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Biological and genetic aspects of wild x domestic hybridization in wild boar and wolf populations

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ABSTRACT

Nowadays, hybridization is recognized as a powerful evolutionary force promoting speciation and shaping adaptation, but also as a serious threat to the conservation of biodiversity.

This thesis is focused on two cases of hybridization between wild and domestic conspecifics, whose effects are mostly unexplored.

In *Sus scrofa*, I sought to expand knowledge about hybridization between wild boar and domestic pig. I investigated the main sources of domestic genes introgression, and assessed hybridization at neutral markers and functional genes at both local and European scale. I also developed a set of new uniparental markers for studying male-specific gene flow, and studied the reproductive phenology of wild populations.

In *Canis lupus*, I investigated patterns of hybridization between wolf and domestic dog in an Italian mountain area, focusing on the assessment of introgression and the food habits of hybrids.

As regards wild boar, I detected introgression all over Europe, also highlighting the role of breeding stations in spreading domestic genes across wild populations. With respect to wolf, a new approach was used to provide complementary (genetic and phenotypic) data on specific individuals and to support hybrid identification. A trophic niche overlap between wolves and hybrids was also proved.

These studies can have relevant management implications, offering new elements of knowledge on different aspects of the hybridization in two worrisome species of the Italian fauna.

RIASSUNTO

Più conosciamo il fenomeno dell'ibridazione, e più ci rendiamo conto del suo aspetto bivalente: da un lato, potente forza evolutiva che favorisce la speciazione; dall'altro, seria minaccia per la conservazione della biodiversità. Questa tesi si occupa in particolare dell'ibridazione tra conspecifici domestici e selvatici, un fenomeno i cui effetti sono lungi dall'esser compresi appieno.

Nella prima parte del lavoro, mi sono concentrato sull'ibridazione tra maiale domestico e cinghiale (*Sus scrofa*). Cercando di chiarire quali fossero le principali fonti di introgressione di geni domestici nelle popolazioni selvatiche, e adottando sia marcatori molecolari neutrali che geni funzionali per rilevare introgressione su scala locale ed Europea. Ho inoltre collaborato allo sviluppo di nuovi marcatori a trasmissione patrilineare, ed allo studio della fenologia riproduttiva in popolazioni selvatiche di cinghiale.

Nella seconda parte della tesi mi sono occupato dell'ibridazione tra lupo (*Canis lupus*) e cane in un'area dell'Appennino toscano, studiando l'entità del fenomeno e le abitudini alimentari degli ibridi.

Per quanto riguarda il cinghiale, l'introgressione è risultata estesa a tutt'Europa, e nella sua diffusione si è rivelato importante il ruolo giocato dagli allevamenti. Relativamente al lupo, il nostro nuovo approccio ha fornito informazioni complementari su genetica e fenotipo di specifici individui, fondamentali per una più accurata identificazione degli ibridi. Abbiamo inoltre evidenziato una forte sovrapposizione della nicchia trofica tra ibridi e lupi, dunque una loro probabile competizione per le risorse.

I risultati ottenuti possono offrire notevoli implicazioni gestionali, ampliando le conoscenze su diversi aspetti dell'ibridazione tra forma domestica e selvatica in due specie 'problematiche' della fauna italiana.

INTRODUCTION

This thesis deals with various aspects of the complex phenomenon of hybridization, increasingly recognized as a powerful evolutionary force promoting speciation and shaping adaptation, but also posing a serious risk to the conservation of biodiversity.
A special case of hybridization is that occurring between wild species and domestic conspecifics, a subject that deserves a special attention for its evolutionary and management implications. This thesis is focused on two cases of wild x domestic hybridization. The former part is focused on the hybridization between wild boar (*Sus scrofa*) and domestic pig (*Sus scrofa domestica*), while the second one deals with the crossbreeding between wolf (*Canis lupus*) and dog (*C. lupus familiaris*). Both genetic/methodological and biological aspects are touched, such as the identification of hybrids, the development of new molecular markers and investigations on the source of introgression, reproductive phenology of the species involved and ecological traits of hybrids.

Hybridization and its effects on fitness

Hybridization was defined as 'interbreeding of individuals from genetically distinct populations, regardless of their taxonomic status' (Rhymer & Simberloff, 1996), while 'introgression' refers to the movement of genes between genetically differentiated forms mediated by backcrossing (Avise 1994).
Accordingly, introgressive hybridization refers to an exchange of genes between evolutionary lineages as opposed to hybridization yielding exclusively inviable or infertile offspring (Seehausen 2004). These broad definitions account for the fact that both inter- and intraspecific hybridization are relevant from a conservation perspective, both providing an extremely tough set of issues for conservation biologists.

Hybridization and introgression are strongly increasing worldwide due to intentional and incidental translocations of plants and animals, habitat modifications (Allendorf et al. 2001), human pressure (Rutledge et al. 2012) and climate change (Garroway et al. 2010).

For example, the introduction of plants and animals outside their native range, the creation of extensive areas of new habitats and the establishment of migration corridors have the effect of breaking down mechanisms of isolation between species and populations, generating opportunities for formerly allopatric taxa to meet and hybridize (Rhymer and Simberloff 1996; Stronen & Paquet 2013). Furthermore, a population decrease caused by anthropogenic factors can promote hybridization among species/populations because of the increased difficulty in finding mates (Allendorf & Luikart 2007). Mallet (2005) reported that at least 25% of plant species and 10% of animal species (6% of European mammals) are involved in hybridization and introgression with other species.

This increasing anthropogenic hybridization is causing extinction of many taxa (species, subspecies and locally adapted populations) by both replacement and genetic mixing (Allendorf & Luikart 2007). Hybrids may displace one or both parental taxa through the production of hybrid swarms (populations in which all individuals are hybrids, after a number of generations of backcrossing with parental types and mating among hybrids; Allendorf & Luikart 2007). Intraspecific hybridization may therefore compromise the genetic integrity of native populations and can homogenize their peculiar genetic characteristics, reducing, in this case, the 'raw material' for future allopatric speciation (Olden et al. 2004).

Nevertheless, the long term evolutionary consequences of introgressive hybridization remain largely unpredictable. Hybridization can have serious consequences on morphology, physiology, behaviour and individual fitness. Hybrid fitness can be influenced by both endogenous or exogenous selection. The former refers to factors acting against certain hybrid genotypes regardless of the environment in which they occur (e.g., meiotic irregularities or physiological/developmental abnormalities in individuals of mixed ancestry). Exogenous selection, instead, refers to environment-specific fitness differences (Burke & Arnold 2001).

In fact, hybrids may show a lower fitness compared to either parental taxa (outbreeding depression) due to loss of local adaptation to environmental conditions (extrinsic outbreeding depression).

For example, hybrids between species or populations with different combinations of coloration and behavior are likely to have reduced fitness because of having the wrong

combination of coloration and behavior (Allendorf & Luikart 2007). Outbreeding depression may also arise through underdominance (heterozygote disadvantage) between alleles of the two parental populations, or through a breakup of coadapted gene complexes (Stronen and Paquet 2013).

Hybridization is less likely to result in outbreeding depression when there is little genetic divergence between the involved populations. But even in this case, there can be important effects on adaptive divergence among populations (Allendorf et al. 2001).

Therefore, most hybrid genotypes tend to be less fit than parental genotypes in parental habitats. However, in some cases, hybrids can show equal or superior fitness in new habitats and, occasionally, even in parental habitats (Seehausen 2004).

If hybrid genotypes are fitter than one or both parents in some environments, then hybridization could make a positive contribution and single alleles that confer an advantage in the alternative environment will introgress quickly, although such introgression may be hard to detect (Barton 2001). Besides the presence of advantageous alleles, hybrids may also outperform their parents due to the sheltering of deleterious recessive alleles, though this effect (heterosis, or hybrid vigor) is mainly observed in F1 hybrids and lost in subsequent generations. In addition, increased heterozygosity will increase the fitness of hybrid individuals for loci where the heterozygotes have a selective advantage over homozygote types (Allendorf & Luikart 2007). In this way, hybridization can lead to the formation of new stable genetic populations potentially kick-starting speciation and adaptive radiation over a very short timescale (Roy et al. 2015). From another point of view, new hybrid taxa showing high fitness may be invasive and displace the parental taxa (Abbott 1992).

Many authors focus on the potential of hybridization as a source of adaptive genetic variation, functional novelty and new species: natural and anthropogenic introgressive hybridization is widespread and plays an important role in the evolution of animal and plant species, both at the inter- and intraspecific level (Burke & Arnold 2001; Largiadèr 2007). Moreover, Intraspecific hybridization in the form of gene flow among populations has traditionally been seen as a cohesive force that holds species together as units of evolution (Mayr 1963).

Conservation programs should aim to preserve the evolutionary potential of species, therefore careful considerations should be taken for a conservation/management of wild

hybrids. According to Stronen and Paquet (2013) it may be helpful to establish conservation priorities for hybrids that take into consideration (1) the extent to which the hybrids in question are natural as opposed to the (likely) result of human activity and (2) their ecological role in the local environment.

Wild x Domestic Hybridization (WxDH)

A particular case of hybridization that deserves special attention is that occurring between wild and domestic conspecifics. Indeed, gene flow between domesticated and wild conspecifics— or closely related species—seems to have greatly increased in the last decades (Randi 2008), because of the positive trend of several wild species (e.g., wolves and ungulates), the widespread occurrence of free-ranging domestic animals (dogs, cats, pigs, goats), accidental escapes of captive individuals, and massive releases of captive-reared game stocks (e.g., galliforms, waterfowl, salmonids). Among European mammals, for example, wild × domestic hybridization (WxDH) occurs between European wildcat (*Felis silvestris silvestris*) and domestic cat (*Felis silvestris catus*), European polecat (*Mustela putorius*) and domestic ferret (*Mustela furo*), mouflon (*Ovis musimon*) and sheep (*Ovis aries*), wolf (*Canis lupus*) and domestic dog (*Canis lupus familiaris*), Alpine ibex (*Capra ibex*) and domestic goat (*Capra aegagrus*), and wild boar (*Sus scrofa*) and domestic pig (Hindrikson et al. 2012; Giacometti et al. 2004; Largiadèr 2007).

In the last decades, WxDH has received a lot of attention because of its serious evolutionary implications. The domestication process generally leads to genetic changes resulting from inbreeding, genetic drift, artificial selection and relaxed natural selection in captivity (Price 1999). They can entail rapid and striking physical, physiological and behavioural changes, such as morphological maladaptations, reduced brain size, increased litter size and alteration of the feeding, antipredator and sexual behaviours (O'Reagan and Kitchener 2005). Significant morphological changes can occur as a by-product of the selection of animals for tameness within as few as 10-15 generations. Hence, captive animals quickly show marked differences from their wild conspecifics, as the selection pressures acting on them are substantially different from those in the wild (O'Reagan and Kitchener 2005).

Domestic genes introgressed into wild populations, therefore, can have deleterious effects on fitness that can pose a potential threat to populations by decreasing viability and increasing the risk of extinction (Bryant & Reed 1999). For example, it has been shown that hybrids between wild and farmed Atlantic salmons (*Salmo salar*) have lower anti-predatory responses (Houde et al. 2010) and that introgressive hybridization between the common quail (*Coturnix coturnix coturnix*) and the domesticated Japanese quail (*Coturnix coturnix japonica*) affects the migratory behavior of the former (Largiadèr 2007).
Furthermore, wild x domestic hybridization may also have strong effects on genetic population structure (Goedbloed et al. 2013b). Additionally, populations of domesticated animals typically exceed those of their wild counterparts by several orders of magnitude, and this may facilitate unidirectional gene flow, posing a threat to the genetic integrity of natural populations (Godinho et al. 2011).

On the other hand, variants that appear in the domestic line may prove to be advantageous and selected for in the wild. It is the case of the melanistic mutation in North American wolves, which derives from past hybridization with domestic dogs, and has been proved to be positively selected in dense forests (Anderson et al. 2009). In other cases, Quantitative Trait Loci (QTL) variants selected in captivity to increase fertility may introgress into wild populations increasing their fitness and invasiveness. For example, García et al. (2011) argued that the invasive potential of the wild boar populations in Uruguay has emerged from introgressive hybridization with domestic pigs.

Interestingly, in many cases, WxDH seems to be a relatively ancient process.
Indeed, domestication has traditionally been viewed as being directed by humans and involving strong bottlenecks in the domestic population and reproductive isolation between wild and domestic forms, but the simplicity of this view is questioned by a growing body of empirical and theoretical work. New models suggest that neither reproductive isolation nor strong intentional selection have been as crucial and widespread as previously thought: domestication is rather seen as a long-term, diffuse process, involving gene flow (during as well as after domestication) between wild and domestic populations and a lack of strong domestication bottlenecks (Larson and Fuller 2014; Frantz et al. 2015).

Methodological aspects

Until the mid 1960s, the detection of hybrid individuals relied upon phenotypic traits only, with the assumption that hybrids will be phenotypically intermediate to parental individuals. However, this is not always the case; in fact hybrids sometimes display a mosaic of parental phenotypes, and they also may be morphologically indistinguishable from one of the parental taxa (Allendorf et al. 2001). Furthermore, morphological traits do not allow one to distinguish among first generation hybrids (F1), backcrosses and later generation hybrids. The use of molecular genetic markers (which started with allozymes in the 1960s) highly simplified the study of hybrids.

Genetic analyses of hybrids are based upon loci showing differences in allele frequencies between the parental taxa. Generally, the identification of recently introgressed hybrids, such as F1, F2 and first-generation backcrosses, could be achieved with a limited number of loci if the allele frequencies at these loci are sufficiently differentiated between the populations (Vähä and Primmer, 2006). Diagnostic loci that are fixed or nearly fixed for different alleles in two hybridizing populations are the most useful to investigate hybridization patterns (Cornuet et al. 1999). Both the amount and direction of gene flow between populations could be investigated, using different types of molecular markers.

In the last decades, the use of the Polymerase Chain Reaction (PCR) and the development of highly polymorphic markers such as autosomal microsatellites (or, short tandem repeats, STRs), represented major advances in the field of population genetics and gave the way to the development of new statistical methods to investigate individual ancestry and population assignment (e.g., Bayesian admixture analysis, see Hansen et al. 2001). Today, high-throughput technologies have improved genomic resources, such as single-nucleotide polymorphism (SNP) arrays and sequence assemblies, and enable the genome-wide genotyping of several species. SNP-based assays have proven to be powerful tools for inferring patterns of hybridization, population history and population structure (for example, Ramos et al. 2009). Although microsatellites and SNPs were found to be extremely useful markers to study ongoing hybridization and to estimate the amount of introgression, sequence analysis of functional genes (e.g., genes involved in regulating melanogenesis) may also be of help, if private or nearly private gene variants exist at this genes in different populations/species (see Fang et al. 2009).

Another interesting aspect which can be investigated with the help of molecular markers is the directionality of hybridization. In fact, often, the hybridization process seems prevalently asymmetric, involving in most cases one sex of a given parental form or species and the opposite sex of the other one (as in the case of the female wolf x male dog hybridization, see Vilà and Wayne 1999, Hindrikson et al. 2012). This asymmetry can be due to physiological, ecological and behavioural factors, and can be studied by employing uniparental markers such as the hypervariable domain of the mtDNA control region (mtDNA CR1) and Y-linked STR haplotypes.

It is noteworthy to mention that today we are able to investigate patterns of hybridization in natural populations using non-invasive sampling methods, which allow genetic studies of free-ranging animals without the need to capture, handle, disturb or even observe them (Taberlet & Luikart 1999). The DNA can be obtained by feces, shed hairs, urine, buccal cells from food wodges, eggshells and other sources. Non-invasive genetics allows the study of elusive or rare species otherwise extremely difficult to sample. However, it has some drawbacks: the main limitations are due to the low quality, and often quantity, of the obtained DNA, which reduce the yields and increase the rate of genotyping errors (e.g., false alleles and allelic dropout). Nevertheless, a set of techniques (laboratory protocols and statistical tools to evaluate the reliability of genotypes) have been developed in order to account for and correct most genotyping errors (e.g., Miller et al. 2002, Kalinowski et al. 2006).

Hybridization between wild boar and domestic pig

The Hybridization between wild boar and domestic pig deserves a special attention for its important economic and management implications. It is difficult to argue that the introgression of domestic pig genes into wild boar populations has led to maladaptation to the local environment. By contrast, wild boar populations have been growing considerably over the past decades in most of Europe (Massei et al. 2015) to the point that at present, this species reaches nearly four million individuals in the continent and is currently considered a pest in many areas (Apollonio et al. 2010). Indeed, the wild boar can cause extensive damages to crops, woodland, and grassland vegetation, as well as vehicle collisions and other issues in urban areas (Geisser and Reyer 2004; Massei et al. 2011). Moreover, wild

boar are known carriers of several parasites and diseases that, in some cases, pose a threat to livestock, wildlife, and human health (Gortàzar et al. 2007). For example, classical swine fever (hog cholera) is of increasing concern in Europe, where wild boar appear to play an important epidemiological role. Cross-infections between wild boar and domestic pigs are a primary cause of swine fever outbreaks in farmed and free-living populations. Domestic pigs are infected due to direct contact or indirect contact, via feeding on contaminated meat (Kramer-Schadt et al. 2007). This constitutes a real threat for the pig farming industry and caused major economic losses in countries with an industrialized pig production, like Germany, the Netherlands, Belgium, Spain and Italy (see Artois et al. 2002). Another major issue relates to the spread of African swine fever (ASF). ASF had an uncontrolled spread across the Caucasus region and the Russian Federation in the last years, and concerns have increased that ASF will spread to many other European countries through wild boar incursions, with possible devastating economic consequences (De la Torre et al. 2015).

The demographic explosion of wild boar has probably been due to a combination of factors, such as the depopulation of rural areas, changes in agricultural practices, reintroduction, reduced hunting pressure, lack of predators, and climatic changes (Massei and Genov 2004), and was favored by the high ecological plasticity and fecundity of the species. However, one of the most challenging hypotheses is that the present spread of wild boar is at least partially due to the gradual acquisition of some advantageous genetic traits by introgression from the domestic form (such as increased growth rate and fertility; see Goedbloed et al. 2013b). For example, García et al. (2011) argued that introgressive hybridization with pigs is one of the reasons of the increasing invasiveness of wild boar populations in Uruguay.

The hybridization between wild boar and domestic pig has probably occurred since the first domestication events (i.e., in western Eurasia and in the Near East, independently, around the ninth millennium BC; Ottoni et al. 2013). Among others, Frantz et al. (2015) highlighted that a continuous and extensive gene flow likely took place between multiple genetically and geographically distinct wild boar populations and European domestic pigs during and after domestication. The two forms have been sympatric for centuries, until intensive farming, a few centuries ago, progressively reduced their possibility to come into contact (Scandura et al. 2011a).

However, hybridization still occurs today, and genetic introgression from domestic pigs into wild boar populations has been detected by various authors (e.g., Frantz et al. 2013; Goedbloed et al. 2013a and 2013b; Koutsogiannouli et al. 2010; Scandura et al. 2011b) in approximately 4–27 % of the analyzed individuals and has been suggested to possibly have important ecological consequences, by altering traits like reproduction rate and immunology (Goedbloed et al. 2013a). Nevertheless, the homogenizing effect of continuous gene flow from the wild boar into domestic pig was likely counteracted by strong positive selection for behavioral and morphological traits, which maintained the genetic basis for the morphological and behavioral dichotomy observed between wild boars and domestic pigs (Frantz et al. 2015).

Wild boar can crossbreed with domestic pigs both in natural conditions (e.g., in Bulgaria, Croatia, Sardinia and many other areas where open-air pig farming is still practiced; Apollonio et al. 2010; Scandura et al. 2008) and in captivity. The latter case implies an intentional hybridization in farmed stocks, usually aimed at increasing litter size and piglet growth rates (Goulding 2001) and/or at producing "wild boar-like" hybrids to be released for hunting purposes (e.g., in Central Italy; Randi et al. 1989). Restocking with reared individuals, frequently crossed with domestic pigs, was also used as a tool to prevent population decline in many countries; therefore, introgression of domestic pig genes into wild boar might be very common in European populations (see Frantz et al. 2013).
In particular, some authors (e.g., Goedbloed 2013b) emphasized the role of wild boar breeding stations in spreading the domestic genes into wild boar populations. For example, Gongora et al. (2003) analyzed animals in two Finnish farms and found high frequencies of domestic alleles in one of them. Koutsogiannouli et al. (2010) detected a higher percentage of hybrids (16.7 %) in a Greek breeding station than in the wild population (5 %). Frantz et al. (2012) proved the admixed origin of the English wild boar population, which originated from animals that escaped from breeding stations over the past few decades.

Today, the availability of large amounts of genomic data offers the possibility to investigate in more detail and appreciate the complexity of the hybridization process. However, much remains to be understood about many aspects of hybridization between wild boar and

domestic pig, like the exact amount of introgression in European natural and farmed wild boar populations, the directionality of hybridization, and its effects on fertility, physiology and behaviour of the wild form.

Hybridization between wolf and dog

Wolf-like canids (genus *Canis*) evolved recently (in the last 2–4 million years ; Von Holdt et al. 2011), and retained the potential to hybridize in nature, giving rise to new taxa that could quickly adapt to prey community, landscape and climate changes (Randi et al. 2014).
However, hybridization among canids can also have negative effects; for example, hybridizing free-ranging or feral dogs (*C. lupus familiaris*) are threatening the survival of endangered species such as the Ethiopian wolf, *C. simensis* (Gottelli et al. 1994). In particular, the process of introgressive hybridization between wolf (*Canis lupus*) and its domestic counterpart has become a growing concern for conservationists in Europe. Indeed, the spread of domestic genes into wolf populations could disrupt local adaptation (Vilà and Wayne 1999), potentially representing a serious threat to the long term survival of genetically pure wolf populations in the wild (Boitani 2003; Hindrikson et al. 2012).

According to Vilà et al. (1997) the dog originated from the wolf more than 100,000 years ago, and its genetic diversity may have been enriched by multiple founding events, followed by occasional interbreeding with wild wolf populations. Other studies suggest a more recent origin for the domestic dog (nearly 15,000 years ago in East Asia; Savolainen et al. 2002), and agreed that wolf–dog interbreeding has an ancient origin (~10,000 years ago, Schwartz et al. 1997).

Wolf × dog hybridization still occurs today, and has been reported for both North America (Muñoz-Fuentes et al. 2010) and Europe. In Europe, wolf dog hybridization has been reported to occur at relatively low frequency in Italy (Randi and Lucchini 2002; Verardi et al. 2006; Iacolina et al. 2010; Lorenzini et al. 2014), Scandinavia (Vilà et al. 2003), and the Iberian Peninsula (Godinho et al. 2011), while it seemed to be more widespread and frequent in Bulgaria, Latvia and Estonia (Randi et al. 2000; Hindrikson et al. 2012). Here,

wolves may have more opportunities to mate with dogs, due to low wolf density, abundance of free-ranging dogs, and high hunting pressure causing disruption of wolf social structure (Hindrikson et al. 2012).

However, the high genetic similarity between dogs and wolves makes the detection of hybrids extremely difficult. For example, it was remarked that to correctly distinguish backcrosses from purebred parental individuals, it is advisable to analyze at least 48 microsatellite loci (Vähä and Primmer, 2006). As most of the cited studies employed less than 30 markers, it is possible that we are currently underestimating wolf x dog hybridization.

The probability of crossbreeding was reported to be favoured by several factors, including a significant presence of free-ranging dogs, and the occurrence of an expansion phase for the wolf population, with their interactions taking place preferentially at the edge of the wolf's distribution (Lorenzini et al. 2014). Such expansion of the wild species has been occurring in many countries in the last decades, favoured by legal protection of wolf and significant increase of wild ungulates (Randi et al. 2014). Additionally, there is usually high imbalance between population sizes of stray/free ranging dog and wolf—the former largely prevailing—and this may facilitate unidirectional gene flow, adding further threats to the genetic integrity of natural populations.

As suggested by studies on variation of uniparental markers (like mtDNA and Y-chromosome microsatellites) and field observations, the hybridization generally involves female wolves and male dogs (Iacolina et al. 2010), and to date, there is little evidence of hybridization in the opposite direction, i.e. between male wolves and female dogs (but see Hindrikson et al. 2012). Wolves involved in hybridization events were suggested to be females without a breeding position in their natal pack, that disperse to establish a new pack (Mech and Boitani 2003). According to Hindrikson et al (2012), various factors may explain the sexual asymmetry in hybridization: (i) male wolves may be avoided by dogs because they are bigger and generally more aggressive; (ii) female wolves seem to be more active than males in seeking for a dog as a partner; (iii) male dogs are usually capable of mating all year round, therefore they are available to breed with a lone female wolf; (iv) if female dogs involved in

hybridization are not feral but just freely ranging, they usually do not bring up their offspring in the wild, and her hybrid offspring may remain undetected in genetic investigations.

Anomalous phenotypic traits may indicate hybridization, however it is often difficult to distinguish between signals of introgression and intraspecific variation; indeed, there is general agreement that it is impossible to identify wolf–dog hybrids with certainty on the basis of morphological features only (Randi et al. 2014). Deviations from wild type coat patterns and from the "typical" body size and proportions, the presence of dewclaws and depigmented nails are generally regarded as clues of hybridization with the dog (Ciucci et al. 2003). For example, the melanistic mutations at the *β-Defensin* gene has been suggested to have originated in dogs and introduced in wolves through introgressive hybridization (Anderson et al. 2009).

To date, much remains to be known on the effects of wolf x dog hybridization on the individual fitness, though some studies indicate that hybrids can represent good competitors for wolves. Caniglia et al. (2013) described a pack of hybrid origin whose members lived for at least 7 years in the wild, preying on wild ungulates and behaving apparently like wolves. This adds further worries about the consequences of wolf-dog hybridization and its threat to the conservation of wolf populations. According to Boitani et al. (2000) every practical measure should be implemented to remove obvious hybrids from the wild (keeping in captivity and sterilization have been suggested; see Ciucci 2012). Therefore, an effective identification of wolf-dog hybrids is absolutely crucial for conservation and management strategies.

Contents of the thesis

The first half of this dissertation is focused on the hybridization between wild boar (*Sus scrofa*) and domestic pig (*Sus scrofa domestica*). The wild boar is a key species for wildlife management, subjected in the past to local extinctions and translocations, and currently enormously and worryingly expanding in many areas, where it can affect ecosystems and impact local economy. It has the widest geographical range of all ungulates and one of the

widest of all terrestrial mammals, being native to Europe, Asia and North Africa, and introduced as a game species in all other continents, with the exception of Antarctica (Scandura et al. 2011a). Its domestic counterpart is considered one of the most valuable domesticated animals, having a crucial economic importance in many countries and being used as a model species in biomedical research.

Given the huge economic and management implications of hybridization in *Sus scrofa*, it is crucial to understand under what conditions the crossbreeding between the two forms occurs. Wild boar can crossbreed with domestic pigs both in natural conditions and in captivity, where intentional hybridization with the pig is often carried out in order to produce "wild boar-like" hybrids with improved litter size and growth rate. In some cases the latter escape or are illegally released for hunting purposes. In **Chapter I**, we evaluated the role of breeding stations in the spread of domestic genes across wild populations. We assessed the degree of admixture in wild boar sampled both within breeding stations and in the surrounding wild populations in Piedmont and Sardinia, two Italian regions with a different history of wild boar presence and pig husbandry. Animals were genotyped with a panel of 16-18 microsatellite loci. Domestic pigs from local breeding stations were also genotyped and used as reference populations in the Bayesian admixture analyses. We specifically addressed the following question: Have captive stocks higher levels of introgression than the surrounding wild population? This could indicate a key role of breeding stations in spreading domestic genes.

In **Chapter II,** we investigated hybridization patterns in *Sus scrofa* on a broader spatial scale and considering different genetic markers (i.e., functional genes). In fact, we analyzed the variation at the melanocortin receptor I (*MC1R*) and nuclear receptor subfamily 6, group A, member 1 (*NR6A1*) genes in wild boars sampled throughout Europe. These two loci influence coat colour and number of vertebrae in *Sus scrofa*, respectively. Both the *MC1R* and *NR6A1* genes have been under strong artificial selection during domestication, therefore it is possible to identify genotypes private to domestic breeds.
The aims of this work were: (i) to evaluate the MC1R genetic diversity of European populations, and (ii) to investigate the presence, frequency and spatial distribution of *MC1R* and *NR6A1* domestic alleles in European wild boar populations.

If autosomal microsatellites and functional genes have proved to be useful molecular markers to investigate WxDH patterns in *Sus scrofa*, they cannot tell us much about male-specific gene flow and possible sexual asymmetry in hybridization. For this purposes, one should employ uniparental markers like microsatellites located on the Y chromosome. Given the current lack of Y-specific polymorphic markers in *Sus scrofa*, in **Chapter III**, we developed 4 new Y-chromosome polymorphic microsatellites. We identified new male-specific sequence variants in *Sus scrofa* starting from the available genome sequences. Then, we designed PCR primers and performed an initial screening on few individuals, optimizing protocols and discarding markers that showed one or more of the following: non-specific amplification; low amplification success; no variation; not clearly readable electropherograms. Four loci were finally selected, and a total of 13 populations were screened for variation at these loci, including wild boars from all over Europe and commercial and local domestic pig breeds of European and Asian origin.

To correctly identify the real partitioning of genetic variation in natural populations (i.e., the actual population structure) may become more challenging in presence of hybridization, because many exotic alleles may be found (belonging to domestic breeds or allochthonous wild conspecifics), biasing allelic frequencies and therefore individual assignment probabilities to different clusters/subpopulations. This may also lead to a wrong assessment of the effects of environmental factors on dispersal patterns and gene flow in natural populations. In **Chapter IV**, we carried out a landscape-genetics analysis aimed to disclose the effects of habitat and infrastructures on dispersal and gene flow in the Sardinian wild boar. In doing so, we accounted for introgressive hybridization between the local wild boar and domestic pigs or allochthonous wild boars. Indeed, we genotyped 368 wild boars with a panel of 16 microsatellites. We performed an initial Bayesian cluster analysis aimed at removing non-negligible distortions in allele frequencies attributable to introgressive hybridization or to introduction of exotic boars. After the removal of putative introgressed individuals from the dataset, we were able to evaluate the influences of environmental and anthropic factors on the genetic structure in the population, by employing an array of statistical approaches and methodologies.

Introgressive hybridization, in particular between wild and domestic conspecifics, has been shown to have various behavioural and physiological effects in many species. For example, for species in which timing and synchrony of reproduction have, at least partially, a genetic basis, the introgression from the domestic form into wild populations' gene pool may lead to altered reproductive patterns. Additionally, studying reproductive phenology in wild populations can also be useful in identifying crucial periods in which reproduction is concentrated, and hybridization may take place. With this in mind, in **chapter V**, we carried out a preliminary investigation on the effects of individual, environmental and social factors on the timing of reproduction in a natural wild boar population, also discussing the possible evolutionary and genetic implications of the patterns detected. Specifically, we analyzed litters belonging to more than 300 pregnant sows culled in a mountain area of Tuscany, determining the conception date (CD) from an estimate of the mean fetal age and the culling date. We then investigated which factors drove the variation in CD, by implementing linear mixed models, Mantel tests and spatial autocorrelation analyses.

The second part of this dissertation is focused on the hybridization between wolf (*Canis lupus*) and dog (*Canis lupus familiaris*) in the Italian region of Tuscany.

The wolf is the most abundant large predator in Europe. Human attitudes toward this species ranged from fierce competition and extermination to admiration (Boitani 2000). Wolves have been exterminated from most of Europe in the last centuries, probably reaching a minimum number around the middle of the 20th century. Depredation on domestic animals has been the main reason for controlling or exterminating the wolf (Boitani 2000), and is seen as the most serious problem in wolf management.

Despite a past persecution, relatively healthy populations survived in all three Mediterranean peninsulas, and in the last decades the wolf populations in several European countries have been increasing in number and distribution range (Boitani 2000). The Italian wolf population suffered severe persecution until 1971, when wolf hunting was forbidden and poison baits banned. A fully protected status was given to the species in 1976. This, together with an increase in wild prey availability enabled the population to grow and

reoccupy its historical range. During its recent expansion, Italian wolves have experienced a prolonged contact with an abundant and widespread population of stray dogs (Iacolina et al. 2010), leading to increasing risk of hybridization.

We still have no precise understanding of the effects of wolf x dog hybridization on the individual fitness, therefore establish the ecological role of hybrids in the local environment could be crucial for developing correct conservation and management strategies.

Accordingly, the aim of **chapter VI** was to get insights on the trophic behaviour of free-ranging hybrids in a mountainous environment (Tuscan Apennines). Data refer to two areas (The Catenaia Alps, AP and the Poti Alps, AP), stably occupied by wolf packs. Non-invasive sampling of scats and hairs and opportunistic collection of tissues from carcasses were carried out.

We assessed the level of genetic introgression from the domestic dog in the two areas by analyzing 12 autosomal microsatellites, the mitochondrial DNA control region and two Y-chromosome microsatellites, with reference populations of pure Tuscan wolves and domestic dogs.

The two areas strongly differed in the level of genetic introgression from the domestic dog: AP showed high introgression, while AC had very low introgression. In natural environment wolves feed mainly on wild ungulates, while dogs mainly act like scavengers or rely on anthropogenic food resources (livestock and garbage), occasionally used by wolves. What about hybrids? Do they show intermediate food habits?

We investigated the winter food habits in AC and AP by scats analysis, in order to verify whether a hybrid status may induce different food preferences, and, in so doing, to evaluate if hybrids may be potential competitors for wolves.

The big concern arisen from the spread of canine genes in wild wolf populations has led to a big effort to optimize diagnostic genetic tools to detect hybrids (e.g., Randi et al. 2014). However, due to the high genetic similarity between dogs and wolves, genetic methods turned out to have strong limits and the adoption of both genetic and morphological criteria is increasingly suggested. Non-invasive genotyping of wolves, for example, leads to individual identification (also called 'genetic fingerprinting') and sexing, but does not allow to associate any morphological information to the sampled individuals. However, having

data on anomalous phenotypic traits (like non-*wild type* coat, dewclaws and white nails) is crucial, in that these characteristics may be sign of domestic gene introgression. In **Chapter VII**, we demonstrate the potential benefits of a simultaneous use of videotrapping and non-invasive genotyping to provide complementary information on individual wolves in a population. This combined approach can help to test the reliability of individual recognition and can give support to hybrid designation.

Finally, I included in Appendix a work on the genetic composition of brown hares (*Lepus europaeus*) inhabiting the province of Arezzo (Tuscany, Italy), that I concluded at the beginning of my PhD. It deals with the effects of the present and past management regime on genetic diversity and population structure, the identification of allochthonous individuals and the inference of the population history.

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FIRST PART

Hybridization between wild boar and domestic pig

CHAPTER 1

Are captive wild boar more introgressed than free-ranging wild boar? Two case studies in Italy

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ORIGINAL PAPER

Are captive wild boar more introgressed than free-ranging wild boar? Two case studies in Italy

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Abstract Hybridization between wild boar (*Sus scrofa*) and domestic pig occurred in the past and still occurs today, having great evolutionary and management implications. In fact, genetic introgression from the domestic form may alter traits like behavior, reproduction rate, and immunology in wild populations, with likely demographic impacts. Thus, it is crucial to understand under what conditions hybridization occurs in *S. scrofa*. Captive crosses with domestic pigs (released or escaped) have been suggested to constitute the major source of the spread of domestic genes into wild boar populations. However, to date, few studies have assessed the degree of admixture in farmed animals in comparison to the surrounding wild populations. With this purpose, we analyzed microsatellite loci in wild boar sampled in breeding stations and in the local wild population in two Italian regions (Sardinia and Piedmont). Both captive populations had lower allelic richness than the corresponding wild population, but a similar expected heterozygosity. In Piedmont, introgression from the domestic form into the wild population seems to be extremely low, while there are significant signs of admixture in the sampled breeding stations. In Sardinia, instead, the captive sample did not differ significantly from the wild population, which showed moderate signs of introgression. We conclude that hybridization in nature seems to play the key role in Sardinia, while intentional hybridization in captivity is the major source of introgression in Piedmont. Our findings emphasize the need for a routine genetic monitoring of wild boar captive populations, coupled with reference data on the neighboring wild populations.

Keywords *Sus scrofa* · Gene flow · Hybridization · Microsatellites · Bayesian cluster analysis

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Introduction

Heritable traits owned by animals raised in captivity may seriously affect wild populations, in case of hybridization subsequent to accidental escapes or intentional releases of captive-bred individuals. This represents a major conservation and wildlife management issue, since the mixing of diverging gene pools can lead to genetic homogenization and cause outbreeding depression and maladaptation to the local environment (Olden et al. 2004). On the other hand, introgressive hybridization may generate admixed genotypes that can be more adapted than their parental populations; this may induce a local increase of species invasiveness (Largiadèr 2007).

The gene pool of a captive population may differ from that of surrounding free-ranging populations for a number of reasons: (i) it arose from the local wild population but diverged because of founder effect and genetic drift, (ii) the captive population was founded by individuals transferred from a distant area of the species' range, and (iii) the captive stock was affected by genetic introgression of non-native genes. In the latter case, the source of introgression can be represented either by another population of the same species or by a related wild/domestic taxon. A strong genetic divergence between wild and captive populations can be the effect of breeding practices aimed at modifying some species' traits (like tameness, reproductive performances, meat quality). In some cases, such changes are obtained through crossbreeding with the domestic counterpart. Domestic genes introgressed in this way into captive animals can thus be transferred to the

wild population, leading to the aforementioned risks and unpredictable consequences on fitness. For example, it has been shown that hybrids between wild and farmed Atlantic salmons (*Salmo salar*) have lower anti-predator responses (Houde et al. 2010) and that introgressive hybridization between the common quail (*Coturnix coturnix coturnix*) and the domesticated Japanese quail (*Coturnix coturnix japonica*) affects the migratory behavior of the former (Largiadèr 2007). On the other hand, variants that appear in the domestic line may prove to be advantageous and selected for in the wild. It is the case of the melanistic mutation in North American wolves, which derives from past hybridization with domestic dogs, and has been proved to be positively selected in dense forests (Anderson et al. 2009).

Gene flow between domesticated and wild conspecifics—or closely related species—seems to have greatly increased in the last decades (Randi 2008), because of the positive trend of several wild species (e.g., wolves and ungulates), the widespread occurrence of free-ranging domestic animals (dogs, cats, pigs, goats), and massive releases of captive-reared game stocks (e.g., galliforms, waterfowl, salmonids). Among European mammals, for example, wild×domestic hybridization (WxDH) occurs between European wildcat (*Felis silvestris silvestris*) and domestic cat (*Felis silvestris catus*), domestic ferret (*Mustela furo*) and European polecat (*Mustela putorius*), wolf (*Canis lupus*) and domestic dog (*Canis familiaris*), Alpine ibex (*Capra ibex*) and domestic goat (*Capra aegagrus*), and wild boar (*Sus scrofa*) and domestic pig (Hindrikson et al. 2012; Giacometti et al. 2004; Largiadèr 2007).

The latter case deserves special attention for its economic and management implications. In fact, wild boar populations have been growing considerably over the past decades in most of Europe (Saez-Royuela and Telleria 1986; Bieber and Ruf 2005) to the point that at present, this species reaches nearly four million individuals in the continent and is currently considered a pest in many areas (Apollonio et al. 2010). Indeed, the wild boar can cause extensive damage to crops, woodland, and grassland vegetation, as well as vehicle collisions and other issues in urban habitats (Geisser and Reyer 2004; Massei et al. 2011). Moreover, wild boar are known carriers of several parasites and diseases that, in some cases, pose a threat to livestock, wildlife, and human health (Gortàzar et al. 2007). The demographic explosion of wild boar has probably been due to a combination of factors, such as the depopulation of rural areas, changes in agricultural practices, reintroduction, reduced hunting pressure, lack of predators, and climatic changes (Massei and Genov 2004), and was favored by the high ecological plasticity and fecundity of the species.

However, one of the most challenging hypotheses is that the present spread of wild boar is at least partially due to the gradual acquisition of some advantageous genetic traits by introgression from the domestic form. Garcia et al. (2011) argued that the invasive potential of the wild boar populations in Uruguay has emerged from introgressive hybridization with pigs (which have been listed among the 100 world's worst invasive species; Lowe et al. 2000).

The hybridization between wild boar and domestic pig (DP) has probably occurred since the first domestication events (i.e., in western Eurasia and in the Near East, independently, around the ninth millennium BC; Ottoni et al. 2013). The two forms have been sympatric for centuries, until intensive farming, a few centuries ago, progressively reduced their possibility to come into contact (Scandura et al. 2011a). However, hybridization still occurs today, and genetic introgression from domestic pigs into wild boar populations has been detected by various authors (e.g., Frantz et al. 2013; Goedbloed et al. 2013; Koutsogiannouli et al. 2010; Scandura et al. 2011b) in approximately 5–27 % of the analyzed individuals and has been suggested to possibly have important ecological consequences, by altering traits like reproduction rate and immunology (Goedbloed et al. 2013).

For all these reasons, it is crucial to fully understand under what conditions WxDH occurs in *Sus scrofa*. Wild boar can crossbreed with domestic pigs both in natural conditions (e.g., in Bulgaria, Croatia, and Sardinia, where open-air pig farming is still practiced; Apollonio et al. 2010; Genov et al. 1991; Scandura et al. 2008) and in captivity. The latter case implies an intentional hybridization in farmed stocks, usually aimed at increasing litter size and piglet growth rates (Goulding 2001) and/or at producing "wild boar-like" hybrids to be released for hunting purposes (e.g., in Central Italy; Apollonio et al. 1988; Randi et al. 1989). Restocking with reared individuals, frequently crossed with domestic pigs, was also used as a tool to prevent population decline in many countries; therefore, introgression of domestic pig genes into wild boar might be very common in European populations (see Frantz et al. 2013). Some authors hypothesized that hybridization in nature could have a minor role in the spread of "domestic genes" into wild populations (Scandura et al. 2011a). Instead, they emphasized the possible role of wild boar breeding stations, where high levels of introgression were occasionally observed. For example, Gongora et al. (2003) analyzed animals in two Finnish farms and found high frequencies of domestic alleles in one of them. Koutsogiannouli et al. (2010) studied the MC1R variation in wild boar in Greece, detecting a higher percentage of hybrids (16.7 %) in a breeding station than in the wild population (5 %). Frantz et al. (2012) proved the mixed wild boar/domestic pig ancestry of the English wild boar population, which originated from animals that escaped from farms in recent decades. Nonetheless, to date, no study has been carried out to directly compare natural and captive wild boar populations in a given area using neutral markers.

