Assessing the spatial extent of wolf-dog hybridization in real-time and at population level using non-invasive DNA sampling

Maria Carolina Pacheco Freitas
Mestrado em Biodiversidade, Genética e Evolução
Departamento de Biologia
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Orientador
Raquel Godinho, Assistant Researcher, CIBIO/InBio, Centro de Investigação em Biodiversidade e Recursos Genéticos
Invited Assistant Professor, Universidade do Porto

Coorientador
José Vicente López-Bao, Post-Doctoral Researcher, Research Unit of Biodiversity (UO/CSIC/PA), Oviedo University, Spain; Grimsö Wildlife Research Station, Swedish University of Agricultural Sciences (SLU), Riddarhyttan, Sweden

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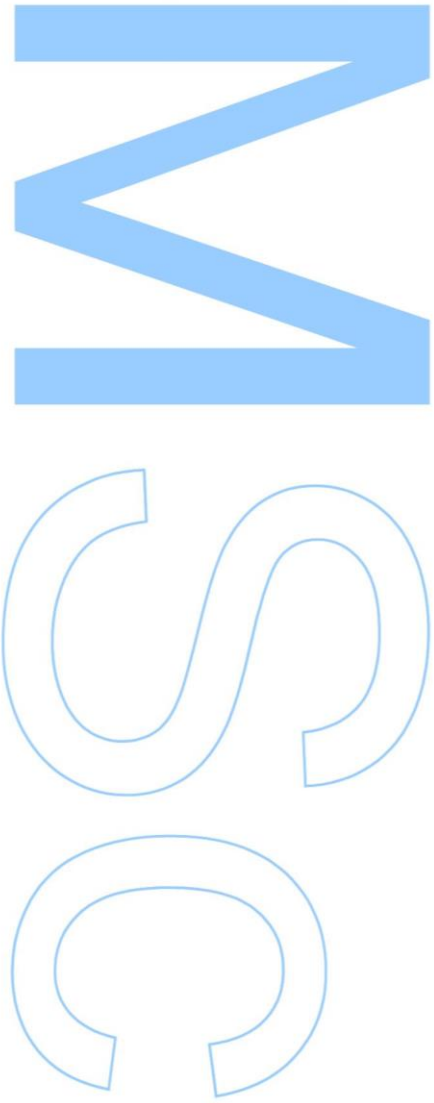

"Try to imagine a small group of wolves sitting at a table engaged vigorous debate. These wolves are from various parts of the globe and are perhaps a bit more scholarly than most. In fact, they are especially knowledgeable about the biology of that notorious two-legged species, *Homo Sapiens*. They have been brought together to document their relationship with humans over the last several millennia. Pause for a few moments and consider what they might say..."

"Wolves and Humans" Fritts *et al.*, in "Wolves. Behaviour Ecology and Conservation"

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Sumário

Na literatura, o cruzamento entre espécies selvagens e os seus homólogos domésticos é regularmente descrito como uma potencial causa para a perda de adaptações locais da população selvagem ou mesmo para a homogeneização genética das populações. No entanto, alguns autores têm vindo a sugerir a existência de um potencial evolutivo neste tipo de hibridação. Estas opiniões contrastantes deram origem a um polémico debate na comunidade científica sobre possíveis implicações deste tipo de cruzamentos na conservação e gestão da espécie selvagem envolvida.

Ao longo da última década, alguns relatos espacial e temporalmente espaçados sobre hibridização entre lobo (*Canis lupus*) e cão (*Canis lupus familiaris*) levaram a uma crescente preocupação entre investigadores e conservacionistas. No entanto, a extensão de hibridação, ou seja, a distribuição geográfica dos eventos de hibridação, a nível populacional raramente é conhecida, o que limita o possível delineamento e implementação de medidas de conservação e gestão. Assim, este trabalho tem como principal objetivo avaliar a extensão de hibridação numa população de lobo localizada numa área extremamente humanizada no Noroeste Ibérico.

