressive conservation measures should be applied. Introgression of domestic genes into the wild genepool is a major concern for conservation, not only for the wildcat but also for other animals such as the wolf (Verardi *et al.* 2006). Therefore, accurate identification of hybrids is mandatory and has several ecological applications, mainly for removal or neutering of admixed and domestic individuals or the identification of genetically pure populations for breeding programs (Vähä & Primmer 2006), and it is considered the most important conservation measure for all *Felis silvestris* subspecies (Driscoll & Nowell 2010).

In the particular case of Iberian populations, two distinct genepools are still detectable but hybridization seem to be considerably higher and geographically more widespread than observed in previous studied (Oliveira *et al.* 2008a). The increase in human populations is associated with the increase of domestic cats and their spread into wildcat territories near villages and farms (Sarmento *et al.* 2009; Ferreira *et al.* 2011). Therefore, conservation measures should focus on controlling the density of feral domestic cats, especially in locations near wildcat territories (Lecis *et al.* 2006), and investing in neutering and vaccinating pet cats in order to avoid the spread of diseases that can be fatal to wildcat populations (Kitchener *et al.* 2005). Moreover, the impact of the presence the domestic cats on the ecologic equilibrium of the wild subspecies should be assessed at population levels to infer if removal of domestic and hybrid individuals is required. It is also important to understand that the elusive behaviour and low densities of the wildcat, similar morphological appearance with the domestic conspecific, and the fact that it poses no risk for people or livestock contributes to a general ignorance of the species (Klar *et al.* 2008). Therefore, it is mandatory to inform local populations and raise awareness for the endangered status of the European wildcat before implementation of any conservation measure (Ferreira

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et al. 2011), because the participation of cat owners and people who have the habit of feeding feral domestic cats is essential to manage free ranging animals, especially considering that these have an increased fitness due to supplementary feeding by humans (Sarmento *et al.* 2009).

Moreover, it should also be considered that past and present habitat destruction is still a huge concern for wildcat conservation. Anthropogenic modification and devastation of some wild habitats might lead to changes in the ecosystem equilibrium and food chains, consequently leading wildcats to approximate human villages searching for food. This can ultimately lead to increase contact with domestic cats. On the other hand, habitat destruction can lead to further fragmentation of wildcat's geographical distribution. Habitat loss and fragmentation can cause isolation of populations with a possible effect on genetic diversity and population fitness, as happens with populations of Florida black bears (Dixon *et al.* 2007). Following fragmentation, populations can become genetically structured due to reduced effective population sizes, especially if there is low gene flow among them, potentially leading to inbreeding, reduction in genetic diversity and ultimately compromising species survival (Martínez-Cruz *et al.* 2007). Top predators as the wildcat are particularly sensitive to population declines and fragmentation of their distribution ranges, given their low densities (Oliveira 2012). Although our results do not demonstrate clear signs of genetic substructure, Oliveira's (2012) results identified geographically and genetically distinct populations and subpopulations in Europe, including in Iberian Peninsula. The author discusses that the high dispersal rate demonstrated by wildcats may counteract this process, but if fragmentation and isolation increases over time it can potentially become a more worrying situation with possible severe declines in genetic diversity. Therefore, it is important to invest in habitat restoration, protection of low disturbance sites and autochthonous forests, enhancing rabbit availability and providing ecological corridors to connect subpopulations (or underpasses to allow wildlife dispersal through anthropogenic barriers), and even translocation of animals if needed (Sarmento *et al.* 2006; Fernandes 2007; Martínez-Cruz *et al.* 2007; Dixon *et al.* 2007; Monterroso *et al.* 2009; Hartmann *et al.* 2013), not only in subpopulations of Iberian Peninsula but also among populations throughout Europe (Oliveira 2012). This would help increase genetic variability through increased gene flow, as well as enhance habitat quality and prey availability that could prevent wildcats to approach villages or farms.