In the present study, we compared the degree of admixture and genetic diversity of wild boar sampled within breeding

stations and in the wild in Piedmont and Sardinia, two Italian regions with a different history of wild boar presence and pig husbandry.

Materials and methods

Two different groups of individuals were considered and analyzed separately. The first dataset (SAR) included 353 wild boar samples from all over Sardinia, shot by local hunters during the period 2001–2011; 28 wild boar sampled in three Sardinian breeding stations (SBS1, n=5; SBS2, n=13; SBS3, n=10); and 128 domestic pigs, including free-ranging individuals from Sardinia and commercial breeds. The second dataset (PIE) consisted of 631 wild boar sampled in Piedmont between 2006 and 2010 (among which, 94 wild boar were killed in road accidents), 9 captive wild boar randomly sampled in three farms (PBS1, n=3; PBS2, n=1; PBS3, n=5), and 94 pigs from local breeding stations. Hairs or tissue samples (ear tips or muscle) were collected.

In both study areas, an increase in wild boar numbers was recorded in the last decades, causing significant problems. In Sardinia, the incidence of wild boar/vehicle collisions is growing, with 2,346 accidents between 2001 and 2012, 53.4 % of which occurred in the last 4 years. In Piedmont, wild boar were responsible for 50 % of the 2,184 car accidents caused by wildlife between 2003 and 2005 and for 72 % of the 41,622 crop damages reported from 2000 to 2005.

Genomic DNA was isolated both from hair follicles using the InstaGene Matrix protocol (Bio-Rad, Hercules, CA, USA) and from 25 mg of ethanol-embedded tissue using the GenElute Mammalian DNA miniprep Kit (Sigma-Aldrich, St Louis, MO, USA) or the NucleoSpin Tissue™ Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocols. In both cases, DNA was eluted in a final volume of 200 μl.

Eighteen microsatellite loci (S002, S005, S068, S097, S101, S155, SW24, SW122, SW1492, SW2021, SW240, SW2406, SW2496, SW2532, SW461, SW72, SW857, SW936; details at www.thearkdb.org) were selected for studying the Piedmont populations (i.e., domestic pigs, wild boar, and animals sampled in breeding stations). All samples in the SAR dataset were genotyped at the University of Sassari with a panel of 16 microsatellites (IGF1, S026, S090, S155, S215, S355, SW122, SW1492, SW2021, SW24, SW2496, SW2532, SW461, SW72, SW857, SW951; details at www.thearkdb.org), already tested on European wild boar populations (e.g., Vernesi et al. 2003; Iacolina et al. 2009; Scandura et al. 2008, 2011b).

Microsatellite genotyping of PIE samples was performed in three multiplexed polymerase chain reactions (PCRs) at the laboratory of the Chamber of Commerce in Torino. Multiplex 1 contained loci SW24, SW122, SW240, SW857, and S068; multiplex 2 contained loci S002, S005, S155, and SW2406; and multiplex 3 contained the remaining loci. Samples were amplified in a 7.5-μl reaction mixture containing 0.05 mM dNTPs, 120–450 nM of each primer, 1× PCR reaction buffer, 3.4 mM $MgCl_2$, 0.1 μg BSA, 0.75 U Platinum® Taq DNA Polymerase (Invitrogen, Life Technologies), and approximately 50 ng of template DNA. Amplification conditions consisted of an initial denaturation step for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s, and then a final extension at 72 °C for 60 min. All the successfully amplified products were analyzed by capillary electrophoresis on an automated sequencer ABI PRISM (Applied Biosystems). Alleles were scored using GeneMapper 3.7 (Applied Biosystems).

For the SAR dataset, PCR analysis of microsatellites was performed applying the reagent concentrations and conditions described in Scandura et al. (2008).

The occurrence of null alleles and scoring errors in the dataset was checked using the software MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). Allele frequencies and standard genetic diversity indices, including observed heterozygosity (H_O), unbiased expected heterozygosity (H_E), and the mean number of alleles per locus (A), were calculated for each group with GENETIX 4.05 (Belkhir et al. 2004). Estimates of allelic richness (AR) and private allelic richness (PAR), adjusted for the lowest sample size (18 genes for PIE and 46 for the SAR dataset), were obtained by the rarefaction statistical approach implemented using HP-RARE (Kalinowski 2005). Weir and Cockerham's (1984) estimators of Wright's F-statistics (f and θ, hereafter F_{IS} and F_{ST}) were computed in GENETIX, and their significance was tested by 1,000 permutations. A factorial correspondence analysis (FCA) was performed to visualize distances among genotypes. Deviations from the Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium were tested by the Markov chain method implemented in GENEPOP 4 (Rousset 2008) setting for both tests 10,000 dememorizations, 100 batches, and 10,000 iterations per batch. Significance levels were adjusted according to the sequential Bonferroni correction for multiple comparisons (Rice 1989).

Additionally, for the two datasets separately, the Bayesian clustering algorithm implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to infer individual genetic ancestry, using the following settings in the prior: admixture model, correlated allele frequencies among populations, and no population information (all other settings as default). Ten independent runs were carried out for a number of genetic clusters varying from K=1 to K=10, with 500,000 iterations following a burn-in period of 500,000 iterations. The most likely value of K was determined according to the method developed by Evanno et al. (2005), and 20 additional runs were performed using this value.

Individual Q values were obtained by averaging the three runs with highest posterior probability and used to compare, for each dataset, the degree of admixture of animals from the breeding stations with that of the local wild population. In order to account for differences in sample size, 1,000 subsets of individuals, randomly chosen from the wild population and having the same size of the corresponding captive sample (N=28 for SAR and N=9 for PIE), were created using the statistical software package R (R Development Core Team 2011). To categorize individuals as "hybrids", two different and relatively stringent thresholds were considered: Q_{WB} (membership to the wild boar cluster) between 0.10 and 0.90, and Q_{WB} between 0.05 and 0.95; otherwise, they were classified as pure wild boar/domestic pigs (this simple approach was possible since the optimal number of clusters turned out to be 2). The mean Q value and the number of hybrids in the 1,000 subsets were calculated using both thresholds. The percentage of cases in which the subset had a number of hybrids equal to or greater than the captive sample was calculated. Similarly, the percentage of cases in which the subset had a mean Q_{WB} equal to or lower than the captive sample was obtained.

Results

MICRO-CHECKER analysis on both wild populations did not detect allele scoring errors caused by large allele dropout. Average estimated frequency of null alleles per locus was 8.7 % (SAR) and 4.7 % (PIE), with a maximum frequency of 14.8 % at locus S355 (SAR) and 7.6 % at locus S068 (PIE). According to simulation studies (e.g., Carlsson 2008), at these frequencies, the bias in the STRUCTURE assignment test caused by null alleles is negligible. Thus, we decided to retain all loci in the analysis. On the contrary, the levels of genetic differentiation (i.e., F_{ST}) and the percentage of loci that shows deviations from HWE expectations can substantially increase in the presence of null alleles (Carlsson 2008; Chapuis and Estoup 2007). Indeed, in both wild populations, no locus was in HWE (with α=5 %), while significant genotypic linkage disequilibrium was found in 23 out of 120 (SAR-wild population (WP)) and in 43 out of 153 (PIE-WP) pairs of loci (after the correction for multiple tests). This lack of equilibrium could also be explained by the presence of population structure in both study areas (already reported for Sardinia in Scandura et al. (2011b)), as suggested by the deficit of heterozygotes and the high value of F_{IS} (Table 1). In farms, F_{IS} turned out to be comparable (in PIE) or lower (in SAR) than in WP.

Genetic diversity indices are shown in Table 1. Expected heterozygosity is similar between captive (CP) and wild populations (WP), in both Sardinia and Piedmont (H_E: SAR-WP 0.604, SAR-CP 0.587, one-tailed paired t test, p=0.197; PIE-WP 0.753, PIE-CP 0.728, one-tailed paired t test, p=0.191). In the presence of high genetic introgression from domestic pigs, captive populations are expected to show high genetic variability. Instead, they had a low allelic richness (AR: SAR-WP 5.84, SAR-CP 4.94, one-tailed paired t test, p=0.001; PIE-WP 5.76, PIE-CP 4.78, one-tailed paired t test, $p<0.001$). There was significant differentiation among all populations in both datasets, and as expected, the lowest genetic distance was found between wild and captive boar populations (Piedmont: F_{ST} DP-WP=0.162, F_{ST} DP-CP=0.149, F_{ST} WP-CP=0.066; Sardinia: F_{ST} DP-WP=0.081, F_{ST} DP-CP=0.100, F_{ST} WP-CP=0.021, all significant at $p<0.001$). Also, F_{ST} values highlight the lower genetic distance between the domestic and wild populations in Sardinia with respect to that observed in Piedmont, as can be also noticed in the FCA in Fig. 1. In the FCA plot, all the individuals from breeding stations (both in SAR and PIE) fell in the WP cluster.

In both datasets, the Bayesian analysis performed in STRUCTURE identified two well-defined clusters, corresponding to wild boar and domestic pigs (K=2 was the most likely

Table 1 Genetic diversity indices and deviation from Hardy-Weinberg expectations at 16–18 microsatellite loci in domestic pigs (DP) and wild boar (captive (CP) and wild populations (WP)) in Piedmont (PIE) and Sardinia (SAR)

		n	H_E	H_O	A	AR	PAR	F_{IS}	HWE
PIE	WP	631	0.753±0.133	0.683±0.127	11.556	5.763	1.266	0.093**	<0.001
	DP	94	0.693±0.124	0.564±0.104	9.111	5.114	1.879	0.186**	<0.001
	CP	9	0.728±0.103	0.661±0.224	4.778	4.778	0.711	0.098*	0.1601
SAR	WP	353	0.604±0.215	0.498±0.192	9.000	5.840	0.546	0.177**	<0.001
	DP	128	0.702±0.189	0.636±0.183	9.938	7.439	2.690	0.093**	<0.001
	CP	28	0.587±0.184	0.555±0.213	5.063	4.937	0.409	0.055*	<0.001

H_E expected heterozygosity, H_O observed heterozygosity, A mean number of alleles per locus, F_{IS} inbreeding coefficient, *AR* allelic richness, *PAR* private allelic richness (rarefaction, PIE=18 genes, SAR=46 genes), *HWE p* value of the test for deviations from the Hardy-Weinberg equilibrium

**$p<0.01$ (highly significant)

*$p<0.05$ (significant)

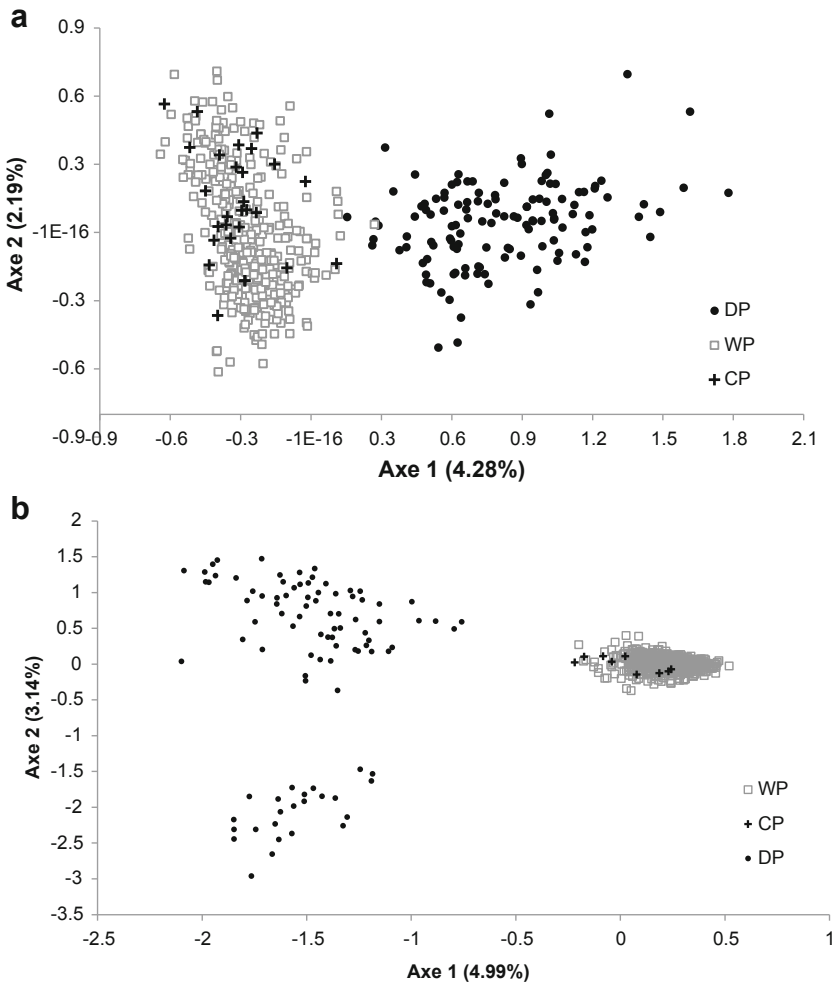

Fig. 1 Factorial correspondence analysis (*FCA*) of microsatellite genotypes of domestic pigs (*DP*) and wild boar captive (*CP*) and natural populations (*WP*) in Sardinia (**a**) and Piedmont (**b**)

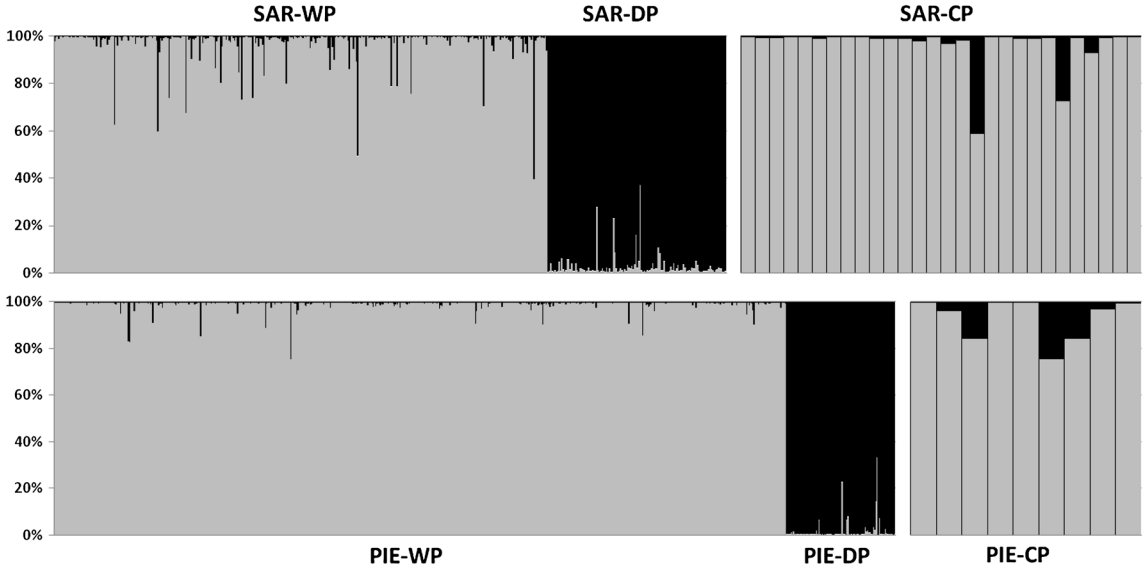

Fig. 2 Estimated proportions of membership to the two clusters inferred by STRUCTURE analysis performed on domestic pig (*DP*), natural wild boar (*WP*), and captive populations (*CP*) in Piedmont (*PIE*) and Sardinia (*SAR*). Each individual is represented by a *vertical bar*

scenario following Evanno et al. (2005); Fig. 2). The admixture between wild and domestic forms turned out to be low (especially in Piedmont, in agreement with the results of the FCA), with high average rates of self-assignment for both WP and DP (mean Q_{WB}: PIE-WP 0.993; SAR-WP 0.973. See Table 2). In the PIE dataset, considering a threshold of 95 % to define a "pure" individual, only 13 wild boar out of 631 (2.1 %) were hybrids (and only 6 with a 90 % threshold). In the Sardinian WP, 33 hybrids (9.3 %) were detected among the 353 sampled wild boar when the threshold was set to 95 % (22 with a 90 % threshold). As concerns the captive populations, three hybrids out of nine individuals (33.3 %) were detected in Piedmont, considering both thresholds, and the mean Q_{WB} was 0.928, being different from the WP. In fact, in none of the 1,000 subsets of nine individuals randomly selected within the PIE-WP, the mean Q_{WB} resulted lower than or equal to that in the PIE-CP (lowest mean-Q_{WB}=0.955). Furthermore, no more than two hybrid individuals per subset were detected in these 1,000 groups, considering both thresholds (Fig. 3).

In contrast, in the SAR dataset, the CP had a higher mean Q_{WB} (0.966), in line with the values observed for the WP (0.973). Indeed, 22.2 % of the 1,000 subsets of 28 individuals randomly selected within the SAR-WP had Q_{WB} lower than or equal to that in the SAR-CP. Only three hybrids (10.7 %) were detected out of 28 Sardinian captive boar considering a 95 % threshold, and two with a 90 % threshold. This result also fits with the rate observed in the wild population: 50.4 % of the 1,000 SAR subsets had a number of hybrids equal to or higher than the SAR-CP, considering a 95 % threshold (53.4 %, with the 90 % threshold).

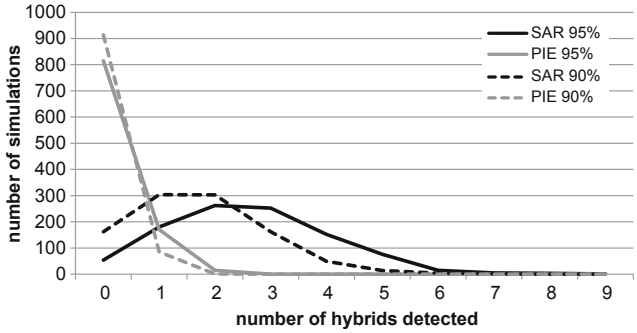

Fig. 3 Number of hybrids (*x*-axis) detected in the 1,000 subsets of individuals randomly selected from each wild population and having the same size of the corresponding captive sample (N=28 for Sardinia (*SAR*) in *black*, and N=9 for Piedmont (*PIE*) in *gray*). Individuals were classified as "hybrids" referring to two different thresholds, namely 95 % (*solid line*) and 90 % (*dashed line*)

Discussion

In order to evaluate the possible role of farmed wild boar as a source of introgression of domestic genes into wild populations, it is crucial to establish whether and to what extent farmed wild boar genetically differ from wild populations.

As concerns genetic variability in the breeding stations sampled in our study, while in Piedmont and Sardinia, expected heterozygosity did not differ between CP and WP, both captive populations had a significantly lower allelic richness than the corresponding wild populations (Table 1). This could be accounted for by a small number of founders and genetic drift in the farms. Recent hybridization with domestic pigs would have increased heterozygosity and allelic richness in the CPs.

Two markedly different situations were highlighted by the results of Bayesian analysis: (1) in Piedmont, the signs of introgression from the pig into the wild population seem to be extremely weak, while the extent of admixture in the sampled breeding stations is not negligible (PIE-CP, mean Q_{WB}=0.928), with three hybrids detected out of nine individuals. Among the 1,000 subsets of nine individuals randomly selected from the PIE-WP, none had such low Q_{WB} and in none more than two hybrids were detected. Thus, admixture in PIE-CP is significantly higher than that in PIE-WP (p<0.001). (2) On the other hand, the Sardinian wild population turned out to be relatively more introgressed (SAR-WP, mean Q_{WB}=0.973), as one might have supposed observing the FCA graph (Fig. 1), but the sampled farms resulted clearly in line with these values (Q_{WB}=0.966, three hybrids detected out of 28 animals). In fact, 222 out of the 1,000 subsets of 28 individuals created randomly from the SAR-WP had a Q_{WB} lower than or equal to that of the SAR-CP, and at a 95 % threshold, 504 subsets had three or more hybrids. Thus, we cannot exclude that the SAR-CP have the same degree of admixture of the SAR-WP.

Therefore, our data suggest that the main source of introgression from domestic pig in the Sardinian wild boar population is not represented by intentional or unintentional releases

Table 2 Membership to the wild boar cluster (Q_{WB})±standard deviation and proportion of hybrids detected in domestic pig (DP), natural wild boar (WP), and captive populations (CP) in Piedmont (PIE) and Sardinia (SAR). Two different thresholds (95 % and 90 %) were used to categorize individuals as "hybrids"

		n	Mean Q_{WB}	Proportion of hybrids (95 %)	Proportion of hybrids (90 %)
PIE	DP	94	0.015±0.045	0.074	0.032
	WP	631	0.993±0.019	0.021	0.010
	CP	9	0.928±0.091	0.333	0.333
SAR	DP	128	0.024±0.048	0.078	0.039
	WP	353	0.973±0.067	0.093	0.062
	CP	28	0.966±0.090	0.107	0.071

of wild boar reared in captivity. Hybridization in nature seems to play the key role; in fact, in several areas of Sardinia, an illegal husbandry system occurs, with pigs being allowed to wander freely all year round, in the absence of fences, human control, and a regular food supplementation. Anyway, it must be taken into account that the actual frequency of the domestic×wild *Sus scrofa* hybridization in Sardinia may have been slightly underestimated. Indeed, our data could be biased by a possible higher mortality of hybrids both in the wild (selection against hybrids) and near villages: especially in the presence of directional hybridization favoring male wild boar×domestic sow crosses, several hybrids could be raised close to villages, receiving supplementary food by humans and then being killed as piglets and used for the traditional cookery.

On the contrary, even if the low number of captive individuals analyzed suggests to be cautious, our results seem to indicate that, in Piedmont, the risk of introgression of domestic genes into the wild population is mainly associated to releases (intentional or unintentional) of animals deliberately hybridized in captivity. Nonetheless, a direct and thorough comparison between the two situations (i.e., Piedmont and Sardinia) would have required the employment of the same set of microsatellites, in order to prevent any possible difference in diagnostic power.

At the European level, on the basis of geographical features and population histories, it is presumable that the most common situation is similar to that found in Piedmont, with farms being the main source of introgression of domestic genes into wild populations.

In fact, even if the "pannage" (i.e., the seasonal practice of releasing pigs in the forest, so that they could feed on fallen acorns and beechnuts) was a common form of pig husbandry in Europe until the Modern Era, providing plenty of opportunities of crossbreeding with wild boar (White 2011), at present, open-air pig farming is commonly practiced only in a few regions of Southern Europe, such as Bulgaria, Croatia, Iberia, Corsica, and Sardinia (Apollonio et al. 2010; Boireau and Vallée 2004; Genov et al. 1991; Rodriguez-Estevez et al. 2012).

Instead, breeding stations are widespread throughout the continent. Local administrations often authorized their establishment without being able to control the source and health conditions of the captive-bred wild boar, which frequently escaped from captivity and/or were illegally released (Carnevali et al. 2009). These animals could affect population structure and species distribution. For example, according to Monaco et al. (2007), the scattered presence of the wild boar in many areas of the Italian Alps is mainly attributable to illegal releases. Also, uncontrolled releases occurred in France, Greece, Belgium, and Luxembourg (Apollonio et al. 2010; Frantz et al. 2013). Furthermore, the current British, Swedish, and Dutch populations originated (completely or mostly) from animals that escaped from farms, the former having been proved to have a mixed wild boar/domestic pig ancestry (Apollonio et al. 2010; Frantz et al. 2012). Occasional escapes were reported also for Denmark and Slovenia (Apollonio et al. 2010). Finally, the Irish "wild boar" population whose origin is attributable to illegal releases and/or farm escapees has been shown to be composed of pigs and hybrids (Mc Devitt et al. 2013). These issues are thus extremely common, and in the case of high introgression from the domestic form, captive-bred animals could spread "domestic genes" into local populations, representing a threat to native gene pools and likely affecting the viability of these populations or increasing their invasiveness. Since, to date, the effects of introgression from the domestic pig on the fitness of wild individuals are not completely clear, we strongly recommend a strict genetic monitoring of wild boar from breeding stations, even though their release into nature is not planned or permitted. We also emphasize that a careful analysis implies the use of genetic reference data on the wild populations present in the same areas. These considerations can be extended to other species in which captive stocks can come into contact with wild conspecifics, both by intentional releases (e.g., for restocking) or accidental escapes from enclosures. In other ungulates, the risk of genetic pollution from captive populations is often linked to the presence of imported animals belonging to the same or to related species. For instance, the spread of sika deer (*Cervus nippon*) genes into native red deer (*Cervus elaphus*) populations is raising major concerns in Europe (Zachos and Hartl 2011). Nevertheless, as recently remarked by Stronen and Paquet (2013), it should be kept in mind that hybridization may also play a positive role in permitting adaptation to and increased chances for survival in human-dominated landscapes.

In the common practice, for technical reasons (e.g., the need to catch and sedate the animals), it is extremely difficult to obtain samples from all of the individuals in a farm. Consequently, when genotyping captive animals, researchers often have to deal with small numbers. In such cases, the approach used in the present study would be recommendable, as it allows comparing the degree of admixture in populations with very different sizes, taking into account such difference, and determining the empirical probability that captive populations were more introgressed than wild ones.

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CHAPTER 2

Lack of polymorphism at *MC1R wild-type* allele and evidence of domestic alleles introgression across European wild boar populations

Lack of polymorphism at *MC1R wild-type* allele and evidence of domestic alleles introgression across European wild boar populations

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Keywords: *Sus scrofa*; wild boar; domestic pig; *MC1R;* coat colour; *NR6A1*; QTL; genetic introgression

Summary

Domestication promotes the emergence of novel phenotypic and behavioural traits in domesticated animals compared to their wild ancestors. We analysed variation at the melanocortin receptor I (*MC1R*) and nuclear receptor subfamily 6, group A, member 1 (*NR6A1*) genes in European wild boar populations, two loci which have been under strong artificial selection during domestication. These loci influence coat colour and number of vertebrae, respectively.

A total of 145 wild boars were sampled throughout Europe, to evaluate frequency and spatial distribution of domestic alleles and patterns of hybridization between wild and domestic forms. Most of the wild boars (94%) were homozygous for the European *wild-type* (E^+) *MC1R* allele. We did not observe any synonymous substitution in the European E^+ allele, confirming its monomorphism even in areas known to be hotspots of wild boar genetic diversity. The remaining wild boars (6%) showed genetic introgression of three different European domestic alleles. No Asian *MC1R* allele was found in our sample.

Furthermore, domestic *NR6A1* alleles were observed in 6% of wild boars. Considering the two loci analyzed, 11% of boars, sampled all over Europe, showed signs of recent or past introgression in their genome.

This data agrees with previous investigations on other molecular markers, confirming that, compared to Asian conspecifics, European wild boars have a relatively low genetic diversity, which is locally increased by the introgression of allelic variants from the domestic counterpart.

Main text

The variation in pigmentation in mammals depends on the spatial distribution of pigmentation across the body and along hairs, and the balance between the black eumelanin and the yellowish pheomelanin. This balance is controlled primarily by two loci, *Extension* and *Agouti*. The *Extension* locus encodes the melanocortin receptor I gene (*MC1R*), a G protein-coupled receptor expressed in melanocytes (Fang *et al.* 2009).

A typical *wild-type* coat colour in mammals is composed of a mixture of eumelanin and pheomelanin. Mutations of the *MC1R* gene leading to a constitutively active receptor are dominant and induce the production of eumelanin only, causing dark/black coat colour. Nonsense and frameshift mutations, that prevent the formation of functional receptors, are recessive and cause the production of pheomelanin only, leading to yellow/reddish coat colour (Suzuki 2013).

In *Sus scrofa*, a striking difference in coat colour patterns exists between the wild and the domestic form, due to purifying selection for camouflage coat colour in natural environment, and strong human selection in domestic lineages (Fang *et al.* 2009, Li *et al.* 2010). Studies on the genetic basis of coat colour variation in pigs had established an allelic series including four alleles at the *Extension* locus: Dominant black (E^D), *wild-type* (E^+), black spotting (E^P) and recessive red (*e*) (Legault 1998). Subsequent sequence analyses on the porcine *MC1R* gene revealed several allelic variants of these four phenotypically-defined alleles (Andersson 2003, Fang *et al.* 2009).

Asian wild boar populations show high diversity, having at least 14 different *wild-type MC1R* allelic variants, in which only synonymous substitutions occur (Li *et al*. 2010); instead, no polymorphism for the *wild-type MC1R* allele has been detected so far in European wild boars (Fang *et al.* 2009). Nevertheless, this lack of polymorphism can be due to the small number and narrow geographic range of the European samples sequenced so far (Kijas *et al.* 1998; 2001; Giuffra *et al*. 2000; Fang *et al.* 2009), excluding populations living in Southern peninsulas, which are known to be hotspots of wild boar genetic diversity (Vilaça *et al.* 2014).

Since the *wild-type* allele is private to wild populations (with the exception of the Hungarian Mangalica domestic breed), DNA polymorphisms at the *MC1R* locus have been successfully used for the traceability of meat products (Fontanesi *et al.* 2014) and to detect introgression of domestic pig genes into wild boar populations (e.g. Koutsogiannouli *et al.* 2010; Frantz *et al.* 2013). Domestic *MC1R* alleles have been found in 5% of 119 free-ranging and 16% of 12 reared wild boars in Greece (Koutsogiannouli *et al.* 2010), and in one out of 153 wild boars from Western Europe (Frantz *et al.* 2013).

Another trait that differentiates wild boars and European commercial pigs is the number of vertebrae: 19 in the wild boar and 21-23 in ameliorated breeds. A proline to leucine substitution at codon 192 (p.Pro192Leu) in the nuclear receptor subfamily 6, group A, member 1 (*NR6A1*) gene was shown to be the most likely causative mutation underlying the QTL effect on the number of vertebrae in the pig (Mikawa *et al.* 2007). The mutant allele is fixed in most European commercial pig breeds, while wild boar populations carry the *wild-type* allele only.

We analysed the variation at the *MC1R* and *NR6A1* genes in wild boars sampled throughout Europe as well as in a sample of local Italian pigs, with the following aims: i) evaluate the *MC1R* genetic diversity of European populations at a wider scale compared to previous studies, in order to investigate the presence of polymorphism at the European *wild-type MC1R* allelic variant; ii) investigate the presence, frequency and spatial distribution of *MC1R* and *NR6A1* domestic alleles in European wild boar populations.

We obtained tissue samples (provided by local hunters) and DNA samples, for a total of 145 European wild boars from 12 different localities (Belarus, *N* = 10; Bulgaria, *N* = 5; Croatia, *N* = 6; France, *N* = 15; Greece, *N* = 10; mainland Italy, *N* = 33; Luxembourg, *N* = 8; Poland, *N* = 7; Portugal, *N* = 11, Romania, *N* = 8; Sardinia, *N* = 21; Spain, *N* = 11). Additionally, we obtained DNA samples from 20 Sardinian and four Italian "Cinta Senese" pigs raised in free or semi-free conditions, possibly affected by introgression of *wild-type* alleles, given their possibility to cross-breed with wild boars.

We amplified the *MC1R* gene as a single 1.2 kb fragment (the entire 963 bp coding region plus 6 bp of the 5'-untranslated region, and 208 bp of the 3'-untranslated region) employing the PCR2 (Fang *et al.* 2009) as reverse and a new forward primer (PCR4: 5'-GGGAGCCATGAGCTGAGCAGG-3'). We used the QIAGEN Fast Cycling PCR kit (Valencia, CA, USA), with 66°C annealing. Both forward and reverse strands were sequenced. Due to the presence of frameshift mutations, putative heterozygotes were cloned and sequenced to confirm the alleles.

The results of sequence analysis revealed that 94% of wild boars were homozygous for the already known European variant of the *wild-type* allele (Tab.1), which had an overall frequency of 96%. We did not find any synonymous substitution at this allele in our sample, supporting the hypothesis that European wild boars are monomorphic at this locus. The first studies on *MC1R* variation in *Sus scrofa* (Kijas *et al.* 1998) highlighted a substantial genetic distance between Asian and European pigs, providing one of the first indications of independent domestication events in the two continents (Andersson & Plastow 2011). Asian domestic pigs likely originated from a more diverse wild stock, whereas European domestic pigs originated from European wild boars, which faced a more pronounced population bottleneck prior to domestication (Groenen *et al.* 2012). This can partially explain the relatively low diversity of both European wild boars and domestic pigs in comparison to Asian conspecifics (Megens *et al.* 2008; Groenen *et al.* 2012), and the absence of variation at the *MC1R* European *wild-type* allele.

Neither Asian wild-type nor Asian domestic *MC1R* alleles were detected among 145 individuals, even if introgression of European pigs/wild boars with Chinese pigs has been widely documented (Megens *et al.* 2008; Groenen *et al.* 2012; Goedbloed *et al*. 2013).

Three already known domestic alleles of European origin were detected among wild boars: dominant black (E^D) and two variant of black spotting (E^P and E^{P2}), with overall frequency of 0.7-3.1%. Regions like Bulgaria and Sardinia, where pigs are often reared in semi-free conditions and may cross-breed with the wild form (Scandura et al. 2008), showed frequencies of domestic alleles up to 10-20%. Interestingly, four of the nine introgressed boars carried two domestic alleles, indicating that introgression may reach high levels at a

very local scale, and/or that intentional hybridization in captivity may be an important source of introgression (see Canu *et al.* 2014).
Additionally, the Sardinian domestic stock was affected by substantial genetic introgression from wild boars, showing a frequency of the *MC1R wild-type* allele of 7.5%, to our knowledge never reported for domestic pigs (with the exception of the Mangalica breed). However, this result is in line with the previous evidence of cross-breeding between wild and domestic *Sus scrofa* in Sardinia.

The *NR6A1* gene polymorphism was investigated by PCR-RFLP with the method described by Fontanesi *et al.* (2014). The occurrence of either mutant alleles in wild boars or *wild-type* alleles in domestic pigs was further verified through sequencing. The *wild-type* allele was fixed in most wild populations, and, like the *MC1R* allele E^+, had an overall frequency of 96% across European wild boar populations. Italian, Spanish, Greek, Bulgarian and French wild boars showed signs of introgression (Tab.1). Conversely, the *wild type NR6A1* allele was detected in 12.5% of domestic pigs (allele frequency = 6.3%), suggesting that present or past gene flow between wild and domestic forms was not negligible and bidirectional, at least in Sardinia and continental Italy.

In the present work, we found no sequence variation at the *MC1R* E^+ allele across an array of European wild populations, which included those inhabiting Southern peninsulas. Considering both the high number of *MC1R* E^+ allelic variants detected in Asian wild boars and the amount of non-synonymous mutations occurring in domestic breeds (arisen after domestication), the complete lack of synonymous substitutions at the *wild-type* allele in Europe is surprising and not fully explained by a pre-domestication population bottleneck. Further advances in the knowledge of the pig genome might provide an explanation.
It is noteworthy that the frequency of the *wild-type* allele is kept high in wild populations across Europe, even in areas which experienced a decline of natural predators, suggesting that selection against non-camouflage coat can be maintained by hunting.

We also detected relatively high genetic introgression from domestic pigs into some European wild boar populations, in agreement with previous studies (e.g., Goedbloed *et al.* 2013). With few exceptions (in Greece and Bulgaria, see Tab.1) signs of introgression at the

NR6A1 locus in wild populations did not match those observed at the *MC1R* locus. Considering both loci, introgression could be found all over Europe. As much as 11% of wild boars carried domestic genes (1.4% in both loci), and the gene flow was bidirectional at least in some areas. The *MC1R* and *NR6A1* loci have proved to be useful markers to complement the information provided by other more widely employed genetic markers (e.g., mtDNA and microsatellites) to disclose patterns of gene flow among wild and domestic European and Asian forms of *Sus scrofa.*

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Conflict of Interests

The authors declare no conflict of interests

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Tables

		MC1R									*NR6A1*			
	Area	E^P/E^P	E^P/E^{P2}	E^P/e	E^D/E^P	E^D/E^D	E^+/E^P	E^+/E^{P2}	E^+/E^D	E^+/E^+	CC	CT	TT	Tot
DP	Italy				1	3						**1**	3	4
	Sardinia	8		2	7	1			**1_A**	**1_B**		**$2_{C,D}$**	18	20
	Tot	8		2	8	4			**1**	**1**		**3**	21	24
WB	Belarus						**1**	**1**		8	10			10
	Bulgaria					**1_E**				4	4		**1_E**	5
	Croatia									6	6			6
	France									15	13	**2**		15
	Greece		**1_F**							9	9		**1_F**	10
	Italy									33	29	**4**		33
	Luxembourg									8	8			8
	Poland						**1**			6	7			7
	Portugal									11	11			11
	Romania									8	8			8
	Sardinia	**2**					**1**			18	21			21
	Spain						**1_G**			10	10	**1_H**		11
	Tot	**2**	**1**			**1**	**4**	**1**		136	136	**7**	**2**	145

Tab. 1

Individual genotypes identified at the *MC1R* locus and at the *NR6A1* g.299084751C>T (p.Pro192Leu) polymorphism in domestic pigs (DP) and European wild boars (WB). Introgressed individuals are shown in bold. Each lower case letter (A-H) identifies a given individual in the population. E^+ = European *wild-type* allele; *e* = recessive red; E^D = dominant black, European form; E^P and E^{P2} = black spotting (respectively, corresponding to the 0101, 0401, 0301, 0501 and 0502 alleles in Fang et al. 2009). CC = individual carrying two wild-type *NR6A1* alleles; TT = individual carrying two domestic *NR6A1* alleles; CT = heterozygous individual.

CHAPTER 3

Novel Y-chromosome STRs in *Sus scrofa* and their variation in European wild boar and domestic pig populations

Novel Y-chromosome STRs in *Sus scrofa* and their variation in European wild boar and domestic pig populations

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Summary

Y chromosome markers are important tools for studying male-specific gene flow within and between populations, hybridization patterns and kinship. However, their use in non-human mammals is often hampered by the lack of Y-specific polymorphic markers. We identified new male-specific sequence variants in *Sus scrofa* starting from the available genome sequences. From a set of 23 male-specific simple tandem repeats, we selected four polymorphic regions (up to 8 alleles), falling respectively in one duplicated and two single-copy loci, which were informative in showing Y chromosome diversity in different wild and domestic populations. A total of 34 haplotypes were found by screening 211 individuals from 13 populations, with a haplotype diversity ranging from 0.00 (±0.00 SD, Duroc breed) to 0.90 (±0.16 SD, Croatian domestic pigs). A significant difference in haplotype frequency was observed between wild and domestic populations. The described Y chromosome variation can be useful to track male inheritance and gene flow in wild and domestic populations, and promises to provide significant insights to evolutionary and population genetic studies in *Sus scrofa*.

Introduction

The Y chromosome, except the pseudoautosomal region, is male specific, haploid and constitutes a non-recombinant block (Randall *et al.* 2010). In humans, Y-DNA haplotypes have proven to be an important tool in detecting male-specific gene flow (Simms *et al.* 2013), reconstructing human origins (Francalacci *et al.* 2013), demographic dynamics and migration events (Marks *et al.* 2012). In these studies both single nucleotide polymorphisms (SNPs) and short tandem repeats (STRs or microsatellites) have been used. Since Hurles & Jobling (2001), the analysis of Y chromosome diversity has been increasingly applied to several mammal species, investigating genetic history and phylogeny (Steiner *et al.* 2012; Wheeldon *et al.* 2013), domestication (Meadows *et al.* 2006; Warmuth *et al.* 2012), population structure and differentiation (Cortes *et al.* 2011; Moska *et al.* 2013), hybridization (Cortes *et al.* 2011; Hindrikson *et al.* 2012), parentage assignment (Katoh *et al.* 2009) and sex bias in gene flow (Musiani *et al.* 2007; Bidon *et al.* 2014). However, because of the distinctive architecture of the Y chromosome and its low genetic variation a substantial methodological effort is required for the development of Y-specific markers, which is also quite often prevented by the lack of accessible sequence data necessary for variants screening (Katoh *et al.* 2009; Greminger *et al.* 2010). The limited variation of the Y chromosome is mainly related to its lower effective population size (one quarter than that of autosomal DNA) and can be enforced by high levels of polygyny, which are common in many mammal species. Moreover, the difficulties in generating sequence data and aligning contigs (Murphy *et al.* 2006) and in finding Y-specific genetic markers are related to the frequent occurrence of gene conversion, degeneration and repetitive sequences on this chromosome (Rozen *et al.* 2003; Skaletsky *et al.* 2003). Though a general lack of variation across the chromosome, variability of Y-linked STRs is thought to be similar to that of autosomal ones (e.g. Kayser *et al.* 2004) and they are likely to represent informative markers within species (Handley & Perrin 2006). Actually, few polymorphic markers combined into haplotypes are sufficient to describe paternal genetic diversity. For example, in humans, only seven variable STRs were sufficient to depict the vast majority of the global haplotype diversity (Greminger *et al.* 2010).

Sus scrofa is one of the most widespread mammal species. The domestic form has a prime interest for both its agricultural and biomedical use, while the wild boar is one of the most relevant game species in Europe. Despite its great importance for humans and the

availability of the full genome sequence (Groenen *et al.* 2012), there are few studies addressing the variability of the Y chromosome in *Sus scrofa*. These studies are mainly related to the identification of markers for sex determination (Fontanesi *et al.* 2008) or focused on gene expression analysis (Domingo-Roura *et al.* 2003). Only two studies implemented the use of Y chromosome markers for the investigation of pig origins (Cliffe *et al.* 2010) and the reconstruction of the history of peripheral modern breeds (Ramirez *et al.* 2009). The latter study showed the presence of a geographical pattern across Eurasian populations, revealing a low male-mediated gene flow between the two main centers of pig domestication. However, as reported by Cliffe and colleagues (2010), much of the diversity within the Eurasian wild boar, either locally or as a whole, has to be disclosed and the potential contribution of ancestral Y lineages to domestic breeds still needs to be fully assessed.

Goal of the present study was to develop a set of variable Y chromosome specific markers to assess male-specific variation within and among populations in *Sus scrofa*. Since the available SNPs seem to suffer low variation (Cliffe *et al.* 2010), we focused on the identification of a panel of STRs that fulfilled the criteria of specificity and variability proposed by Greminger and colleagues (2010); however, considering that some multilocus markers can be diagnostic and are currently included in forensic panels (see Diegoli 2015 for a review), we did not limit our selection to single-copy regions.