Para tal, foi realizada uma amostragem não invasivas numa área de aproximadamente 5000 km^2 correspondente ao território de 13 alcateias incluindo uma previamente descrita como hibrida. Tendo em vista a identificação de eventos de hibridação e a compreensão da sua dinâmica foi selecionado um painel de 18 microssatélites em combinação com uma sequência de DNA mitocondrial, que foi posteriormente utilizado em diversas análises bayesianas e de parentesco. Das 332 amostras iniciais foi possível identificar 140 indivíduos, dos quais 78 foram atribuídos à população de lobo, 58 à população de cão, e por último 4 indivíduos apresentaram proporções intermediárias de atribuição às duas populações e foram identificados como híbridos. Assim, e apesar da elevada prevalência da espécie doméstica na área de estudo, identificámos uma taxa de hibridação baixa (5%) e semelhante à encontrada num estudo anterior realizado com toda a população ibérica. À semelhança de trabalhos anteriores, tanto a população doméstica como a selvagem apresentam-se como duas identidades genéticas independentes e distintas. Adicionalmente, os indivíduos híbridos encontram-se dispersos geograficamente ao longo da área amostrada, mostrando o caracter espacial da hibridação, enfatizado pela identificação de diferentes alcateias de origem para dois dos híbridos.

Em suma, estes resultados mostram um cenário sem antecedentes de múltiplos e dispersos eventos de hibridação, bem como evidenciam o possível potencial das populações selvagens em manter a sua identidade genética associada a baixas taxas

de hibridação mesmo quando estão em permanente contacto com a espécie doméstica, como é o caso de populações de lobos em áreas extremamente humanizadas. Deste modo, apesar de este trabalho nos permitir enriquecer o nosso conhecimento sobre padrões de hibridação a nível populacional, é necessário realizar mais estudos interdisciplinares de forma a melhor compreendermos os processos que poderão estar na base desta dinâmica, como por exemplo processos ecológicos ou mesmo comportamentais.

Palavras-Chave: Lobo cinzento; cão doméstico; hibridação, introgressão, amostras não-invasivas, estratégias de gestão; conservação

Abstract

The crossbreed between wild species and their domestic counterparts has been described in the literature as potential threat to the species conservation, since it may lead to the disruption of local adaptations or to the genetic homogenization through introgressive hybridization. Nevertheless, some authors have been suggesting that this type of hybridization could enhance the evolutionary potential in the wild. Thus raising a controversial debate in the scientific community concerning the possible consequences of wild-domestic hybridization.

In the last decade, sporadic but spatially spread reports of hybridization events between wolves (*Canis lupus*) and dogs (*Canis lupus familiaris*) have led to a growing concern among researchers, stakeholders and conservationists. However, the geographical patterns of the hybridization events at population level is rarely known, limiting the design and implementation of conservation and management measures. Therefore in this work we focused on assessing the geographic extent of hybridization events in northwestern Iberia, an area where the wolf population, despite the human-dominated landscape, persisted to the severe regression that the European populations suffered.

This was accomplished through a non-invasive genetic survey of an area of 5000 km^2, covering the territory of 13 packs including an earlier described hybrid pack. A combinations of a panel of 18 microsatellites and a fragment from the mtDNA control region was used in different Bayesian and pairwise relatedness analysis in order to properly identify hybrids and assess the dynamic of these events. From a total of 332 collected samples, we were able to identify 140 distinct individual genotypes, from which 78 were assigned to the wolf population, 58 to dog, and 4 presenting intermediate assignment values were identify as hybrids. Thus, and independently from the high presence of the domestic species in the sampling area, we were able to asses a low hybridization rate (5%), similar to a previous study for the entire Iberian Peninsula population. Also in line with previous studies, both wild and domestic populations represent independent and distinct gene pools. Additionally, the detected hybrids were dispersed throughout the sampling area, and further the identification of two distinct and spaced home-packs of two hybrids shows the spatial component of hybridization events in the sampling area.