Studies combining eco-ethological and population genetics research would be useful to better understand the factors influencing cat hybridization and what is causing different admixture rates throughout Europe (Lecis *et al.* 2006; Oliveira *et al.* 2008a; Randi 2008; O'Brien *et al.* 2009; Hartmann *et al.* 2013). Oliveira and colleagues

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(2008a, b) discussed that the ecological, ethological and genetic characteristics of Iberian wildcats are still poorly studied, which is consistent with other authors' opinions on the lack of detailed information on wildcat biology and spatial ecology throughout Iberian Peninsula (Sarmento *et al.* 2006; Monterroso *et al.* 2009). Furthermore, widespread studies regarding the occupation of wildcat territories by feral domestic cats are also lacking (Sarmento *et al.* 2009). Although the genetic features are now better understood, a multidisciplinary approach combining this knowledge with ecological and ethological studies is needed, since it would help assess the causes of the breakdown of reproductive barriers (Pierpaoli *et al.* 2003), and the real genetic and ecological dynamics of the hybridization process. For example, understanding temporal and space use patterns of the two subspecies and of their hybrids is crucial to identify the behavioural processes that influence interbreeding (Germain *et al.* 2008). Also, a more exhaustive analysis of the geographical location of hybrids can provide information about the possibility of crossbreeding between wild and domestic cats being restricted to peripheral areas of wildcat subpopulation's range where wildcat density is low compared to high density of domesticates, as previously observed in Italy (Randi *et al.* 2001; Lecis *et al.* 2006), or if admixture events can also take place in the core of the species distribution (Oliveira 2012). In fact, the thorough study of hybrids' ecological and behavioural characteristics would be an excellent source of information about hybridization dynamics, and the influence of hybrids in wild populations.

African wildcats might be facing the same threats as European wildcats, but the lack of genetic, ecological and ethological information is more distressing than for its European conspecific. Moreover, as aforementioned, studies in areas of overlapping ranges such as Near East, would provide interesting information on both subspecies, the interactions among them and with the domestic cat.

Considering the threats that jeopardise the long time survival of African and European wildcats as distinct subspecies, general conservation topics are proposed by several authors and authorities following IUCN guidelines (Driscoll & Nowell 2010). This entity's priorities for the conservation of *Felis silvestris* subspecies rely essentially in increasing studies for achieving more thorough information on genetics and ecology, and to optimize an accurate method for the distinction of domestic, hybrid and wild individuals, in order to identify genetically pure populations and prevent hybridization by neutering and removal of feral domestic cats. In order to achieve these objectives public campaigns are required to raise awareness about the wildcat status; wildcat populations should be regularly monitored to check their densities, distribution, and to evaluate mortality by illegal hunting and road kills; and investments should be made to

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protect large suitable habitats with adequate ecological corridors and healthy prey populations to prevent wildcats to approach human settlements (Stahl & Artois 1991; Fernandes 2007; Oliveira *et al.* 2008b; Sarmento *et al.* 2009). Also, hybridization should be carefully studied, mostly when populations have shown a severe decline in the last years or are currently small and isolated; in areas where wildcat colonization is recent or the habitat has recently gone through considerable changes; and when human population in a particular area is largely increasing with consequent growth in density of domestic cats (Stahl & Artois 1991; Nowell & Jackson 1996).

4.5. Marker improvement and future perspectives

Understanding anthropogenic hybridization and its causes and consequences on natural populations is considered by most authors one of the key element for the protection of the endangered wildcat, and for the elaboration of proper conservation plans at a regional, national or international level. Although the use of highly polymorphic microsatellites and advanced software have improved significantly the study of this phenomenon through the last decade, the increasing need of more detailed and specific knowledge on the impact of introgression on the wildcat genome, its effect on fitness and, ultimately, on the dynamics of natural populations, is promoting the development and improvement of innovative molecular markers.

The identification of pure wildcats and hybrids is essential for the study of hybridization but also for other ecological purposes, like the correct inference of distribution areas (Fernandes 2007). The genetic similarity of domestic and wildcats, especially domestic and northern African wildcats, and lack of accuracy for the detection of backcrossed individuals among domestic/European wildcat hybrids encourage the use of more effective and diagnostic markers (Nussberger *et al.* 2013). Oliveira (2012) discusses that one of the most important conclusions that can be drawn from the published studies about cat hybridization in Europe is that the distinction between domestic and wildcat is usually possible with a low number of microsatellites, but a higher number is needed to increase the resolution of admixture analyses. In fact, the use of a small set of microsatellites, as the 14 used in this work and other similar numbers used in other studies (Randi *et al.* 2001; Oliveira *et al.* 2008a; b; O'Brien *et al.* 2009; Hertwig *et al.* 2009; Eckert *et al.* 2010), can provide an accurate distinction between the two subspecies, but is not informative enough for the correct distinction of hybrid classes and for distinction of backcrossed individuals (Vähä & Primmer 2006). This is evident in our results, since our set of markers did not provide accurate results