Methods

A portion of 1,637,716 bp of the Y-chromosome sequence (Blott *et al.* 2003; Sscrofa10.2; accession number CM001155) was screened for the presence of STRs with a specifically designed PERL (www.perl.org) script. The identified regions were analysed with BLASTN 2.2.28 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) to check for homologies with the X-chromosome (accession number NC010461). PCR primers were designed in PRIMER3PLUS (Untergasser *et al.* 2007) for haploid regions. Primers, with a length of 18-25 bp and 40-60% GC content, were designed to produce a PCR product in the range 100-350 bp to allow their use even with low quality DNA. Products specificity was tested with PRIMER-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and only the best primer pair for each considered STR was selected (see Table 1). Initial screening was performed on a set of seven

males and seven females from different European countries. PCRs were initially conducted with standard protocols and then optimized for single loci (see Appendix S1 for PCR conditions). PCR-amplified STR alleles were sized using ABI PRISM 3130XL automatic sequencer (Applied Biosystems, Carlsbad, CA) and internal ROX-500 size standard (Applied Biosystems) at BMR Genomics (Padua, Italy). Allele size was subsequently determined using PEAK SCANNER V1.0 (Applied Biosystems).

Markers that showed multilocus amplification were further investigated to identify the amplified regions, and only the one that presented a clear duplication pattern on the Y-chromosome was retained (Appendix S2).

Because sequences from clones have a considerably higher error rate due to misincorporation of nucleotides (10-100 times higher; Loewen & Switala 1995) confirmation of the presence of a STR region within the PCR product was obtained by direct sequencing of both strands in a subsample representing the different observed alleles. PCR conditions were the same described in Appendix S1, but unlabeled primers were used. PCR products were sequenced on both strands using the BigDye Terminator kit v3.1 (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. Runs were performed at BMR Genomics. Sequences were visualised and manually checked in FINCHTV V.1.2.0 (Geospiza Inc., Seattle, WA) and aligned in MEGA 5.2 (Tamura *et al.* 2011).

The selected markers were subsequently used to genotype 221 male individuals, including wild boars from eight different regions in Europe and domestic pigs belonging to local and commercial breeds of both Asian and European lineages (see Table 2 for sampling regions). The occurrence of rare alleles was validated by at least two independent repetitions.

As the Y-chromosome is assumed to behave as a single segregating unit, alleles at the different loci were combined into haplotypes. The ARLEQUIN 3.5 software (Excoffier *et al.* 2005) was used to verify linkage disequilibrium (LD) between loci (Markov chain length: 10,000 iterations and 1,000 dememorizations) and haplotype diversity (H_D; Nei 1987) in each sampling region. Additionally, to account for different sample sizes, we calculated allelic richness (AR - rarefaction to 5 individuals) in CONTRIB 1.02 (Petit *et al.* 1998). ARLEQUIN was also used for an analysis of molecular variance (AMOVA; 10,000 permutations), based on F_{ST} estimation. In this analysis three hierarchical levels (groups of populations, populations and individuals) and two grouping structures were tested: *i*) wild vs. domestic European populations and *ii*) commercial breeds, local breeds, Western wild boar populations and

Eastern wild boar populations (see Table 2 for group subdivision). Pairwise F_{ST} values and their significance, calculated in ARLEQUIN, were used to assess differences in haplotype frequencies between populations.

In order to investigate the relationships among haplotypes, a median joining (MJ) network (Bandelt *et al.* 1999) was built using the default settings in the program NETWORK 4.6.1.1 (Fluxus Techonolgy, Suffolk, UK). Following Bannasch *et al.* (2005), markers were weighted according to their variance as follows: YLI01a had a variance of 0.005 and was assigned a weight of 10, YLI04 had a variance of 0.323 and was given a weight of 8, YLI01b had a variance of 1.009 and was weighted 5, and YLI10 had a variance of 71.205 and was weighted 1.

As sequence differences at ubiquitin-specific protease 9 (*USP9Y*) and amelogenin (*AMELY*) genes were discovered to have an uneven distribution between Europe and Asia (Ramirez *et al.* 2009), these two regions were sequenced in one sample for each detected haplotype following the authors' protocol.

Results

The analysis of a portion of the Y-chromosome led to the identification of 13 STRs, which did not present homologies with X-chromosome regions. On the basis of the product specificity results in PRIMER-BLAST, we selected nine primer pairs for the initial screening. Subsequently to the validation process three primer pairs were selected to compose the genotyping panel (Appendix S2 and Table S1), two of which amplified single-copy STRs (respectively YLI04 and YLI10), while the third one (YLI01) amplified two inverted regions of the chromosome, separated by about 37 kilobases (Appendix S3 and Table S2). The two loci of marker YLI01 were named *a* and *b*, following the convention used for human loci (see Gusmão *et al.* 2006), and it was not possible to assign alleles to either of the two co-amplified loci. A maximum of eight alleles per locus were found in the 221 analysed individuals (Table 1). YLI01 and YLI04 showed a very frequent and geographically widespread allele (234 and 230, respectively). Marker YLI10 was the most variable and did not show a predominant allele (Fig. 1).

LD between YLI01 and YLI04 was confirmed ($p < 0.005$). Locus YLI10 was not in LD with loci YLI01a/b, but it was strongly associated ($p < 0.001$) with YLI04. Considering such

associations, we pooled the three markers (four loci) for haplotype construction. By combining alleles, 34 haplotypes were obtained (Table 2). Overall H_D amounted to 0.902 (±0.010 SD), the lowest value was found in Duroc breed (0.000±0.000 SD), where a single haplotype was present, while the highest were identified in Croatian domestic pigs (0.900±0.161) where four haplotypes were detected from only five individuals (Table 2). Significant differences in haplotype frequencies were observed between domestic pig breeds and wild boar populations with F_{ST} values ranging from 0.193 to 0.888 (almost all pairs $p < 0.05$, Table 4). Between wild populations F_{ST} values ranged from -0.045 (Estonia-Austria comparison) to 0.282 (Estonia-Croatia). The AMOVA analysis between wild and domestic European populations showed that most (59.41%) of the variation is within populations, but a relevant percentage of variation was observed between the two groups (25.77%). When considering four groups, inter-group variation was fairly similar (21.74%) while within population variation increased (65.37%). NETWORK analysis showed concordant results, with many haplotypes shared among populations and a very weak geographic pattern (Fig. 2). Additionally, we observed the presence of a minor group of unique wild boar haplotypes, which was fairly distant from the main group shared between wild and domestic animals. Of the three most frequent haplotypes (H32, H12 and H11, respectively 22.62%, 11.76% and 10.86% in frequency), the first one was exclusive of European commercial and Sardinian domestic pigs, the second was found in wild boar samples only and was spread across European countries, while the last one was shared between wild boars and Sardinian domestic pigs. Haplotypes of commercial and Asian domestic pigs were interconnected and quite peripheral in the diagram, while those found in free ranging pigs were shared with both wild boars and commercial breeds or were closely related to their haplotypes. Haplotype H15 (6.79% frequency), in a central position in the network, was shared between wild boars and domestic pigs (but commercial breeds).

In our study we observed a single HY3 haplotype in the Asian domestic pig population (10%) and it was absent in both domestic and wild European samples. HY1 was the only detected haplotype in commercial breeds, and it was dominant in both European wild boars and local breeds (68% and 88% respectively). HY1 and HY2 haplotypes were spread across the network and did not show any structuring, while the single HY3 individual is in an intermediate position.

Discussion

The isolation and characterization of neutral variable markers on the Y-chromosome can contribute to understand the processes underlying evolution and domestication in *Sus scrofa*. They may also reveal useful to increase the resolution in parentage tests and hybridization analyses and contribute to the investigation of population structure and phylogeography.

In this study we successfully identified three variable markers, corresponding to four STR loci, that led to the identification of 34 different haplotypes in a sample of 211 individuals of different European wild and domestic populations, together with 10 Asian domestic pig samples. The analysis of *USP9Y* and *AMELY* genes was consistent with an European origin of most of the STR-Y haplotypes observed in European wild and domestic *S. scrofa*. Yet, this cannot be conclusive due to haplotype sharing.

The observed diversity levels are in the range of what reported with Y-linked STRs in other domestic (*Ovis aries*, Ferencakovic *et al.* 2013; *Canis lupus familiaris*, Bannasch *et al.* 2005) and wild (*Pan paniscus*, Eriksson *et al.* 2006; *Ovis musimon*, Ferencakovic *et al.* 2013) mammal species. Interestingly, haplotype diversity is concordant with values published by Vilaça and colleagues (2014) for the D-loop region of the mitochondrial DNA (mtDNA) in European wild boar. However, the geographic pattern found with mtDNA was not detected with Y STR data. Although the limited sample size requires caution, this discrepancy between the two classes of uniparental markers would suggest a higher gene flow in the Y-chromosome, which is coherent with a mostly male-biased dispersal in this species (Keuling *et al.* 2010).

On the opposite, unlike the high similarity reported in several mtDNA studies (Scandura *et al.* 2011; Ramirez *et al.* 2009), a relevant genetic differentiation between pigs and wild boars was observed (25.72% of variation in AMOVA analysis), with only five (out of 12) pig haplotypes in common with the sampled European wild populations. The high variation of the developed Y-chromosome markers in wild populations and the observed divergence between wild and domestic lineages make this panel a useful tool to evaluate the male contribution to the current diversity in swine breeds, as well as to assess the extent and directionality of hybridization between the two forms (Scandura *et al.* 2011; Goedbloed *et al.* 2013).

Finally, based on these preliminary results, the described markers will contribute to disentangle several aspects of the species' biology, where the implementation of polymorphic paternally-inherited markers can be crucial (e.g., male reproductive success and dispersal patterns).

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Conflict of Interests

The authors declare no conflict of interests.

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Supporting Information

Appendix S1. PCR conditions.

Appendix S2. Primers' product validation.

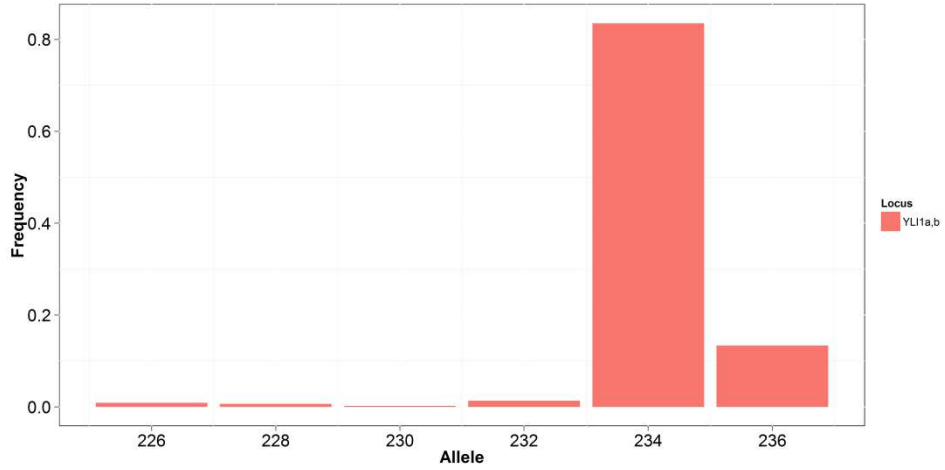
Appendix S3. YLI01 loci investigation.

Table S1. Information on discarded primer pairs.

Table S2. Information on primers developed to verify the presence of the duplicated region.

Figure 1. Allele frequency distribution of the three Y-chromosome markers in the cumulated sample of 221 *Sus scrofa* individuals. a) Alleles of YLI01 are jointly shown, though they refer to two STR loci, located in a duplicated region of the Y chromosome. b) YLI04. c) YLI10.

a)



b)

c)

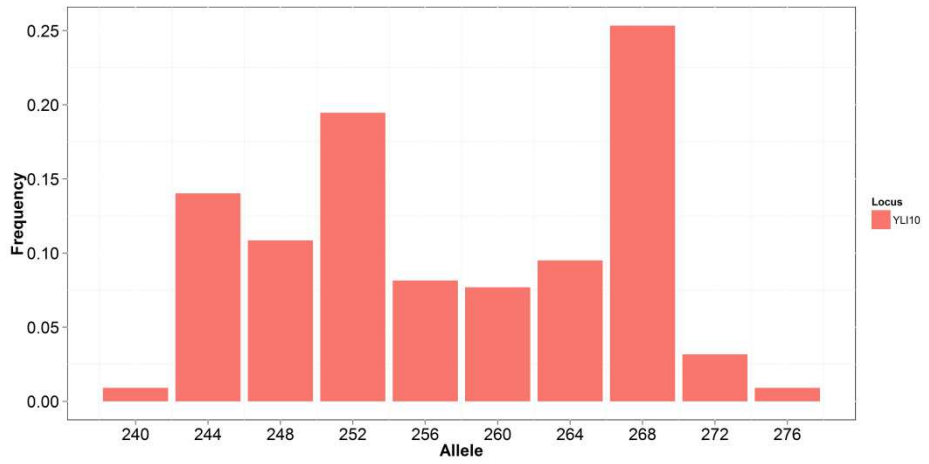


Figure 2. Median-joining network showing phylogenetic relationships among Y chromosome haplotypes computed by weighing microsatellite loci by their variance. Circles are proportional to haplotype frequency and number of mutations separating nodes are shown (dashes). Colours correspond to wild boar or domestic pig populations where haplotypes were observed.

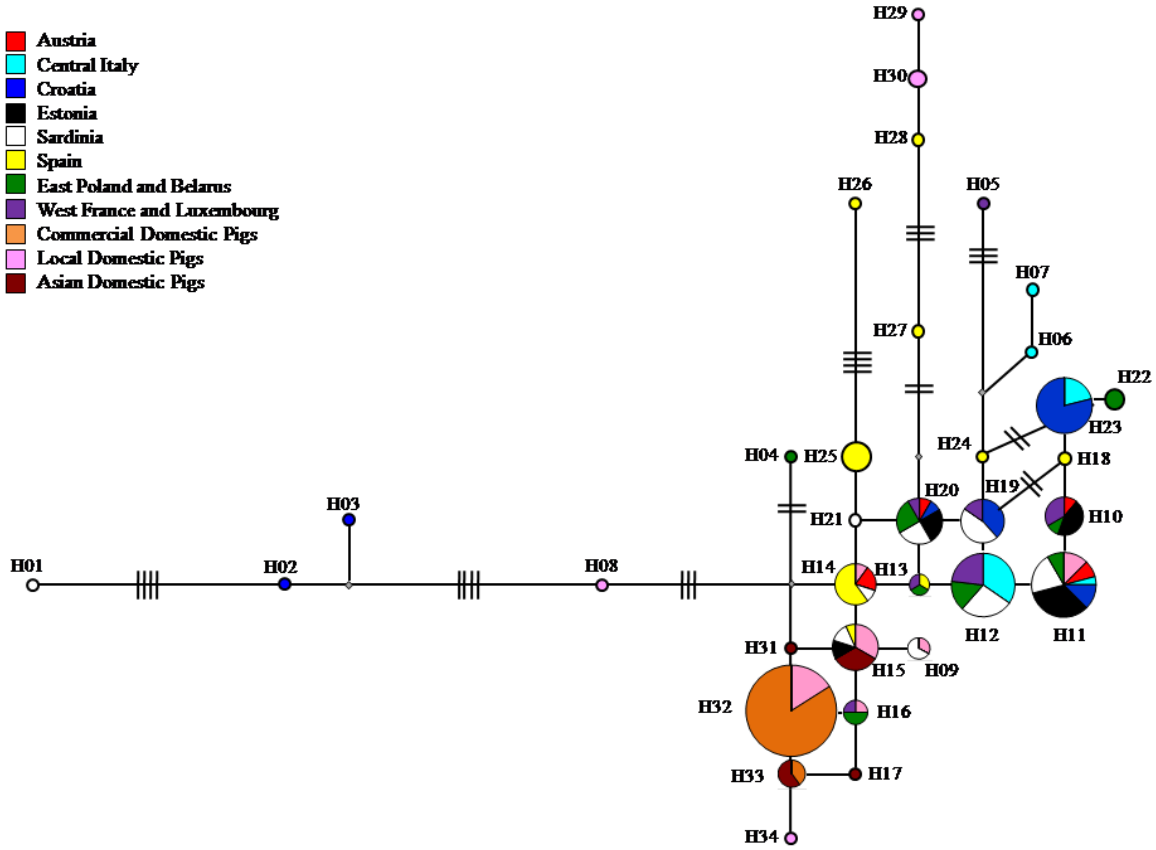

Austria
Central Italy
Croatia
Estonia
Sardinia
Spain
East Poland and Belarus
West France and Luxembourg
Commercial Domestic Pigs
Local Domestic Pigs
Asian Domestic Pigs
H01
H02
H03
H04
H05
H06
H07
H08
H09
H10
H11
H12
H13
H14
H15
H16
H17
H18
H19
H20
H21
H22
H23
H24
H25
H26
H27
H28
H29
H30
H31
H32
H33
H34

Table 1. Primer sequences, annealing temperature, number of detected alleles, repeat motif and allele size range of the developed Y chromosome STRs.

Locus		Primer 5'-3'	Ta (°C)	No. of alleles	Repeat motif	Allele size (bp)	Position	Dye
YLI01a/b	F	TTTGCATCACCCATATAGATACAGA	65	6*	$(GT)_9$	226-236*	454452/	FAM
	R	GCTGTAGGTGTGGCCCTAAA					569183	
YLI04	F	TCCCAATGGTGGTGCTATTT	54	7	$(GT)_{19}$	228-240	79244	HEX
	R	GGGAGGACTCACCTGTAGAA						
YLI10	F	CTAGAATGTCGCAGAAGT	59	8	$(CCTT)_{11}C(CTTT)_{16}$	240-276	927613	NED
	R	CCCCAATACACAAAGAAAGA						

*due to the duplicated nature of this marker, alleles cannot be attributed with certainty to either of the two loci.

Table 2. Genetic diversity at the developed Y chromosome STR markers in a array of *Sus scrofa* populations. GroupA and GroupB indicate the clustering used for AMOVA analyses.

GroupA	GroupB	Population	N	Nr. Haplotypes	AR[5]	H_D (±SD)
WB	EWB	Austria	6	4 (0)	2.667	0.867 (±0.129)
WB	WWB	Central Italy	16	5 (2)	1.751	0.650 (±0.108)
WB	EWB	Croatia	27	7 (3)	1.878	0.664 (±0.088)
WB	EWB	Estonia	17	4 (0)	1.963	0.721 (±0.079)
WB	WWB	Sardinia	28	9 (2)	2.839	0.865 (±0.034)
WB	WWB	Spain	18	9 (5)	2.702	0.837 (±0.066)
WB	EWB	East-Poland and Belarus	16	8 (2)	3.087	0.900 (±0.046)
WB	WWB	West-France and Luxembourg	14	6 (0)	2.417	0.791 (±0.089)
DP	CoDP	Large White	19	2 (0)	0.468	0.199 (±0.112)
DP	CoDP	Duroc	25	1 (0)	0.000	0.000 (±0.000)
DP	LoDP	Sardinian Domestic Pigs	19	6 (2)	2.202	0.760 (±0.070)
DP	LoDP	Croatian Domestic Pigs	5	4 (2)	3.000	0.900 (±0.161)
-	-	Asian Domestic Pigs	10	4 (2)	1.913	0.711 (±0.118)
		TOT	221	34	4.156	0.902 (±0.010)

Group: WB – wild boar, DP - domestic pig, EWB - Eastern wild boar, WWB - Western wild boar, CoDP - commercial domestic breed, LoDP - local domestic breed; N – sample size; Nr. Haplotypes - number of observed haplotypes, in parenthesis the number of private haplotypes is given; AR[5] - allelic richness with a rarefaction of 5; H_D - haplotype diversity, SD - standard deviation.

Table 3. Y chromosome haplotypes found in wild boar and domestic pig samples at the three developed markers (allele size expressed in base pairs). HY haplotypes correspond to those defined by Ramirez *et al.* (2009).

Haplotype	N	YLI01a/b*	YLI04	YLI10	HY haplotypes
H01	1	226/226	230	252	HY1
H02	1	226/232	228	256	HY2
H03	1	226/232	228	264	HY2
H04	1	236/236	230	256	HY1
H05	1	230/234	234	244	HY2
H06	1	232/232	234	252	HY2
H07	1	232/232	236	252	HY2
H08	1	228/234	230	260	HY1
H09	3	234/234	228	264	HY1
H10	9	234/234	230	244	HY1
H11	24	234/234	230	248	HY2
H12	26	234/234	230	252	HY1
H13	3	234/234	230	256	HY1
H14	10	234/234	230	260	HY1
H15	15	234/234	230	264	HY1
H16	4	234/234	230	268	HY1
H17	1	234/234	230	272	HY3
H18	1	234/234	232	244	HY2
H19	13	234/234	232	252	HY1
H20	12	234/234	232	256	HY1
H21	1	234/234	232	260	HY1
H22	2	234/234	234	240	HY1
H23	19	234/234	234	244	HY2
H24	1	234/234	234	252	HY1
H25	5	234/234	234	260	HY1
H26	1	234/234	234	276	HY1
H27	1	234/234	238	256	HY1
H28	1	234/234	240	264	HY1
H29	1	234/234	240	272	HY1
H30	2	234/234	240	268	HY1
H31	1	234/236	230	264	HY1
H32	50	234/236	230	268	HY1
H33	5	234/236	230	272	HY1
H34	1	234/236	230	276	HY1

*due to the duplicated nature of this marker, alleles cannot be attributed with certainty to either of the two loci. To be conservative, the smaller allele of the pair was attributed to the locus YLI01a and the larger to YLI01b.

Table 4. Pairwise F_{ST} values between populations.

	CIT	WFL	SAR	SPA	CRO	AUS	EST	EPB	DDP	SDP	CDP	LDP	ADP
CIT	-												
WFL	0.041	-											
SAR	0.092*	0.021	-										
SPA	0.221*	0.262*	0.198*	-									
CRO	0.174*	0.266*	0.244*	0.211*	-								
AUS	0.174*	0.126	0.077	0.091	0.246*	-							
EST	0.234*	0.177*	0.133*	0.259*	0.282*	-0.045	-						
EPB	0.086*	0.020	0.032	0.150*	0.201*	-0.003	0.060	-					
DDP	0.754*	0.823*	0.702*	0.779*	0.730*	0.888*	0.802*	0.696*	-				
SDP	0.309*	0.300*	0.278*	0.331*	0.407*	0.202*	0.250*	0.193*	0.431*	-			
CDP	0.287*	0.320*	0.250*	0.217*	0.306*	0.220*	0.327*	0.165*	0.887*	0.271*	-		
LDP	0.677*	0.742*	0.641*	0.710*	0.675*	0.786*	0.726*	0.610*	0.077	0.329*	0.775*	-	
ADP	0.338*	0.358*	0.294*	0.363*	0.429*	0.282*	0.305*	0.241*	0.791*	0.097	0.397*	0.660*	-

* - significant value ($p < 0.05$); ADP = Asian domestic pigs; AUS = Austria; CDP = Croatian domestic pigs; CIT = Central Italy; CRO = Croatia; DDP = Duroc; EPB = E-Poland and Belarus; EST = Estonia; LDP = Large White; SAR = Sardinia; SDP = Sardinian domestic pigs; SPA = Spain; WFL = W-France and Luxembourg.

Appendix S1. *PCR conditions*

PCR conditions were the following: 3 µl of DNA solution, 0.1 U Taq DNA polymerase (Sigma-Aldrich, Spruce, St. Louis, MO), 1x PCR buffer (Sigma-Aldrich, Spruce, St. Louis, MO), 3mM $MgCl_2$, 100 µM of each dNTP and 1.6 pmol of each primer (forward primers were labelled with a ABI fluorescent dye for sizing purposes) for a total volume of 10 µl. The amplification profile was set up with an initial step of denaturation at 95°C for 5 min, followed by 35 cycles of 92°C for 45s, annealing temperature (see Table 1) for 45s, and 72°C for 30s. A further extension step of 72°C for 10 min concluded the reaction.

Appendix S2. *Primers' product validation*

Primer pairs YLI6, YLI7 and YLI9 did not produce a reliable PCR product and were discarded.

Primer pairs YLI03, YLI05, YLI08 produced more than a single amplification product and were further investigated.

YLI03 and YLI05 presented a single expected amplification product according to PRIMER-BLAST. As it was not possible to find where the duplication was located with GenBank the markers were discarded.

Marker YLI08 produced high quality amplicons but showed confused peak patterns and was thus excluded from analyses.

Appendix S3. *YLI01 loci investigation*

Primers of marker YLI01 annealed in two different regions of the Y chromosome, 37 Kbp apart, and their amplification, in some samples, produced two products, resembling a status of heterozygote. Unfortunately, the involved region is long about 48Kbp and highly conserved. For this reason, it was not possible to amplify selectively the two different loci, respecting the criterions stated in the Methods section. However, as the duplication (i.e., two alleles) was observed only in a portion of the analyzed sample, mainly domestic pigs, we verified its occurrence also in the apparently homozygote samples. We thus developed two primer pairs (Table S1), targeting the extremes of the duplicated regions, so to include both the terminal part of the region and a flanking non-duplicted sequence in the product. They were expected to produce two fragments in case the duplication was present and a single one if the duplication was missing. PCR conditions were the following: 3 µl of DNA solution,

0.1 U Taq DNA polymerase (Sigma-Aldrich, Spruce, St. Louis, MO), 1x PCR buffer (Sigma-Aldrich, Spruce, St. Louis, MO), 3mM $MgCl_2$, 100 µM of each dNTP and 1.6 pmol of each primer for a total volume of 10 µl. The amplification profile was set up with an initial step of denaturation at 95°C for 5 min, followed by 35 cycles of 92°C for 45s, 60°C for 45s, and 72°C for 30s. A further extension step of 72°C for 10 min concluded the reaction. PCR products were visualised on a 2% agarose gel (Sigma-Aldrich, Spruce, St. Louis, MO). In this way the duplication was confirmed in all the analysed individuals. Therefore, when no difference in product size could be observed (i.e., 'apparent homozygotes'), we assumed that the animal was carrying two STR alleles of the same size.

Table S1. Primer sequences, annealing temperature and repeat motif and position of the discarded primer pairs.

Locus		Primer 5'-3'	Ta (°C)	No. of alleles	Repeat motif	Position
YLI03	F	CAAAAATTCCGTCAAACGTG	62-53	D	AC	781945
	R	TGGTCATTTGTCATGGGGTA				
YLI05	F	ATGCTGGGAACAACAGACTT	62-53	D	CA	179283
	R	TTCTTCATCTTGTCGTGGGT				
YLI06	F	ATGCTTGGGAACTTTGTAAG	/		TTCT	800573
	R	AGGAAGGAAGGAAGGAAGA				
YLI07	F	GCAACTGACTTCTGAATGTT	/		TG	837114
	R	GAGCAGCACAAGAAATAGC				
YLI08	F	GGTGCAGCCCTAGGAAAGA	65	M	GT	1108097
	R	CCTGGAAACGTCTGTATGCC				
YLI09	F	TGAGCTGTGGTGTAGGTT	/		AT	1402181
	R	TTCATTTGTTCTGGGTCTTG				

/ = no amplification; D = duplication; M = Multicopy; P = palindrome

Table S2. Primer sequences, annealing temperature, length of the fragment and position of the primer pairs developed to verify the presence of the duplicated region in the Y chromosome of the analyzed samples.

Primer		Primer 5'-3'	Ta (°C)	Fragment length (bp)	Position
PalEnd_1F4	F	GTGTAGGTCAAGAATGTGGCT	60	99	577918
PalEnd_1R24	R	TATGGAAGTTCCCAGGCTATTGC			
OrigEnd_1F28	F	TAGAGCCTCACCTCCAAGGTA	60	113	490919
OrigEnd_1R2	R	GTATTTGAGAGCCCTGAATGGCA			

CHAPTER 4

Isolation by distance, by barrier and by resistance have shaped a sharp genetic structure in a island wild boar population.

Isolation by distance, by barrier and by resistance have shaped a sharp genetic structure in a island wild boar population.

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Abstract

Genetic diversity within animal populations can be evenly distributed or, more often, be structured by the simple effect of distance or by the existence of breaks in landscape connectivity. Additionally, such human activities as restocking with allochthonous individuals and anthropogenic hybridization can deeply affect genetic make-up and structure of populations, sometimes partially concealing natural patterns of variation.

We studied the genetic differentiation within the wild boar (*Sus scrofa*) population in Sardinia island (Italy), and tested for the existence of isolation by distance (IBD), isolation by resistance (IBR) and isolation by barrier (IBB), accounting for the effects of local genetic introgression from continental boars and domestic pigs.

A total of 368 Sardinian wild boar samples were analysed with a set of 16 microsatellites. Signals of genetic introgression were identified through a Bayesian cluster analysis which also included 214 reference wild boars from several European countries and 114 domestic pigs.

Almost 25% of individuals sampled in the island were recognized as putative hybrids. After their removal from the dataset, the genetic structure in the purged population was investigated by using different statistical approaches.

Blind (STRUCTURE and PSMIX) and spatially explicit methods (GENELAND and STRUCTURE) supported a sharp partition into three discrete subpopulations. A significant IBD pattern was detected. Nevertheless, the correlation between genetic distances and geographic distances

increased when we took land use into account, by employing a matrix of 'effective distances' between individuals (least-cost paths).

In addition, genetic discontinuities between subpopulations were also explained by the presence of the main motorway (S.S. 131), crossing the island from north to south.

Interestingly, the combined effect of the barrier (S.S. 131) and landscape features seems to have limited the gene flow in the island, protecting western subpopulations from the spread of exotic genes, mostly introgressed in the eastern subpopulation. This study reveals how human-transformed landscapes can strongly impact the genetic connectivity even in large-sized and highly mobile animal species.

Key words: *Sus scrofa meridionalis*, Sardinia, landscape genetics, microsatellites, gene flow, road impact

Introduction

Connectivity and habitat fragmentation can have a strong impact on the onset of genetic differentiation between and within populations of the same species. Indeed, the presence of barriers may lead to a disjunction and, sometimes, a complete isolation of part of a population, leading to genetic drift and, consequently, divergent genetic composition.

Usually, the presence of barriers and absence of corridors promotes the limitation of gene flow, the reduction of genetic diversity and the increase of inbreeding (Balkenhol & Waits, 2009). In the last decade numerous analytical approaches have been developed to infer microevolutionary processes driven by environmental fragmentation and human infrastructures, giving rise to a field named 'landscape genetics' (Manel et al., 2003; Storfer et al., 2010). Briefly, landscape genetics analyses consist in correlating genetic variation with environmental characteristics, in order to estimate the effect of the environment on the population genetic structure (Storfer et al., 2007). Landscape genetics has been widely adopted to evaluate the influence of natural and (especially) anthropogenic barriers on the gene flow of animal and plant species. Since urban and suburban development and road network extension are among the primary causes of habitat fragmentation, these approaches can be of help in planning management practices for species conservation (Holderegger & Di Giulio, 2010).

For this purpose, several studies on the landscape genetics of large mammals have been conducted (e.g. roe deer *Capreolus capreolus* Coulon et al., 2006; red deer *Cervus elephus* Pérez-Espona et al., 2008; mountain lion *Puma concolor* Castilho et al., 2011; giant panda *Ailuropoda melanoleuca* Zhu et al., 2010; tiger *Panthera tigris* Sharma et al., 2013; caribou *Rangifer tarandus* Weckworth et al., 2013). Such studies, using different statistical approaches, indicated that landscape features could explain a large proportion of genetic differentiation that is not explained by spatial distance only.

Increasing urbanization and the development of transport infrastructures in Europe may have affected the spatial behaviour of many large mammals, like wild ungulates, and consequently the genetic structure of their populations. In spite of social relevance and diffusion of wild ungulates, only few studies have been aimed at evaluating the effect of anthropogenic barriers on ungulate population structure (bighorn sheep *Ovis canadensis*

Epps et al., 2005; roe deer Coulon et al., 2006; Kuehn et al., 2007; Hepenstrick et al., 2012; red deer Šprem et al., 2013, wild boar *Sus scrofa* and red deer Frantz et al., 2012). Due to scarcity of information, it is difficult to establish the real impact of such barriers, since they could have various levels of permeability depending on the species' behavioural characteristics. For example Frantz et al. (2012) showed how the presence of a motorway could affect differently two ungulate species in Belgium. In the latter study, the motorway seemed to act as a barrier for the red deer, leading to a genetic differentiation between subpopulations inhabiting the opposite sides of the road, while it did not seem to severely affect gene flow in the local wild boar population.

Population structure and genetic diversity within managed ungulate populations can also be greatly affected by harvest (Allendorf et al., 2008), translocation of individuals (e.g., DeYoung et al., 2003, Frantz et al., 2006) and by the introduction of exotic conspecifics (or closely related species) that creates the conditions for introgressive hybridization (Simberloff 1996). Additionally, the occurrence of hybridization between wild and domestic conspecifics may have particularly strong effects on genetic population structure (Goedbloed et al. 2013).
As a consequence, geographical patterns of genetic variation in a highly managed species such as the wild boar, should be evaluated with caution (Scandura et al., 2011).

The wild boar is one of the most important and widespread ungulate game species in Europe. It shows an opportunistic behaviour and is adaptable to almost any type of environment. Climate represents the main limiting factor for wild boar through its effect on the species' physiology or through its indirect effects on food availability and accessibility (Geisser & Reyer, 2005; Melis et al., 2006).
The wild boar is considered a sedentary species with a small-scaled use of the space (Keuling et al., 2008), regardless of the habitat occupied. Dispersal is male-biased, females are philopatric and form matriarchal social groups, while adult males stay mostly isolated. The available data indicate that wild boar dispersal takes place between 11 and 16 months of age and covers limited distances (mostly < 20 km, Briedermann, 1990; Truvè & Lemel, 2003; Keuling et al., 2010). Dispersal patterns are influenced by various factors such as population density, habitat structure and quality, and climate (Dardaillon & Bougnon, 1987; Keuling et al., 2010). In addition, human activities can have an impact on different aspects of the

species ecology and behaviour. For instance, wild boar is known to modify its activity and spatial patterns in relation to human disturbance. Undisturbed wild boars tend to be active during the day, while under hunting pressure and high human disturbance they shift their activity to nocturnal (Boitani et al., 1994; Podgórski et al., 2013). Nevertheless, thanks to its plasticity, a tendency to adapt to human presence and infrastructures is observed around urban centres (Cahill et al., 2012; Osashi et al., 2013).

Our study is focused on the wild boar population inhabiting Sardinia, a scarcely populated island and still underdeveloped in terms of main infrastructure, if compared to other regions of continental Europe. The Sardinian wild boar is considered a dwarf form of the European wild boar, which originated during the Neolithic, and it is currently classified as a distinct subspecies (*Sus scrofa meridionalis* Major 1883), on the basis of both morphological and genetic evidences. The long-lasting isolation of the Sardinian population produced a relevant genetic differentiation, observed using different types of genetic markers by Scandura and colleagues (2008, 2009, 2011) and Iacolina and colleagues (2015). Furthermore, Scandura et al. (2011) detected appreciable levels of genetic introgression from domestic pigs and continental wild boars, and a relevant genetic structure, with three subpopulations: one in the east of the island, one in the north-west and the last in the south-west. The authors concluded that the sharp east-west genetic differentiation could not be explained by isolation-by-distance only and suggested that landscape features could have played an important role in creating the observed genetic pattern.

In the present study, we analysed the Sardinian wild boar population, increasing the number of individuals and genetic markers compared to previous studies, with the aim to investigate the role of natural and anthropogenic environmental variables in shaping the observed genetic structure in the island and preventing a genetic shuffling among subpopulations. Isolation by distance (IBD), isolation by resistance (IBR) and isolation by barrier (IBB) were tested using different landscape genetics approaches and considering various environmental features, as suggested by Balkenhol et al. (2009) and Frantz et al. (2012).

Materials and Methods

Study area

Sardinia is the second largest island in the Mediterranean sea (24,100 km^2). Its population density is relatively low for Europe (1,640,379 inhabitants, around 68/km^2), with most people living in the five main cities and along the coast, while the hinterland is characterized by small villages and large uninhabited areas.

The climate is mediterranean-temperate at low elevations and along the coast, and continental in the inland at higher elevations. Temperature is mild and relatively constant during the year (on average 18°C, ranging between a mean of 7°C in winter and 25°C in summer). Precipitations mostly fall during autumn and winter, being more frequent in the northern and western sides of the island. Annual precipitations range from less than 400 mm in the dry south to almost 1500 mm in the eastern mountains.

The island is relatively dry, and major waterways have mostly the features of streams in summer. A single small natural lake (Lake Baratz, 0.6 km^2) and some tens of artificial basins (the biggest one being Lake Omodeo, 29 km^2) are also present, as well as a number of ponds and lagoons along the coast. Because of the island size, there is a wide variety of habitats. Coasts (1,849 km long) are generally high and rocky, interspersed by a number of sandy shores. Mountains occupy only 13.6% of the territory and are mainly concentrated in the central-eastern part of the island. The main mountainous massifs are the Gennargentu, in the central-eastern side (reaching 1,834 m a.s.l.) and the chains of Marghine and Goceano, crossing the island from north-east to south-west. Plateaus and flatlands occupy 18,5% of the territory, with the main flatland located in the south-west (the Campidano Plain, a human-modified landscape dominated by cultivations, especially cereal crops, fruit trees and vineyards).

The wild boar is a game species widespread in the island, due to its ecological plasticity (it is rare in the Campidano Plain only). Estimates of population size are rough and affected by large confidence intervals. Local densities were estimated by using hunting data from 168 hunting grounds spread throughout the island, with 0-33 culled heads/km^2. On the basis of habitat suitability analyses higher densities are expected to occur in the central and northern part of the island (Autonomous Region of Sardinia 2012).

To evaluate the impact of land use on the dispersal of wild boar and its ultimate effect on the genetic structure, we used the 4th Level CORINE Land Cover at scale 1:50,000 (Heymann et al., 1994). We divided the island into 6451 cells of 2x2 km and for each cell we calculated the percentage surface of each land cover category. Cover categories were pooled according to their similarity and effectiveness to act as barrier to the species' movements (Tab. 1). Considering the lack of information regarding the Sardinian population, we relied on studies in Mediterranean areas that have stressed the importance of seasonal availability of food and water, and the presence of refuge areas (Boitani et al., 1994; Massei et al., 1997; Fernandez-Llario & Carranza, 2000; Focardi et al., 2008). In addition to such natural variables, also the distribution of human activities and infrastructures has a strong impact on the presence of the wild boar. Railway and road networks are in most cases crossed by wild boar, but if busy and associated to permanent shields may become effective barriers, limiting the species' dispersal. Only one main road with the mentioned features occurs in Sardinia: the S.S. 131 'Carlo Felice', a superficial motorway with 4 lanes and with very few crossing points for wildlife. Its trail was firstly set in the XIX century, but it was paved in its present form in the last 50 years. It crosses the island from north to south connecting the two main cities, Cagliari and Sassari.

Sampling and microsatellite genotyping

A total of 368 wild boar samples were obtained from all over Sardinia by local hunters during the period 2001-2011 (Fig. 1a). Muscle or hair samples were collected from hunted animals and stored, respectively, in absolute ethanol or frozen until analysis. Sampling locations were mapped using ArcGIS v. 10 (ESRI, Redlands, CA, USA). Accuracy of spatial information differed among samples: punctual GPS locations were available for some animals only, whereas for most of them hunters reported either the municipality or the hunting ground where the animal was shot (i.e. polygons in the range 26-547 km^2, median size 79 km^2). In the latter case, geographical coordinates of the geometric centre of the area were used for statistical analyses.

DNA was extracted using Genelute kit (Sigma-Aldrich, St Louis, MO, USA) for tissue samples and Instagene Matrix (Bio-Rad, Hercules, CA, USA) for hair samples, and then stored at -20°C. All samples were genotyped with a panel of 16 microsatellites (STR; Short Tandem Repeat): S090, SW122, SW2532, S355, SW1492, SW461, IGF1, SW951, SW2021, SW2496,

S026, S215, SW72, SW857, S155 and SW24 (details at www.thearkdb.org). Each PCR was performed in a 10 µL reaction volume, containing 3 µL of DNA solution, 0.5 U of Taq DNA polymerase (Euroclone, Pero, Italy), 1× PCR buffer (Euroclone), 2.5 mM $MgCl_2$, 100 µM of each dNTP and 2 pmol of each primer. The forward primer of each pair was labelled with an ABI fluorescent dye (6-FAM, HEX or NED). The amplification profile was set up with an initial step of denaturation at 95 °C for 3 min, followed by 35 cycles of 92 °C for 45 s, Ta (54–62 °C) for 45 s, and 72 °C for 30 s. A further extension step of 72 °C for 10 min concluded the reaction. Amplicons were sized using capillary electrophoresis in an ABI PRISM 3100 and 3730XL Avant automatic sequencer (Applied Biosystems, Carlsbad, CA, USA) by the BMR-Genomics sequencing service (Padua, Italy). Peak Scanner software v. 1.0 (Applied Biosystems) was used to analyze electrophoretic data.

In order to evaluate possible signatures of genetic introgression from both allochthonous wild conspecifics and the domestic form, Sardinian wild boars genotypes were compared with 214 reference wild boars from different European countries and with 114 Sardinian domestic pigs, including commercial breeds and local free-ranging individuals (Tab. S1).

Microsatellite data analysis

In order to detect evidences of null alleles, stuttering or large allele dropout, data were checked with MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004).

Deviations from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (LE) were tested in the Sardinian population using GENEPOP v. 4.2 (Raymond & Rousset, 1995). Tests for HWE employed the Markov chain method proposed by Guo and Thompson (1992), with the following chain parameters: 10000 dememorizations, 100 batches and 10000 iterations. Deviations from LE were tested for each pair of loci. Significance levels were adjusted according to the sequential Bonferroni correction for multiple comparisons (Rice 1989).

The occurrence of imported exotic boars along with the signature of genetic introgression from continental populations (Italian peninsula or central Europe) and from domestic pigs was recently proven in Sardinia by Scandura and co-authors (2011). They stressed that these factors could lead to biased inference on the population genetic structure in the island. On account of those findings, in the present study we preliminarily screened all Sardinian genotypes in order to remove non-negligible distortions in allele frequencies attributable to human-mediated introgressive hybridization or to introduction of exotic boars.