Concluding, these results show not only a previously unseen scenario of multiple and widespread hybridization events, but also support the hypothesis that wolf populations can present overlapping distributions with their domestic counterparties without having high hybridization rates or similar gene pools. Nevertheless, it is important

to continue improving knowledge, focusing now on better understanding the underlying process (e.g. ecological or behavior traits) of hybridization dynamics.

Key-Words: Grey wolf; Domestic Dog; wolf/dog hybridization, introgression, noninvasive samples, management strategies

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List of Figures

List of Tables

List of Abbreviations

AIMs – Ancestry Informative Markers

AMOVA – Analysis of Molecular Variance

Bp – Base Pair

CM – Costa da Morte

F_{st} – Fixation index (Wright's F-statistics)

Kb – Kilo-base pair (= 1,000 nucleotides)

Ma – Million year

mtDNA – Mitochondrial DNA

NIS – Non-invasive sample

NW – Norwest

q – Posterior Probability

q_i – Admixture proportion

r – Pairwise relatedness value

SNP – Single Nucleotide Polymorphism

STR – Short Tandem Repeat

ya – Years ago

Chapter 1 – General Introduction

1.1. Hybridization and Evolution

Hybridization involves the successful mating between individuals from two populations, that regardless of their taxonomic status are distinct in at least one heritable character (Anderson 1948). When applied to species, this is a controversial topic among biologists as it violates the biological species concept that postulates reproductive isolation between species (Mayr 1942). Thus, the possibility of hybridizing creates a conflict between the need to interrupt gene flow to allow for population divergence (Mayr 1963; Coyne & Orr 2004) and its potential to generate adaptive variation, functional innovation and new species (Anderson 1949; Stebbins 1959; Lewontin & Birch 1966).

Historically, zoologist and botanists have considerable distinct vision on the role of hybridization in evolution. Whereas zoologists have tended to consider hybridization as a rare process or, rather, a taxonomical problem, botanists considered it as an evolutionary process, promoting diversity and adaptive potential of species (Anderson 1948, 1949; Stebbins 1959; Grant 1981), with the potential for speciation (Arnold 1997). One of the main factors contributing to this difference of opinions is the widespread presence of allopolyploidy (more than two sets of chromosomes derived from different species) throughout plant taxa that proves their hybrid origin (Soltis & Soltis 1995), emphasising the potential of hybridization as a source of speciation. On the contrary in animal taxa allopolyploidy is restricted to some fish and frog species, and commonly hybridization occur between closely related species with the same number of chromosomes (Seehausen 2004).

Hybridization is commonly perceived negatively sustained in that it decreases individual fitness (Dobzhansky 1940; Mayr 1963). Such argument associated with the biological species concept hampers *a priori* the recognition of hybridization as a driving force of species evolution (Arnold 1992). Yet, hybridization is common in recently diverged species where reproductive isolation may not yet be complete (Wu 2001).It's worth notice that, generally, few individuals actually undergo natural hybridization and so it could be considered rare, although when accounting for the number of species that undergo natural hybridization the term rare is no longer appropriate (Mallet 2005). In fact, Arnold (1997) reveal that hybridization influences the evolutionary trajectory of around

25% of plant species and 10% of animal's species, above all young, recently diverged species.

Nowadays natural hybridization is increasingly recognized as an important contributor to biological diversification, through the formation of new hybrid taxa and also through adaptive introgression after successive backcrosses to parental lineages (Abbott *et al.* 2013; Seehausen *et al.* 2014). Despite the difficulty that sometimes exists in distinguished hybrid speciation from adaptive introgression, it has been recognized in different animal taxa, mainly in fish and insects (Mavarez & Linares 2008), but also mammals (Lavrenchenko 2014, and references therein).