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in the distinction of hybrid classes, even demonstrating high heterozygosity and allelic richness. The same happens in other hybridizing species as the wolf and dog (Randi 2008). With the set of 38 microsatellites used by Oliveira (2012) no hybrid individuals resulting from backcrosses with wildcat were misidentified as pure wildcats. However, there was still some difficulty in the accurate distinction of hybrid classes and identification of individuals resulting from backcrosses with domestic cats, and, therefore, hybridization might still be underestimated and its real dynamics not completely understood (Oliveira 2012). Moreover, such number of microsatellites requires higher laboratory and economic effort, as well as a high quantity of extracted DNA to amplify several multiplex reactions that is not compatible with non-invasive DNA limitations (Beja-Pereira *et al.* 2009; Guichoux *et al.* 2011). Therefore, some authors propose the development and optimization of SNP markers for hybridization studies, based on their aforementioned advantages (Beja-Pereira *et al.* 2009; Oliveira 2012; Nussberger *et al.* 2013).

Although SNPs have a lower mutation rate and, consequently, are less polymorphic than microsatellites, their efficiency and diagnostic power rely on highly differentiated allele frequencies, technical advantages and widespread distribution across the genome (Morin *et al.* 2004; Guichoux *et al.* 2011; Nussberger *et al.* 2013). These markers can also provide lower genotyping errors (Nussberger *et al.* 2014; Kraus *et al.* 2014) and might be particularly good options for amplification in non-invasive DNA samples, given that smaller amplification fragments are needed (Broquet *et al.* 2007; Fabbri *et al.* 2012; Nussberger *et al.* 2013, 2014). In addition, their use require a higher number of *loci* than microsatellites for diverse studies, but overall, the possibility of selecting and genotyping a huge quantity of SNPs with cost-effective methods might overcome this limitation (Morin *et al.* 2004).

Oliveira and colleagues (2008b) suggested a genome-wide study for the development of diagnostic *loci* related to genes that suffered changes during the domestication process, mainly associated with reproduction, coat colour and pattern, disease resistance and behaviour, pointing out the advantages of SNPs to overcome some errors associated to microsatellites, like homoplasy. Considering this, Oliveira (2012) developed 158 SNPs for cat hybridization inference. This set provided very accurate identification of parental genotypes, first and second generation hybrids and backcrosses. A smaller subset of 35 most polymorphic SNPs was also tested, since the use of a large number of makers (158) might not be viable especially for non-invasive samples, and also presented accurate identification of all parental and hybrid classes, with very few misidentifications (Oliveira 2012).

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Nussberger and colleagues (2013) also developed single nucleotide polymorphisms for wildcat/domestic cat hybridization study and selected a subset of 48 with the highest F_{ST} (higher than 0.8). The authors tested the assignment power of this small set for the usual 6 hybrid categories (parental wild and domestic, F1, F2, BxW and BxD), but also for additional four – [BxW]xW, [BxW]xF1, [BxD]xD, [BxD]xF1. With the set of 48 SNP markers, and even with smaller sets of 32 or 24, the authors were able to accurately distinguish almost all parental, first, second and third generation hybrids. Plus, no hybrids were misinterpreted as parental, which demonstrate the potential of SNPs for the identification of admixed and pure individuals.

Single nucleotide polymorphisms are already being developed and tested for the study of hybridization in other species, such as introduced rainbow trout and native westslope cutthroat trout (Hohenlohe *et al.* 2011), including in some studies that use non-invasive sampling for monitoring species, for example in wolf/dog hybridization (Seddon *et al.* 2005; Fabbri *et al.* 2012; Kraus *et al.* 2014). High discriminating SNP sets may bring new insights to the study of cat hybridization and its population dynamics, given the high accuracy of identification for the different hybrid classes (Oliveira 2012). Mitochondrial DNA SNPs have also been suggested (Driscoll *et al.* 2011), which might provide interesting results regarding maternally inherited *loci* to be used as a complement to biparently inherited markers.