With this in mind, we implemented a Bayesian cluster analysis in STRUCTURE v. 2.3.4 (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009). Firstly, we performed 10 independent Monte Carlo Markov chain (MCMC) runs simulating a number of subpopulations (K) ranging between 1 and 10, with the following settings: admixture model, no population information, correlated allele frequencies, 200,000 burn-in and 200,000 iterations of data collection. Then, we selected the smaller value of K that allowed the discrimination among the following four groups: Sardinian wild boar (Sardinian WB), mainland Italy wild boar (Italian WB), other European wild boars (European WB) and domestic pigs. Accordingly, we assessed the nature of each individual sampled in Sardinia in relation to the possible occurrence of gene introgression from the other wild and domestic populations. The degree of admixture was individually evaluated by referring to the Q-values obtained in the run with highest likelihood at the selected K-value. To be conservative, only individuals showing >90% membership to the Sardinian cluster (Q_{Sar}) were retained for further analyses. In so doing we admit the erroneous exclusion of non-introgressed individuals that can be misclassified, but we expect that real immigrants and first-generation hybrids will be correctly classified ($Q_{Sar} < 0.90$) and excluded (see also Frantz et al., 2013).

The pruned dataset of Sardinian wild boars was hence used to infer population structure. In agreement with Balkenhol et al., (2009) and Frantz et al., (2012) who suggested the use of multiple approaches to investigate population structure, two different Bayesian clustering algorithms (STRUCTURE v. 2.3.4 and GENELAND v.4.0.3, Guillot et al., 2008, 2012) and a general maximum likelihood method (PSMIX, Wu et al., 2006) were implemented.

Two procedures were adopted in STRUCTURE: a 'blind' simulation, neglecting any prior information, and an 'informed' analysis, where samples were attributed 'a priori' to one of the subpopulations identified in the previous study (Scandura et al., 2011). In the latter, the attribution to a subpopulation was based only on the geographic position of samples. Samples collected in municipalities that stretch in between two subpopulations were omitted. In total, 165 individuals were ascribed to the Eastern (ES), 50 to the North-Western (NWS) and 62 to the South-Western (SWS) subpopulations.

The first ('blind') analysis was performed with the following settings: K = 1-10, 10 independent Monte Carlo Markov chain runs for each K, admixture model, no population information, correlated allele frequencies, 200,000 + 200,000 iterations (burn-in + data

collection). The optimal K-value was chosen according to the ΔK statistics developed by Evanno et al., (2005).

In the second ('informed') analysis the same settings were used, except for the incorporation of population information in the prior (usepopinfo function) and the number of K, which was fixed to 3 (equal to the number of expected subpopulations). Thereby we forced the algorithm to calculate the assignment probability to three inferred clusters, which were expected to correspond to the three assumed subpopulations.

To visualize the genetic population structure across geographic space we used the inverse distance weighted (IDW) interpolation in Spatial Analyst tool in ArcGis (as in Vandergast et al., 2011) to interpolate Q-values.

A further 'informed' analysis was implemented in GENELAND, a Bayesian clustering algorithm that use both geographic and genetic information to estimate the number of subpopulations, to assign each individual to the subpopulation of origin and to identify possible migrants between subpopulations. The method used by the program to identify spatial patterns is in the Poisson-Voronoi tessellation (Muche, 2005). It assumes that there is an unknown number of polygons that approximate the true partition into subpopulations across space. The area covered by each subpopulation can be approximated by the union of polygons. We determined the membership of individual to each population by running the algorithm 10 times, with the following settings: K = 3, 100,000 MCMC iterations, with thinning of 50 and 500 burn-in, admixture model, correlated allele frequencies, amount of uncertainty on spatial coordinates equal to 500 metres. The run providing the highest average posterior probability was considered for population membership.

Additionally, we used PSMix (Population Stratification inference via Mixture model; Wu et al., 2006), an R package based on maximum likelihood methods using the Expectation-Maximization (EM) algorithm (Dempster et al., 1977). Program settings were: K = 3 with 100,000 iterations in the EM algorithm, admixture model and a convergence criterion eps = $1 \times e^{-10}$. Results consisted in estimates of individual membership to the three inferred clusters.

Finally, we performed a FCA (Factorial Correspondence Analysis) in GENETIX v. 4.05 (Belkhir et al., 2004) to visualize non-introgressed genotypes in a three-dimensional space on the basis of their genetic proximity.

Isolation by distance, by resistance and by barrier

As the presence of a strong isolation by distance (IBD) pattern in the population can induce to overestimate the real number of genetic clusters (Frantz et al., 2009; Guillot, 2009; Schwartz and McKelvey, 2009), we used GenAlEx 6.4 (Peakall & Smouse, 2005) to evaluate the occurrence of IBD in the Sardinian population by Mantel test (Mantel 1967). A genetic distance matrix and a Euclidean distance matrix were constructed for this purpose.

Euclidean distances, however do not usually reflect the real routes used by species. In fact, individual movements are influenced by different landscape elements that are not taken into account when using linear distances. Accordingly, to account for the influence of landscape features on gene flow in the island, we calculated the least-cost distances (LCD) between sample locations. The LCD is an "effective distance", calculated as the shortest distance between two points, corrected by considering the cost of the species movement across different habitat patches (i.e. the habitat resistance). In order to obtain the LCDs, we assigned a weight to each land cover category (Tab. 1), with regard to the habitat permeability to wild boar and its use of the habitat (feeding, refuge), using Spatial Analyst Tool in ArcGIS (Fig. 1b). For each of the 6451 2x2 km cells we then calculated a crossing cost, by multiplying the percentage surface of each land cover category for its weight. In this way, we created a map of (presumed) resistance to the wild boar movements and we were able to calculate the LCDs in ArcGis. The matrix of pairwise LCD was then used as distance matrix and compared with genetic distance in Mantel tests.

In addition to the effect of land cover on gene flow, we also evaluated the influence of physical barriers represented by conspicuous infrastructures. In particular, we considered the S.S. 131 'Carlo Felice'. In order to test isolation by barrier (IBB) due to the presence of the motorway, we produced two different matrices to be compared with the genetic matrix. Firstly, we produced a binary model considering the presence/absence of the barrier between two samples (1 = presence; 0 = absence). Secondly, we considered the number of times an individual should cross the barrier to reach a different area in the island. In this case the matrix included three values: 0 = no crossing, 1 = single crossing and 2 = double or multiple crossing. To evaluate the relative effectiveness of Euclidean distances, least-cost paths and physical barriers in explaining the observed genetic pattern, the four different spatial distance matrices were used to perform Partial Mantel tests (Smouse et al., 1986)

using the vegan package (Oksanen et al., 2007) in R. Significant correlations were determined by the calculation of Pearson product-moment correlation using a permutation test with 999 replicates. Monte Carlo *p* -values were calculated to determine the significance of partial Mantel tests.

Finally, we also evaluated the presence of IBB by detecting barriers to gene flow with the software BARRIER v. 2.2 (Manni et al., 2004), which implements the Monmonier's (1973) maximum difference algorithm. The software detects edges associated with the highest genetic diversity, statistically tested by resampled bootstrap matrices of molecular data. The graphical pattern of the genetic boundaries is computed by the Delaunay triangulation (Brassel and Reif 1979). In the BARRIER analysis, the pruned Sardinian STRs dataset was divided into 16 sampling areas on the basis of the geographic proximity of samples and a F_{ST} distance matrix was calculated among areas to detect the most conspicuous genetic boundaries (i.e., only 16 geographic locations were considered in this analysis due to the limits of the algorithm).

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Results

The total number of different alleles in the Sardinian sample was 154, with a range of 6-16 and an average of 9.63 ± 3.18 (standard deviation, SD) per locus. Missing alleles represented 2.17 % of the dataset. MICRO-CHECKER did not find scoring errors in the dataset or evidence of large allele dropout. Not surprisingly for a structured population, a significant deviation from Hardy-Weinberg equilibrium was detected in the overall population (all loci $P<0.01$) and several pairs of loci resulted in linkage disequilibrium (51/120 at $\alpha=0.05$ and 45/120 at $\alpha=0.01$).

Identification of immigrant/introgressed individuals

Similarly to the results obtained by Scandura et al. (2011), the Bayesian analysis in STRUCTURE sharply distinguished the four main populations (Sardinian WB, Italian WB, European WB, and domestic pigs) in the overall sample of 696 individuals. However, this result was not achieved at K = 4, but when K was equal to 5. Each population was univocally assigned to one of the inferred clusters (with $Q>0.9$), with the exception of Sardinian wild

boars which were mainly assigned to two clusters (I and III, summed to obtain a "Sardinian cluster", Q_{Sar}), corresponding to a cumulative average membership of 0.886 (Fig. S1 Suppl. Mat.). Hence, to conservatively assess which individual was a possible recent immigrant or affected by introgression, we applied the threshold of Q_{Sar} >0.9.

A relevant degree of introgression was found in the Sardinian sample: 75.8% of individuals (n=279) had Q_{I+III} > 0.90 and were attributed to the Sardinian population; 12 samples were attributed to the domestic pig cluster with a Q_V > 0.80, while 36 individuals showed 0.80 < Q_{I+III} < 0.90. The remaining wild boars (n=41) showed an admixed ancestry and could not be assigned to any population (all Q-values < 0.8, Fig. 2). For further analyses we thus removed from the dataset 89 admixed individuals (=12+36+41), with the addition of other two individuals, that, because of uncertainty on the death place, could not be attributed *a-priori* to any subpopulation. Consequently the final pruned dataset was composed by 277 individuals.

Genetic structure of the Sardinian population

The 'blind' Bayesian analysis in STRUCTURE on the pruned dataset confirmed the results obtained by Scandura and coauthors (2011), detecting K = 3 as the most likely partition and identifying three subpopulations in the island: one in the north-west (NWS), one in the south-west (SWS), and one including all eastern Sardinia (ES, Fig. 3). In total, 160 individuals were assigned (on the basis of the highest Q-value) to the ES cluster, 58 to the NWS, and 59 to the SWS. Only 17 individuals (6.1%) were assigned to a different population from what expected on the basis of the sampling site. Our results confirmed the presence of an abrupt genetic discontinuity in coincidence with the S.S. 131 and the Campidano plain.

The 'informed' analysis at fixed K=3, as expected, improved the allocation of samples to the respective sampling area. All samples but two (99.3%) were assigned to the expected subpopulation. The same result was obtained with the analysis in GENELAND (Tab. 2). PSMIX showed a similar partition into subpopulations to that obtained with STRUCTURE without any prior geographical information. In this case, 23 samples (8.3%) were not attributed to the expected subpopulation (Tab. 2).

The consistency of results obtained by different analytical approaches points to a sharp structuring in the island, with a limited ongoing gene flow between subpopulations. Pairwise differentiation was maximum for the NWS-SWS pair (F_{ST} = 0.161) and minimum for ES-NWS

(F_{ST} = 0.089), as confirmed by the FCA plot (Fig S2 Suppl. Mat). As expected (see Scandura et al., 2011), HWE and LE analyses within subpopulations revealed a sensitive reduction of significance with respect to the global population (HWE: 3/16 deviations at α=0.01 in ES, no deviation in NWS and 4/16 deviations at α=0.01 in SWS; LE: 1/120 loci pair at α=0.05 in ES, 2/120 at α=0.05 in NWS, 4/120 at α=0.01 in SWS).

Influence of distance and landscape on gene flow

The Mantel test performed using the Euclidean distance matrix showed the presence of a weak degree of IBD in the population (R_{xy} = 0.099, p < 0.001; Fig. 4a), suggesting a more complex genetic pattern in the island. In fact, there was a huge gain in correlation between genetic data and spatial distances when the least cost matrix was used in the Mantel test instead of the Euclidean distance matrix (R_{xy} = 0.337, p < 0.001, Fig. 4b), indicating an IBR pattern (e.g., influence of land use variables on the gene flow). A similar correlation, however, was obtained using either the binary barrier matrix (R_{xy} = 0.365, p <0.001) or the matrix accounting for the number of crossings (R_{xy} = 0.337, p <0.001), underlining the key role of the S.S.131 in shaping the observed spatial pattern of variation. Such results were confirmed by partial Mantel tests, where genetic distances were well explained by least-cost and barrier matrices, even when controlling for the effect of Euclidean distance (Tab. 3).

In accordance with our previous results, the barrier predictor analysis using the Monmonier's maximum difference algorithm identified two major barriers (Fig. 5). The first barrier separated sampling locations belonging to the SWS subpopulation from the rest of samples, emphasizing its strong genetic differentiation. The second barrier separated the NWS subpopulation from sampling sites occupying the south-western and the eastern part of the island. Again, both putative barriers matched quite well the extension of the motorway S.S. 131.

Discussion

This study reveals how the joint effect of distance and landscape features can generate genetic discontinuities across a large mammal population. A weak IBD pattern was revealed in the Sardinian wild boar population. Contrarily, IBR and IBB were shown to be important in

shaping the observed genetic pattern, the former due to the effect of environmental variables on gene flow and the latter being associated to the effect of human infrastructures.

In the present study we have enlarged the dataset used in Scandura et al. (2011) by including a larger sample of Sardinian wild boars (from 210 to 368) and increasing the number of autosomal markers (from 10 to 16). Nonetheless, new results confirmed the partition into the same three subpopulations (ES, NWS and SWS) that had been previously detected. Three different statistical approaches (STRUCTURE, GENELAND and PSMIX) gave full support to such genetic structure, thus accomplishing recommendations given by Balkenhol et al. (2009) and Frantz et al. (2012).

A signature of recent gene introgression from continental wild boars and domestic pigs was also confirmed. Specifically, gene introgression seemed to affect mainly the eastern subpopulation, while the north-western and south-western ones were marginally interested. In total, almost 25% of individuals sampled in the island were recognized as putative hybrids and their exclusion from population structure analyses prevented the confounding effect possibly arising from the local occurrence of exogenous alleles.

An IBD pattern was observed in the population and was evident even neglecting landscape features (i.e. using Euclidean distances between sampling sites). Nonetheless, the presence of a sharp genetic structure suggested the existence of barriers to gene flow in the island. IBD was observed in other European wild boar populations at a local scale, whereas it appeared to be absent at a continental scale (Scandura et al., 2008). Both Frantz et al. (2009) and Goedbloed et al. (2013) found a IBD pattern in populations of Central-Western Europe, detected in spite of the non-negligible genetic introgression from alien or domestic sources (see also Frantz et al., 2013).

As expected, the genetic diversity within the Sardinian population was better explained when landscape features, i.e. land use and the main motorway (S.S. 131), were taken into account. This result suggests that the presence of unsuitable habitats and man-made infrastructures can effectively limit wild boar movements in the island. Particularly, the sudden genetic differentiation between western and eastern subpopulations seemed to occur in conjunction with the motorway S.S. 131 (Fig. 3).

When the assignment tests were performed using the geographical information on the individuals as prior, the majority of individuals (> 96%) were assigned to the respective sampling subpopulation. In contrast, in the analysis based only on the genetic information, the number of misassigned individuals increased and included especially individuals sampled near the motorway. Such result would suggest that wild boar could disperse across the motorway, but at a very low rate.

The impact of the motorway seemed to be stronger in the south, where the major barrier was detected. This is supported by the records of road accidents along the S.S. 131. During the last 10 years (2001-2011, Autonomous Region of Sardinia, unpublished data), 106 casualties involving wild boars were recorded through the complete extent of the S.S. 131. Most of them occurred in the road section separating NWS and ES subpopulations. Therefore, the northern part of the highway seems to be more frequently crossed by the wild boar.

Furthermore, the reduced gene flow between SWS and ES can also depend on the high resistance to the species given by the Campidano plain (Fig. 1b). In this area, the motorway connects two major urban centres (Cagliari and Oristano), crossing a lowland characterized by important suburban and industrial surfaces as well as agricultural crops, more affected by human disturbance. Hence, in this case a pattern of isolation by resistance (McRae 2006) is likely to prevail.

The NWS and SWS populations appeared reciprocally isolated (i.e., no recent gene flow), probably due to the breaking presence of the urban area of Oristano, delimited in the west by the coast and in the east by the S.S. 131.

Notably, such barriers in Sardinia have also prevented the spread of introgressed genes from the eastern subpopulation to the rest of the island and probably safeguarded the genetic integrity of the SWS subpopulation (seemingly the purest one). This is a very interesting case in a conservation viewpoint, as only negative effects are typically associated to anthropogenic barriers.

But can the current landscape, which has been mostly influenced by recent transformations, explain the high genetic differentiation observed across the island?

Actually, although a long time lag is usually expected between a causal factor and a detectable genetic effect, simulations have proved that a limited number of generations (as small as 15) can be sufficient for the genetic signature of a landscape barrier to become detectable (Landguth et al., 2010). Accordingly, several studies exploring genetic discontinuities linked to linear barriers have documented the relevant effect of infrastructures built only 10-40 years before (Epps et al., 2005; Pérez-Espona et al., 2008; Hepenstrick et al., 2012). This time span is similar to that elapsed from the broadening of the S.S. 131, which is likely to have played an important role in shaping the observed genetic pattern.

However, unlike our study, Frantz et al., (2012) found that a motorway had no influence on the genetic structure of a wild boar population in Belgium. In contrast to S.S. 131, this road had many subways and underpasses available for wildlife. Furthermore, despite the lack of population sub-structure between the two sides of the road, Frantz et al. (2012) did not exclude that the road acted as a barrier, but that other factors (like a large N_e) could have masked it. Likewise, Vassant et al. (1993) argued that wild boars were not impacted by the motorway A5 in France, as marked individuals regularly crossed the road taking advantage of wildlife corridors. In our case the Sardinian motorway is almost devoid of corridors that facilitate movements of the local fauna for an entire stretch of about 200 kilometres. Unfortunately, to date, nothing is known about the spatial behaviour and habitat preferences of the Sardinian subspecies and we cannot exclude differences from the continental counterparts. Future projects should be addressed to combine population genetics and ecology to test the actual impact of land use and roads on the spatial patterns of *Sus scrofa meridionalis*.

Our results have direct consequences on the management of wild species in Sardinia. Given that the motorway has seemingly an important role as barrier to gene flow in the wild boar population, it could as well represent a cause of fragmentation for other species, promoting local genetic isolation. The effect on other species should be tested by targeted studies, as the same infrastructure might have different impact on different species (see for instance Frantz et al., 2012).

Finally, possible long-term detrimental effects (small population size, inbreeding, genetic drift) of habitat fragmentation should be carefully evaluated in the wild boar, in order to promote a sustainable management of its genetic resources.

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Table 1. Land cover categories used to describe the Sardinia environment. Each category was associated to a specific weight, assigned on the basis of the permeability to the species and the use of the habitat by wild boar (feeding, refuge).

Land use category	Description	Categories weight
Anthropic areas	Urbanized and industrialized areas	100
Wetland	Rivers, water basins, swamps	80
Open areas	Nude land, rocks	60
Cultivated areas	Arable land, fruit and olive trees, vineyards	30
Pastures	Meadows dedicated to livestock	20
Shrubs	Unused land with low and bushy vegetation	10
Maquis	Typical Mediterranean maquis	2
Forest	Deciduous, coniferous and mixed forests	0

Table 2. Results of genetic assignment tests of Sardinian wild boars to three subpopulations (Eastern, E; North-Western, NWS; South-Western, SWS) on the basis of different statistical methods. Stru1- 'blind' Bayesian assignment in Structure (no population information), Stru2 – 'informed' Bayesian assignment in Structure (geographical information), GL – Bayesian assignment in Geneland (geographical information), PM – maximum likelihood assignment in PSMix (no population information).

		Area of assignment											
		ES				**NWS**				**SWS**			
		Stru1	Stru2	GL	PM	Stru1	Stru2	GL	PM	Stru1	Stru2	GL	PM
Sampling area	**ES**	**94%**	**99%**	**100%**	**89%**	5%	1%	0%	9%	1%	0%	0%	2%
	NWS	4%	2%	4%	2%	**96%**	**98%**	**96%**	**98%**	0%	0%	0%	0%
	SWS	5%	0%	0%	2%	3%	0%	0%	3%	**92%**	**100%**	**100%**	**95%**

Table 3. Results of partial Mantel tests: correlation between genetic distance and different spatial distances in the Sardinian wild boar population. Geo = Euclidean distance matrix; Bar = either matrix of presence/absence (1/0) of physical barrier, or matrix accounting for the number of barrier crossings (2/1/0); LeastC = least-cost distance matrix.

Partial Mantel Tests						
			Barrier 0/1		Barrier 0/1/2	
Matrix 1	Matrix 2	Controlled variable	*R*	*P*	*R*	*Pval*
Gen	Geo	Bar	0.081	0.001*	0.076	0.001*
Gen	Geo	LeastC	0.075	0.001*		
Gen	Bar	Geo	0.361	0.001*	0.331	0.001*
Gen	Bar	LeastC	0.193	0.001*	0.145	0.001*
Gen	LeastC	Bar	0.123	0.001*	0.143	0.001*
Gen	LeastC	Geo	0.331	0.001*		

Legends to figures

Figure 1. Maps of Sardinia showing information used in landscape genetic analysis. (a) Map showing land use categories, sample locations and main roads. (b) Map showing cell resistances used to calculate least cost paths among sampling sites (in red).

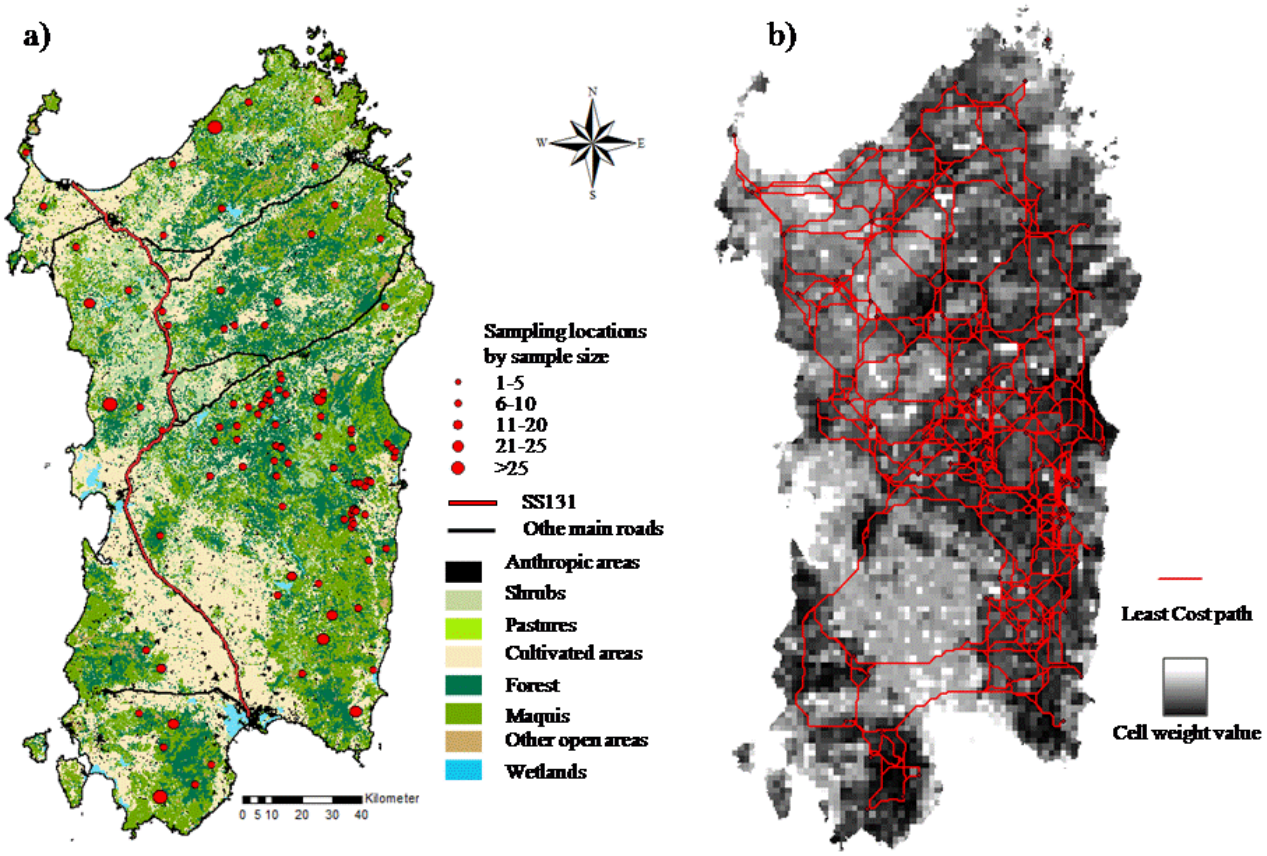


Figure 2. Assignment proportions obtained by STRUCTURE for each of the 368 Sardinian wild boar genotypes. Individuals are represented by thin vertical lines, showing the membership (Q) to the clusters inferred by the program (colored segments). Membership to clusters I and III (in yellow), both exclusive to the Sardinian population, were pooled. Only individuals that were univocally assigned to the Sardinian component ($Q_{I+III} \geq 0.9$, i.e. left to the solid black line) were identified as non-introgressed members of the Sardinian population and used for the inference of population structure (n = 277).

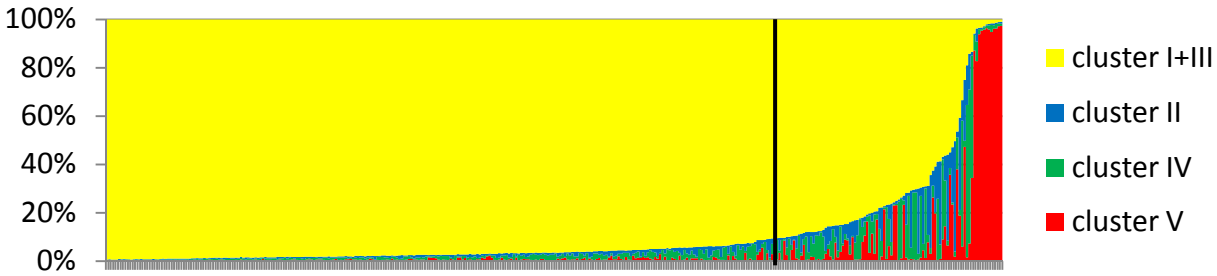


Figure 3. Genetic structure of the Sardinian wild boar population inferred by STRUCTURE. (a) Log-likelihood values for the different K values in the simulation and corresponding outcome of the Evanno's method. Three subpopulations were identified: North-Western (NWS), South-Western (SWS) and Eastern Sardinia (ES). (b) Graphical representation of individual membership proportions of wild boars to the three subpopulations, obtained by the Inverse Distance Weight (IDW) interpolation method.

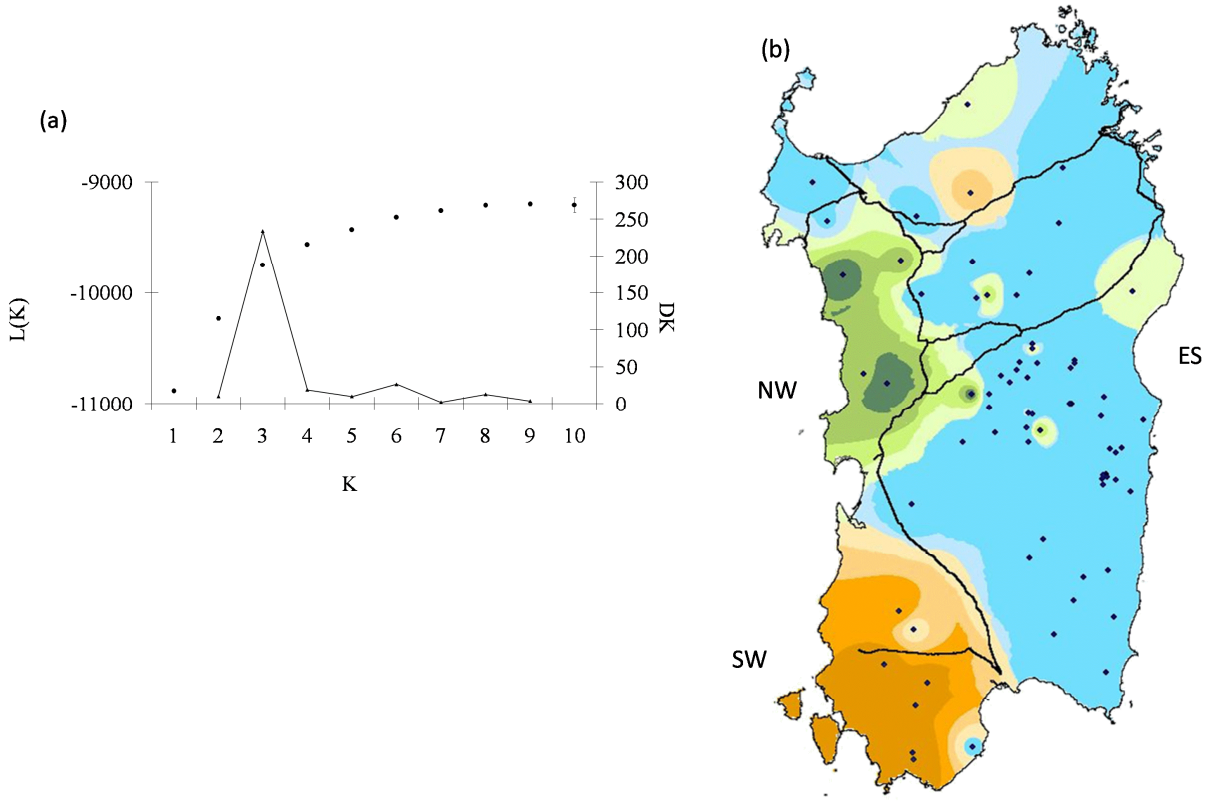


Figure 4. Correlation between genetic and geographic distance in the Sardinian wild boar population, as resulting from Mantel tests in Genalex, excluding putative hybrids (purged dataset, n = 277). In the upper plot (a) geographic distances refer to the Euclidean distances between sampling sites; in the lower plot (b) geographic distances refer to least-cost distances.

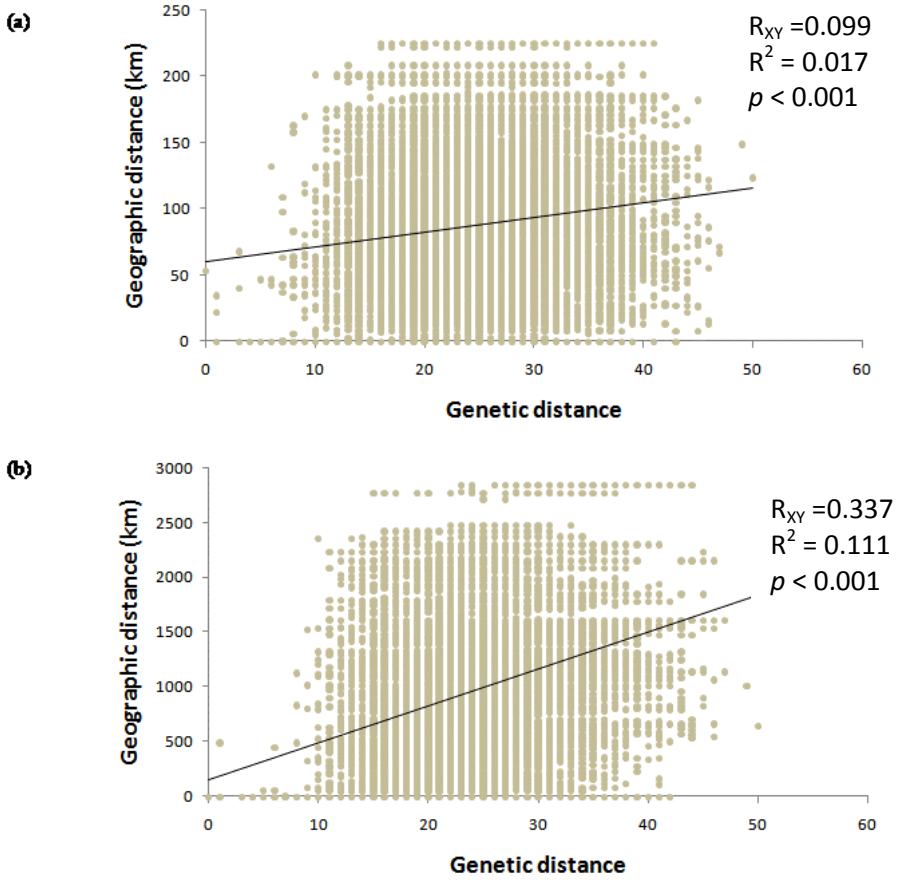
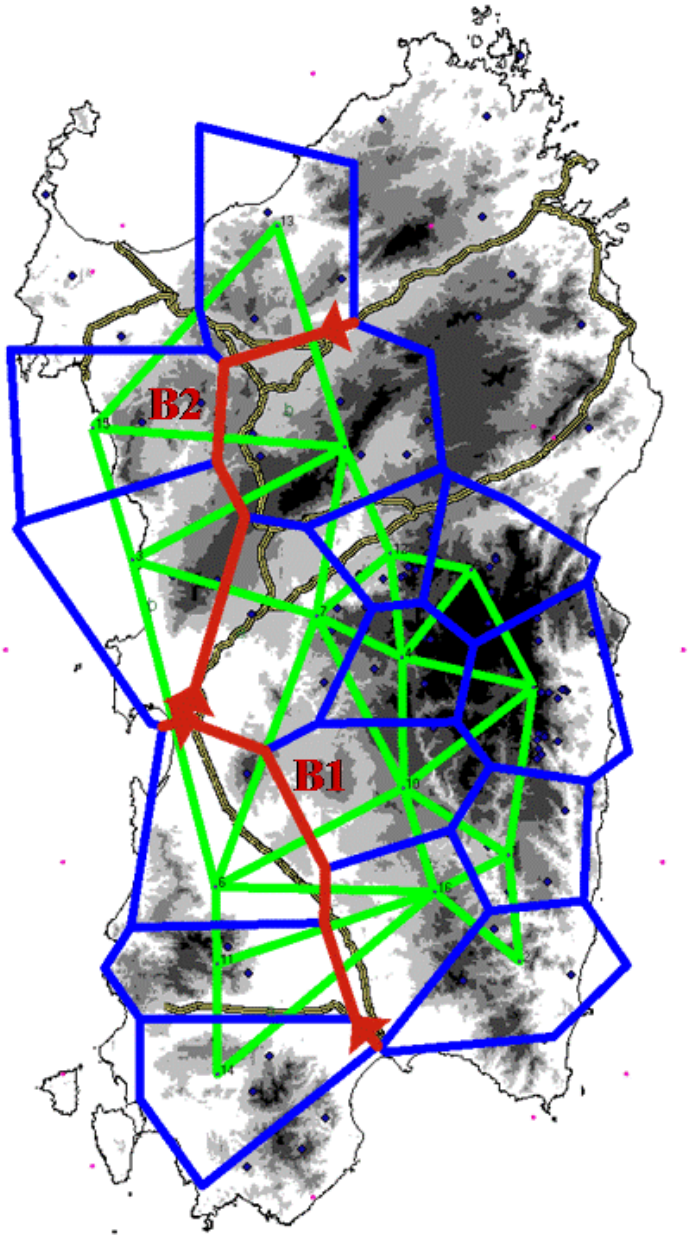


Figure 5. Barriers to gene flow identified by the Monmonier's algorithm in the Sardinian wild boar population. Major barriers are represented by red lines (B1 and B2). Green and blue polygons represent respectively the Delaunay triangulation linking sampling sites and the Voronoi tessellation used by the program BARRIER.



Supplementary Materials

Table S1. List of samples of continental wild boar and domestic pig used as reference populations for assignment tests.

Reference population	Country/breed	Number of individuals
Italian WB	Central-Southern Italy	75
European WB	Spain	15
	France	15
	Luxembourg	10
	Austria	13
	North-East Italy	19
	Poland	43
	Belarus	24
Domestic pigs	Commercial breeds	16
	Sardinian free-ranging	98

Figure S1. Assignment proportions to the five clusters (I-V) identified by Structure for the four populations in the global dataset (n=698), namely: Italian wild boar (WB-Ita), European wild boar (WB), domestic pigs (DP) and Sardinian wild boar (WB-Sar).

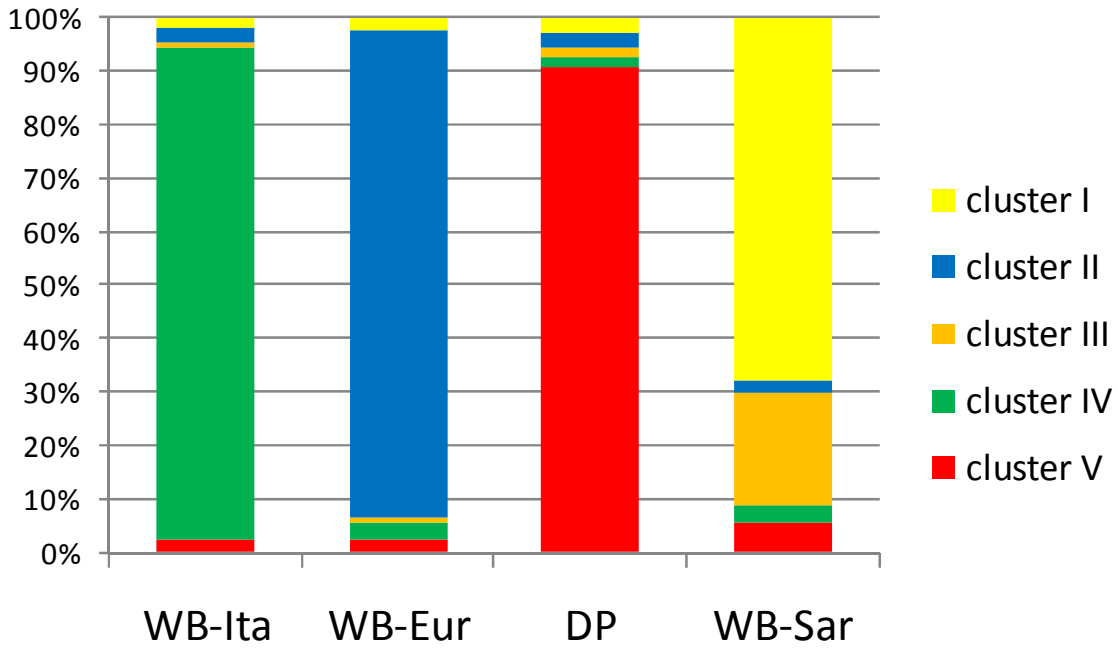
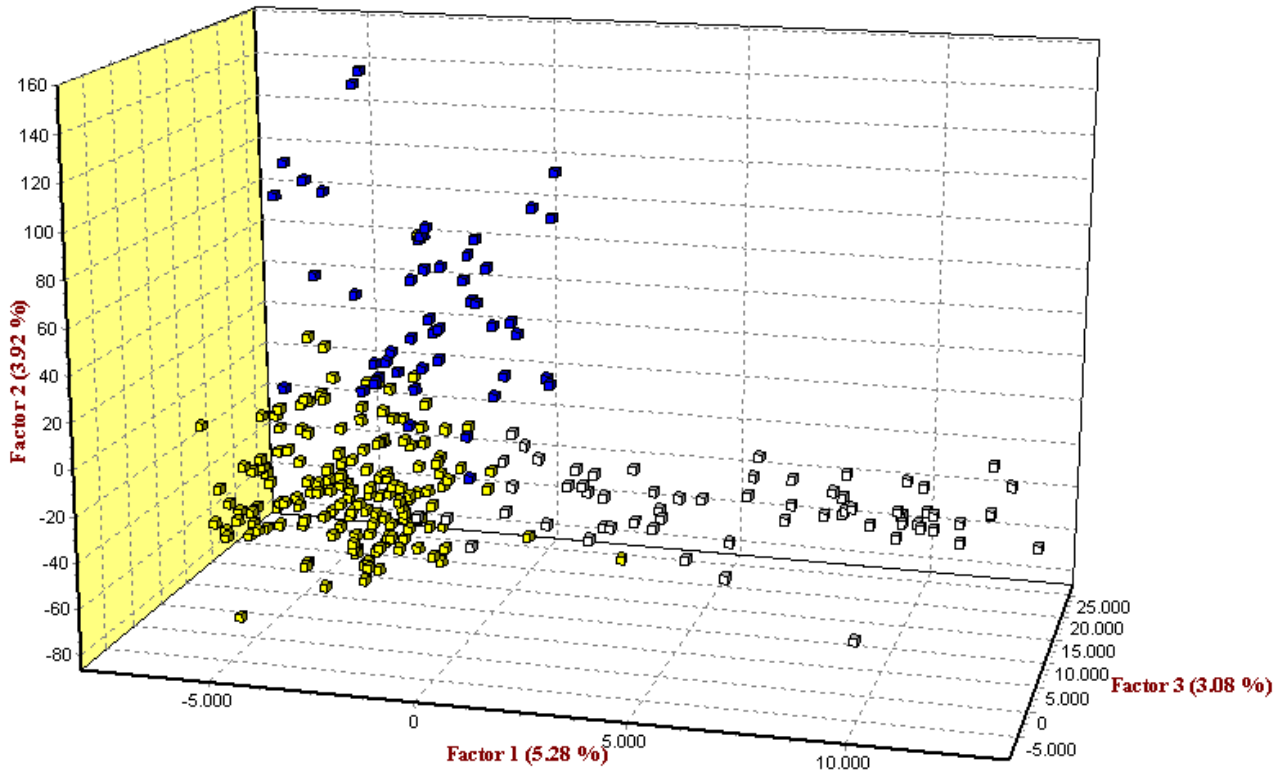


Figure S2. Factorial Correspondence Analysis (FCA) plot of multilocus genotypes belonging to the three subpopulations (ES – yellow, NWS – blue, SWS – white).



CHAPTER 5

Reproductive phenology and conception synchrony in a natural wild boar population

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Research Article

Reproductive phenology and conception synchrony in a natural wild boar population

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Abstract

Reproductive synchrony among gregarious mammals has a strong adaptive value and may lead to cooperative behaviors aimed at maximizing offspring survival. Additionally, temporal clustering of estrus has important implications on individual mating tactics and ultimately affects the degree of polygamy in a population. Although several studies have examined the reproductive biology of wild boar (*Sus scrofa*), much remains to be understood about the patterns of timing and synchrony of reproduction in natural populations. We analyzed the spatiotemporal distribution of conception dates in an Italian wild boar population taking into account the effects of environmental and individual factors, in order to determine the main variables influencing the timing of reproduction and to detect the signs of a socially-driven reproductive synchrony. Specifically, for each litter belonging to 354 pregnant sows culled between 2006 and 2013 in a mountain area of Tuscany, we determined the conception date (CD) from an estimate of the mean fetal age and the culling date. We then investigated which factors drove the variation in CD, by implementing linear mixed models, Mantel tests and spatial autocorrelation analyses. The selected model showed significant effects of rainfall, temperatures, and previous and current productivity on CD, as well as a strong correlation of CDs among sows culled in close spatial and temporal proximity (i.e., in the same hunting ground and hunting season). Likewise, autocorrelation analyses and Mantel tests consistently indicated that closer sows had similar conception dates. Overall, our results confirm the effect of resource availability and climate on wild boar reproductive phenology, and suggest socially-driven reproductive patterns, in spite of a high turn-over in social groups due to hunting. Finally, possible advantages and evolutionary implications of reproductive synchrony in wild boar are discussed.