1.1.1 Hybridization, Humans and Evolution

Although natural hybridization and introgression in animals are currently recognized as a source of biological diversification and evolution (Abbott *et al.* 2013; Seehausen *et al.* 2014; Lavrenchenko 2014), recent anthropogenic interference in natural ecosystems have had an impact on species abundance and distribution, thus creating new scenarios that force the coexistence of species/populations that otherwise would be isolated (Reusch & Wood 2007). Changes in species distribution associated to humans (e.g. invasive species) are promoting anthropogenic hybridization substantially, translating into a conservation problem for many wild species (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). This conservation concern around hybridization has resulted in a dramatic increment in the number of reports of this type of hybridization and introgression worldwide (Allendorf & Luikart 2007). According to Allendorf *et al.* (2001) three different (but not exclusive) outcomes can come from anthropogenic hybridization: i) hybridization without introgression; ii) widespread introgression; and iii) complete admixture. Different implications for the parental species arise in the different stages previously mentioned, and so the identification of the appropriate category for each scenario is important for the correct development of effective conservation strategies (Allendorf *et al.* 2001).

Anthropogenic hybridization is now common in a wide range of species and contexts. For example, food and pet trade promotes the contact of exotic with native species (e.g. turtles of the genus Mauremys in Taiwan; Fong & Chen 2010) or intentional translocation of captive-reared stocks, such as the case of the red-legged partridge (*Alectoris graeca* and *A. rufa*; Barilani *et al.* 2007), increases the probability of

anthropogenic hybridization. Non-native taxa can be even more problematic when the taxa is useful to humans, and therefore more adapted to current changing environments, since it will have a selective advantage over the native taxa that could ultimately become extinct (Mooney & Cleland 2001; Wolf *et al.* 2001)

The widespread occurrence of free-ranging domestic animals, and massive releases of captive-reproduced game stocks, are factors which directly promote hybridization between wild and domestic species (Randi 2008). The outcome of this cross-breeding may lead to the introgression of domestic alleles - shaped by artificial selection - into wild populations with potential negative consequences for species such as genetic homogenization and/or disruption of local adaptation (Rhymer & Simberloff 1996; Wolf *et al.* 2001; Allendorf *et al.* 2001; Verardi *et al.* 2006; Anderson *et al.* 2009; Oliveira *et al.* 2009; but see Musiani *et al.* 2007).

Hybridization between wild and domestic forms affects a wide range of species and families worldwide. For example, in the Order Carnivora, some examples can be found in Mustelidae, in which the American mink (*Neovision vison*) population is currently threatened by the overwhelming presence of domestic mink and their hybridization in natural populations (Kidd *et al.* 2009). Also the European polecat (*Mustela putorius*) is threatened by sympatric domestic ferrets (*Mustela putorius furo*), with which it hybridizes since it was first introduced in nature for controlling rabbit populations in Britain (Davison *et al.* 1999). In the Suidae family, genetic introgression from domestic pigs into European populations of wild board (*Sus scrofa*) is common, may be having ecological consequences by altering traits like reproduction rate or immunology of wild boars (Goedbloed *et al.* 2013; Canu *et al.* 2014; Murakami *et al.* 2014). But a more dramatic example occurs in the Felidae family with the European wild cat (*Felis silvestris silvestris*), where cryptic hybridization has been reported throughout all their distribution, especially in Hungary and Scotland where extensive hybridization and introgression might lead to the species extinction (Pierpaoli *et al.* 2003; Witzenberger & Hochkirch 2014).

1.2. A paradigmatic example: The Wolf

The grey wolf (*Canis lupus* Linnaeus, 1758) is one of the most charismatic species around the world, not only for all the mysticism surrounding the species, but also for the ecological value of this species as an apex predator. Originally, wolves ranged

over the entire Northern Hemisphere north of 13º-20º latitude, including central Mexico, the Arabian peninsula, and southern India (Kumar & Rahmani 2000; Mech & Boitani 2010). However, due to conflicts with humans, the species has been legal and illegally persecuted throughout centuries (Boitani 2003), leading to an significant reduction of the species distribution. Nowadays wolves occupy most of their former range in Asia, Alaska and Canada but have become extinct in most of Central and Western Europe, Mexico and most of the USA.