Molecular markers, particularly SNPs, are already being developed using next generation approaches (for example, Nussberger *et al.*, 2013; Oliveira, 2012). The development of a massive number of molecular markers with the use of these advanced techniques is very promising to increase efficiency and diagnostic power of markers. Particularly, the study of wildcat populations (as well as other felid species) will benefit tremendously from all information originated from the increasing knowledge of the cat genome (Pontius *et al.* 2007; Pontius & O'Brien 2007; O'Brien *et al.* 2008; Menotti-Raymond *et al.* 2009; Mullikin *et al.* 2010; Tamazian *et al.* 2014), that enable the study of specific parts of the genome that contrast between wild ancestors and the domesticated relatives, possibly involved in the domestication process (Oliveira 2012). In particular, the identification of specific mutations that appear in the domestic cat and are predictably absent in natural wild populations – like the ones determining morphological (variable coat colours and patterns) and physiological diversity – and others that benefit wild populations and that remain fixed in wild but not in domestic cats – like camouflage patterns and hunting behaviours – provide potential diagnostic candidate genetic variants for distinguishing wild and domestic cats (Oliveira 2012). Moreover, the possibility of analysing the structure of hybrid genomes, particularly the size and distribution of blocks derived from one or both parental species, might provide

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very interesting insights into the hybridization process (Abbott *et al.* 2013). This will also provide an opportunity to assess differential rates of introgression throughout different genomic regions (Oliveira 2012).

Next generation techniques are evolving to be more and more common, accessible and widely applied, and the cost of the procedures are decreasing (Allendorf *et al.* 2010). Therefore, it is possible that in a near future these will become routine procedures for several ecological and conservational purposes, including the assessment of hybridization, possibly optimized to be used in low quality non-invasive samples.

Future analyses should include the amplification of uniparentally inherited *loci*, such as mitochondrial DNA genes, to infer the direction of hybridization and understand the hybridization dynamics in natural populations, as also stated by Oliveira (2012). Nevertheless, mtDNA markers have to be carefully chosen to avoid the amplification of mtDNA introgressed in the nuclear genome (*numts*; Antunes *et al.*, 2007; Lopez *et al.*, 1996). Also, for analyses based on non-invasive genetic sampling, it must be assured that the resulting fragment is short enough to be easily amplified in fragmented DNA.

The efficiency of nuclear markers for admixture analyses has to be carefully tested, particularly with studies comparing microsatellites and SNPs for wildcat/domestic cat hybridization research, to fully understand which markers are more informative and most cost-effective. Moreover, the particularities of non-invasive sampling should be taken in consideration when comparing the efficiency of nuclear markers. A combination of different types of markers, representing neutral and non-neutral variation could be an adequate option to obtain accurate results on genetic structure, admixture analyses and individual identification (Fabbri *et al.* 2012; Oliveira 2012).

Moreover, an optimization of non-invasive sampling procedures should be considered, either by collection of other types of non-invasive samples, like hairs, or focusing collection in areas with low densities of red fox, for example. This should be done in order to achieve an efficient sampling that allows the study of hybridization at population level, and further comparative research to investigate what ecological features of the populations can influence hybridization rates.

Lastly, the majority of markers now used were selected or even developed for the distinction of European wildcats and domestic cats, and most were not tested for their power in distinction of other subspecies, including the African wildcat (for example, Nussberger *et al.*, 2013). Given their high genetic similarity, it is important to select and optimize markers specifically for the distinction of African wildcats and

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domestic cats. Considering the endangered status of northern African wildcats and evidences of possible hybridization with the domestic cat, it is a priority to use informative molecular markers to thoroughly understand their population genetic dynamics.

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5.   Conclusions and final remarks

The domestic cat is one of the most charismatic pet species. It has been, through times, an inspiration for arts and literature, and is currently spread through almost all continents, either occupying an important position in our households or living independently. However, its wild conspecifics are almost unknown to the majority of human population, mostly due to their elusive behaviour, low densities, high morphological similarity with the domestic form, and little influence on human life.

The domestication process is relatively recent and evolved with continuous interbreeding among the wild and domestic subspecies, which contributed for their high similarity, not only in their morphological aspect, but also in genomic information. Nowadays, due to numerous threats, mostly human related, hybridization with the domestic subspecies might be threatening the long time survival of the wildcat, contributing to their endangered status. This situation has to be studies thoroughly for an accurate identification of its real impacts. However, the high morphological and genetic similarity between the two *taxa* complicates the hybridization analyses and identification of introgressed individuals. During the last decade, several studies based on microsatellite markers and advanced software provided a more comprehensive overview of the hybridization scenario across Europe, identifying areas with low impact of admixture (Randi *et al.* 2001; O'Brien *et al.* 2009; Hertwig *et al.* 2009; Eckert *et al.* 2010), and others with widespread hybridization (Beaumont *et al.* 2001; Pierpaoli *et al.* 2003; Lecis *et al.* 2006). In Iberian Peninsula, the wild and domestic subspecies showed distinct genepools with few hybridization events in Portugal (Oliveira *et al.* 2008a; b), but a more widespread study was still needed, with a more extensive sampling effort. In North Africa, although some evidences of hybridization exist (Phelan & Sliwa 2005; Driscoll *et al.* 2007), this situation was not yet thoroughly studied, and the northern African wildcat (*F. s. lybica*) remains poorly known.