Introduction

Reproductive or breeding synchrony refers to the tendency of individuals to carry out some stages of the reproductive cycle (e.g., courtship, mating, birth) at the same time as other individuals of the population (Findlay and Cooke, 1982). Among gregarious mammals, breeding synchrony usually occurs by adjustment of the timing of estrus, but may also occur by gestation adjustment (Berger, 1992). Reproductive synchrony may have a strong adaptive value in that individuals capable of synchronizing their activities with neighboring conspecifics may have a number of selective advantages. For example, high density of newborns may lead to the satiation of predators (swamping), while decreasing the likelihood for a given newborn to be preyed upon (Plard et al., 2014). Moreover, breeding synchrony may account for an increased efficiency in food localization (Findlay and Cooke, 1982), a higher probability of detecting and repelling predators, and the possibility for the young to be communally nursed (e.g., adoptions, allosuckling etc.; Ims, 1990). Reproductive synchrony may have evolved as a mechanism to enforce monogamy in certain species. However, in systems in which males have the capability to monopolize spatially aggregated females, female reproductive synchrony can result in polygyny rather than monogamy (Ims, 1990).

On a population level, reproductive synchrony is especially common in seasonal environments, where individuals may select the same favorable time for reproduction in relation to climate and resource availability (Ims, 1990). Indeed, especially in these environments, reproductive phenology of individuals is a key determinant of fitness, with the timing of reproduction affecting their reproductive output and future performance (e.g., lifetime reproductive success; English et al., 2012). In wild ungulate populations, inter-annual changes in resource availability (English et al., 2012), plant phenology (Post et al., 2003; Owen-Smith and Ogutu, 2013), and variations in rainfall (Moe et al., 2007; Ogutu et al., 2014; Plard et al., 2014) were shown to be associated with the timing and synchrony of births. Nevertheless, reproduction is often much more synchronous than expected if environmental seasonality alone were taken into consideration. Indeed, the temporal pattern of reproduction may also be shaped by many physiological, ecological and socio-biological processes (Ims, 1990). In fact, in several ungulate species, individual factors such as age, female condition and previous annual reproductive output were reported to affect spatiotemporal variation in ovulation and/or birth date (e.g., Garel et al., 2009; Plard et al., 2014). Moreover, exogenous factors such as hunting pressure and natural predation may play a role in determining spatiotemporal patterns in reproduction (e.g., Wissel and Brandl, 1988; Post et al., 2003). In some cases, the possibility that individuals have to adjust the timing of reproduction is constrained: in bighorn sheep (*Ovis canadensis*), for example, parturition date was reported to be partly heritable (Feder et al., 2008). However, the driving factor is often to be found in any biological interactions that may lead to tight clustering of reproductive events. This translates into reproductive synchrony on a social unit level. In several species of birds and mammals (including humans), social stimuli exchanged between neighboring females were found to induce reproductive synchrony (Ims, 1990; Mc Clintock, 1998). In particular, as for mammals, estrus synchrony can be achieved through

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the exchange of pheromones among females, or through the exposure to a male (Ims, 1990); indeed, the role of chemical signals was confirmed by experiments with hormone-like compounds (Thompson and Monfort, 1999; Jacob et al., 2004). Also spatial patterns of reproductive events can suggest that reproductive synchrony may be socially induced. For example, reproduction was shown to be more synchronized among neighboring individuals than among more distant ones in black-headed gull (*Larus ridibundus*) (Wissel and Brandl, 1988), wildebeest (*Connochaetes taurinus*) (Estes, 1976) and musk deer (*Moschus sifanicus*) (Meng et al., 2003). In red deer (*Cervus elaphus*), related females tend to group together, and this association was found to lead to synchronous estrus within kin groups (Iason and Guinness, 1985).

Despite many studies conducted in the past, much remains to be understood about the patterns of reproductive synchrony in several ungulates, including wild boar (*Sus scrofa*), a key species for wildlife management. Wild boar populations have been growing considerably over the past decades in both their native and introduced ranges (Massei et al., 2015), thus affecting both community structure and ecosystem function, but also impacting local economy, by causing extensive crop damages and vehicle collisions, and by transmitting diseases to livestock and wildlife (Schley et al., 2008; Apollonio et al., 2010; Barrios-Garcia and Ballari, 2012). Compared with other European ungulates, the wild boar is characterized by such peculiar life-history traits, as early onset of puberty (between 5 and 10 months of age; Fonseca et al., 2011), high fertility (with mean litter size ranging from 3.05 to 6.91 in different European populations; Bywater et al., 2010), and a relatively short gestation period (around 115-122 days; Henry, 1968; Vericad, 1983). Females have an estrus cycle of about 21-23 days, are receptive for 1-3 days (Henry, 1968), and generally produce one litter per year. Births have been reported from February to November, generally with a single peak in spring or late winter. However, a bimodal distribution was observed in some years (Markina et al., 2003; Maillard and Fournier, 2004), possibly due to resource availability and genetic introgression from the domestic pig. Habitat quality, climatic conditions, photoperiods, hunting pressure, amount of resources (particularly acorn and chestnut mast; Maillard and Fournier, 2004), and supplementary food were shown to affect many reproductive parameters in wild boar, including the timing and synchrony of births (e.g., Šprem et al., 2011). Servanty et al. (2009) demonstrated that the breeding probability and the onset of estrus in females respond to variations in female body mass at different ages under varying conditions of climate and resources availability. Furthermore, they showed that multiparous females can adjust the timing of their estrus, inducing a time delay in the reproduction, so that gestation may start in a different month every year.

However, little is known about sociobiological patterns of reproductive synchrony in wild boar. Their social organization is centered around philopatric adult females (Podgórski et al., 2014). Although the usual social unit is composed of a matrilineal group with one or more related adult females and one or more cohorts of offspring, deviations from this pattern were found (Iacolina, 2009). An outstanding contribution to the understanding of reproductive processes in wild boar was given by Meynhardt (1984), who made observations on mating habits and social behavior in several groups of free-living wild boar in Germany in the 1970s and 1980s. Meynhardt documented the existence of group-specific and highly repeatable (from year to year) dates of reproduction, and observed a very high within-group estrus synchrony, with the greatest intra-group difference in the date of estrus amounting to 8 days. Meynhardt also emphasized the key-role of the group leader sow: after her death, the group of females can experience a delay in reproduction and a loss of estrus synchrony. Furthermore, he observed no reproductive synchrony among different social units within a population, and no influence of mast production and group age structure on the timing of reproduction. Likewise, Dardaillon (1988) observed a good synchronization of births in wild boar groups in Camargue, and Delcroix et al. (1990) reported the occurrence of accurate within-group synchronization in reproductive processes in two groups of female wild boar kept in semi-natural conditions.

Nevertheless, given the number of factors which can affect ovulation time, as discussed above, and the possible variation of such factors in different ecological contexts, we expected population-specific deviations from the scenario described by Meynhardt (1984).

In the present study, we analyzed the spatiotemporal distribution of conception dates in an Italian wild boar population living in a less predictable environment in comparison to Central Europe, and exposed to a very high hunting pressure (with a substantial turnover in the social group composition, see Iacolina, 2009). In these conditions, within-group reproductive synchrony could be either reduced or not even observed.

We tested the occurrence of two alternative patterns of conception dates in the study population:

H0: Random distribution of conception dates (no reproductive synchrony is seen on a group level, and there is no influence of environmental factors);

H1: Conception dates respond to social and/or environmental factors, creating a spatial pattern in our study area.

The occurrence of similar conception dates on a local scale (patchy pattern) may indicate within-group estrus synchrony and/or the presence of local patterns in some environmental factors influencing the wild boar reproductive phenology. We accounted for the effects of environmental and individual factors, in order to detect the signs of reproductive synchrony with a possible sociobiological basis. According to the literature, we predict that higher age (Gethöffer et al., 2007), good body conditions (Servanty et al., 2009) and favorable environmental conditions (e.g., see Aumaître et al., 1984 and Maillard and Fournier, 2004 for the effect of a greater food availability; see Plard et al., 2014 for the effect of rainfall) would anticipate the reproduction in the wild boar population.

Materials and methods

Data collection and reproductive tracts analysis

Data were gathered in an area of the Tuscan Apennines (Province of Arezzo, Central Italy), which extends over 134 km^2 and includes a protected area, the Oasi Alpe di Catenaia (OAC, 28 km^2). Elevation ranges from 330 to 1414 m a.s.l., with main peaks within the OAC. The study area is mainly covered with deciduous forests (67%), consisting of beech (*Fagus sylvatica*) at altitudes higher than 900 m, and Turkey oak (*Quercus cerris*) and chestnut (*Castanea sativa*) at lower altitudes. Conifer forests of black pine (*Pinus nigra*), silver fir (*Abies alba*) and Douglas-fir (*Pseudotsuga menziesii*) represent 7% of the area, while cultivated areas cover around 16%, and shrubs and pastures 7%. This area has a continental climate, with hot and dry summers, cold and rainy winters, high humidity rate and occasional snow from October to April above 100 m a.s.l.. Red fox (*Vulpes vulpes*) and wolf (*Canis lupus*) inhabit the study area, with the wild boar representing the most important prey for the latter (Bassi et al., 2012). Three hunting districts surround the OAC, subdivided into a total of 45 hunting grounds (Fig. 1) with a mean surface of 237±145 ha, where the wild boar is regularly hunted from October to January by means of battues (i.e., dog drives), with 30-50 hunters and several hounds. Data from culled wild boar were gathered in 28 of these areas (with a minimum of 3 and a maximum of 41 observations in each area) during eight hunting seasons from 2006/2007 to 2013/2014.

The weight of each wild boar and the place and date of culling were recorded (when no precise location was available, the centroid of the hunting ground was used in the data analyses). All individuals were aged according to their tooth eruption and replacement pattern, as well as their tooth consumption (Briedermann, 1986), and assigned to one of the following age classes: juvenile (< 12 months), yearling (12-24 months), and adult (> 24 months).

The reproductive tracts (uteri and ovaries) of 2313 females were collected and examined in the laboratory. The uteri were dissected for examination and the fetuses in each uterus of pregnant females were counted, weighed, and sexed (when possible). The fetal age (FA, in day) was then estimated by using the Huggett and Widdas (1951) formula, which had already been applied to the wild boar by Vericad (1983). Vericad

measured the gestation length in a sample of captive sows and the birth weight of piglets, and used fetuses of known age to validate the equation:

$$FA = \frac{(\sqrt[3]{mW} + 2.3377)}{0.097}$$

with mW being the mean weight of fetuses in a litter. For each female with at least one weighed fetus, an estimate of the conception date (CD) was determined by using the date of culling and the mean estimated age of the litter obtained with the Vericad method. CD was then converted into a numeric variable, by setting, for each year, March 01 (year x) = 0, and February 28 (year x+1) = 364. Differences across years in the mean and distribution of CD were tested by Kruskal Wallis test in R.

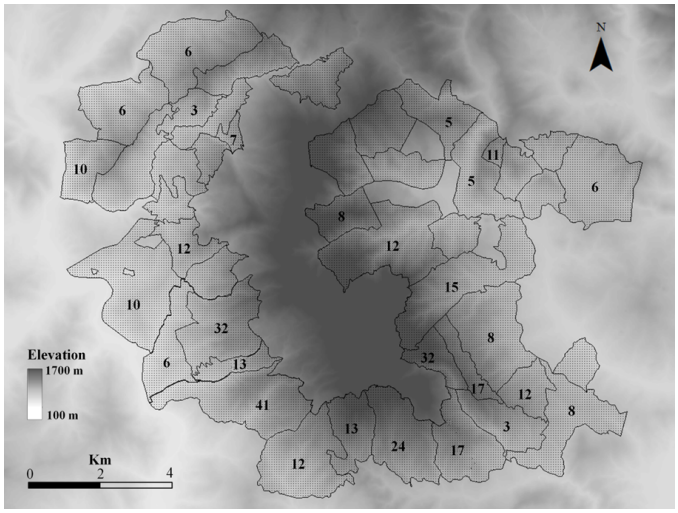

Figure 1 – Map showing the 45 hunting grounds (dotted) in the Italian Apennine study area and the distribution of the 354 culled sows used for the analyses. The protected area (Oasi Alpe di Catenaia), with higher elevations, is located among the hunting grounds.

Linear mixed models

CD was used as response variable in linear mixed models fitted by using R version 2.15.3 (R Core Team, 2013), with the following environmental and individual factors as fixed effects:

- (i-ii) individual factors (i.e., varying on an individual level): (i) BODY MASS, the weight of each individual; (ii) AGE, the age class of each individual (either subadult or adult). We removed juveniles from the dataset, since only two juvenile females with measurable fetuses were collected;

- (iii-iv) variables measured on a hunting ground scale, representing environmental heterogeneity within the study area: (iii) HABITAT, a categorical variable with two levels, summarizing the environmental features of each hunting ground. It was obtained by calculating the relative abundance of eight habitat types (i.e., deciduous, conifer and mixed forests, shrubs, sparse vegetation, crops, meadows/pastures and urban areas) in each hunting ground using ArcGis 10.1 (ESRI Inc., Redlands, CA, USA). A Hierarchical Cluster Analysis was then performed in R, and its consistency was ascertained by using the ClValid package (Brock et al., 2008), comparing the results of different clustering methods (hierarchical, divisive hierarchical, k-means, and model-based clustering) and groupings (2-6 groups of areas), by using the internal validation measures. A Principal Component Analysis was then performed in R on the same data, to help interpret the clustering obtained (i.e., which habitat types drove the clustering); (iv) BCT COVER, the sum of the relative abundances of beech, chestnut and Turkey oak, calculated for each hunting ground;

- (v-x) variables measured on an annual basis, referred to the whole study area, representing annual variations in climate, population density and resource availability: (v-vi) SEED PROD and PREV.SEED PROD, representing the annual seed production of beech, chestnut and Turkey oak in the study area in the current and the previous year, respectively. The data for seed production were available from three permanent plots of beech, chestnut and Turkey oak in the Alpe di Catenaia study area, each 1 ha in size (data available from Consiglio per la Ricerca e la sperimentazione in Agricoltura, Forestry Research Centre - CRA-SEL - Arezzo; Tab. 1 in Cutini et al. 2013 lists the main stand characteristics of each plot). Estimates of the annual seed production were obtained by using the litterfall method, whose procedures, sampling strategy, reliability and accuracy are described by Chianucci and Cutini (2013); (vii): CULLED, for each year, the total number of boars culled in the three hunting districts, recorded and reported to the Fish and Wildlife Service of the Province of Arezzo, which checked and validated data. Assuming a constant effort over years, we considered hunting bag records as a proxy of wild boar population density (see Davis et al., 2012; Cutini et al., 2013 for further details on the relationship between annual census and hunting bag data); (viii-x): T.MAX, T.MIN, and RAINFALL, for each year, the average maximum temperature (°C) during the hottest month, the average minimum temperature during the coldest month (°C), and the total annual rainfall (mm), respectively. Values were obtained by averaging the data on temperatures and precipitations from four weather stations located in the study area (Ufficio Territoriale per la Biodiversità, Pieve S. Stefano, Province of Arezzo, official data).

Additionally, the variable HGROUND/YEAR was included as a random effect in all the models, representing groups of sows culled in the same hunting ground during a given hunting season, in order to evaluate specifically the correlation in conception dates among close animals. The hunting grounds were designed according to the landscape morphology, and their surfaces were relatively small and comparable to the size of the annual home range of a wild boar female group in the study area (Apollonio et al., 2007). Moreover, family groups are only weakly spatially affected by hunting disturbance (Keuling et al., 2008), and, therefore, two females culled in the same hunting ground within a tight time window are far more likely to belong to the same social group than two animals randomly selected from the database. Thus, we considered HGROUND/YEAR a rough proxy of social group. Hence, as in the case of other ecological studies focusing on heterogeneity, the random effect HGROUND/YEAR represented an actual variable of interest (see Bolker et al., 2009). Individuals with missing values in one or more variables were removed from the database, in order to have the same number of observations in all the models considered. All the quantitative variables were centered at their mean value. Four biologically meaningful interactions were also included in our full model (T.MAX×RAINFALL; BODY MASS×AGE; SEED PROD×BCT COVER; PREV.SEED PROD×BCT COVER).

We tested the inclusion of the random term HGROUND/YEAR in the model by performing a likelihood ratio test between two nested models via the anova command in R: we compared two full models (i.e., where the fixed component contained all explanatory variables and as many interactions as possible), with and without the random term, both fitted with restricted maximum likelihood estimation (REML), as suggested by Zuur et al. (2009). We found the optimal fixed component of the model by using the backwards selection approach illustrated in detail in Zuur et al. (2009). We started from the full model fitted by using maximum likelihood by dropping one variable at a time. We performed likelihood ratio tests between the full model and each nested model obtained in this way, each time removing the less significant variable (highest *p*-value) until all the variables were significant at the 5% level. The final model obtained was then refitted by using the REML estimation and validated by checking the assumptions of normality, homoscedascity and independence, by inspecting the standardized residuals plots as described in Zuur et al. (2009). Then, we calculated the intraclass correlation coefficient ICC (which provides the measure of the correlation among the observations from the same year and the same area) as $d^2/(d^2 + \sigma^2)$, where d is the standard deviation of the random intercept, and σ is the residual standard deviation (Zuur et al., 2009). To quantify the goodness of fit of each model, we estimated R^2 following Magee (1990): R^2 = 1 – exp (- 2/n ($logLM$ - $logL0$)), where n is

the number of observations, $logLM$ is the standard log-likelihood of the model (which includes fixed and random effects) and $logL0$ is the standard log-likelihood of the null model (containing intercept and random effects only). In presence of a patchy spatial pattern in conception dates, not exclusively due to environmental heterogeneity and at least partially caused by social interactions, we predicted the inclusion of the random term in the final model, and a high ICC (i.e., a large amount of variance explained by HGROUND/YEAR). On the contrary, in presence of a spatial pattern solely due to environmental heterogeneity in the study area, we predicted the inclusion of the variables iii and/or iv in the model, and a low effect of the random term HGROUND/YEAR. Neither the random term nor environmental factors are expected to be included in the selected model, if the distribution of conception dates were completely random in our study area (H0).

Mantel tests and spatial autocorrelation analysis

In addition, in order to reveal possible spatial patterns in reproduction, two dissimilarity matrices between individuals were constructed for each hunting season: a matrix of distance in conception date (REPR matrix), and a matrix of geographic distance (GEO). Then, the matrix correlation between REPR and GEO was calculated by the Mantel statistic r, as implemented in the R package Vegan (Oksanen et al., 2010), by using 999 permutations to test for significance. We expected non-significant Mantel tests in the presence of no spatial pattern, and significant tests in the presence of a patchy spatial pattern in conception dates on account of similar conception dates among neighboring individuals.

Furthermore, we calculated the autocorrelation in conception dates through space (over multiple distance classes) by performing a spatial autocorrelation analysis with GENALEX 6.41 (Peakall and Smouse, 2006) using the REPR and GEO matrices. Eight distance classes were considered, each being 1500 m wide, except for the first smaller one (500 m) meant to include only individuals culled closely to each other (and therefore more likely to belong to the same social unit), and the last wider one (5500 m) meant to include a sufficient number of observations (Tab. 3). A total of 999 permutations and 999 bootstraps were run so as to generate 95% confidence intervals around the null hypothesis (no autocorrelation) and around the estimated value (r), respectively. This analysis allowed to evaluate whether and how much the data correlation varied with distance. In the presence of a patchy pattern in conception dates, we expected strong autocorrelation mainly in the first distance class.

Results

Of the 2313 females examined, 742 (32.08%) were pregnant. Of these, 382 had at least one fetus weighed. The conception dates estimated had different mean, median and distribution among years, with some years showing a clear bimodal shape (Fig. 2). Mean and distribution of conception dates differed among years (K-W test: χ^2=117.39, df=7, p<0.001), with means ranging between October 10 and November 19.

Of the aforementioned 382 females, 354 (194 adults and 160 subadults) were included in our models, having no missing data in any of the variables considered. The results of the analysis performed with ClValid indicated that K=2 was the optimal number of clusters to summarize the environmental features of the hunting grounds, and the PCA allowed us to identify deciduous forests as the habitat type that drove the clustering. The likelihood ratio test between the full model with and without the random term was highly significant (CD, L-ratio 33.07, p<0.001). Therefore, the inclusion of HGROUND/YEAR in our models was strongly supported, thus indicating correlation in conception dates on a local scale. The final model selected (MS) included T.MAX, T.MIN, RAINFALL, SEED PROD, PREV. SEED PROD, and the interaction T.MAX×RAINFALL as fixed factors, thus confirming a significant environmental and climatic influence (on an annual basis) on wild boar conception dates, as it could have been hypothesized considering the noticeable fluctuations of CD among years (Fig. 2). This model was refitted with REML and found to meet the assumptions of normality, homoscedasticity and independence. The hypothesis of random distribution of CD in our study area (H0), corresponding to the intercept-only model, was then rejected.

The summary of MS is reported in Tab. 1. The variance for the random intercept was 13.04 and the residual variance was 15.92, thus giving a relatively high intraclass correlation (ICC = 0.402). In other words, 40.2% of CD variance was observed among groups, each composed by animals culled in the same hunting season and in the same hunting ground. The fixed part of the model accounted for 15.3% of variance (R^2 = 0.153). The slopes of all the predictors significantly differ from 0 at the 1% level, except for the slope of T.MIN, which was significant on the 5% level (Tab. 1). With all the other predictors at baseline (i.e., average values of rainfall, past and current productivity, temperatures), the estimated conception date was October 17 (corresponding to the intercept 230).

Table 1 – Summary of Linear Mixed Model MS, explaining the variability of conception dates estimated with the Vericad method in an Italian Apennine wild boar population. Proportion of explained variance (R^2), number of parameters, Intraclass Correlation Coefficient (ICC), residual standard deviation (RSD), standard deviation of the random intercept (RISD), parameter estimates with corresponding standard errors (SE) and t-test are reported.

n	**Parameters**	R^2		
354	9	0.153		
Fixed effects				
	Value	SE	t-value	p
(Intercept)	229.927	3.246	70.825	0.000
T.MAX	25.485	4.896	5.205	0.000
T.MIN	3.343	1.419	2.355	0.020
RAINFALL	-4.960	1.012	-4.903	0.000
SEED PROD	-72.596	12.912	-5.623	0.000
PREV.SEED PROD	59.463	10.109	5.882	0.000
RAINFALL×T.MAX	0.171	0.035	4.928	0.000
Random effects				
HGROUND/YEAR				
RSD	15.917			
RISD	13.041			
ICC	0.402			

The conception date was earlier in years with high productivity (β = -72.60 $\pm$ 12.91), and later with high values of both productivity in the previous year (β = 59.46 $\pm$ 10.11) and high temperatures, (T.MAX and T.MIN, β = 25.49 $\pm$ 4.90 and 3.34 $\pm$ 1.42, respectively), with T.MAX seemingly having a greater effect than T.MIN. Conception dates were also anticipated in rainy years (RAINFALL, β = -4.96 $\pm$ 1.01), though

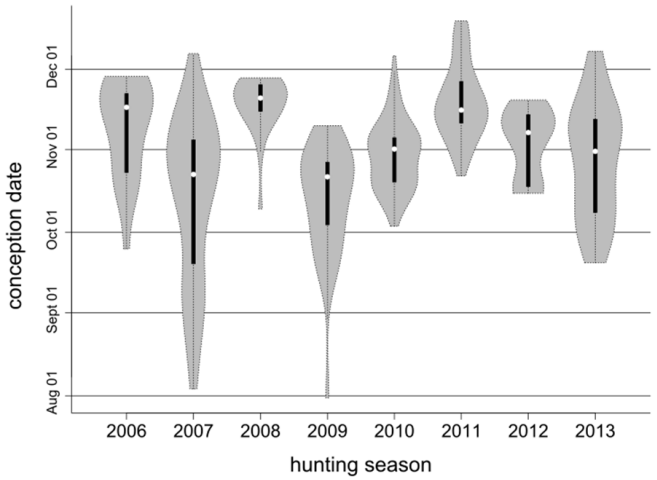

Figure 2 – Conception dates in a sample of 382 litters belonging to wild boar females culled during eight hunting seasons in an Italian Apennine population. The violin plots show median (white point), quartiles and distribution of conception dates estimated with the Vericad method, for each year.

this effect was reduced in case of hot summers (T.MAX×RAINFALL, $\beta = 0.17 \pm 0.03$).

The results of Mantel tests (Tab. 2) indicated a weak though significant correlation between the dissimilarity matrices of conception date (REPR) and geographic location (GEO) in six out of eight years. Significant r ranged between 0.095 and 0.221. Hypothesizing intra-group reproductive synchrony, we did not expect very high r values, since even though close animals are assumed to have similar conception dates, distant groups do not necessarily have different CD (indeed, the group effect is assumed to be random and normally distributed around 0). Only in 2006 and 2012 no significant correlation was found, but these were also the years with the lowest number of observations.

Table 2 – Correlation between geographic distance and distance in conception dates in female wild boar of the Italian Apennine study population. For each year, number of individuals (n), Mantel's correlation (r) and its *p*-value are reported. Significant tests are shown in bold.

YEAR	n	r	*p*
2006	25	0.075	0.142
2007	79	**0.113**	0.008
2008	29	**0.197**	0.033
2009	41	**0.212**	0.015
2010	65	**0.221**	0.002
2011	58	**0.120**	0.012
2012	22	0.056	0.150
2013	63	**0.095**	0.020

Moreover, significant spatial autocorrelation of conception dates occurred in the first distance class (0-500 m, Tab. 3) in seven out of eight years (highly significant in six cases), with relatively high r (significant r between 0.089 and 0.524, with mean across all years equal to 0.271). This indicated that the timing of reproduction was similar in sows sampled in the same area and in the same year (rejection of H0), possibly suggesting within-group synchronization, in agreement with the model results. Significant autocorrelation was also found in four out of eight years in the second and third distance classes, but generally with lower r.

Discussion

Conception dates had high within-year variance (with a maximum in 2007, Fig. 2), possibly due to the high ecological plasticity of the species and to the favorable climatic and environmental conditions in the study area which enable different groups/individuals to adopt different strategies (e.g., to delay the reproduction). The bimodal distribution of births in some years and their peak in late winter-spring previously reported for other regions (Markina et al., 2003; Maillard and Fournier, 2004) match what we observed in our study population. However, it should be remarked that the conception dates we obtained did not give us a comprehensive picture of the wild boar reproductive phenology through the year, because the sampling was only carried out between October and January, and the gestation period lasts around 120 days in *S. scrofa*.

Mean and distribution of CD varied significantly across years. This result was somewhat unexpected, given that conception dates in wild boar social units were reported to be highly repeatable from year to year (Meynhardt, 1984). The strong hunting pressure in the study area and the resulting high turnover in social units may represent one of the possible explanations for our findings. We may speculate that a significant number of leader sows (whose role in determining the group-specific date of reproduction is crucial, Meynhardt, 1984) was culled each year in the study area.

Actually, as highlighted by our model, the main causes of CD variation among years are to be found in inter-annual variation in productivity, rainfall, and temperatures. High seed production and rainfall can create favorable conditions for the wild boar. Our model predicted that, in these conditions, sows tended to anticipate the conception date, possibly to ensure that their offspring could take full advantage of resource abundance and grow fastly prior to the onset of the harsh season. Conversely, warmer years (i.e., with higher maximum temperatures during the hottest month, or higher minimum temperatures during the coldest month) may correspond to unfavorable conditions (e.g., drought) and turned out to be associated with a delay in the timing of reproduction. Our model is consistent with Aumaître et al. (1984), who reported that in exceptionally good years the wild boar birth peak can occur up to two months earlier than usual. Moreover, Servanty et al. (2009) highlighted the effect of resource availability on the wild boar reproductive phenology. Our results are also consistent with Plard et al. (2014), who showed that rainfall is often associated with anticipated parturition date in other ungulate species, and Ogutu et al. (2014), who reported that dry conditions may lead to delayed births in African ungulates. However, our findings are inconsistent with Fernández-Llario and Mateos-Quesada (2005), who showed that dry summers and autumns are associated with an early period of conception in a Spanish wild boar population, and with Meynhardt (1984), who suggested no influence of mast production on the timing of reproduction. Since the sensitivity to certain environmental conditions can have a genetic basis, different population histories leading to different genetic make-up of populations may therefore imply local differences in behavioral or physiological responses to similar environmental stimuli. Our model also showed that high values of productivity in the previous year delayed the mean conception date. This can be related to an increase in the time required for the recovery of body condition, following a great maternal investment in the previous year (see Servanty et al., 2009). Previous works (e.g., Feder et al., 2008; Servanty et al., 2009) provided evidence for the effects of body condition and age on parturition date in wild boar and other ungulates. Interestingly, neither individual variables (AGE and BODY MASS) nor habitat differences in our study area (BCT COVER and HABITAT) were found to explain a significant amount of variance in conception dates.

Overall, our results indicated the occurrence of a patchy spatial pattern in the wild boar reproductive phenology across the study area, a case falling in our hypothesis H1. On a local scale, closer sows showed similar conception dates. Indeed, for six out of eight years, the Mantel tests revealed weak to moderate (significant) spatial autocorrelation for the conception dates estimated. This spatial relationship was not statistically significant in the two years with the lowest n, possibly due to the sampling of a low proportion of females belonging to the same social group. The patchy pattern was confirmed by the results of the spatial autocorrelation analysis: females sampled at 0-500 m from each other had correlated conception dates (Tab. 3).

Accordingly, the linear mixed model results highlighted the primary importance of HGROUND/YEAR in explaining the conception date variance. Considering that the effect of individual and environmental factors was accounted for in our analysis, the high correlation (40%) in conception dates among sows culled in the same year and in the same area may indicate intra-social group reproductive synchrony, as observed by Meynhardt (1984) in Germany, and suggested by Mauget (1980) and Briedermann (1986). These findings are also consistent with the study of Delcroix et al. (1990), who observed estrus synchrony in wild boar in captivity.

Spatial patterns of reproductive synchrony have been shown for many mammal species and argued to be a consequence of socio-sexual interactions. For instance, Mc Clintock (1971) pointed out the role of pheromones in inducing estrus synchrony in humans. The stimuli involved seemed to have mainly an olfactory nature, often originating from the male. For example, Whitten (1956) observed that the presence of a male caused a synchronization of estrus in mice (*Mus musculus*), and this was also demonstrated in sheep (*Ovis aries*) and goats (*Capra hircus*) (Underwood et al., 1944; Shelton, 1960). On the contrary, the synchronization appeared to be caused by interactions between females in red and musk deer (Iason and Guinness, 1985; Meng et al., 2003), as well as in captive wild boar (Delcroix et al., 1990). Similarly, in American bison (*Bison bison*), unmated females were observed to use olfactory cues to explore the status of other females prior to their own estrus, but not afterwards (Berger, 1992).

Table 3 – Results of spatial autocorrelation analysis of conception dates of wild boar litters in the Italian Apennine study population. The number of pairs, autocorrelation coefficient r and its significance for each year and distance class (the end point, in km, is shown in the first row) are reported. Highly significant values ($p < 0.01$) are shown in bold.

		0.5	2	3.5	5	6.5	8	9.5	15
2006	n	26	28	53	33	28	46	19	67
	r	**0.524**	**0.270**	-0.053	-0.106	0.140	0.022	0.147	-0.319
	p	0.001	0.007	0.785	0.872	0.082	0.389	0.106	0.999
2007	n	125	299	392	404	592	577	382	310
	r	**0.291**	0.013	0.053	-0.021	0.038	-0.040	-0.115	0.036
	p	0.001	0.316	0.041	0.795	0.025	0.960	1.000	0.094
2008	n	54	16	31	129	25	47	54	50
	r	**0.384**	**0.300**	0.003	-0.306	0.162	0.091	**0.169**	-0.179
	p	0.001	0.005	0.525	1.000	0.027	0.058	0.006	0.987
2009	n	39	123	130	115	203	100	76	75
	r	0.048	-0.009	**0.124**	-0.050	-0.027	-0.053	0.019	-0.029
	p	0.258	0.577	0.008	0.882	0.827	0.862	0.362	0.731
2010	n	131	165	402	328	363	339	203	149
	r	0.089	-0.017	**0.072**	-0.047	0.007	-0.016	-0.018	-0.060
	p	0.042	0.708	0.005	0.952	0.377	0.761	0.721	0.946
2011	n	86	109	154	195	362	211	259	277
	r	**0.209**	0.145	**0.139**	-0.023	-0.091	-0.013	0.007	-0.041
	p	0.006	0.013	0.002	0.777	0.998	0.665	0.406	0.932
2012	n	29	21	25	28	41	19	24	44
	r	**0.457**	-0.127	-0.297	0.194	-0.434	**0.418**	-0.428	**0.305**
	p	0.007	0.843	0.982	0.038	1.000	0.004	0.995	0.001
2013	n	137	205	256	156	364	239	159	437
	r	**0.169**	0.080	0.037	-0.033	0.009	-0.007	0.093	-0.140
	p	0.005	0.032	0.100	0.817	0.402	0.616	0.017	1.000

Reproductive synchrony in wild boar on the social group level is highly adaptive, in that it offers a number of possible advantages:

(i) piglets can be communally nursed. Both adoptions and allosuckling (i.e., suckling from a female other than the mother) are known to be extremely common in wild boar (Delcroix et al., 1985; Meynhardt, 1987). Allosuckling was observed in other ungulates (e.g., fallow deer *Dama dama*, Ekvall, 1998; red deer, Landete-Castillejos et al., 2000; reindeer *Rangifer tarandus*, Engelhardt et al., 2014), and found to be a means for pups to broaden their passive access to antibodies after birth (Garnier et al., 2013), but is rarely adopted as a group strategy. This is not the case of wild boar, among which allosuckling is widespread and lactation (occurring approximately at hourly intervals) can be synchronized within a group (Massei and Genov, 2000). This behavior can foster a better development of the piglets through an optimal feeding and may significantly increase their survival. This is especially advantageous in Southern Europe, where summer is the limiting season and summer drought can cause substantial losses among piglets (Fernández-Llario and Mateos-Quesada, 2005).

(ii) Synchronous births enhance the mobility potential of a given social unit by reducing the time span during which the group movements are constarined by the presence of small piglets. Indeed, it was shown that the female daily home range in the weeks immediately prior and following the birth is drastically reduced in wild boar (from 40-80 ha to 1-3 ha according to Janeau and Spitz, 1984). A similar reduction in female home range was observed in many ungulates (*Odocoileus virginianus* Schwede et al., 1993; *Dama dama* Ciuti et al., 2006; *Capra ibex* Grignolio et al., 2007; *Capreolus capreolus* Bongi et al., 2008) where it is often related to a hider strategy.

(iii) synchronized births may contribute to a collective and consequently more efficient defense of the young against such predators as the wolf and the red fox, that are common in the study area and in most of the European range of wild boar; furthermore, a stronger dilution effect reduces the individual probability of piglets to be killed by a predator. Both communal defense and dilution effect are well-known anti-predatory strategies adopted by ungulates (Jarman, 1974). Piglets are quite vulnerable to predation if not properly assisted by sows; in the study area, they frequently occur in the diet of both wolves and foxes (Bassi et al., 2012). Conversely, a group of sows is able to successfully cope with predators, thus reducing predation upon piglets. In this regard, even such effective predators as the wolf were seen to be chased by wild boar groups (pers. obs.). Predator harassment can be an effective anti-predator strategy (Mukherjee and Heithaus, 2013), and was observed in some ungulate species (Jarman, 1974; Berger, 1979; Carbyn and Trottier, 1987; Berger and Cunningham, 1995; Prins, 1996). Indeed, adult wild boar weighting more than twice their predator and having sharp canine teeth are very likely to exploit this option.

Moreover, reproductive synchrony may also have evolved to favor polygamy (Ims, 1990) and this can, in turn, influence genetic variation and quality of newborns. Prior to the rutting period (falling mainly in late autumn and early winter), adult male wild boars get restless and increase their marking behavior and fights with other males. In the rutting period, photoperiodism and possibly other environmental factors trigger the seasonal increase in the endocrine activity of testes (Šprem et al., 2011). Males travel long distances in search of a group of sows, fighting against potential rivals and persistently chasing the sows (Dardaillon, 1988; Massei and Genov, 2000). Once joined a female group, a male is able to maintain a monopoly over the aggregated females (though this does not seem to be the rule for all populations, see Poteaux et al., 2009), until the arrival of a stronger male, or until he has mated with all the females (Massei and Genov, 2000). In the presence of high estrus synchrony within a social group, the probability of the latter occurrence increases. Furthermore, since the time spent with a single group is reduced, a male can maximize the number of groups visited, thus increasing the number of matings. As a consequence, high reproductive synchrony can increase the fitness of dominant males and promote the dissemination of their genes across a wider area. Actually, after mating with all the synchronized females, a boar may spend additional time with the group, trying to prevent other males from gaining access to still receptive females (post-copulatory mate guarding), as argued by Delgado et al. (2008), or it may decide to leave and approach another female group. In the latter case, other (subordinate) males may have the opportunity to mate with groups of still receptive unguarded females. In this scenario, multiple mating by females (i.e., polyandry) may be promoted, inducing a post-copulatory competition among mating boars, in which the fertilization success of a given male depends on the relative amount/quality of semen it can ejaculate (i.e.,

sperm competition; Aguilera-Reyes et al., 2006). By this way, high estrus synchrony would promote sperm competition and possible multiple paternity within litters. Multiple paternity was observed by Delgado et al. (2008) and Poteaux et al. (2009) in Portuguese and French wild boar, respectively, and had been previously documented in feral pig populations in Australia (Spencer et al., 2005). Preliminary data revealed its occurrence also in our study area (Iacolina, 2009). Interestingly, Aguilera-Reyes et al. (2006) showed that, in domestic pigs, the sow can influence the possibilities of success of the ejaculations from different males, slanting the paternity towards the male with the higher genetic variability (strategy known as "cryptic choice"). Both single paternity by a dominant male and multiple paternity may have genetic advantages for females, by leading to a possible inheritance of "good genes" by the litter in the former case and increasing the genetic diversity among sibs in the latter. In highly unpredictable environments, the second strategy may be adaptive, as it improves the chance to have a successful progeny.

Finally, the possible effect of kinship on estrus synchrony in sows remains unknown. In red deer, female relatives associate together, and this association leads to synchronous estrus within kin groups which is not due to kinship per se (Iason and Guinness, 1985). Conversely, kinship may influence reproductive patterns in bighorn sheep, in which the parturition date was shown to be partly heritable (Feder et al. (2008)). If the timing and synchrony of reproduction have, at least partially, a genetic basis, even the introgression from the domestic pig into the wild boar gene pool may lead to altered reproductive patterns. Wild boar can crossbreed with domestic pigs both in natural conditions (where open-air pig farming is still practiced) and in captivity (see Canu et al., 2014). In fact, genetic introgression from domestic pigs into wild boar populations was detected by various authors (e.g., Koutsogiannouli et al., 2010; Scandura et al., 2011; Frantz et al., 2013; Goedbloed et al., 2013) and was suggested to have important ecological consequences, by altering such traits as behavior and reproductive performances (Goedbloed et al., 2013). Further genetic studies together with reproductive data are recommended to investigate the possible heritability of such life history traits as timing and synchrony of reproduction in wild boar, also considering their impact on the species' demography and its management.

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SECOND PART

Hybridization between wolf and dog

CHAPTER 6

Do wolves and free-ranging wolf x dog hybrids share the same food niche? First insights from Apennine mountains, Italy

Do wolves and free-ranging wolf x dog hybrids share the same food niche? First insights from Apennine mountains, Italy

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ABSTRACT

Hybridization can have serious consequences on morphology, physiology, behaviour and individual fitness, especially when wild and domestic conspecifics are involved. Domestic genes introgressed into wild populations can pose a threat to populations by altering their genetic make-up, with a possible impact on their long-term viability. However, hybrids may also represent ecological competitors for the parental wild form, with unpredictable consequences on trophic cascades and ecosystem equilibria. The understanding of the ecological role of hybrids could thence be crucial for developing appropriate conservation strategies.

The Italian wolf (*Canis lupus*) population has been isolated for around one century and underwent a severe bottleneck, which led to a peculiar genetic composition. Today, however, its genetic integrity is threatened by the spread of canine genes due to hybridization with stray dogs in the wild.

Aim of the present study was to get insights on the ecological role of free-ranging hybrids, by investigating their winter food habits and their feeding niche overlap with wolves in a mountain area of Central Italy (Tuscan Apennines). Levels of genetic introgression were essayed in two adjacent areas, occupied by putative wolf packs, by analyzing non-invasive samples and carcasses collected therein with a set of uniparental and bi-parental markers (12 autosomal microsatellites, the mitochondrial DNA control region and two Y-chromosome microsatellites). Individuals from the two areas strongly differed in the level of genetic introgression from the dog and were accordingly classified as hybrids and wolves.

The analysis of 339 scats for each area, collected in winter time, showed that the main prey species was wild boar in both areas, followed by roe deer. Packs inhabiting both areas selected wild boar weighting between 10 and 35 kg, and juvenile roe deer. We did not detect significant differences in the diet composition between the two areas: independently from their admixture level, individuals showed a trophic behaviour similar to other

previously studied Apennine wolf populations, confirming the role of wolf–dog hybrids as potential competitors for wolves. This study represents the first investigation on the food habits of free-ranging wolf-dog hybrids. Further studies on different aspects of their biology and ecology are recommended, in order to improve the appraisal of the impact of hybridization on natural wolf populations.

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Keywords: *Canis lupus italicus*, genetic introgression, wild x domestic hybridization, food habits, trophic niche overlap

INTRODUCTION

Introgressive hybridization (i.e., exchange of genes between evolutionary lineages as opposed to hybridization yielding exclusively inviable or infertile offspring, Seehausen 2004), due to intentional and incidental translocations of plants and animals, habitat modifications (Allendorf et al. 2001), human pressure (Rutledge et al. 2012) and climate change (Garroway et al. 2010),is an expanding phenomenon which is compromising the genetic integrity of native populations and causing the extinction of many taxa (Allendorf & Luikart 2007; Allendorf et al. 2013).