Being a generalist species, wolves show high adaptability to contrasting and extreme scenarios (Mech & Boitani 2010), which is reflected in the wide variability of phenotypic traits. For instance, body size in this species range from small (around 13 kg) in deserts to large sizes (over 78 kg) in the northern latitudes (Mech & Boitani 2010). Also coat color vary across the entire black-white spectrum, with mottled grey being the most common phenotype (Gipson *et al.* 2002). As a social creature, wolves lives in packs, normally formed by family groups consisting of a breeding/dominant pair, 2 to 3 sub-adults and 4 to 6 pups (Mech 1970), although they can also leave as lone individuals. Additionally, wolves can travel long distances, in fact, there are evidence of wolf journeys of over 1000 km (Linnell 2005).

The natural history of European wolf populations has been characterized by a dramatic decline in numbers during the past centuries (Boitani 2003). Throughout the 19th century, intense persecution (*e.g.* organized hunting, poison, bounties) associated with reduction of wild prey populations, lead to significant reduction in wolf distribution and abundance. As a result, by the end of that century, wolves were only present in the southern peninsulas (Iberia, Italy and the Balkans) and in Eastern regions, where it persisted until legal protection was established in most European countries in late 20th century, which in association with rural depopulation and wild prey recovery, lead to the recent and well-documented wolf re-colonization (Boitani 2003). Although in most EU countries wolves are strictly protected at present, still there are some exceptions were wolves are partially protected or they are a game species (Boitani 2003; Linnell & Boitani 2011, and references therein).

In the Iberian Peninsula, wolves currently inhabit north-western Spain (Galicia, Leon and Asturias) and North-eastern Portugal in a continuous population, whereas two isolated populations occur one in Andalusia, Southern Spain, and other south of the Douro river, in Central Portugal (Blanco & Cortés 2002; Alvares *et al.* 2005, Blanco & Cortés 2012). Iberian wolf population is estimated in ca. 2000-2500 individuals (Alvares *et al.* 2005). In Spain, wolves in north of river Douro are in Annex V of the EU Habitats

Directive (92/43/EEC) being either game species, fully protected or a species with a special regime (no game species) depending on each Spanish autonomous region; whereas in south of river Douro the species is protected being in Annexes II and IV of the EU Habitats Directive. In Portugal, the species is fully protected (Annexes II and IV of the EU Habitats Directive) in all the country (Trouwborst 2014)

Phylogenetic analysis of DNA sequenced from mitochondrial protein-coding genes and control region have revealed (Gottelli *et al.* 1994; Wayne *et al.* 1997; Vilà *et al.* 1999) that wolf genus *Canis* is a monophyletic group that includes the Asian wild dog (dhole; *Cuon alpinus*). The grey wolf, coyote (*C. latrans*), and the Ethiopian wolf (*C. simensis*) have a single common ancestry, forming a monophyletic clade, with the golden jackal (*C. aureus*) as the most likely sister taxon.

Molecular analyses both with nuclear and mtDNA revealed an absence of large-scale geographic structure in Eurasian and North American wolf populations. Recent genetic studies at regional scales have found unexpected cryptic diversity in grey wolf populations (Carmichael *et al.* 2001; Geffen *et al.* 2004; Pilot *et al.* 2006; Musiani *et al.* 2007; Hindrikson *et al.* 2013), which can be explained by a combination of factors such as habitat type, climate, an prey specialization. These findings evidence a more complex patterns of genetic diversity than was previously thought, and that the high mobility wolves and the associated gene flow might not prevent genetic structuring under specific local conditions. Regarding the identification of subspecies of *Canis lupus*, this has been under some controversial and several proposals have been made by several authors (*e.g.* Hall 1981; Sokolov *et al.* 1985; Nowak 2003). However recently thirty subspecies of wolves have been recognized (Wozencraft 2005), including the Iberian wolf subspecies, that was first proposed by Angel Cabrera in 1907 (*C. l. signatus*) based on divergence in morphological traits, and much more recently with molecular data (Lucchini *et al.* 2004).