Considering this, we delineated two main objectives for this work that focused on the thorough study of hybridization in Iberian Peninsula, including at population scale, and an overview of the hybridization scenario in North Africa.

We were able to access the hybridization situation in the Iberian Peninsula, given that the selected microsatellites were successfully amplified in invasive and non-invasive samples, after optimization of PCR reactions to overcome the limitations of each sample type (Beja-Pereira *et al.* 2009). Our results confirm the presence of two distinct genepools for the wild and the domestic subspecies, but also reveal the existence of hybridization events geographically widespread through the sampling

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area. Overall, a hybridization rate of 20% was determined for the whole Iberian Peninsula, much higher than the rates determined in previous studies performed in this location (Oliveira *et al.* 2008a; b). It was also possible to infer a high hybridization rate in the population of Muniellos Natural Reserve, where domestic cats were also found, highlighting the capacity of domestic cats to proliferate to wild territories and live in sympatry with their wild conspecifics. However, it was not possible to accurately identify the hybridization classes, and the simulation analyses indicate that some backcrossed individuals might remain unidentified, which emphasize the need of further analyses.

Moreover, the optimized microsatellite panel was also successfully used for the amplification of DNA extracted from northern African wildcat samples. The three subspecies showed high levels of genetic diversity. Domestic cats and African wildcats demonstrated a very high genetic similarity, compatible with the African ancestry of the domestic cat (Vigne *et al.* 2004; Driscoll *et al.* 2007). On the other hand, European wildcats proved to be well differentiated from domestic and African wildcats, which is in accordance with the early splitting of European wildcats compared with the other subspecies (Driscoll *et al.* 2007). The distinction between northern African wildcats and domestic cats was possible, but their high genetic similarity prevented a completely clear differentiation, which requires further analyses with more diagnostic markers. Nevertheless, it was possible to identify a potential evidence of admixture between domestic cats and northern African wildcats, which supports the importance of further hybridization studies focused in North Africa. However, this admixed genotype might also be an artefact of the inaccurate distinction between these two subspecies, and therefore requires further investigation.

Considering our results, conservation and management plans should mostly focus on preserving sufficiently large and suitable habitats to maintain healthy populations that assure the preservation of the genetic variability. The restoration and preservation of natural habitats with healthy prey populations will also contribute to avoid contact between wild and domestic cats, since wildcats will not look for food and shelter near farms or other human settlements so often. Nevertheless, it is also essential to invest in neutering and vaccinations plans for free ranging domestic cats, in order to avoid interbreeding and spread of diseases into wild populations. Above all, it is mandatory to start campaigns to raise awareness about the wildcat situation, especially focussed towards people living near wildcat territories. These conservation measures are equally important for both European and African wildcats, although the African subspecies still need more thorough studies regarding their ecological and genetic features.

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Although our marker set successfully identified pure and admixed individuals, the limitations of microsatellites on the accurate identification of hybrid classes and, particularly, of backcrossed individuals should be considered, mostly due to the potential underestimation of hybridization. Therefore, it is important to develop and optimize more informative markers that allow the accurate distinction of these hybrid classes. Moreover, the correct distinction between African wildcats and domestic cats should also be regarded as a priority, and more informative markers should also be selected for this specific purpose. Single nucleotide polymorphisms might provide more accurate results and overcome the limitations of microsatellites, and are already being developed and tested for wildcat hybridization studies. Also, Next Generation Sequencing approaches are already being used for marker development, and might be an important tool for investigation of the genomic implications of hybridization, or even as a standard procedure for the identification of admixed individuals in a near future.