Hybridization can have serious consequences on morphology, physiology, behaviour and individual fitness, but the long term evolutionary consequences of introgressive hybridization remain largely unpredictable.

Hybrids may show a lower fitness compared to either parental taxa (outbreeding depression) due to loss of local adaptation to environmental conditions (Allendorf and Luikart 2007). However, in some cases, hybrids can show equal or superior fitness in new habitats and, occasionally, even in parental habitats (Seehausen 2004). In this case, single alleles that confer an advantage in the environment will introgress quickly (Barton 2001). Hybridization can also lead to the formation of stable genetic populations potentially kick-starting speciation and adaptive radiation over a very short timescale (Roy et al. 2015). For these reasons, many authors focus on the potential of hybridization as a source of adaptive genetic variation, functional novelty and new species (Burke & Arnold 2001; Largiadèr 2007).

Wolf-like canids (genus *Canis*) evolved recently (in the last 2–4 million years ; Von Holdt et al. 2011), and retained the potential to hybridize in nature (Wayne et al. 1997), giving rise to new taxa that could quickly adapt to prey community, landscape and climate changes (Randi et al. 2014a). There are several examples of canid hybridization, such as that between dog (*Canis lupus familiaris)* and Ethiopian wolf (*Canis simensis*, Gottelli et al. 1994), between red wolf (*Canis rufus*) and coyotes (*Canis latrans*, Wayne and Jenks 1991), between eastern wolves (*Canis lycaon*) and eastern coyotes (see Benson et al. 2014), and between coyote and gray wolf (*Canis lupus*, Lehman et al. 1991, Benson et al. 2014). In particular, the process of introgressive hybridization between the gray wolf and its domestic counterpart has become a growing concern for conservationists in Europe. Indeed, the spread of domestic genes into

wolf populations could disrupt local adaptation (Vilà and Wayne 1999), potentially representing a serious threat to the long term survival of genetically pure wolf populations in the wild (Boitani 2003; Hindrikson et al. 2012).

Wolf-dog hybridization has an ancient origin (~10,000 years ago, Schwartz et al. 1997). Voluntary wolf-dog crossbreeding was widespread (Iljin 1941) in order to create different wolf-dog breeds. Furthermore, the number of accidental wolf-dog hybridization events in nature highly increased in the last 20 years, mainly due to anthropogenic factors. The risk of hybridization was often assumed to be higher near to human settlements, where stray and village dogs are abundant (Boitani 1983, and Blanco et al. 1992), and in areas where wolves are rare, or strongly persecuted (Randi et al. 2000, Andresone et al. 2002, and Heiler and Leonard 2008). The occurrence of an expansion phase for the wolf population was also reported to be an important factor favouring hybridization (Lorenzini et al. 2014).

In Europe, wolf-dog hybridization has been reported to occur at relatively low frequency in Scandinavia (Vilà et al. 2003) and the Iberian Peninsula (Godinho et al. 2011), while it seemed to be more widespread and frequent in Bulgaria, Latvia and Estonia (Randi et al. 2000; Hindrikson et al. 2012).

Because of the extensive contact with dogs (Boitani 1993), Vilà and Wayne (1999) agreed that even in Italy the probability of wolf-dog hybridization was high, and subsequently, many authors found increasing evidences of its occurrence (Randi and Lucchini 2002, Verardi et al. 2006, Iacolina et al. 2010, Caniglia et al. 2013, Lorenzini et al. 2014, and Randi 2014b).

Due to the past persecution, the Italian wolf population suffered of severe bottleneck, which, in association with its prolonged isolation, led to genetic erosion (Lucchini et al., 2004). Even if in recent decades both its population size and range have increased, the Italian wolf is still considered vulnerable, and wolf-dog hybridization is recognized as a major issue for its conservation in the national action plan for wolf conservation (Genovesi 2002).

Several studies have been conducted on wolves and dogs independently, in order to better understand their behaviour and their role in ecological communities (Mech 1970, Okarma 1995, Mech and Boitani 2003, Butler et al. 2004, Macdonald and Sillero-Zubini 2004, Huges and Macdonald 2013, Gompper 2014, Vanak et al. 2014), as well as their possible interactions (Kojola et al. 2004, and Lescureux and Linnell 2014).

Donadio and Buskirk (2006) argued that between wolf and dog should exist an intense competition in case of sympatry. In natural environments wolves feed mainly on wild ungulates (Okarma 1995, Vucetich et al. 2005, Peterson and Ciucci 2003, Wikenros et al. 2009, Ripple et al. 2010, Mattioli et al. 2011, Bassi et al. 2012, Palmegiani et al. 2013), while it has been observed that dogs mainly act like scavengers or share food resources like livestock and garbage with wolves (Boitani 1983, Ovsyanikov and Poyarkov 1996, and Vanak and Gompper 2009).

However nothing is known about the feeding ecology of hybrids. Despite an increasing number of studies focused on genetic aspects in order to assess the amount of introgression in natural populations, Lescureux and Linnell (2014) warned about the lack of data on the behaviour and ecology of wolf-dog hybrids under free-ranging conditions.

The aim of the present study is to provide preliminary information about the ecological role of hybrids in free-ranging conditions, checking for differences in food habits with respect to sympatric wolves. In order to reach this goal, we first assessed by molecular markers the degree of introgression in packs living in two adjacent areas in a mountainous environment (Italian Apennines), one of them hosting individuals showing anomalous morphological traits.

MATHERIAL AND METHODS

Study area

The study area is located in the Arezzo province, north east of Tuscany, Italy Here the Apennines form a number of minor massifs, two of which are named 'Alpe di Catenaia' (AC) and 'Alpe di Poti' (AP), and extend from north to south (Fig. 1).

The two ridges cover an area of 458 km^2 (AP: 234 km^2; AC: 224 km^2). The AC area includes a protected area of 27 km^2 where hunting is banned. Altitude ranges from 300 to 1414 m above sea level within AC, and between 200 and 990 m a.s.l. in AP. In both areas vegetation cover is mainly composed of mixed deciduous hardwoods (73% and 65% of total area for AC and AP respectively), the main tree species being represented by beech (*Fagus sylvatica*) oak (*Quercus* spp.), and chestnut (*Castanea sativa*), with a prevalence of beech in AC and oak in AP.

The climate of both areas is temperate and seasonal with hot, dry summers, and cold, wet winters. Snowfall usually starts in October and may occur until April.

In both areas wild ungulates are represented mainly by roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*); red deer (*Cervus elaphus*) has been occasionally observed in AC. The only resident lagomorph is the brown hare (*Lepus europaeus*). A number of farms surround the study area, mostly raising sheep (*Ovis aries*), constituting a potential additional source of prey for wolves and hybrids.

The wolf population in the Arezzo province was continuously monitored since 1998 by direct observations, wolf-howling, snow-tracking (see Scandura et al. 2011) and, more recently, by camera trapping. Since then, the AC area was interested by the presence of a resident wolf pack. The AP area, instead, is inhabited by wolves since 2004 only. Therefore, data on the two areas that are presented here refers to the period 2004-2013.

Hybridization assessment: sampling and genotyping

Scats and hairs were collected in AC and AP, as well as in the rest of the province, along trails chosen on the basis of documented wolf presence. We also opportunistically sampled tissues from carcasses recovered in the province and nearby areas. For each carcass, close examination was carried out in order to detect the presence of anomalous phenotypic traits (like melanism, spur in hind-legs, abnormal coat colour patterns), possibly due to introgression of domestic genes.

Samples were stored at -20°C in 96% ethanol. Since the start of genetic monitoring, a total of 1,148 samples were analyzed, including 1,109 invasive and non-invasive wolf (or presumed wolf) samples from the Arezzo province and 49 samples from other provinces in Tuscany (scats, hairs, and tissues). DNA was extracted from scats according to Gerloff et al. (1995). The GenElute Mammalian DNA miniprep Kit (Sigma-Aldrich, St Louis, Missouri) and the InstaGene Matrix (Bio-Rad, Hercules, CA, USA) were employed for extracting DNA from tissues and hairs, respectively. For laboratory analyses, we followed the recommendations provided by Budowle at al. (2005) for animal DNA forensic.

Samples were PCR-amplified and genotyped at 12 unlinked autosomal microsatellites: six dinucleotides (CPH2, CPH4, CPH5, CPH8, CPH12; Fredholm and Wintero 1995; C09.250 Ostrander et al. 1993) and six tetranucleotides (FH2004, FH2079, FH2088, FH2096, FH2132 and FH2137; Francisco et al. 1996). PCR conditions were optimized for each primer pair and are available upon request. Non invasive samples were genotyped by NGB Genetics Srl

(Bologna, Italy) using a multiple-tube protocol as implemented by Lucchini et al. (2002), and consensus genotypes were reconstructed using GIMLET 1.3.3 (Valière 2002). Gender of non-invasively sampled individuals and non-sexed wolf carcasses was determined by employing the Amelogenin marker as in Randi et al. (2014a). The genotyping led to identification of more than 200 different putative wolf genotypes, corresponding to different individuals on the basis of the probability of identity for sibs ($P_{(ID)sib}$,Waits et al. 2001).

For each individual, in addition, a portion of the hypervariable domain of the mtDNA control region 1 (CR1) was amplified and sequenced following Vilà et al. (1999). Males were analyzed following Iacolina et al. (2010) at two Y-chromosome microsatellite loci (MS34A and MS34B), and alleles at the two non-recombining loci were combined to construct haplotypes.

All the aforementioned analyses were also carried out for a sample of 39 dogs (either owned dogs or stray dogs kept in kennels) from the same area.

Hybridization assessment: admixture analyses

The first step to assess the degree of admixture of wolves sampled in the AC and AP areas was creating a reference dataset of supposedly pure Italian wolves and dogs. As regards wolves, we considered all the different individuals successfully genotyped at ≥ 10 loci, except those sampled in AC and AP. Then, to be conservative, we removed from the dataset each animal showing a sign of possible introgression. Therefore, each individual having a canine haplotype in Y-chromosome microsatellites, or not showing the Italian wolf haplotype w14 at the CR1 (Randi et al. 2014a) was discarded. Additionally, genotypes were removed if associated to any anomalous phenotypic trait (for carcasses). A total of 37 wolves were retained. Their microsatellite genotypes, along with that of the 39 dogs, were included in a first dataset with the aim to identify and remove any additional individual showing introgression at autosomal microsatellites. For this purpose, we performed a first analysis employing the Bayesian clustering algorithm implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000), with the following settings in the prior: admixture model, uncorrelated allele frequencies among populations, and no population information. Ten independent runs were carried out for a number of genetic clusters varying from K=1 to K=10, with 250,000 iterations following a burn-in period of 250,000 iterations. The most likely value of K was determined according to the method developed by Evanno et al. (2005), in order to verify

the prevailing partition into two main groups (dogs and wolves). Our results, as expected, indicated K=2 as the most likely partition, identifying a dog and a wolf cluster. We considered the Q-values of the run with highest posterior probability, and removed all the individuals showing less than 97.5% of membership to a single cluster. In this way, we obtained the reference populations of pure wolves (n=34) and dogs (n=37), showing no evidence of introgression at any considered marker. A second dataset was then created, including the reference populations and 52 different genotypes from AC (n=26) and AP (n=26) areas, sampled between winters 2004/05 and 2012/13. Subsequently, STRUCTURE was run 10 times, with K fixed at 2, with 250,000 iterations and 250,000 burnins, admixture model, uncorrelated allele frequencies and the option "update p from pop flag only" activated. In this way, the estimated allele frequencies of the wolf and dog reference clusters were not affected by the allele frequencies of the other samples to be classified. The degree of admixture of individuals inhabiting the AC and AP areas was assessed as the individual membership to the two inferred clusters (respectively Q_{WOLF} to wolves and Q_{DOG} to dogs), by considering the run with the highest posterior probability.

Prey abundance

Densities of wild boar and roe deer within the study area were estimated by the Provincial Administration of Arezzo with drive censuses every May (described in Mattioli et al. 2004). Population surveys took place each spring in both the protected and non-protected portions of the study area, encompassing about 80% of the wooded area and 20% of the other cover types; between 13 and 19 (AC) and between 16 and 19 (AP) forest blocks were sampled each year.

Animal density within each forest block surveyed was estimated as described in Davis et al. (2012). Post-birth abundance of roe deer was estimated on the basis of their spring counts, percentage of adult females in the population and female fertility. The percentage of adult females in the population was derived from direct observations during the drive censuses, while female fertility was estimated by counting the fetuses found in females shot by hunters (Hunting Plan Provincial Administration of Arezzo). Wild boar population structure was derived from direct observations, while sex ratio in the population and fertility parameters were obtained through an analysis of fetuses found in the uteri of the females shot.

Diet assessment and trophic niche analysis

From 2004/2005 to 2012/2013, a total of 515 and 339 winter scats were collected respectively in AC and AP. In order to have a comparable sample size between the two areas, we randomly created and used a subsample of 339 winter scats for AC.

The canid diet compositions were determined by means of scat analysis. Scats were washed in a sieve of 0.5 mm and the macroscopic prey remains (e.g. hairs and bones), fruit and grasses found in every scat were dried at 68°C for 24 h. Prey categories included wild boar, roe deer, hare, small rodents, and livestock (goats, sheep and cattle). Prey remains were identified through comparison to a reference collection of mammal hairs, bones, and teeth. We identified the prey species and age or weight class (for ungulates only), when possible. This identification was based on the macroscopic characteristics of hairs and bones following Mattioli et al. (2011). Boar remains were assigned to one of the following three weight classes: newborn piglet (<10 kg), piglet (10–35 kg), and adult (>35 kg). Roe deer remains were classified into two classes: juvenile (<4 months) and adult (>4 months), see Bassi et al. (2012) for the methodology. The ability of operators to discriminate among samples from different species and age/weight classes was verified by means of a blind test employing "artificial scat samples'', using a collection of 200 bags containing prey remains from a variety of species (all potential prey in the study area), with different weight and age classes. A total of 50 bags were randomly assigned to each operator (with further 25 random samples specifically aimed at identifying wild boar weight classes). Only operators who correctly identified all test samples were selected to analyze the collected scat samples.

In order to estimate the contribution of each species in the diet, we calculated the average percentage volume (AV%), and the relative biomass value for each food item. AV% was defined as Vi/N×100 where Vi is the total volume of a given food item i, and N is the total number of scats. The relative biomass was calculated for each species using the relative volume values and applying the biomass models of Weaver (1993) y= 0.008x + 0.439, and Ciucci (2001) y = 0.009x + 0.39, where y represents the biomass (kg) of prey for each collectable scat and x is the live weight of prey.

The trophic niche breadth and overlap between hybrids and wolves were evaluated through Levins' index (1968) and Pianka's index (1973) respectively, applied to the volume of food categories in their respective diet.

Levins' formula is: $B = 1/ \Sigma pi^2$, where pi is the contribution of each item in the total diet. The index B could achieve value from 1 (strong specialization on one category) to n, where n represents the total number of food categories (extremely opportunistic foraging behaviour). The Pianka's formula is: $O_{wh} = \Sigma p_{iw}p_{ih}/\sqrt{\Sigma p_{iw}^2 \Sigma p_{ih}^2}$, where O_{wh} is the measure of overlap between the diet of two species (here, wolves and hybrids), p_{iw} is the proportion of the resource *i* out of the total resources used by wolves, while p_{ih} is the proportion of the resource *i* out of the total resources used by hybrids. *i* could range from 1 to n, where n is the total number of food items considered. The index O could range from 0 (no overlap) to 1 (full overlap).

Additionally, different statistical tests were performed in order to compare the overall composition of the diet in the two areas. First of all we performed Chi-Square tests to evaluate if a significant difference existed in the diet composition between the two areas (both overall and considering different weight/age classes of wild boar and roe deer). Secondly, we compared presence-absence data for the prey categories using non metric multidimensional scaling based on a Bray–Curtis dissimilarity matrix; the value obtained from this analysis ranges from 0 (identical diet composition) to 1 (totally different composition). For a more detailed analysis, we grouped prey items into three broad categories: wild boar, cervids, and accessory preys. Thirdly, an analysis of similarities (ANOSIM) and the similarity percentage analysis (SIMPER) were carried out, in order to understand if the use of food items differed between the two areas, and which prey category contributed most to the observed similarity (Clarke 1993), respectively.

Finally we calculated Manly's selectivity index (Manly et al. 1972) to determine the preference of wolves and hybrids for the two main prey species (i.e. wild boar and roe deer), for every winter season (n=7).

To estimate selection by wolves, we used the estimated density values for wild boar. The complement of the parameters estimated for wild boar applies to roe deer. For each winter season, we inferred the relative use of boar and its relative availability, as described by Davis et al. (2012). The formula we used was the following: $\alpha_i = r_i/n_i \ (1/\sum(r_j/n_j))$ where α_i represented the preference index for prey type *i*; r_i and r_j the proportion of prey type *i* or *j* in the diet (*i* and *j* = 1, 2, 3, … m); n_i and n_j the proportion of prey type *i* or *j* in the environment; m the number of possible prey types. When $\alpha_i = 1/m$, feeding was not selective. Prey species *i* was preferred when $\alpha_i > 1/m$ and avoided when $\alpha_i < 1/m$. All the analyses were performed

in R 3.1.2 (http://cran.r-project.org/).

RESULTS

Genetic analysis

Out of 52 different individuals obtained in the two areas in the period 2004-2013, three non-invasively sampled specimens from AP and two from AC were classified as dogs by the STRUCTURE analysis, showing more than 90% assignment to the dog cluster (Q_{DOG} >0.90), and were not considered in subsequent calculations. No AC individual was classified as hybrid using a threshold of 90% membership to the wolf cluster (Q_{WOLF}), whereas as much as 14 out of 23 AP individuals showed an admixed ancestry (Fig. 2). These values increase to 1 and 16 individuals in AC and AP, respectively, using a 95% threshold. The mean Q_{WOLF} was 0.985 in AC and 0.797 in AP. The difference among Q_{WOLF} values in the two areas was highly significant (Wilcoxon rank sum test: W = 488, p < 0.001). Uniparental markers did not evidence any striking difference between AC and AP: 100% of animals in both areas carried the Italian wolf haplotype w14. Canine Y-chromosome haplotypes (i.e., H3, Iacolina et al. 2010) occurred in males of both areas at a similar frequency (0.10 in AC and 0.125 in AP).
Nevertheless, canine Y-haplotypes were associated with Q_{WOLF} > 0.99 in AC and with Q_{WOLF} <0.50 in AP, possibly indicating a past introgression in the former area, and more recent hybridization in the latter, as also suggested by the observed Q values.
According to these genetic results, for simplicity, we classified the individuals frequenting the AP area as hybrids, and the individuals frequenting the AC area as wolves.

Diet analysis

We identified 8 different food categories for the AP area , and 11 for the AC area (Table 1). The two diets had a very similar composition. Wild ungulates were the principal consumed category in both areas (in total AV% = 95.00% and biomass% = 98.73% in AP, and AV% = 92.23% and biomass% = 94.41 in AC, Table 1), and among them, wild boar represented the main item (AV% = 47.23% in AP and AV% = 62.76% in AC), followed by roe deer (AV% = 47.08% in AP and AV% = 29.24 in AC). Hares, small mammals, livestock and vegetables can be considered as accessory food items, amounting in total to 5.00% (AV%) for the AP and

7.77% (AV%) for the AC areas. These results are further confirmed by the calculation of the niche breadth; indeed Levins index indicated a specialization on at maximum two main items in both areas, i.e., wild boar and roe deer (Table 2). Moreover, the Pianka's index revealed an almost complete overlap, for both method of diet quantification (OAV% = 0.94, Obiom%=0.97, table 2). Chi-square tests confirmed that no significant difference in diet composition was present between AC and AP ($\chi^2_{AV\%}$ = 11.54, p > 0.05, and $\chi^2_{biom\%}$= 7.8, p > 0.05).

We also analyzed the volume represented by wild ungulates only (Fig. 3 a and b); even if roe deer are more used in AP than in AC (Table 1), it is possible to notice that the different weight and age classes of wild boars and roe deer are used in similar proportions in the two areas. As regards wild boar, the most used class was piglets (i.e., animals weighting 10-35 kg; AV% = 41.49% in AP and AV% = 51.56% in AC), followed by adults (AV% = 3.11% in AP and AV% = 6.03% in AC) and newborn piglets (AV% = 2.63 in AP and AV% =5.17% in AC). As regards roe deer, the most used age class is represented by juveniles (AV% = 25.96% in AP and AV% = 14.67% in AC), followed by adults (AV% = 21.12% in AP and AV% = 14.57% in AC). Even in this case we did not detect a significant difference between the two areas neither in the use of wild boar ($\chi^2_{AV\%}$ = 0.66, p > 0.05, and $\chi^2_{biom\%}$=3.04, p >0.05), nor in the use of roe deer ($\chi^2_{AV\%}$ = 0.17, df=, p > 0.05, and $\chi^2_{biom\%}$=0.17, p >0.05).

The results from the Bray–Curtis dissimilarity matrix confirmed the above-mentioned trend: indeed the outputs of this analysis varied from 0.14 to 0.20, indicating very low dissimilarity between the two areas (Table 3).

The analysis of similarity did not show difference in the use of wild boar and roe deer in the two areas (p > 0.1), while there was a difference in the use of accessory items (R = 1, p = 0.01), possibly due to an higher use of livestock in AC (cumulative AV%: = 0.76% in AP and 2.38% in AC). The similarity observed seemed to be explained mainly by a similar use of wild boar (simper results: 0.58% of the overall similarity, p = 0.01), while the roe deer use did not result explanatory (p > 0.1).

Moreover, the results of Manly's selectivity index, calculated for each winter season, underlined a strong positive selection for wild boar in both areas, with values of α ranging between 0.89 and 0.96 for AC, and between 0.75 and 0.92 for AP (Table 4).

DISCUSSION

Already in 1993, Boitani pointed out that the risk of hybridization between the wild and domestic form of *C. lupus* was high in Italy, due to the small wolf population size and to the extensive contact with free-ranging dogs. Indeed, in the last years, an increasing number of hybrid individuals was diagnosed (Lorenzini et al. 2014, Randi et al. 2014).
Accordingly, in the present study, we detected evident signs of introgression from the dog in resident wolves inhabiting a mountain area of Central Italy. We detected strikingly different levels of introgression in two adjacent areas over the study period: individuals sampled in AP resulted highly introgressed, as a consequence of presumably recent hybridization events, while those inhabiting AC showed only weak signs of a seemingly less recent introgression of canine genes. As expected considering the known asymmetry in the hybridization process (male dog x female wolf, Hindrikson et al. 2012), introgression from the dog into the local wolf population appeared in autosomal and patrilineal markers only.

So far, no studies have been conducted on behavioural traits of free-ranging hybrids, thus no evidence is reported of possible deviating habits that can be associated to the genetic introgression from the domestic form. Neither it was investigated the possible ecological effects of the spread of introgressed individuals. In the present study we analyzed, in a direct comparative way, the trophic ecology of wolf-dog hybrids, in order to understand if, from a trophic point of view, they could pose a threat for the wolf itself, competing with it for the same resources.

Our results showed that, in similar ecological conditions, wolf-dog hybrids have the same food preferences as wolves, with the tendency to feed mainly on wild ungulates, with wild boar representing the main prey species. As stressed by all the statistical analyses, independently from the degree of canine introgression, individuals belonging to the AC and

AP areas used the same prey categories, and also similarly relied on the different age and weight classes of their preys.

As regards wild boar, individuals living both in AC and AP relied mainly on piglets between 10 and 35 kg and, among roe deer, juveniles represented the target class. The Manly's selectivity index pointed out a strong selection for wild boar in both study areas, emphasizing that individuals living in both territories actively preyed on wild boar, even if roe deer was the more abundant and available species. These results are in agreement with the optimal foraging theory (Stephens and Krebs, 1986). This intensive use of wild boar could depend on its gregarious behaviour: wild boar not only may result easier to detect than roe deer (which is solitary or in small groups), but the clustering of individuals may also allow the predator to focus on a medium-sized and more vulnerable weight class (piglets between 10 and 35 kg). Concerning roe deer (second food item), juveniles were mostly used in the two areas, in agreement with several studies (Salvador and Abad 1987, Okarma 1995, Jędrzejewski et al. 2002, and Mattioli et al. 2011), in which young ungulates were shown to be usually the preferred prey, because they are generally slower, less dangerous, and more inexperienced with predators than adults (Mech 1970).

From which parental line this trophic behaviour has been inherited? Several studies have been conducted on dog and wolf food habits separately.

As regards dogs, even if some studies have shown the capability of free-ranging dogs to kill and feed on wildlife (Kuuk and Snell 1981, and Campos et al.2007), and their ability to prey large-sized mammals (Boitani 1995; Bluter and du Toit 2002), the majority of researches highlighted the tendency of dogs to rely mainly on anthropogenic food. Indeed, the use of livestock and garbage is very common (Butler and du Toit 2002, Atickem 2003, Butler et al. 2004, and Vanak and Gompper 2009).

Concerning wolf, there is plenty of studies showing that this carnivore feed mainly on wild ungulates, with a small percentage of its diet made up by accessory items, according to its opportunistic behaviour (Okarma 1995, Kubarsepp and Valdmann 2003, Peterson and Ciucci 2003, Gazzola et al. 2005, Lanski et al. 2011, Nowak et al. 2011, Bassi et al. 2012, and Palmegiani et al. 2013). The used prey size and species varied from place to place according to many factors: environmental features (i.e., weather, terrain, etc.), preys (i.e. species,

availability, vulnerability, density, social behaviour, etc.), and predators (i.e. experience, pack size, tradition, individual preference, etc.) (Huggard 1993, and Mech and Peterson 2003). All these factors made wolves able to specialize on some preys (usually 1-2) in different regions (Jędrzejewski et al. 2012). In Southern Europe ungulates were the main preys, with wild boar and roe deer both representing main food items (Peterson and Ciucci 2003). This pattern is confirmed in the Italian peninsula; here several authors observed a specialization of wolves on wild ungulates (Meriggi et al. 1996, Capitani et al. 2004, Gazzola et al. 2007, and Marucco et al. 2008), the wild boar often being the main prey species (Mattioli et al .1995 and 2011, Meriggi et al. 2011, and Milanesi et al. 2012).

Finally, there are very few researches conducted on the competition between wolf and dogs, with opposing results. Jhala (1993) observed competition between dogs and Indian wolves (*Canis lupus pallipes*) for the blackbuck fawns (*Antelope cervicapra*), while Atickem (2003), argued that the trophic competition was not important between free-ranging dogs and Ethiopian wolf (*Canis simensis*).

All the studies conducted on wolf and dog food habits underlined that both predators have an opportunistic feeding behaviour (Boitani 1983, Gibson 1983, MacDonald and Carr 1995, Meriggi and Lovari 1996, Capitani et al. 2004, Reed et al. 2006, Campos et al. 2007, and Vanak and Gompper 2009). According to our results, the food habits shown by the hybrids clearly overlapped those observed in local wolves.

This result can be easily interpreted. The majority of field observations have reported hybridization between female wolves and male dogs (Ishadov 1977, Ryabov 1978, Boitani 1982, Bondarev 2002), while crosses between female dogs and male wolves remain very rare (Hindrikson et al., 2012). This sexually asymmetric pattern in hybridization (see also Vilà et al., 2003, Iacolina et al., 2010; Godinho et al., 2011) has been confirmed by genetic studies also (Randi et al. 2000, Andersone et al. 2002, Vilà et al. 2003, Verardi et al. 2006, Randi 2008). As usually a male dog neither assist the female in pup rearing and care (Boitani et al. 1995, Vilà and Wayne, 1999), nor forms long-term bonds with her; in case of mating between a he-dog and she-wolf, pups are therefore likely to be reared by the wolf mother only, which transmits them her habits.

It has been observed that the element of learning, tradition, and individual preference are involved in prey species preferences, and usually packs in natural environment maintain long

traditions of hunting routes and habits (Haber 1996). Due to their sociality, wolves show a considerable potential for cultural transmission (Boitani 2003) and the transmission of all the experiences allows them to gain a keen knowledge of the prey in their territory and to develop habits which increase their hunting efficiency (Mech and Peterson 2003).

All the above-mentioned factors can therefore explain why wolf-dog hybrids in our study area show a wolf-behaviour. Indeed, hybrid offspring, raised under free-ranging conditions, learn the feeding habits transmitted by the wolf mother, more likely to be advantageous in the local environment.

Nevertheless, given the seemingly high trophic overlap, the presence of hybrids could represent an ecological (and not only genetic) threat to the local wolf population in the long-term.

Actually, a wolf-like trophic behaviour of hybrids could represent one of the factors facilitating the spread of introgressed individuals among the Italian wolf population. Behaving like wolves, in fact, hybrids can result perfectly adapted to the natural environment, can find wolf mates and form new introgressed packs (this seem to be the case for AP). This might also explain the high frequency of backcrossed individuals among the identified hybrids in Italy (Randi et al. 2014) and is confirmed also by our genetic results, that show signatures of past hybridization events, revealing a dilution of canine genes into the wolf population gene pool.

Several studies demonstrated that natural predation could regulate herbivores abundance (Hairston and Hairston 1993, Eberhardt 1997, and Krebs et al. 1999). Both wolves and hybrids turned out to rely mainly on wild ungulates, likely contributing to regulate their populations in our study area. However, the main factor driving ungulate populations trends in the area seems to be represented by hunting (unpublished data). Moreover, the impact of wolf-hybrids on the local community of wild ungulates cannot be easily considered as additive with respect to the impact of wolves. Indeed, hybrid packs are likely to replace wolf packs, not causing a local increase in predator density.

Interestingly, our data do not highlight a higher predation by the hybrids on livestock. Given that changes in behaviour in the dog compared to its wild ancestor have a genetic basis (Saetre et al. 2006), a possible consequence of hybridization is a less elusive behaviour, leading to a higher frequentation of human-modified landscapes and a higher impact on

livestock. This does not seem the case in our study area, possibly because of a limited availability of domestic preys or of the effectiveness of prevention measures.

To date, there are no studies about the actual competition between wolf and wolf-dog hybrids, and the present study represents the first research on the feeding ecology of free-ranging hybrids. The results we obtained stressed that in natural environment wolf-dog hybrids may share the same trophic preferences with wolves, therefore indicating hybrids as potential competitors. Further studies on different aspects of the ecology and socio-biology of wolf-dog hybrids (e.g., reproductive behaviour, sociality, territoriality, interaction with prey and environment, etc.) are highly recommended, in order to improve the appraisal of the impact of hybridisation on natural wolf populations. Widening the knowledge about wolf-dog hybrids could be crucial for the conservation of the Italian wolf population.

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FIGURES AND TABLES

Table 1. Diet composition for the two areas (AP: Alpe di Poti; AC: Alpe di Catenaia). Results are reported as average volume percentage (AV%) and as percentage of biomass for both areas.

Food item	AP		AC	
	AV%	**biomass %**	**AV%**	**biomass %**
Wild boar	47.23	55.09	62.76	65.34
Roe deer	47.08	42.49	29.24	28.65
Cervids	0.68	1.15	0.23	0.42
Ungulates total	**95.00**	**98.73**	**92.23**	**94.41**
Hare	0.00	0.00	1.77	1.34
Small mammals	0.15	0.10	0.46	0.33
Sheep	0.15	0.22	1.23	1.95
Goat	0.00	0.00	0.31	0.40
Cow	0.00	0.00	0.31	0.66
Livestock	0.61	0.94	0.54	0.90
Vegetables	3.56	-	2.31	-
Other	0.53	-	0.85	-
Other categories total	**5.00**	**1.27**	**7.77**	**5.59**

Table 2. Calculation of niche breadth for both areas by means of Levin index, and trophic niche overlap between the two areas by means of Pianka index. For the calculation of these indexes, we used both the AV% and the relative biomass values. AP: Alpe di Poti; AC: Alpe di Catenaia.

	Levin index (B)		**Pianka index(O)**
	AP	AC	
AV%	2.24	2.08	0.94
biomass%	2.06	1.96	0.97

Table 3. Results from the Bray–Curtis (BC) dissimilarity matrix calculation. Analysis were performed using both the methods of diets quantification (AV% and relative biomass) and considering wild boar and roe deer as macro-categories first (i.e., without distinction according to weight and age classes), and then considering them divided into classes.

Method		BC value
Macro-categories	AV%	0.20
	biomass%	0.14
Weight/age classes	AV%	0.20
	biomass%	0.19

Table 4. Manly's selectivity index for wild boar in wolves (Alpe di Catenaia - AC) and hybrids (Alpe di Poti - AP). Analysis were performed using the biomass method for diet quantification and obtaining available biomass from density data obtained by drive censuses. The index was calculated only for winters in which we had >20 scats for each pack.

		2004/05	2005/06	2006/07	2010/11
Area	AC	0.90	0.89	0.96	0.96
	AP	0.83	0.78	0.92	0.84

Figure 1. Study area. On the left, the gray shade represents the Arezzo province, which is zoomed on the right. The Alpe di Catenaia (AC) and Alpe di Poti (AP) areas are bordered respectively by a grey line and a black line; The red line represents the border of the protected area within AC.

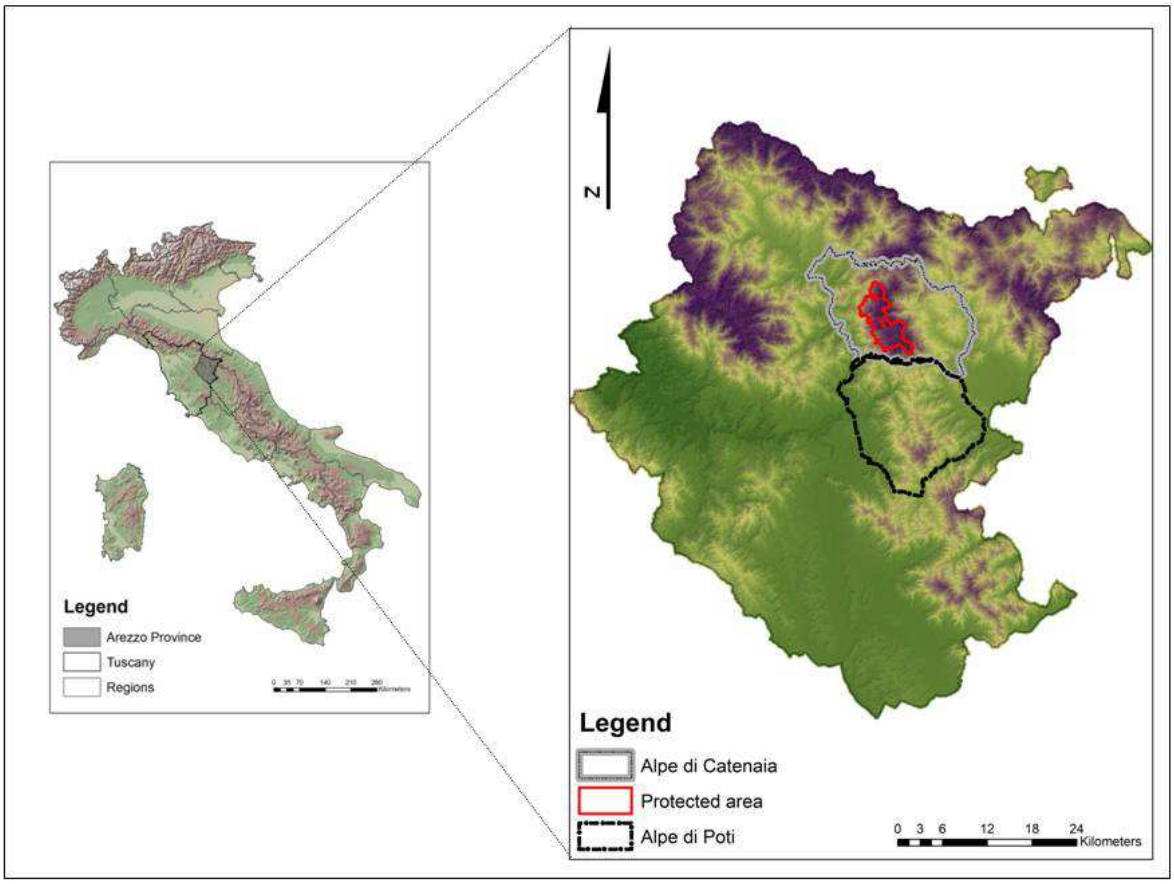


Figure 2. Estimated proportions of membership to the two clusters inferred (dog: dark gray; wolf: light grey) by STRUCTURE analysis, performed on 47 putative wolves sampled in the Alpe di Catenaia (AC, on the left of the dotted line) and the Alpe di Poti (AP, on the right) areas. Each individual is represented by a vertical bar.

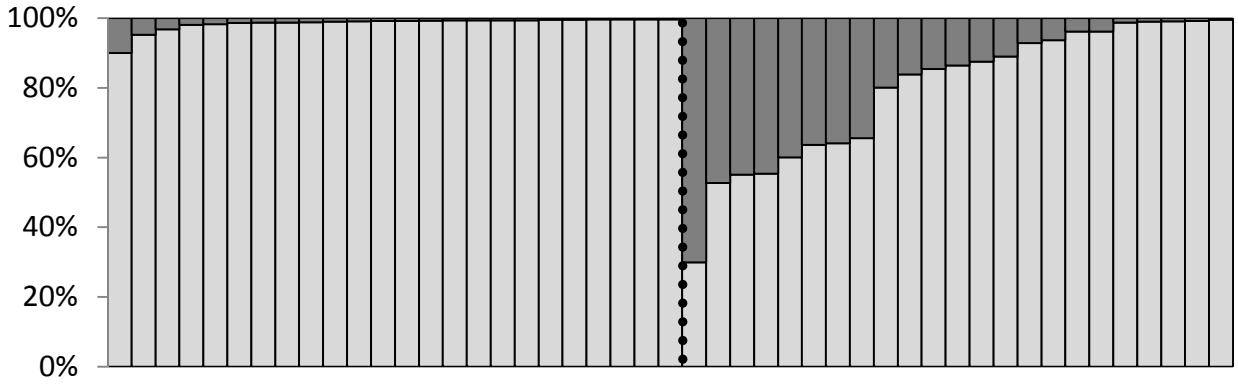
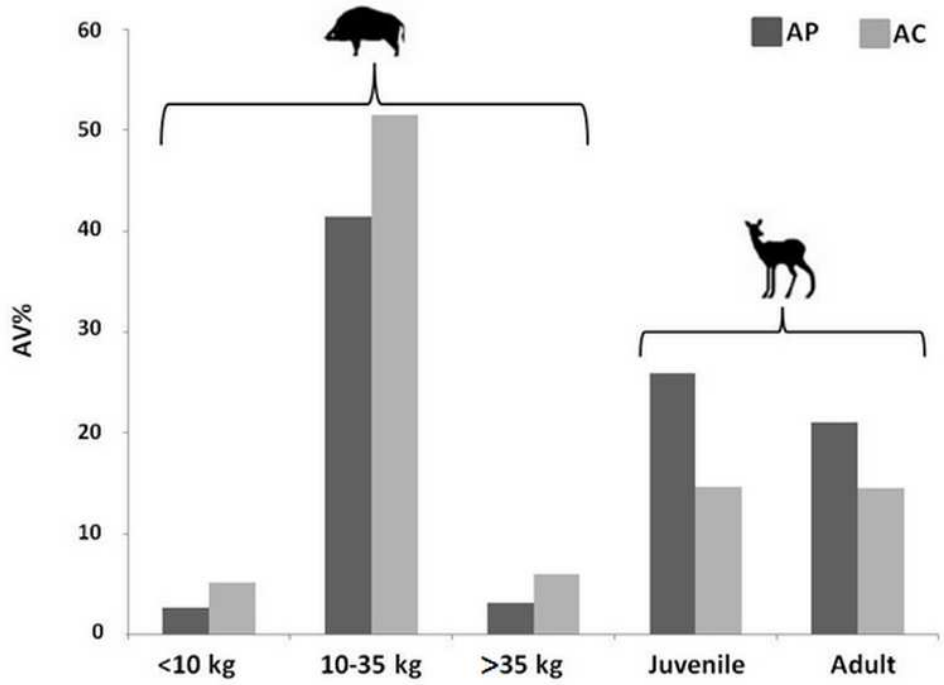
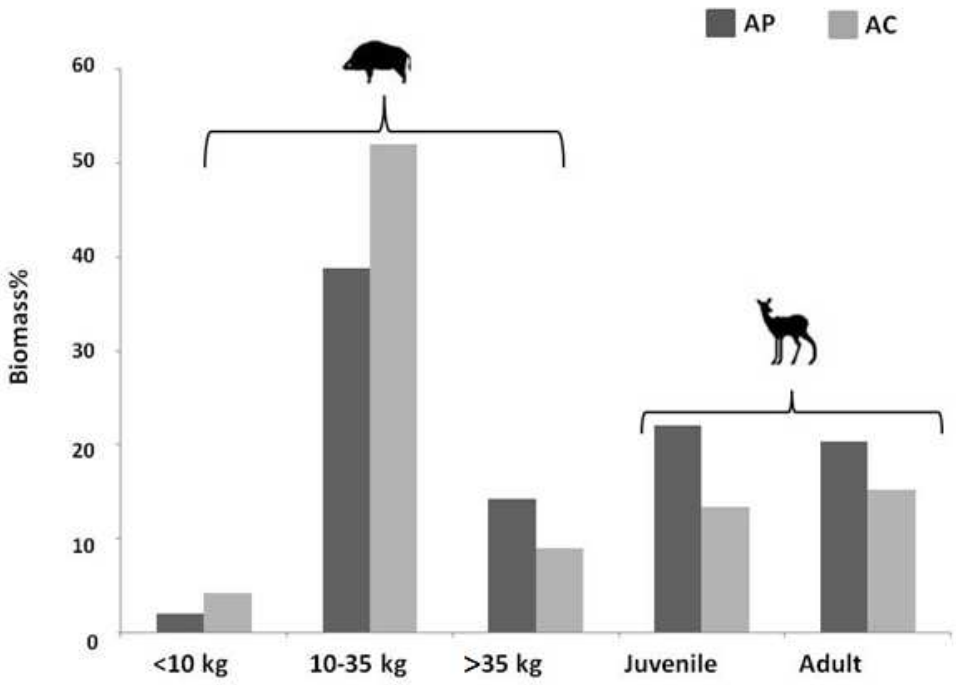


Figure 3. Use of different weight and age classes of the two main prey species by packs in the two areas (AC, Alpe di Catenaia; AP, Alpe di Poti), reported either in terms of AV% (figure 3a), or in terms of relative biomass (figure 3b). Wild boars are divided into three weight classes (<10 kg, 10-35 kg, and >35 kg), while roe deer in two age classes (juvenile, and adult).

a)



b)



CHAPTER 7

Video-scats: joining camera trapping and non-invasive genotyping as a tool to confirm hybrid assessment and individual identification

Video-scats: joining camera trapping and non-invasive genotyping as a tool to confirm hybrid assessment and individual identification

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Abstract

The use of motion-activated video cameras and non-invasive genetic sampling has become very common to remotely obtain outstanding information on wild populations of rare or elusive carnivores. The two approaches are usually implemented separately, and when they are integrated, it happens at a population level (mostly for population size estimation). Here we present the advantages offered by the integration of camera trapping and non-invasive genotyping at an individual level, in a monitored Italian wolf population affected by introgression from domestic dogs. We recorded 16 events of defecation at camera traps and analysed the collected scats in order to get an univocal individual determination (by analyzing sex markers, 12 autosomal microsatellites, two Y-chromosome microsatellites and the control region of the mitochondrial DNA). Genetic data for 13 scats were combined to morphological and behavioural traits observed in the videos and compared to other data from ongoing genetic monitoring, finally allowing to assess: pack membership, breeding status, morphological traits (included those referable to hybridization), resampling history, and genetic purity. We discuss drawbacks and possible solutions, that can suggest to promote the use of 'video-scats' as an opportunistic source of valuable information.