1.3. Wolf Domesticated version - The Dog

Domestication underlies the human control of almost all aspects of the animal's life leading to changes in the animals morphological and behaviour traits. Over time, many studies have tried to disentangle the history behind wolf domestication. Although the exact process by which wolf became domesticated is not yet understood, nowadays it is recognized that wolves are the dog ancestral. Nevertheless, it wasn't until the end of

the twentieth century that the emergence of modern genetics enabled the confirmation of such theory. Vilà *et al.* (1997) compared for the first time sequence of mitochondrial control region showing that domestic dogs presented four distinct mtDNA (mitochondrial DNA) wolf lineages, suggesting four independent domestication events or alternatively a single event followed by successive backcross with female wolves. This last study lead not only to the acceptance of the grey wolf as the ancestral of the domestic dog, but also to it further recognition as a subspecies: *Canis lupus familiaris* (Tsuda *et al.* 1997; Vilà *et al.* 1997, 1999; Leonard *et al.* 2002; Savolainen *et al.* 2002).

Archaeological studies indicate that the dog was the first species of animal to be domesticated, occurring in the end of the last Ice Age, alongside with hunter-gathers (Clutton-Brock 1995), although the exact date is controversial. The vast majority of archaeological studies placed domestication about 12.000 to 15.000 ya (years ago, Benecke 1987; Hedges *et al.* 1998; Napierala & Uerpmann 2012). However a recent study, based on a comparative morphological analysis of dog-like canid remains found in Siberia and Belgium dated to about 30.000 ya, suggests theses as in fact the earliest domesticated forms (Ovodov *et al.* 2011).

On the other hand, first genetic studies based on mtDNA data (Vilà *et al.* 1997) argued for multiple domestication events occurring over 100.000 ya. However this estimation was based on many assumptions concerning mutation rates and wide confidence intervals, being latter refuted by other mtDNA-based study that argued for a single domestication event occurring 15.000 ya in East Asia (Savolainen *et al.* 2002).

Recently, Germonpré *et al.* (2009) suggested that domestication had started 31.700 ya in Europe based on archaeological and genetic data. Interestingly, and even more recently, two studies with similar genome sequencing approaches reveal two very distinct results, Wang *et al.* (2013) concluded that dogs and wolves diverged 32.000ya, while Freedman *et al.* (2014) place hybridization 11.000 to 16.000 ya. This wide difference between studies is mainly related with the dependence on molecular evolutionary rates, once there is a lack of knowledge on the dog-specific mutation rate, and so the application of different estimation of the mutation rate in different studies, lead discrepancies between results (Freedman *et al.* 2014; Larson & Bradley 2014).

Furthermore, the geographical location of the first domestication events is also controversial. Archaeological studies tend to better accept the hypotheses of multiple origin location, due to the geographical dispersion of the earliest dog remains found (Larson & Bradley 2014). On the other hand, most of the genetic studies tend to accept

the hypotheses of a single domestication event, commonly in East Asia (e.g. Savolainen *et al.* 2002; Pang *et al.* 2009). However other studies have been suggesting the contribution of different wolf population to the modern dog breeds, but again some disparity exist between studies. Whereas vonHoldt *et al.* (2010) suggest a significant contribution of Middle Eastern wolf populations to dog genome and, for certain breeds, European wolf populations, with a similar approach Larson *et al.* (2012) conclude that it was the East Asian and Near Eastern wolf populations the ones that most contributed to modern dog breeds. However, both studies suggest that ancient crossbreed between domesticated dogs and local wolf populations, may be affecting the discrimination of the point of origin through genetic data. Concluding, more studies are needed in order to better understand dog origins, nevertheless is important to understand that both archaeological and genetic approach have limitation and so the combination of the two fields of knowledge could be the better way to approach this subject (Larson & Bradley 2014).