In conclusion, conservation of wildcats is dependent on a better understanding of all ecological and ethological factors influencing hybridization, and on the development of adequate, diagnostic molecular markers to thoroughly study the hybridization dynamics and its influence on the natural equilibrium of their populations. The conservation measures proposed for European and northern African wildcat might as well be favourable for other wildcat subspecies that are also endangered by similar threats (Nowell & Jackson 1996; Driscoll & Nowell 2010), but much more research is needed to understand their specific requirements and local threats.

It is important to shift our concept of the wildcat species to a more comprehensive view that takes into account its capacity of adaptation to the changing habitats, evolving within its contemporary environment. Therefore, conservation should focus not on the eradication of hybridization, but on constructing management plans that fit the unique requirements of each population, in order to preserve the ecological function of the wildcat in the ecosystem equilibrium. Overall, we should always keep in mind that "the more we know about hybridization and the factors involved, the better we will be able to assess each situation" (Genovart 2008).

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Supplementary material

I

Table S 1 – Location of the collected samples and number of invasive (N $_{Invasive}$) and non-invasive (N $_{Non-invasive}$) samples for each location in Portugal, Spain and North Africa. *All samples collected in Iberian Peninsula with dubious morphological identification (putative hybrids) or without morphological information (scats) were considered as *F. s. silvestris*.

Subspecies	Location		N $_{Invasive}$	N $_{Non-invasive}$
*F. s. silvestris**	Portugal	Barrancos	1	-
		Estremoz	1	-
		Montemor-o-Novo	1	-
		Moura	1	-
		Peneda-Gerês National Park	-	6
		Trás-os-Montes	1	-
		Guadiana Valley Natural Park	8	4
		Vila Nova de São Bento	1	-
		Unknown	3	-
	Spain	Asturias	1	-
		Cabañeros National Park	8	1
		Ciudad Real	3	-
		Serrania de Cuenca Natural Park	3	1
		Coruña	-	12
		Granada	1	7
		Guadalajara	1	-
		Huelva (PND)	1	-
		León	3	-
		Madrid	1	-
		Muniellos Natural Reserve	-	34
		Segovia	2	-
		Serra do Xurês	-	9
		Sevilla	1	-
		Tarragona	-	6
		Toledo	4	-
		Valencia	-	10
		Valladolid	8	-
F. s. catus	Portugal	Almodôvar	2	-
		Loulé	6	-
		Moura	9	-
		Mourão	3	-
		Silves	3	-
F. s. lybica	North Africa	Algeria	2	-
		Niger	2	-
		Morocco	6	-
		Mauritania	6	1
		Senegal	1	-
		Tunisia	1	-
		Western Sahara	1	-
		Unknown	3	-
Total			**99**	**91**

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II

Table S 2 – Distribution of microsatellite *loci* in multiplexes for a) invasive samples and b) non-invasive samples and
their respective primer tails labelled to fit the multiplex arrangement and avoid overlapping results, and
concentration of primer in each PCR reaction (in µM). The concentration of primer for non-invasive PCR
reactions was equal for the first (pre-amplification) and second amplification reactions. All forward primers
in amplification reaction of invasive samples were used with a 10x dilution.

a)

MixI			**MixII-A**			**MixII-B**		
Locus	**Primer tail**	**Conc (µM)**	**Locus**	**Primer tail**	**Conc (µM)**	**Locus**	**Primer tail**	**Conc (µM)**
FCA023	PET	0.26	FCA035	VIC	0.46	FCA126	FAM	0.26
FCA043	VIC	0.14	FCA220	FAM	0.32	FCA149	PET	0.24
FCA096	VIC	0.20	FCA310	NED	0.20	FCA229	PET	0.16
FCA097	NED	0.28				FCA391	VIC	0.28
FCA132	FAM	0.46						
FCA223	PET	0.26						
FCA698	NED	0.32						

b)

MixNI1			**MixNI2**			**MixNI3.1**			**MixNI3.2**		
Locus	**Primer tail**	**Conc (µM)**	**Locus**	**Primer tail**	**Conc (µM)**	**Locus**	**Primer tail**	**Conc (µM)**	**Locus**	**Primer tail**	**Conc (µM)**
FCA023	PET	0.12	FCA035	VIC	0.12	FCA126	FAM	0.08	FCA149	PET	0.10
FCA043	VIC	0.08	FCA096	VIC	0.08	FCA698	NED	0.08	FCA229	PET	0.12
FCA097	NED	0.12	FCA220	FAM	0.18				FCA391	VIC	0.14
FCA132	FAM	0.36	FCA310	NED	0.08						
FCA223	PET	0.12									