Keywords: *Canis lupus*, camera trapping, non-invasive genetic sampling, hybridization, individual identification

Introduction

Camera trapping (CT) and non-invasive genetic sampling (NGS) have become common tools in monitoring wild populations of elusive or rare species. CT has been especially advantageous to document the presence of a cryptic species (Linkie et al. 2013), to achieve an estimation of population size or density (Karanth et al. 2006, Sollmann et al. 2013), to reveal relevant behavioural traits (Harmsen et al. 2010), or to provide high quality images of specific individuals in the population (Hiby et al. 2009, Courtney et al. 2015). On the other hand, the genotyping of non-invasively collected samples provides information on the presence and spatio-temporal distribution of individuals, leading to achieve key data on the population (population size, social structure, genetic diversity, dispersal patterns, occurrence of hybridization and diseases, diet composition – see Wait & Paetkau 2005 for a review).

While in some species individuals can be unambiguously recognized on the basis of easily detectable traits (usually pelage patterns, e.g. tiger *Panthera tigris,* Hiby et al. 2009; snow leopard *Panthera uncia,* Janečka et al, 2011, or Iberian lynx *Lynx pardinus*, Gil-Sánchez et al. 2011), in other species distinguishing individuals is really challenging (Güthlin et al. 2014, Alonso et al. 2015). This limitation compromises the use of remotely-collected camera data for population counts, due to the violation of one of the main assumptions of mark-recapture models (i.e. each individual can be univocally recognized).

NGS is based on 'second choice' biological material (mostly faeces or hairs/feathers) and leads to taxonomic and individual identification (the so called 'genetic fingerprinting'), but does not allow to associate any additional information to the sampled individuals (Waits & Paetkau 2005). This represents a strong limitation, since some individual features cannot be assessed in absence of visual data (for instance age, reproductive status, social status, etc.).

In gray wolf (*Canis lupus*) morphological variation among individuals is usually very limited (higher in North America, lower in Eurasia) and this has hampered the implementation of mark-recapture methods for the estimation of population size, which rely on individual recognition from remote sensing images. However, in some regions, where hybridization with domestic dogs occurs (Italy – Verardi et al. 2006, Spain – Godinho et al. 2011, 2015, Baltic Countries – Hindrikson et al. 2012), inter-individual variation may increase as a consequence of the introgression of canine genes into the wild population. Under such

circumstances, the morphological identification of individuals may become feasible, enabling the application of a new (non invasive) technique to the estimation of population size.

The big concern arisen from the spread of canine genes in wild wolf populations has, on one hand, pushed to the optimization of genetic tools to detect hybrids, but, on the other hand, it has highlighted their limits, due to the high dilution of domestic genes in backcrossed individuals (which represents the vast majority of hybrids) and to the uncertainty of their assignment to the parental populations (Lorenzini et al. 2014, Randi et al. 2014). Similarly, the adoption of morphological criteria only can reveal misleading because of the possible occurrence of 'asymptomatic' hybrids (i.e. showing a wild-type phenotype, Lorenzini et al. 2014) and because of the difficulty to distinguish the effect of introgression from intra-specific phenotypic variation.

As a consequence, wherever possible, the adoption of both genetic and morphological criteria to assess hybridization is strongly advised. But, so far, this has only been possible for captured or dead wolves.

In the present study, we demonstrate the potential benefits of a simultaneous use of motion-activated video cameras and non-invasive genotyping to provide complementary information on individual wolves in a wild population. Its utility can be twice: i) it can give support to hybrid identification in target packs, ii) it can help to test the reliability of individual recognition for the application of mark-recapture methods of population size estimation.

Material and Methods

Study area

The study was conducted in a mountain area located in Tuscany (Italy), including the massifs named Alpe di Catenaia and Alpe di Poti (north-west to the city of Arezzo). Elevations are comprised between 250 and 1414 m a.s.l.. The main land cover of the area is represented by mixed deciduous hardwoods, dominated by oak (*Quercus* spp.), chestnut (*Castanea sativa*) and beech (*Fagus sylvatica*). The wolf is the only large carnivore occurring in the area, while wild ungulates are represented by the ubiquitous wild boar (*Sus scrofa*) and roe deer (*Capreolus capreolus*), plus a limited number of red deer (*Cervus elaphus*). Wolf presence in the area was monitored since 1998, with summer sessions of wolf howling (Passilongo et al.

2010), non-invasive genetic sampling (Scandura et al. 2011) and, more recently, by video-trapping. In the last years, signatures of wolf-dog hybridization were detected in recovered carcasses and non-invasively genotyped individuals (Iacolina et al. 2010).

Remote camera trapping

CT was conducted in the study area between 2013 and 2015. This period included two sessions of standardized CT surveys: 35 cameras were used from 19 January to 28 August 2014 (2838 trap days), 49 cameras from 1 January till 15 June 2015 (2918 trap days). Outside these time windows a number of 3-11 cameras were opportunistically deployed (for a total of 1135 trap days). Remote motion-activated cameras were placed at known scent marking sites used by resident wolves and situated along dirt roads (mostly at crossing points). This was done to maximize both the detection probability and the permanence of wolves in front of the camera. Each camera trap was 24-h active and was visited at variable intervals (usually 2-20 days) to change batteries and SD cards. No bait was employed at trapping sites.

Three models of inbuilt HD digital cameras were used: Buschnell trophy cam HD, UVision UV 562 and UV 572, provided by Fototrappolaggio s.r.l.. All three had passive infrared sensor (PIR) and LED flash. Cameras worked on video mode with a duration of 60 seconds and 1 second interval between consecutive videos.

After removing SD cards, videos were screened to select those containing wolf images. These videos were coded (combining consecutive videos) and carefully watched to record pack identity and number of individuals; for each individual, whenever detectable, the following information was recorded: sex, pack identity, marking behavior, morphological anomalies possibly associated to canine introgression (e.g. coat color pattern or melanism, dewclaws, floppy ears, etc.), and other peculiar traits to be used for individual recognition. Finally, the focus was posed on the defecating individuals, which were identified and their social rank evaluated (either breeding adult or non-breeding pack member), even according to their occurrence and behavior in other videos.

Genetic analysis

Scats deposited on the ground in front of video cameras ('video-scats'), for which the producing individual was filmed, were collected if the elapsed time from defecation was not > 10 days (giving an expected yield <75%, Santini et al. 2007). The collector, wearing sterile gloves, removed a piece of a few centimeters from the scat and transferred it into a 25-ml

plastic tube subsequently filled with 5-10 volumes of absolute ethanol. The sample was stored at room temperature until its shipment to the genetic lab for the analysis. Genotyping was realized by NGB Genetics (Bologna, Italy). DNA was isolated from faecal samples using the Qiagen QIAamp DNA Stool Kit (Qiagen GmbH., Hilden, Germany) and following the producer's protocol. Genotyping was based on the amplification, in three replicates, of one marker (Amelogenin gene) for gender determination and 12 unlinked autosomal microsatellites (dinucleotides: C09.250, CPH2, CPH4, CPH5, CPH8, CPH12 – Ostrander et al. 1993, Fredholm & Wintero 1995; tetranucleotides: FH2004, FH2137, FH2088, FH2096, FH2079, FH2132 – Francisco et al. 1996). Successful samples were sequenced at 350 bp of the control region of the mitochondrial DNA (CR-mtDNA – Vilà et al. 1999) and, if males, were also typed at two Y-chromosome microsatellites (MS34A and MS34B – Sundqvist et al. 2001). Amplification conditions are available upon request. Amplification (PCR) success was calculated as the percentage of successful single-locus PCRs.

For each analysed scat sample, a consensus genotype was constructed from the three replicates, accepting as heterozygote any locus showing two different alleles in at least two independent repetitions, and as homozygote that showing one single allele in all three repetitions. Basing on the consensus alleles, individual error rate was estimated as the number of correct single-locus genotypes divided by the total number of obtained single-locus genotypes. In so doing, whenever possible, we considered as reference 'correct' genotype the matching genotype in the database (see below).

The obtained genotypes were compared to those previously obtained during the genetic monitoring of the Arezzo population (83 genotypes deriving from NGS or from recovered carcasses in the period 2005-2015). The software GIMLET v. 1.3.3 (Valière et al. 2002) was used to identify possible matches between a given new genotype and those already in the database. For each genotype the probability of identity (either $P_{id[random]}$, i.e. calculated for random dyads in the population, or $P_{id[sibs]}$, i.e. calculated between siblings – Waits et al. 2001) was calculated, indicating the probability of occurrence of the same allele combination in a different individual in the population. This probability typically increases with decreasing number of typed loci.

CR-mtDNA sequences were compared in BLAST (https://blast.ncbi.nlm.nih.gov) with other published sequences in order to ascertain their matching with the diagnostic Italian wolf

haplotype W14 (Randi et al. 2000), while Y-chromosome haplotypes were classified according to Iacolina et al. (2010).

Finally, with the aim to assess the degree of introgression from domestic dog in the genotyped individuals and to associate it to the detected morphological traits, a Bayesian cluster analysis was performed in STRUCTURE v. 2.3.4 (Pritchard et al. 2000). As reference 'wolves', we considered 34 genotypes that had no sign of introgression – at autosomal microsatellites, CR-mtDNA, Y-chromosome or (in case of carcasses) morphology – in previous analyses (see Chapter VI). In so doing we excluded individuals sampled in the study area. Similarly, 37 local domestic dogs (half from private owners, half from kennels) were included as reference genotypes in the analysis (popflag=1). Genotypes obtained from scat samples were also included, with popflag=0. STRUCTURE was run 10 times, with fixed K = 2, 250,000 burn-in followed by 250,000 iterations as data collection, admixture model, uncorrelated allele frequencies between populations and the option "update p from pop flag only" activated. The proportion of admixed ancestry of a given individual referred to its membership to the two clusters inferred by the program, by considering the run with highest posterior probability.

Results and Discussion

Between 2 March 2013 and 19 July 2015, over a total of 6891 trap days, 37 defecation events were recorded (on average one event every 186.2 trap days).

Sixteen video-scats were collected by 10 days from deposition and were sent to the lab for genetic analyses. Their age varied between 16 hours and 10 days. For 13 scats a reliable multilocus genotype was obtained (Tab. 1). Amplification success ranged individually between 6% and 100% (on average 78.8%), while error rate was between 0% and 20.7% (on average 2.7% for successful samples) and was a function of the sample age (Fig. 1). All detected errors were due to allelic dropout. The analysis in GIMLET revealed that the 13 consensus genotypes corresponded to 10 different individuals, 5 males and 5 females (Tab. 1), 6 of which were already 'known', i.e. they had previously resulted from the analysis of other non-invasive samples in the area. $P_{id[random]}$ for them ranged between 3.36×10^{-12} and 1.12×10^{-7}, whereas $P_{id[sib]}$ ranged between 5.18×10^{-5} and 2.69×10^{-3}. Therefore each genotype had a negligible chance to be shared by two individuals in the population.

Wolf identification by NGS matched that assessed by CT (Tab. 2): each time a particular individual was recognized in more than one video, the genetic analysis of the corresponding scat samples identified the same genotype. The identified wolves belonged to four different packs and were filmed either alone or accompanied by other 1-3 individuals. Since trapping sites had been selected in correspondence of previously known marking points, an intensive marking behavior was shown by animals. Apart from fecal marking, most filmed wolves showed other types of marking, especially raised-leg urination and ground scratching. According to their dominance and marking behavior, most defecating wolves (n=6) were recognized as breeding adults. All of them but one (MF05) had resulted from >1 fecal sample (AP37 and MF03 were represented respectively 3 and 2 times in video-scats) over up to 4 consecutive years of genetic monitoring.

All videos but one were nocturnal, this making more difficult to evaluate morphological anomalies that could be attributed to hybridization with domestic dog. However, at least two individuals showed deviating phenotypes (AP11 and AC55), and the most evident genetic signatures of introgression: female AP11 (alias aF1-PS) had a low membership proportion to the wolf cluster (Qw=0.928), male AC55 (alias aM1-AC), although showing a Qw=0.99, was carrying a canine Y haplotype (H03) as a legacy of past introgression.

The results of this preliminary study put emphasis on the utility of the integration of simultaneous data from two so far disjoint sources of information for wolves (video images and scats). Especially in areas where hybridization with domestic dog is spreading canine genes across the wolf population, it is of crucial importance to collect evidences of the presence of hybrid packs and on the breeding status of introgressed individuals in a pack. In our study area, for instance, through the combined use of CT and NGS, we identified two individuals, in distinct packs, which had signatures of introgression and were member of the breeding pair. This evidence raises a special concern, as their successful breeding would lead to a further spread of canine genes in the population. Successful mating by wolf-dog hybrids in the wild has been already documented in Tuscany (Caniglia et al. 2013) and was suspected from the high proportion of backcrosses observed in other areas of Italy (Randi et al. 2014).

The combination of remote sensing and genetic analysis of biological material non-invasively collected on-site is not new. In most cases, however, the two methods were used in the same area just to non-invasively collect as many data as possible on local populations of

elusive carnivores: this is the case of wildcat (*Felis silvestris*) in Italy (Anile et al. 2012, Velli et al. 2015). Galaverni and colleagues (2012) have compared the information obtained by NGS and CT within a wolf pack territory in Italy. They outlined the complementarity of the two methods in providing information on pack composition and size, as well as individual morphology and behavior.

However, the main purpose of these studies was often the estimation of population size or density in the area, and not the collection of complementary information on specific individuals.

The main limitation of our approach was the low frequency of defecation events at CT sites. During our surveys, although cameras were located along trails used by wolves, the chance to film a wolf defecating was quite low (0.54 per 100 trap days). In our surveys, no lure was used to attract wolves at the trap sites. The use of a bait, possibly represented by alien scats or urine, can therefore increase the visitation rate. Moreover the utility of 'video-scats' depends on i) the quality of video, ii) the frequency of visits by the operator. If the camera works in bad visibility conditions, videos can become unsuitable, as important traits of the target animal cannot be confidently evaluated. Most of the wolf videos collected were nocturnal (see Fig. 2), thus with a lower definition. The frequency at which camera traps are checked is usually not very high in order to minimize site perturbation. On the other hand, if the site is visited too rarely the collected scat can become unsuitable for genotyping. We observed a marked decline in the amplification success of fecal DNA after around one week from deposition. This result is similar to what obtained by Santini and colleagues (2007) in experimental conditions, though the relationship between scat age and PCR success appeared more linear than in our study.

The genetic analysis of video-scats has confirmed the identity of individuals that had been previously recognized on the basis of phenotypic traits by one of the authors (LM). Two individuals that were sampled multiple times (AP37 and MF03) by video-scats had been correctly identified on the basis of morphological cues only. This confirmation is promising, meaning that intra-population phenotypic variation could allow individual recognition in wolves, possibly facilitated by the introgression of canine genes (yet the two resampled individuals did not show signs of admixture). This would open to the application of mark-recapture approaches based on CR data for population size estimation in wolves.

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Table 1 - Results of genotyping the 16 'video-scats'. The following data are reported: sex (genetically-assessed by the Amelogenin gene), the consensus 12-microsatellites genotype (3 repetitions), the CR-mtDNA haplotype (W14 is the typical Italian wolf haplotype), 2-microsatellites Y-chromosome haplotype (H01 and H02 are widespread in Italian wolves, H03 in Italian domestic dogs) and the probability of identity associated to each multilocus genotype as calculated for random dyads ($P_{id[random]}$) and sibling pairs ($P_{id[sibs]}$).

Sample	Sex	CXX250	FH2004	FH2137	FH2088	FH2096	CPH02	CPH08	FH2079	FH2132	CPH12	CPH04	CPH05	mtDNA	Y-haplotype	Genotype	$P_{id[random]}$	$P_{id[sibs]}$
N313	F	0/0	110/110	0/0	93/117	92/96	96/98	0/0	271/275	0/0	192/192	0/0	116/118	W14	-	AP40	1.12×10^{-7}	2.69×10^{-3}
N317	F	0/0	0/0	0/0	0/0	0/0	98/100	0/0	0/0	0/0	0/0	0/0	0/0	-	-	-	-	-
N321	-	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-	-	-	-	-
N322	M	133/133	110/176	154/156	0/0	92/100	100/100	203/207	271/275	260/296	192/192	145/145	118/118	W14	H02	AP37	1.04×10^{-11}	1.55×10^{-4}
N329	M	133/133	110/164	178/178	0/0	96/100	92/92	203/203	263/263	318/318	192/206	145/145	118/128	W14	H01	AP34	4.42×10^{-9}	4.23×10^{-4}
N339	F	129/133	110/110	156/164	93/125	92/100	100/100	203/203	263/275	260/318	192/210	145/145	118/118	W14	-	AP43	5.41×10^{-11}	1.42×10^{-4}
N340	M	133/133	110/176	154/156	125/125	92/100	100/100	203/207	271/275	260/296	192/192	145/145	118/118	W14	H02	AP37	3.36×10^{-12}	9.50×10^{-5}
N346	F	129/133	106/164	164/168	93/125	96/100	92/100	195/203	263/267	318/318	192/192	145/147	120/128	W14	-	MF01	1.35×10^{-10}	9.22×10^{-5}
N353	-	0/0	0/0	0/0	0/0	0/0	92/100	0/0	0/0	0/0	0/0	0/0	0/0	-	-	-	-	-
N383	M	133/133	106/176	168/178	93/125	96/100	92/100	203/203	263/263	318/318	192/192	145/147	128/128	W14	H01	MF03	2.05×10^{-10}	1.33×10^{-4}
N384	M	133/133	106/176	168/178	117/125	96/100	92/98	195/203	263/263	318/318	192/192	147/147	118/120	W14	H01	MF04	1.92×10^{-11}	7.43×10^{-5}
N385	F	129/133	110/164	164/168	93/125	96/100	92/100	203/209	0/0	0/0	0/0	145/147	116/118	W14	-	AP11	7.51×10^{-8}	9.38×10^{-4}
N393	M	133/133	110/176	154/156	125/125	92/100	100/100	203/207	271/275	260/296	192/192	145/145	118/118	W14	H02	AP37	3.36×10^{-12}	9.50×10^{-5}
N394	M	133/133	106/176	168/178	93/125	96/100	92/100	203/203	263/263	318/318	192/192	145/147	128/128	W14	H01	MF03	2.05×10^{-10}	1.33×10^{-4}
N395	F	133/137	110/110	160/178	93/125	92/96	92/96	203/203	263/271	318/322	192/206	145/145	116/118	-	-	MF05	7.35×10^{-11}	9.06×10^{-5}
N408	M	133/133	110/110	160/178	117/117	92/100	92/98	195/207	263/267	314/322	192/206	145/147	118/118	W14	H03	AC55	7.40×10^{-12}	5.18×10^{-5}

Table 2 - Information on the analyzed 'video-scats' and on the defecating individuals filmed by remote cameras. Sample age refers to the time elapsed between defecation and collection time. Wolf ID is the univocal code given to the specific individual recognized in the video (it incorporates information on social status, sex and pack). Total nr. of individuals is the number of different wolves observed in the video. Marking behavior refers to known types of marking adopted by wolves to mark the territory (FM = fecal marking, RLU = raised leg urination, STU = standing urination, FLU = flexed-leg urination, SQU = squatting urination, GSC = ground scratching). Qw is the assignment probability to the 'wolf' cluster inferred by the software STRUCTURE. Total samplings account for the overall number of resampling of the specific genotype over the whole genetic monitoring of the population. Sampling years is the period over which such resampling had occurred. n.d. = not determined

SAMPLE ID	TRAP ID	DEPOSITION DAY	COLLECTION DAY	SAMPLE AGE	PACK	WOLF ID (video)	TOTAL NR. IND	MARKING BEHAVIOUR	MORPHOLOGY	INDIVIDUAL DIAGNOSTIC TRAITS	GENOTYPE	SEX	PCR SUCCESS	ERROR RATE	Qw	Y-HAPLOTYPE	mt-HAPLOTYPE	TOTAL # SAMPLINGS	SAMPLING YEARS
N313	PO5	12/08/2013	17/08/2013	5 days	Favalto	aF1-MF	2	FLU + FM	wild type	no	AP40	F	64%	2%	0.982	-	W14	4	2
N317	LG1	26/02/2014	08/03/2014	10 days	Lignano	n. d.	1	FM	wild type	no	n.d.	F	25%	-	-	-	-	-	-
N321	PO5	12/03/2014	16/03/2014	4,5 days	Poti Sud	bM1-PS	2	FM + GSC	wild type	Pale coat, reduced black streaks on front legs	n.d.	M	6%	-	-	-	-	-	-
N322	PO9	11/03/2014	12/03/2014	18 hours	Poti Sud	aM1-PS	4	FM	wild type	Long-haired "flag" tail	AP37	M	100%	3%	0.988	H02	W14	8	3
N329	PO5	27/03/2014	30/03/2014	3 days	Favalto	aM1-MF	4	FM	wild type	no	AP34	M	100%	3%	0.994	H01	W14	6	3
N339	PO22	28/08/2014	28/08/2014	17 hours	Poti Sud	bF1-PS	4	SQU + FM	anomalous	reduced white mask, almost absent black streaks on front legs	AP43	F	100%	0%	0.978	-	W14	5	2
N340	PO22	28/08/2014	28/08/2014	17 hours	Poti Sud	aM1-PS	4	STU + FM	wild type	Long-haired "flag" tail	AP37	M	92%	2%	0.988	H02	W14	8	3
N346	MF1b	06/12/2014	07/12/2014	16 hours	n. d.	n. d.	3	FM	wild type	no	MF01	F	100%	0%	0.974	-	W14	1	1
N353	PO5	13/01/2015	23/01/2015	10 days	Poti Sud	aF1-PS	1	FM + GSC	anomalous	Floppy ear, dark coat with streaks, reduced white mask	n.d.	F	11%	-	-	-	-	-	-
N383	MF1a	11/03/2015	14/03/2015	3,5 days	Favalto	aM2-MF	2	FM + GSC + RLU + GSC	wild type	no	MF03	M	89%	3%	0.993	H01	W14	4	1
N384	MF1a	09/03/2015	14/03/2015	5,5 days	Favalto	bM1-MF	1	RLU + GSC + FM + GSC	wild type	no	MF04	M	97%	1%	0.984	H01	W14	1	1
N385	PO22	10/03/2015	15/03/2015	5 days	Poti Sud	aF1-PS	3	FM	anomalous	Floppy ear, dark coat with streaks, reduced white mask	AP11	F	81%	12%	0.928	-	W14	10	4
N393	PO24	25/03/2015	29/03/2015	4 days	Poti Sud	aM1-PS	1	FM + GSC	wild type	Long-haired "flag" tail	AP37	M	100%	0%	0.988	H02	W14	8	3
N394	MF7	20/03/2015	21/03/2015	22 hours	Favalto	aM2-MF	2	RLU + FM + GSC	wild type	no	MF03	M	100%	0%	0.993	H01	W14	4	1
N395	MF7	20/03/2015	21/03/2015	22 hours	Favalto	aF-MF	2	FM + FLU	wild type	no	MF05	F	100%	0%	0.982	-	-	1	1
N408	ACN8	13/04/2015	13/04/2015	16 hours	Catenaia	aM1-AC	2	RLU + GSC + FM	anomalous	Spur on the hind legs, dark coat, marked white mask, long-haired tail	AC55	M	97%	0%	0.990	H03	W14	3	2

Figure 1 – Amplification (PCR) success of the analyzed video-scats as a function of scat age (i.e. time elapsed between defecation and collection).

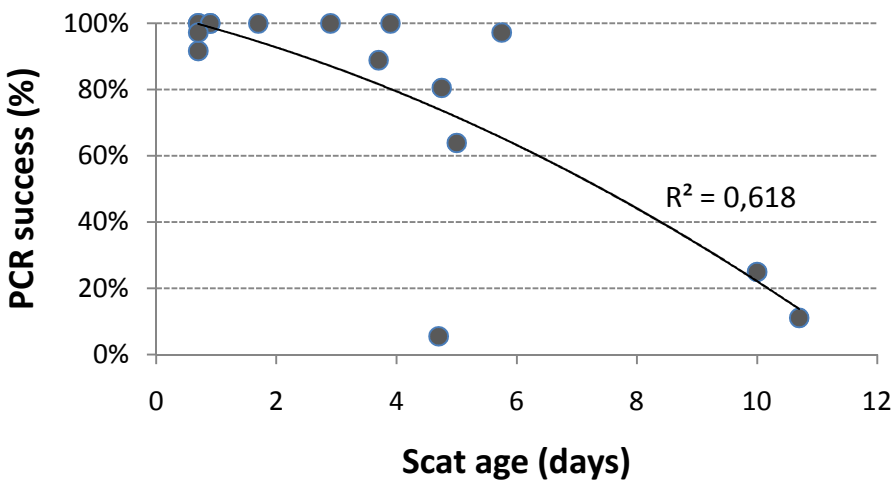

Figure 2 – Two images of defecating wolves extracted from videos recorded in the study area during remote camera trapping surveys (2013-2015).

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CONCLUSIONS

In this thesis, I contributed to the understanding of several aspects related to the broad problematic of hybridization between wild and domestic conspecifics. Specifically, I assessed the presence of ongoing hybridization, leading to the introgression of domestic genes in wild populations of *Sus scrofa* and *Canis lupus*, by analyzing different types of molecular markers and, when possible, taking into account the individual phenotype. In my researches I contributed to the development of new molecular markers and new approaches, investigated the source of hybridization, and also explored some biological aspects of both wild populations and hybrids, that can help to the comprehension of the possible ecological consequences of the observed genetic introgression.

In the first part of my thesis, I sought to expand knowledge about the phenomenon of hybridization between wild boar and its domestic counterpart, the domestic pig.

First of all, I tried to identify the source of hybridization.
Wild boar can crossbreed with domestic pigs both in natural conditions (where open-air pig farming is still practiced) and in captivity, where intentional hybridization with the pig is often carried out in order to produce "wild boar-like" hybrids with improved fertility. But which of these two circumstances represents the main source of domestic genes introgression into wild populations?

In chapter 1, we specifically evaluated the role of farmed wild boars as a source of introgression of domestic genes into wild populations. In order to do so, we analyzed a set of neutral autosomal microsatellites and compared the degree of admixture of wild boar sampled in breeding stations and in the surrounding local wild population in Piedmont and Sardinia, two Italian regions with a different history of wild boar presence and pig husbandry. Given the huge difference in population size between the compared gene pools (wild vs. captive), a statistical approach that allowed to test for dissimilar degrees of admixture accounting for such difference was employed.
Our study revealed that the relative importance of the two sources of introgression can vary greatly across areas.

In Piedmont, introgression from the domestic form into the wild population seemed to be very low, while there were significant signs of admixture in the sampled breeding stations. In Sardinia, instead, both the wild and captive populations showed moderate signs of introgression, and there was no significant difference between their admixture level.
We concluded that hybridization in nature seems to play the key role in Sardinia, while intentional hybridization in captivity, followed by the release of captive hybrids for restocking wild populations, is likely to be the major source of introgression in Piedmont.
At the European level, on the basis of geographical features and population histories, it is presumable that the most common situation is similar to that found in Piedmont, with farms being the main source of introgression. In fact, at present, the opposite situation (that we detected in Sardinia) is possible where open-air pig farming is a common practice; and this happens only in a few regions of Europe. On the contrary, breeding stations are widespread throughout the continent, and local administrations often authorized their establishment without being able to control the source and health conditions of animals.

In chapter 2, the focus was posed on the detection of introgression at functional genes. Indeed, we analyzed variation at *MC1R* and *NR6A1* genes, that influence coat colour and number of vertebrae, respectively. *MC1R* and *NR6A1* genes are particularly useful in the study of hybridization patterns, since they have been under strong artificial selection during domestication and this led to the occurrence of different gene variants in wild and domestic form of *Sus scrofa*. Considering that previous studies had assayed variation across domestic breeds, we included in the analysis a total of 20 domestic pigs and 145 European wild boars samples from 12 different European countries.
Sequence analysis revealed that almost all European wild boars (94%) were homozygous for a single variant of the *MC1R* wild-type allele, which is characteristic of European wild populations and differ from all the variants observed in Asian wild populations. Surprisingly, we did not find any synonymous substitution in the sequence of this allele in our sample, supporting the hypothesis that European wild boars are monomorphic at this locus.
Three domestic *MC1R* alleles of European origin were detected among the remaining fraction (6%) of the analyzed wild boars, proving the introgression of non-neutral gene variants into the European wild population. Introgression was locally high: regions like Bulgaria and Sardinia, where pigs are often reared in semi-free conditions and may cross-

breed with the wild form (Scandura et al. 2008), showed frequencies of domestic alleles up to 10-20%. These results were confirmed by the *NR6A1* analysis: 6% of wild boars carried the domestic variant of this gene, which determines the formation of supernumerary vertebrae.
Considering both loci, introgression was found all over Europe, and proved to be very frequent: as much as 11% of wild boars carried domestic variants at either locus. Moreover, introgression seemed to be bidirectional: in fact, domestic pigs also were affected by genetic introgression of wild variants, showing a not negligible frequency of both the *MC1R* and the *NR6A1 wild-type* alleles (6.25%).
We discussed the consequences of the induced modifications at the investigated quantitative loci in wild boar populations, where especially pelage colours deviating from the wild type may undergone a strong selection and be quickly purged. Their occurrence is therefore likely to indicate ongoing or very recent hybridization.

In chapter 3, given the current lack of Y-chromosome-specific polymorphic markers in *Sus scrofa,* we described a new set of 4 variable Y chromosome microsatellites that we developed to address an important, and so far unexplored, aspect of wild boar x domestic pig hybridization: the sex directionality of crosses.
With the aim to assess male-specific variation at a continental scale, more than 200 male individuals were genotyped at these loci, including wild boars from eight different regions in Europe and domestic pigs belonging to local and commercial breeds of both Asian and European lineages. A maximum of eight alleles per locus were found in the analysed individuals, and 34 haplotypes were obtained by combining alleles (because of the non-recombinant nature of the region).
Significant differences in haplotype frequencies were observed among populations, and especially between domestic pig breeds and wild boar, with F_{ST} values ranging from 0.193 to 0.888. Haplotypes of commercial and Asian domestic pigs were quite peripheral in our network, and the analysis of additional markers (*USP9Y* and *AMELY* genes, that have an uneven distribution between Europe and Asia; Ramirez et al. 2009) in a subset of samples was consistent with an European origin of most of the STR-Y haplotypes observed in European wild and domestic *S. scrofa.*
Haplotype diversity in European wild boar was concordant with values published by Vilaça and colleagues (2014) for the maternally-inherited D-loop region of the mitochondrial DNA,

but the geographic pattern found with mtDNA was not reflected by Y STR data, confirming higher gene flow in the Y-chromosome and male-biased dispersal in this species.
The high variation of the developed Y-chromosome markers and the observed divergence between wild and domestic lineages make the panel of microsatellites we developed a useful tool to evaluate the male contribution to the current diversity in swine breeds, as well as to assess the extent and directionality of hybridization between wild and domestic forms.

In **Chapter 4** we focused on the introgressed Sardinian wild boar population. It represents a nice model to check the impact of introgressive hybridization on the genetic structure of a population (Scandura et al. 2011). The first part of the study was aimed at the identification of introgressed individuals in a large dataset of 368 Sardinian wild boar, typed at 16 neutral microsatellites. A Bayesian admixture analysis was performed to check signatures of introgression by comparison with local domestic pigs and continental wild boar (a second source of possible introgression in the island). Introgression in the island was widespread (more than 24% of individuals with admixed ancestry) and was attributable to hybridization with both allochthonous wild boars and domestic pigs. Once identified and removed the introgressed samples, we were able to identify the correct population structure, by Bayesian cluster analysis, and we revealed which variables had influenced gene flow in the island. The presence of three different subpopulations was ascertained: one occupied the eastern part of the island and two (more pure and disjointed) occurred in western Sardinia. Isolation-by-distance appeared not sufficient to explain such genetic differentiation. According to the knowledge of wild boar spatial behaviour, we modelled the effects of land cover categories and main infrastructures. Our results showed that the correlation between genetic and spatial distance was stronger when environmental characteristics and the presence of a motorway crossing the island from north to south (SS131) were taken into account. In particular, anthropogenic barriers, though relatively recent, seemed to play the major role in shaping the observed diversity. Interestingly, our results highlighted the role of the motorway in preventing the spread of allochthonous and domestic genes to the western subpopulations, thus paradoxically preserving an important portion of the native gene pool.

Chapter 5 deals with a crucial aspect of the biology of the species, which can be severely affected by hybridization. Comprehending how environmental and social cues can induce a

different timing of reproduction at the individual level is preliminary to understand the possible effect of genetic changes (including introgression of domestic genes). Here we described the variation in the phenology of reproduction in an Italian wild boar population and its dependence on environmental and social factors.

Specifically, for each litter belonging to more than 300 pregnant sows culled over 8 years in a mountain area of Tuscany, we determined the conception date (CD) from an estimate of the mean fetal age and the culling date. We then investigated which factors drove the variation in CD, by implementing different statistical approaches (linear mixed models, Mantel tests and spatial autocorrelation analyses).

We found significant effects of rainfall, temperatures, and previous and current mast production on CD, as well as a strong correlation of CDs among sows culled in close spatial and temporal proximity, suggesting intra-group reproductive synchrony as already observed in wild boar in other environmental contexts (e.g., Meynhardt 1984). Therefore, in our area, resources, climate and social factors seemed crucial in creating spatio-temporal patterns in reproduction, while individual factors seemed less important.

The model we obtained may predict birth peaks in wild boar populations living in environmental conditions similar to our study area, providing that environmental data are known.

It would also allow to identify crucial periods in which matings are more frequent and the hybridization risk may be higher. However, given that introgression of domestic genes into the wild

boar gene pool may lead to altered reproductive patterns, future studies addressing the effects of such introgression on timing of reproduction in wild boar are recommended.

The second part of my thesis deals with wolf-dog hybridization, indicated as one of the major threats to the wolf conservation in the Action Plan for the conservation of the wolves in Europe (Boitani 2000) and, specifically, a threat to the conservation of the genetic integrity of the Italian wolf (Genovesi 2002).

Action Plans suggested the assessment of the genetic identity of local wolves in view of assessing/preventing wolf-dog hybridization (e.g., by removing hybrids and stray dogs) because of its possible negative effects. Boitani (2000) also suggested to intensify research on wolf feeding habits (including especially interactions of wolves with game animals and

livestock). Indeed, one of the most important issue in the wolf management is represented by conflicts between wolves and farmers due to the inevitable predation on livestock.

With these premises, in **Chapter 6** we compared levels of canine introgression and feeding ecology between wolves inhabiting two adjacent areas in the Italian Apennines, given the current lack of data on behaviour and ecology of wolf-dog hybrids under free-ranging conditions.

Levels of genetic introgression in the two areas were investigated by analyzing non-invasive samples and carcasses collected therein with a set of uniparental and bi-parental markers. Individuals inhabiting the first area resulted to be highly introgressed, as a consequence of presumably recent hybridization events, while wolves of the second one showed only weak signs of a seemingly less recent introgression of canine genes. Introgression appeared in autosomal and patrilineal markers only, confirming that wolf-dog hybridization is mostly an asymmetric process (Hindrikson et al. 2012).

We then analyzed the diet in the two areas from the fecal content of more than 300 scats collected in winter in each area. No significant difference in the diet composition between the two areas was detected: independently from their admixture level, individuals relied mostly on juvenile roe deer and wild boars, showing a trophic behaviour similar to other previously studied Apennine wolf populations.

Our study represents the first investigation on the food habits of free-ranging wolf-dog hybrids. Given the seemingly high trophic overlap, the presence of hybrids could represent an ecological (and not only genetic) threat to the local wolf population in the long-term. Furthermore, our results do not confirm the worries about a possible higher impact of hybrids on livestock, that could be the result of a less elusive behaviour, linked to the introgression of domestic genes.

In Chapter 7, we evaluated the utility of a novel approach, combining camera trapping (CT) and non-invasive genotyping (NGS) to collect complementary information on specific individuals in a monitored Italian wolf population affected by introgression from domestic dogs.

Between 2013 and 2015, wolf scat deposition was recorded 37 times by remote camera trapping. A total of 16 fresh scats were collected and genotyped at 12 autosomal

microsatellites, the Amelogenin gene, two Y-chromosome microsatellites and the control region of the mtDNA, for sex and individual identification and to assess introgression of canine genes.

Additionally, the videos were screened, in order to identify defecating individuals, evaluate their social rank, and record their phenotypic traits (some of them possibly associated to canine introgression, like anomalous coat color patterns, dewclaws, floppy ears, etc.). In this way we were able to associate genetic, phenotypic and behavioural traits of specific individuals of the population under monitoring.

Wolf identification by NGS matched that assessed by CT: each time a particular individual was recognized in more than one video, the genetic analysis of the corresponding scat samples identified the same genotype. This confirmation is promising, meaning that intra-population phenotypic variation could allow individual recognition in wolves (to our knowledge, not currently employed), possibly facilitated by the introgression of canine genes.

The identified wolves belonged to four different packs and were filmed either alone or accompanied by other 1-3 individuals. Notably, in two different packs, the information referred to a member of the breeding pair which showed signatures of introgression. This evidence raises a special concern, as the successful breeding of hybrid individuals would lead to a spread of canine genes in the population.

The novel approach we used proved to be useful to provide complementary information on individual wolves in a wild population and supporting hybrid identification in target packs. Furthermore, this method can help to test the reliability of individual recognition for the application of mark-recapture methods of population size estimation.

All in all, my studies offer new pivotal elements of knowledge on different aspects of the hybridization in two worrisome species of the Italian fauna: the wild boar and the wolf.

Wild x domestic introgressive hybridization is a complex multi-facial process whose effects are far from being fully understood. Some outstanding points which were considered in my study, but that needs to be further investigated are:

1. the source of domestic genes and the conditions promoting introgression. In this regard, we highlighted the role of wild boar breeding stations in spreading domestic genes across wild populations in some areas, but the actual situation at a national and European level has to be investigated; therefore we strongly recommend a routine genetic monitoring of wild boars in breeding stations. In wolves special effort should be spent in preventing the contact between wild individuals and stray dogs, representing the main source of introgression;
2. methodological tools to assess the introgression at both individual and population levels. In this regard, we developed new markers (Y-chromosome microsatellites) and new approaches (joining camera trapping and non-invasive genotyping). In the future, new techniques designed to assay large sub-section of the genome, in association with next-generation sequencing technologies, will allow more detailed genome-wide hybridization and introgression studies (Twyford and Ennos 2012);
3. ecological and evolutionary effects of introgression. This is one of the most challenging and interesting aspects to be investigated in the future. From our side, we provided the first insights into the food habits of wolf x dog hybrids, but further research on the ecological role of hybrids is necessary.

Researches addressed towards these points are particularly urgent for their relevant management implications: on one hand, they could provide crucial information to achieve a long-term conservation of the Italian wolf; on the other, they can allow to predict, and possibly contrast, the demographic trend of an invasive species, the wild boar, in Europe.

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APPENDIX

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ORIGINAL PAPER

Influence of management regime and population history on genetic diversity and population structure of brown hares (*Lepus europaeus*) in an Italian province

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Abstract In many areas, the management of overexploited populations of brown hare (*Lepus europaeus*) is based on annual restocking. While in some cases exotic hares are introduced, in some others hares are captured locally within protected areas and subsequently released into hunting grounds. We evaluated the genetic effects of this management regime in an Italian province where the brown hare population has recovered in the last few decades, by sequencing the hypervariable domain 1 of the mitochondrial control region and by genotyping eight autosomal microsatellites in hares sampled in both hunting and non-hunting areas. Both nuclear (H_e=0.68 and H_o=0.65) and mitochondrial variability (h=0.853 and π=0.012) were in line with other European populations. When comparing our data with mitochondrial sequences retrieved from GenBank, out of the 21 detected haplotypes, 14 were private to our study area. While 4.6 % of the individuals were found to carry haplotypes attributable to past introductions, 41.5 % grouped within a well-supported lineage, previously identified with a presumed native Italian taxon, *L. e. meridiei*. Despite the detectable geographic partitioning of mitochondrial haplotypes across the province, no genetic structure resulted from microsatellites analysis, indicating that no reproductive barriers exist among hares carrying different mitochondrial lineages. In conclusion, the local management seems to have contributed to the recovery of the species and to a full admixture of nuclear genes in the province. However, neither the extensive translocations nor the possible introductions of exotic heads seem to have completely undermined the local mitochondrial lineages.

Keywords *Lepus europaeus meridiei* · Microsatellites · MtDNA · Population structure · Restocking · Translocations

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Introduction

Wildlife restocking aims at revitalizing a declining/threatened population ("conservation restocking") or, more often, at enhancing future harvest or at making a given harvest regime sustainable over time, thus providing economic and social benefits. However, these practices can have several consequences, such as the spread of pathogens and changes in the genetic makeup, morphology, demography, and behavior of the recipient populations (Champagnon et al. 2012; Laikre et al. 2010). Depending on the amount and nature of the released individuals and on the degree of post-release admixture, restocking can have opposite evolutionary implications. On one hand, the sudden influx of new genes increases local variation and, if accompanied by intraspecific hybridization, may produce novel allelic combinations thus providing fresh

genetic material for evolution. In fact, admixed individuals may exhibit heterosis, particularly if they come from inbred populations (Allendorf and Luikart 2007). On the other hand, large-scale releases often homogenize the genetic features of geographically distinct populations, reducing the overall adaptive potential of the species, and—through outbreeding depression and genetic swamping—may impact on demography and genetic variation, finally depressing the evolutionary potential of local populations (Laikre et al. 2010). In addition, budget-guided evaluations in translocation plans leads to the frequent use of captive-raised individuals, which can be affected by the relaxation of natural selection and by man's selection on specific traits (Champagnon et al. 2012). From a conservation viewpoint, such negative evolutionary effects are challenging, as they entail a higher vulnerability to invasion and a lower capacity to adapt to environmental changes (Olden et al. 2004). As the consequences of population mixing depend also on the genetic divergence between the parental populations (Allendorf et al. 2001), it was remarked that genetic aspects should not be disregarded when planning restocking programs and that a post-release monitoring is required (Bertorelle et al. 2009).

Given its relevance as a game species, the brown hare (*Lepus europaeus* Pallas, 1778), is one of the most managed and translocated mammals in Europe. During the 1970s and 1980s, restocking was usually conducted using heads from breeding stations established in Bulgaria, Slovakia, Hungary, and Poland (Stamatis et al. 2009). For instance, autochthonous brown hares in France and Denmark have been completely replaced by animals introduced from Eastern Europe (Kasapidis et al. 2005; Suchentrunk et al. 2006). Occasionally, these practices have caused health concerns, such as the introduction of parasites (Amori et al. 1996) and diseases (e.g., the European brown hare syndrome, Williams et al. 2002).

According to Pierpaoli et al. (1999, 2003), overhunting and releases of brown hares in Italy began in the early 1900s and continued until the 1990s, causing the replacement of many local populations of *L. europaeus*. Nowadays, the brown hare is present across the whole Italian peninsula, with very high variation in population density, ranging from less than 1 to more than 100 heads/km^2 (Trocchi and Riga 2005). In compliance with the Italian legislation, networks of no-hunting areas (named "zone di ripopolamento e cattura" or ZRC) were established to promote the increase of local wildlife. They are intended as source areas for natural dispersal and artificial restocking at a local scale. However, even though hares from these areas can provide as much as 65 % of the annual hunting bag (Trocchi and Riga 2005), restocking with imported exotic individuals is still common (Modesto et al. 2011). As a consequence, the gene pools of brown hare populations may become a mix of native and exotic lineages.

Some authors argued that the introduction of exotic brown hares could have caused the extinction of an endemic subspecies, *L. e. meridiei* Hilzheimer 1906, once present in central and northern Italy, northern Croatia, and south-eastern France (Amori et al. 1996; Angelici 1998; Pierpaoli et al. 1999, 2003). Its taxonomic value, though, still deserves further evaluations. Pierpaoli et al. (1999, 2003) sequenced the mitochondrial DNA control region 1 (mtDNA CR-1) of European brown hares, finding three distinct and phylogenetically basal haplotypes (Leu1-3) in samples collected in secluded areas in the Italian Apennines. They argued that these haplotypes could represent the remnants of an ancestral population, isolated in Italy during the last glaciation (the subspecies *L. e. meridiei*), while considering all other Italian haplotypes as a possible result of more recent colonization events. More comprehensive studies (Kasapidis et al. 2005; Stamatis et al. 2009) revealed close similarities between Leu1-3 and haplotypes found in Greece and Bulgaria, and hypothesized a spread of this lineage from the Balkans via the northern Adriatic land bridge during the late Pleistocene–early Holocene glaciations. Such haplotypes are hence expected to be found in descendants of native Italian populations. Nevertheless, the origin of these haplotypes from recent translocations from Southern Balkans cannot be completely excluded (Stamatis et al. 2009).

Anyway, the mtDNA provides information only about the female germ line, and the patterns of variation detected in the mitochondrial genome may differ from those arising from morphology and nuclear markers (Koutsogiannouli et al. 2012; Mamuris et al. 2010), also on account of the repeated introgression of mtDNA among hares (Melo-Ferreira et al. 2012) and of its lower effective population size. Furthermore, any inferred phylogenetic tree based on a single locus does not necessarily agree with the actual evolutionary pathway of the species involved (e.g., Pamilo and Nei 1988).

In the present study, we investigated the genetic composition of brown hares in an area of Central Italy (Arezzo Province), where the resident population had recovered after a demographic decline with the contribution of intensive restocking from local ZRCs. In particular, we assessed the levels of genetic diversity, the population structure, the proportion of private haplotypes, and their spatial distribution in the study area by analyzing a partial sequence of the mitochondrial CR-1 region and eight autosomal microsatellite loci.

Materials and methods

Sampling areas and population history

The Arezzo Province covers 3,235 km^2, including forested mountains up to 1,658 m a.s.l. and lowlands, dominated by cultivated fields. Remarkable environmental differences are present between the northern (mountainous) and the southern (flatter) part of the province. The territory is subdivided

into three hunting districts (HD 1–3). Since the early 1980s, in application of a national law (nr. 968/1977), several ZRCs were established. According to anecdotal information, at that time, brown hares had strongly diminished in the province due to overexploitation, especially in the southern plains. Therefore, restocking programs started with captivity raised hares of local origin and hares imported from abroad (e.g., Argentina), which were also released within the ZRCs (Provincial Administration of Arezzo 1991). In 1994, after the failure of such programs (due to the high mortality of released individuals), a new management scheme was adopted. The ZRC hare populations in the south of the province (Val di Chiana) were revitalized by the translocation of individuals from well-preserved areas in the north and by indirect sustaining activities like predator control (especially towards the red fox, *Vulpes vulpes*) and habitat improvement. As a routine of the new management plan, annual count of ZRC populations, subsequent hare captures in early winter, and translocation of the captured animals to hunting grounds in the rest of the province were implemented. An average of 669±128 heads per year (the 84 % of which were from Val di Chiana ZRCs) were translocated in the period 1996–2012, representing more than 15 % of the average annual hunting bag in the province (4,051±657 between 2004 and 2009; Provincial Administration of Arezzo 2012). No introduction of exotic hares was reported within this time span in the province.

Sample collection and DNA extraction

A total of 664 brown hares were captured between December 2006 and January 2007 in 10 out of 13 ZRCs, all located in the southern part of the province (9 out of 10 in the HD 3, Fig. 1), at altitudes lower than 350 m. Each animal was sexed, aged (according to the ossification features of the distal *epiphyseal cartilage* of the *ulna*, Stroh

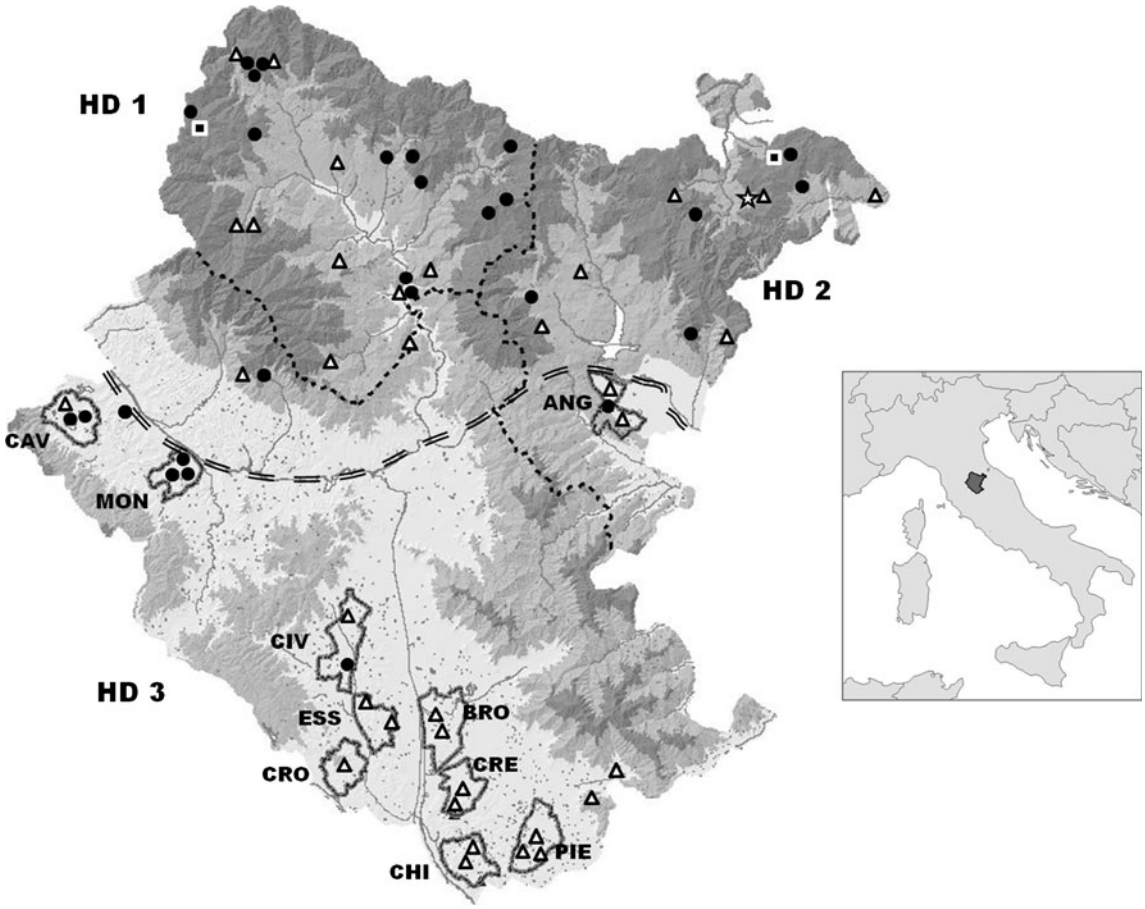

Fig. 1 Map showing the 10 ZRCs of the Arezzo Province considered in the present study (*solid lines*, *ANG*=Anghiari, *n*=20; *BRO*=Brolio, *n*=20; *CAV*=Cavriglia, *n*=15; *CHI*=Chianacce, *n*=19; *CIV*=Civitella, *n*=20; *CRE*=Creti, *n*=21; *CRO*=La Croce, *n*=20; *ESS*=Esse, *n*=15; *MON*=Montevarchi, *n*=5; *PIE*=Pietraia, *n*=20) and the three hunting districts (separated by a dashed line: HD 1, n=40; HD 2, n=15; HD 3, n=19). The *double dashed line* splits the province into North and South. The *three shades of gray*, from the lightest to the darkest, correspond to the altitudinal classes <350, 350–700, and >700 m, respectively. The geographic distribution of the 65 hares selected for mtDNA analysis is also shown, using different symbols for haplogroups (*black circle*, haplogroup M; *triangle*, haplogroup Euh-A; *star*, AMh haplogroup; *square*, Balkan haplotypes)

1931), weighed, and hair samples were plucked for genetic analysis.

One hundred seventy-five specimens were selected, so as to compensate for sampling disparity among areas. Additionally, tissue samples from 78 hares shot in the three HDs (21 different municipalities) during the hunting seasons 2006–2007 and 2007–2008 were analyzed. The term "population" is used hereafter to indicate each of the 13 pools of individuals from the 10 ZRCs and the three HDs (Fig. 1).

Genomic DNA was isolated both from hair follicles using the InstaGene matrix protocol (Bio-Rad, Hercules, California), and from 25 mg of ethanol-embedded tissue using the GenElute Mammalian DNA miniprep Kit (Sigma-Aldrich, St Louis, Missouri), according to the manufacturer's protocol. In both cases DNA was eluted in a final volume of 200 μl.

MtDNA sequencing and data analysis

A subset of 65 hares, representing the whole provincial territory, was selected for mtDNA analysis. A partial sequence of the CR-1 was sequenced using primers Le.H-Dloop and Le.L-Dloop (Kasapidis et al. 2005), which bind, respectively, to positions 15,907 and 15,418 of the *L. europaeus* complete mtDNA (Arnason et al. 2002). Polymerase chain reaction (PCR) amplifications, purification of amplicons, and the following sequencing protocol are described in Scandura et al. (2007).

The sequences obtained were aligned using the ClustalW algorithm (Thompson et al. 1997) implemented in MEGA 5.05 (Tamura et al. 2011). In order to maximize the sequences length and number, including reference hares from Europe and Middle East, a shorter alignment was created by retrieving 346 *L. europaeus* homologous sequences (Kasapidis et al. 2005; Stamatis et al. 2009; Fickel et al. 2005, 2008; Vernesi et al., unpublished) from GenBank, plus one *Lepus timidus* (Waltari et al. 2004) and one *Lepus corsicanus* (Pietri et al. 2011) as outgroups (Table 1).

New haplotypes were identified by collapsing sequences in Collapse 1.2 (Posada 2004). Evolutionary relationships among haplotypes were investigated by constructing a median-joining (MJ) network with NETWORK 4.6.1.0 (Bandelt et al. 1999) and a neighbor-joining (NJ) tree with MEGA, with interior-branch test based on 1,000 bootstrap replications. Estimates of haplotype diversity (h) and nucleotide diversity (π), as well as the number of polymorphic sites (S) and the mismatch distribution in the study population were computed using ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010). Additionally, diversity parameters were estimated for the northern and southern part of the study area (Fig. 1), and the amount of differentiation among them was assessed by analysis of molecular variance (AMOVA) on Φ_{ST}-statistics in ARLEQUIN, using 20,000 permutations to test for significance.

Since haplotypes of the *meridiei* lineage have been previously detected in mountainous areas (Pierpaoli et al. 1999) and a strong population decline occurred in the plains, a different haplotype composition might be expected at different altitudes. Thereby, an AMOVA was also performed considering three groups of individuals, subdivided by the altitude of the sampling point ("plain" <350 m elevation, n=26; "hill" 350–700 m, n=18; "mountain" >700 m, n=21).

Microsatellites genotyping and data analysis

Eight microsatellites, isolated and characterized in the rabbit *Oryctolagus cuniculus* and already tested on *L. europaeus* (e.g. Andersson et al. 1999, Fickel et al. 2005), were selected for use in this study: Sat2, Sat8, Sat12, Sat13 (Mougel et al. 1997), Sol03, Sol08, Sol30 (Rico et al. 1994), and Sol33 (Surridge et al. 1997).

PCR amplifications were performed in 10 μl of reaction mixture containing 0.05–0.15 mM dNTPs, 0.1–0.2 μM of each primer, 1× PCR reaction buffer, 2.5–4 mM $MgCl_2$, 0.05–0.1 U/μl EuroTaq DNA polymerase (EuroClone,

Table 1 Sampling localities, reference and accession numbers of the mtDNA CR-1 sequences included in the alignment in the present study

Taxon	Geographic area	GenBank accession codes	Reference	Number of sequences
Lepus europaeus	Italy, Arezzo Province	KC555540-KC555556	Present study	65
Lepus europaeus	Greece, Bulgaria, Cyprus, Israel	AY466782-853	Kasapidis et al. (2005)	72
Lepus europaeus	Europe, Middle East	DQ469642-710	Stamatis et al. (2009)	69
Lepus europaeus	Germany	AY103494-531; AY154661-666	Fickel et al. (2005)	44
Lepus europaeus	Italy, Spain, Poland, Sweden, Serbia, Slovakia, Germany	AY163356-76; AY300032-36; EU435388-91; EU435393-403; EU435405-07; EU435409; EU435411-14; EU496871; EU496874-75; EU496880;EU496882	Fickel et al. (2008)	54
Lepus europaeus	Italy	HM120879-950; HM120957-991	Vernesi et al., unpublished	107
Lepus timidus	Sweden	AY422309	Waltari et al. (2004)	1
Lepus corsicanus	Corsica	HQ174270	Pietri et al. (2011)	1

Siziano, Italy), 3 μl of template DNA, and distilled water. Reagent concentrations were optimized for each locus.

Amplification conditions consisted of an initial denaturation step at 95 °C for 3 min., followed by 35 cycles of denaturation at 92 °C for 45s, annealing at locus-specific temperatures (52 °C for Sol33, 56 °C for Sat13, 60 °C for Sat8, 62 °C for Sol03, 64–56 °C for Sol30, 65–55 °C for Sol08, Sat12, and Sat2) for 45 s, and extension at 72 °C for 30s; then a final extension step at 72 °C for 10 min. Amplicons were visualized under UV light on a 2 % agarose gel stained with ethidium bromide. All the successfully amplified products were analyzed by capillary electrophoresis on an automated sequencer ABI PRISM (Applied Biosystems) along with an internal size standard (ROX 500, Applied Biosystems). Alleles were scored using GENEMAPPER 3.7 (Applied Biosystems). Complete genotypes were obtained for 249 hares. Amplification and scoring errors in the dataset were identified using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004).

Allele frequencies and standard genetic diversity indices, including observed heterozygosity (H_o), unbiased expected heterozygosity (H_e), and the mean number of alleles per locus (A) were calculated for each locus with GENETIX 4.05 (Belkhir et al. 2004). Allelic richness and private allelic richness were estimated using HP-RARE (Kalinowski 2005), for each ZRC and HD, based on a minimum sample size of 28 genes. Because of the relatively small sample size, one of the ZRCs (Mon, n=5) was excluded from this computation. Deviations from Hardy–Weinberg and genotypic linkage equilibria were tested in GENEPOP 4 (Rousset 2008). Markov chain parameters for both tests were set at 10,000 dememorizations, 100 batches, and 10,000 iterations per batch. Significance levels were adjusted according to the sequential Bonferroni correction for multiple comparisons (Rice 1989). Furthermore, Weir and Cockerham's (1984) estimators of Wright's F-statistics (f and θ, hereafter F_{IS} and F_{ST}) were computed in GENETIX. Their significance was tested by permuting genotypes among populations (1,000 permutations), while 95 % confidence intervals of F_{IS} were obtained by the bootstrap method (Felsenstein 1985; 1,000 bootstraps). A factorial correspondence analysis (FCA) was performed to visualize distances among genotypes.

Moreover, the Bayesian clustering algorithm implemented in STRUCTURE 2.1 (Pritchard et al. 2000) was used in order to detect a possible population structure (i.e., the most likely number of genetic clusters (K)). Ten independent runs were carried out for each value of K (1–10), with 300,000 iterations following a burn-in period of 200,000 iterations, assuming admixture and correlated allele frequencies among groups. The most likely value of K was determined according to the method developed by Evanno et al. (2005).

The fine-scale genetic structure of the Arezzo hare population was investigated by performing a spatial autocorrelation analysis with GENALEX 6.41 (Peakall and Smouse 2006) on the matrices of pairwise genetic distances, based on allele sharing, and pairwise geographic distances between individuals. Ten distance classes with even sample size were chosen. A total of 999 permutations and 999 bootstraps were run so as to generate 95 % confidence intervals around the null hypothesis (no autocorrelation) and around the estimated value (r), respectively. This analysis was performed for the entire dataset and for the two sexes separately, in order to test for sex-biased gene flow in the population. The same software was used to test for isolation by distance by Mantel test (999 permutations).

As the clustering model in STRUCTURE is not well suited in case of isolation by distance (i.e., results are strongly influenced by the sampling scheme and the genetic structure may be overestimated, Schwartz and McKelvey 2009, Frantz et al. 2009), BAPS 5.3 (Corander et al. 2008) was used to support inferences about the population structure, by performing a "spatial clustering of individuals" (in which the prior distribution for clustering depends on the spatial pattern of the observed data), with the a priori upper boundary for the number of clusters ranging between 5 and 25 (K=5, 10, 15, 20, 25, each repeated 10 times).

Results

MtDNA

Twenty-one haplotypes (AR01–AR21), defined by 58 polymorphic sites and two indels, were detected in the 441-bp alignment of Arezzo hare sequences. They were checked for identity with published sequences using the BLAST tool on the NCBI website (www.ncbi.nlm.nih.gov). Four haplotypes matched with as many *L. europaeus* sequences reported in Fredsted et al. (2006) (GenBank accession codes: DQ645432-33, DQ645443, DQ645447), while the other 17 were novel sequences (acc. codes: KC555540–KC555556). One haplotype was found to be very common (AR02, with a frequency of 35.4 %), while 13 of them occurred only once, and the other seven showed a frequency between 3.1 and 10.8 %. Haplotype and nucleotide diversity amounted to 0.853±0.037 and 0.023±0.012, respectively. The mean number of pairwise differences between CR-1 sequences was fairly high (10.18±4.71), showing the presence of individuals belonging to different haplogroups, as visualized in the MJ network in Fig. 2. As expected, the mismatch distribution (not shown) is ragged and multimodal. The two most common haplogroups included 95.4 % of the individuals (27 and 35 hares, accounting for 5 and 13 different haplotypes, respectively) and were separated by a minimum of 13 mutational steps. The three remaining hares carried isolated haplotypes (AR10, AR13, AR20). All the

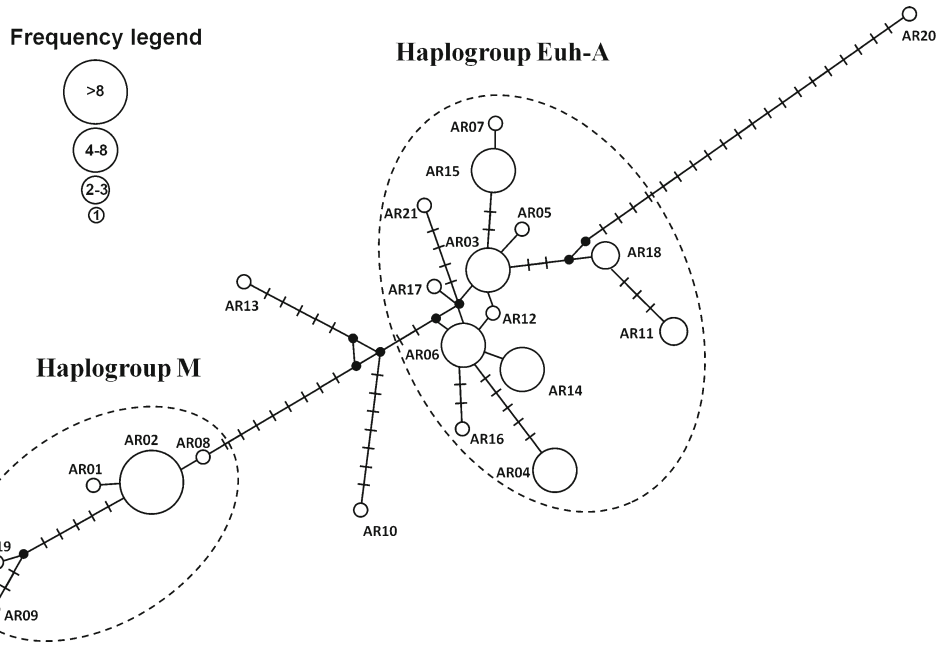

Fig. 2 Median-joining network showing the relationships among the 21 Arezzo CR-1 mtDNA haplotypes. *Perpendicular dashes* correspond to mutational steps (not drawn in the case of a single mutation); putative unsampled haplotypes are represented by *solid black circles*

21 haplotypes were confirmed considering the shortest (338 bp) alignment including 413 hare sequences sampled in different regions in Europe and in the Middle East, collapsing to a total of 247 different haplotypes, 14 (67 %) of which turned out to be private of the province of Arezzo. In the resulting NJ tree (Fig. 3), the 13 haplotypes of the most common haplogroup clustered with the majority of Central European sequences into the clade Euh-A, defined by Stamatis et al. (2009). AR10 clustered with Macedonian/Bulgarian sequences, AR13 grouped mainly with haplotypes from the Aegean region (Peloponnese, Crete, Kythira Island), while AR20 grouped with Anatolian/Middle Eastern sequences (the basal AMh clade in Stamatis et al. 2009). The remaining five haplotypes (four of which were private of the province, and the other already sampled in Northern Italy) formed a well-supported (98 %) clade (named M in Fig. 3), together with other eight haplotypes from Italy (six) and the southern Balkans (two, Thrace and Lefkada Island).

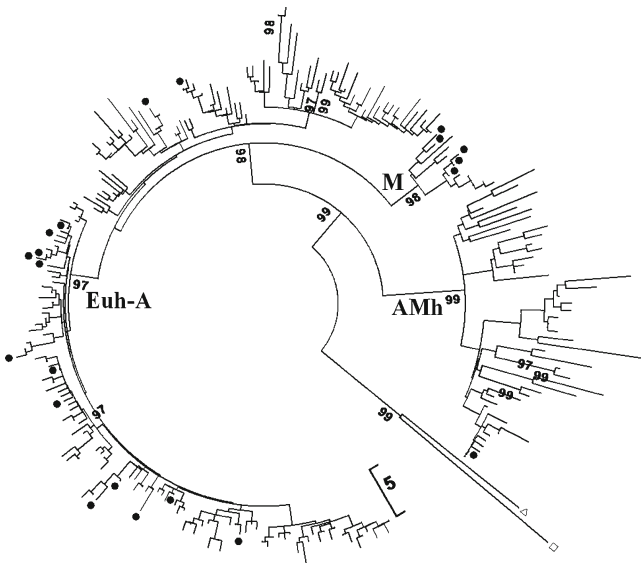

Fig. 3 Neighbor-joining phylogenetic tree, based on 247 hare CR-1 sequences (from Fickel et al. 2005, 2008; Kasapidis et al. 2005; Stamatis et al. 2009; Vernesi et al., unpublished), showing the position of the Arezzo haplotypes (*black circles*). Bootstrap values (expressed as percentages of 1,000 replications) >97 % are shown at branch points. *L. timidus* (acc. code: AY422309, *empty square*) and *L. corsicanus* (acc. code: HQ174270, empty triangle) were used as outgroups. *EUh-A*, European-type haplogroup, subtype A; *AMh*, Anatolian/Middle Eastern type haplogroup; *M*, *meridiei*-type haplogroup

So as to clarify the position of the M clade, a second NJ tree was generated using shorter sequences (248 bp) and including 22 CR-1 haplotypes obtained by Pierpaoli et al. (1999) (data not shown). The most common M haplotype, AR02, matched with the CR-1 sequence Leu2, described by Pierpaoli et al. (1999), which was one of the three phylogenetically basal haplotypes, interpreted as the ancestral mitochondrial lineage corresponding to the subspecies *L. e. meridiei*. Thus, 4.61 % of the samples analyzed carried a probable exotic haplotype and 41.5 % (27/65) had one of the five *meridiei*-type (M) haplotypes. Of these, four out of five are found only in the northern part of the province. The three individuals carrying exotic haplotypes were shot in the same area. Nonetheless, the amount of differentiation between the northern and southern areas of the province was not significant (AMOVA, intergroup ΦST=0.003, p=0.316). Conversely, genetic differentiation in relation to altitude was detected (AMOVA, ΦST among the three groups=0.060, p=0.004). Indeed, the proportion of M haplotypes appears to increase at higher altitudes, being about twice as high in the mountains (plain 30.8 %, hill 33.3 %, mountain 61.9 %), although the null

hypothesis of equality of proportion among groups cannot be rejected (χ^2 5.328, p=0.070). Since most of the M haplotypes were represented by AR02, haplotype diversity at higher elevations was fairly low (plain 0.871, hill 0.902, mountain 0.681).

Microsatellites

MICRO-CHECKER analysis revealed that two loci could be affected by null alleles (Sol03 and Sat2, with null alleles frequencies estimated to be 14.8 and 5.8 %, respectively). No indication of allele scoring errors caused by stuttering or large allele dropout was found. In addition, linkage disequilibrium was detected between Sol03 and Sol30 (p<0.001, significant after Bonferroni correction). In the light of the above findings, locus Sol03 was removed from the statistical analysis.

In total, 88 alleles were observed from the remaining seven loci surveyed. Genetic variability indices are shown in Table 2. The number of alleles per locus ranged from 2 (Sol33, Sat8) to 18 (Sol30, Sat2). Hares from HD 2 had the highest allelic richness and private allelic richness (*Ar*=7.11, *Par*=0.43). Observed heterozygosity ranged from 0.58 (Ess) to 0.72 (Cre) and did not differ from the expected values (one-tail paired t test, $H_o - H_e$, p=0.169). In fact, significant departures from HWE were detected only in two populations (HD 1 and ZRC Chi) and were attributable to a deficit of heterozygous individuals. Accordingly, mean F_{IS} values were significantly higher than 0 (p<0.05) in three populations, including the former two (HD 1, HD 3, and ZRC Chi).

Genetic distances between individuals are illustrated in the FCA in Fig. S1. Two individuals (from HDs 2 and 3) were clearly separated from the others, being presumably introduced hares. As expected, the 13 populations did not form 13 distinct clusters. However, one population (the ZRC Cro) was slightly differentiated from the others, with pairwise F_{ST} values ranging from 0.012 to 0.077 (significant in 11 out of 12 comparisons, seven times at p<0.01 and four times at p<0.05).

Overall genetic variation among populations was low, as indicated by the estimate of F_{ST} across all loci and populations (0.018, p<0.01). This genetic homogeneity was confirmed by the results of Bayesian clustering analyses.

In fact, no population structure was detected by STRUCTURE (K=1 was the most likely scenario following Evanno et al. 2005, Fig. 4). Since the isolation-by-distance pattern revealed by the Mantel test was fairly weak (r^2=0.004, p=0.008), STRUCTURE results should not be considered excessively misleading; moreover, they were supported by the spatial mixture analysis performed using BAPS (number K of groups in optimal partition=1, with log marginal likelihood=−5684.14). Moreover, significant spatial autocorrelation of microsatellite data occurred only in the first distance class (0–9 km: n=4,761 comparisons, r=0.021, p<0.01; Fig. 5). In the third distance class, despite a p value less than 0.05 (13–20 km: n=2,976 comparison, r=0.06, p=0.043), the 95 % confidence interval of the estimated autocorrelation coefficient included 0; therefore, the null hypothesis of no spatial genetic structure could not be rejected. A rough estimate of the extent of nonrandom positive autocorrelation is provided by the first x-intercept (11.86 km). Similar autocorrelation patterns were observed in the two sexes separately (significant autocorrelation in the first distance class only, data not shown).

Discussion

In the present study, we investigated the genetic makeup of a managed brown hare population in Italy, focusing on the

Table 2 Genetic diversity and deviation from HWE expectations (p values) at seven microsatellite loci in brown hares from 3 HDs and 10 ZRCs in the Arezzo Province (Italy). H_e=expected heterozygosity; H_o=observed heterozygosity; A=number of alleles per locus; *Ar* and *Par*=allelic richness and private allelic richness (rarefaction, 28 genes); HWE=p value of the test for deviations from the Hardy–Weinberg equilibrium

	n	H_e	H_o	*A*	*Ar*	*Par*	HWE
HD 1	40	0.652±0.245	0.617±0.237	9.14	6.3	0.27	0.043
HD 2	15	0.688±0.234	0.667±0.255	7.29	7.11	0.43	0.962
HD 3	19	0.687±0.197	0.610±0.223	7.14	6.7	0.39	0.288
ANG	20	0.695±0.149	0.691±0.187	6.86	6.18	0.21	0.171
BRO	20	0.628±0.275	0.657±0.371	6.86	6.13	0.11	0.104
CAV	15	0.656±0.251	0.681±0.269	6.43	6.27	0.15	0.810
CHI	19	0.699±0.177	0.631±0.154	6.43	6.14	0.05	0.025
CIV	20	0.684±0.150	0.686±0.204	6.14	5.72	0.00	0.256
CRE	21	0.692±0.193	0.721±0.193	6.14	5.68	0.10	0.700
CRO	20	0.657±0.212	0.643±0.197	5.57	5.26	0.18	0.127
ESS	15	0.621±0.236	0.581±0.246	6.29	6.15	0.32	0.536
MON	5	0.625±0.201	0.686±0.227	3.57	–	–	1.000
PIE	20	0.675±0.191	0.643±0.197	6	5.48	0.20	0.762
Tot	249	0.680±0.208	0.651±0.203	12.57	–	–	<0.001

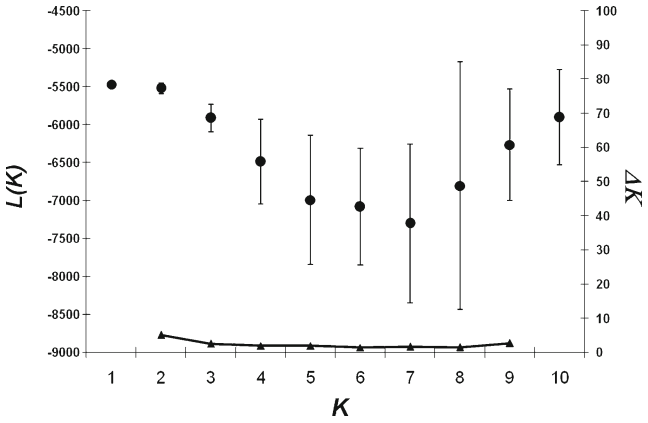

Fig. 4 Results of the STRUCTURE cluster analysis for the study hare population. Mean (±SD) posterior probability of the data ($L(K)$, left *Y*-axis) and standardized second order rate of change of $L(K)$ (ΔK, right *Y*-axis; Evanno et al. 2005), calculated for K values ranging from 1 to 10 over 10 runs

possible effects of the past management regime on the levels of genetic diversity and on population structure.

Although the hare population of the Arezzo Province was mostly restocked with animals from a single area, variation at nuclear microsatellite loci was close to the highest values reported for other brown hare populations (H_e: Arezzo Province, 0.68; Germany, 0.51–0.65, Fickel et al. 2005; Sweden, 0.52–0.69, Thulin et al. 2012; Bulgaria and Iberia, 0.70 and 0.56, Estonba et al. 2006). A high allelic richness was found in the three HDs, which have a larger extension than the ZRCs and, presumably, a higher population size. The higher variation observed in the HDs, though, can also be due to their higher turnover and to the mixing of individuals from different ZRCs.

Furthermore, the Arezzo Province hares exhibited high nucleotide diversity (π=0.023) compared to other conspecific populations (Germany, 0.007, Fickel et al. 2005; northern Italy, 0.015, Fickel et al. 2008; Denmark, 0.004, Andersen et al. 2009), but relatively low haplotype diversity. A past reduction in effective population size could account for the loss of several haplotypes, whereas the presence of different mitochondrial lineages would have implied minor effects on nucleotide diversity. This is consistent with the population decline of the brown hare reported to have occurred after World War II (Trocchi and Riga 2005).

A high genetic similarity among the considered territorial units is suggested by the overlap of individuals in the FCA plot (Fig. S1). Accordingly, no population structure was detected by the Bayesian cluster analysis of microsatellite data, as suggested by both STRUCTURE and BAPS results. Conversely, a certain degree of mitochondrial differentiation was found among hares living at different altitudes, as highlighted by the AMOVA results. This seems to be due to an increased frequency of M haplotypes with elevation (in line with previous studies, reporting the *meridiei* lineage in mountainous areas; Pierpaoli et al. 1999), although this is not supported by statistical tests.

A lack of concordance between nuclear and mitochondrial DNA geographical structuring was remarked in other genetic studies on *L. europaeus* (Fickel et al. 2005; Mamuris et al. 2010; Suchentrunk et al. 2003) and on other hare species (i.e., in *Lepus capensis*, Ben Slimen et al. 2008 and Canu et al. 2012; in *L. timidus*, Hamill et al. 2007), and can be likely due to the different effective population size of the two genomes, but also to different dispersal patterns in the two sexes. In our study, the former factor seems to play a more significant role, as we found no sex bias in the spatial autocorrelation patterns of microsatellite markers. For both sexes, in fact, we detected significant autocorrelation only in the first distance class (0–9 km), in agreement with published data on brown hare dispersal (1–8 km in the majority of individuals according to Trocchi and Riga 2005; on average less than 2 km, as reported by Bray et al. 2007). Given the prevalence of short-range mobility in this species, the genetic homogenization observed across the province at a nuclear level suggests that some factors (e.g., artificial translocations) have locally promoted gene flow. On the other hand, the slight mtDNA structuring could simply result from lineage sorting, driven by stochastic processes that are likely to occur in mountain areas where

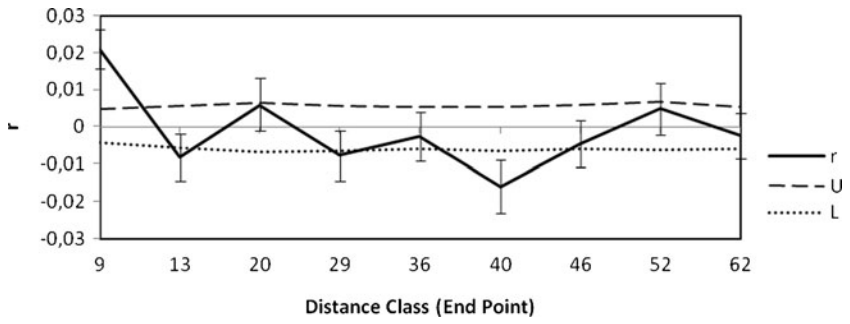

Fig. 5 Results of spatial autocorrelation analysis in the Arezzo hare population. The autocorrelation coefficient, *r*, with its 95 % confidence interval (*error bars*) is shown in relation to distance (in kilometer). The 95 % confidence interval (U=upper bound, L=lower bound) about the null hypothesis of no spatial genetic structure is also shown to check for significance

low hare densities further limit the mitochondrial effective population size. Nonetheless, since few generations of random mating (or high gene flow) can wipe out any trace of a past population structuring at microsatellites, the presence of a legacy of native mitochondrial diversity in some areas cannot be excluded.

Actually, in our survey, we explored the possibility to track native lineages. Unlike previous phylogenetic data reporting a similarity between private Italian haplotypes and south-eastern European sequences (i.e., clade SEEh, Stamatis et al. 2009), the five M haplotypes detected in the study area clustered in the NJ tree with six Italian, one Thracian, and one Greek haplotypes, and diverged from all other mitochondrial sequences of south-eastern Europe (Fig. 3). This clade, supported by a bootstrap value of 98 %, could have presumably spread from the Balkans to central Italy via the northern Adriatic land bridge during the late Pleistocene–early Holocene glacial ages, or, in alternative, by more recent translocations, as argued by Stamatis et al. (2009) and Kasapidis et al. (2005). In the light of our new analyses, three lines of evidence seem to support the former hypothesis: (1) M sequences are presently rare in the Balkan peninsula (3 individuals out of 98 analyzed by Kasapidis et al. 2005; none among the 28 Balkan haplotypes published by Stamatis et al. 2009); (2) four M haplotypes out of the five detected were private to the Arezzo Province; and (3) these haplotypes turned out to be relatively common in peninsular Italy (41.5 % in Arezzo, 6.5–9.5 % in Pierpaoli et al. 1999; 15.9 % in Vernesi et al., unpublished data). These high frequencies may have been reached through a process called "allelic surfing" during the aforementioned late Pleistocene range expansion. In fact, at the edge of an expansion wave, genetic drift can easily bring rare alleles to high frequencies, which can be propagated during a subsequent demographic expansion (Melo-Ferreira et al. 2011). In addition, the two M haplotypes detected in the Balkans might also derive from translocated individuals, since restocking in Greece using Italian brown hares has been reported (Mamuris et al. 2001; Kasapidis et al. 2005). Overall, the high frequency of private haplotypes and, particularly, of M haplotypes (the highest ever reported) could suggest high levels of native mitochondrial diversity in the study area.

The origin of the 35 individuals bearing the 13 Euh-A haplotypes is more questionable because this clade is widespread in Europe and the occasional release of imported hares was reported for the study population in the early 1990s. Yet, we cannot rule out the possibility that many of the Euh-A sequences descend from the native Italian population. On the contrary, three individuals (4.6 %), not belonging to M or Euh-A clades, were attributable to the introduction of exotic stocks and were found in peripheral areas, where five hares introduced from Romania (identified by ear-marks) were recently shot (2009–2011; Provincial Administration of Arezzo 2012).

Although different histories can be tracked in the maternal line, individuals carrying diverging CR-mtDNA haplotypes (like, for instance, Euh-A and M) did not show any detectable difference at nuclear markers. Therefore, our data do not support the idea that a different subspecies (*L. e. meridiei*) has ever occurred and still exists in the Italian Apennines (Angelici 1998; Pierpaoli et al. 1999, 2003).

In order to determine the actual degree of autochthony of the population, nuclear data from local hares should be compared to other populations within a phylogeographic framework. However, selecting these reference populations is not a trivial matter because the genetic composition of each of them could be affected by intraspecific hybridization triggered by translocations, and/or introgression from other hare species. For example, restocking has been cited as a threat to regional gene pools in Greece and Spain (Modesto et al. 2011) and has seriously affected the genetic integrity of the Danish and the French brown hare populations (Kasapidis et al. 2005; Suchentrunk et al. 2006). In this framework, Italy has played the dual role of recipient (e.g., Pierpaoli et al. 1999) and source of hares (e.g., Andersen et al. 2009, Mamuris et al. 2001).

Conclusions

This case study represents a contribution to understand the genetic impact, in the long run, of management practices aimed at restocking brown hare populations.

Our results indicate high levels of genetic diversity that can be accounted for by the admixture of native and introduced gene pools. They also show a high genetic homogenization at nuclear markers but not in the female germ line, with high frequencies of a presumed native lineage, especially in mountainous areas.

The management regime operating during the last two decades in the Arezzo Province could have contributed to generating this pattern. A full recovery of the population and high densities have been reached in the flat and hilly areas in the south, where the most productive ZRCs are located (on average, 5.5 hares/km^2 captured per year between 1996 and 2012; Provincial Administration of Arezzo 2006, 2012). Local gene flow is seemingly high in these areas and strongly affected by both natural and human-mediated radiation from ZRCs. On the contrary, lower densities are observed in the mountainous and forested areas in the north, where gene flow is more likely sustained by translocated hares from ZRCs, rather than by natural immigration.

In natural conditions, assuming female philopatry (Fickel et al. 2005; Mamuris et al. 2010), a stronger genetic discontinuity between the two areas would be expected in

mitochondrial rather than in nuclear markers. But artificial gene flow could mitigate this pattern. If compared to natural dispersal, human translocations are, in fact, characterized by a longer range and a balanced contribution of the two sexes. This can explain the overall lack of evidences of both genetic structure and sex-biased dispersal at nuclear markers. Nevertheless, the weak structure at mitochondrial DNA and the seemingly high frequency of private haplotypes (especially over 700 m, while being very rare in the Val di Chiana ZRCs) would suggest that the artificial gene flow was not so strong as to completely dilute local maternal lineages.

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