Concerning the events that led to the domestication of this taxa not much is known, however some authors suggest that wolf may have taking the first step towards domestication driven by an opportunistic scavenging behaviour, contradicting their natural instinct and draw nearer human settlements (Crockford 2000; Pang *et al.* 2009).

Since their domestication the dog as became “man’s best friend”, playing different roles in society, from protection to hunting companions to pets, serving even in certain cultures as food resource (Ostrander & Wayne 2005; Pang *et al.* 2009). Although the domestic dog history started thousands of years ago, modern breeds have only been established in the past two centuries (Parker *et al.* 2004; vonHoldt *et al.* 2010). Nevertheless, now a days there are more than 350 breeds, showing a wide diversity of body sizes and shapes, coat colours, and also behavioural predispositions (Ostrander & Wayne 2005). Additional, the extreme selection that dogs underwent also resulted in the appearance of strong reproductive barriers between breeds, which significantly increased genetic similarity within breeds as opposed to between breeds (Parker *et al.* 2004).

1.4. Hybridization in *Canis*

The close relationship between all the species of the genus Canis (same number of chromosomes, 2n = 78; Wurster-Hill & Centerwall 1982; Wayne *et al.* 1987) makes hybridization a relatively common event among this clade. Both intra and interspecific

hybridization occur within this taxa, and in all cases the resulting hybrids and subsequent generations are viable and fertile (Iljin 1941). Some examples of interspecific hybridization is the cross-breeding between the coyote (*Canis latrans*) and the grey wolf (*Canis lupus*; Monzón *et al.* 2014); the Ethiopian wolf (*Canis simensis*) and the domestic dog (*Canis lupus familiaris*) Gottelli *et al.* 1994); and also between the red wolf (*Canis rufus*) and the coyote (*Canis latrans*; Bohling & Waits 2011). In the case of intraspecific hybridization, one of the most emblematic cases is between the grey wolf and the domestic dog.

Since the wolf domestication, dog have interbred with wolf several times (Vilà *et al.* 1997). Commonly throughout the taxa history, this crossbreeding was mediated by humans as a method to improve the domestic taxa vigour and better serve as sled dogs (Schwartz 1998). In fact, in the end of the nineteenth century Aristotle wrote about the occurrence of such cross-breeding in the fourteenth century, and across time, other evidence of wolf-dog hybridization was also found in cultures as the American Indians and Eskimos (Schwartz 1998). Nowadays this practice is still common, having been the basis for the establishment of seven different dog breeds (e.g. Alaskan malamute, German shepherd dog, poodles), aiming to enhance features such as body robustness and also to accomplish some particular tasks for humans.

In the wild, hybridization between wolves and dogs is believed to be most frequent near human settlements, where wolf density is low and feral or free-ranging domestic dogs are common (Boitani 1984; Blanco *et al.* 1992). As a consequence, some authors suggest that the increase in the Italian wolf population in recent times was reinforced by intensive hybridization with dog, emphasizing introgression as a common event (Boitani 1984), and further that European wolf populations were mainly constituted by wolf-dog hybrids (Butler 1994). Nevertheless, these studies were based on anecdotal events and latter refuted by more recent molecular studies, that shown that wolf-dog hybridization was rare in the wild and that there was no evidence of significant introgression of domestic genes into wolf populations (Vila & Wayne 1999).

Almost all wolf populations are nowadays expanding (Kaczensky *et al.* 2013) and thus occupying areas of high human pressure, favoring the contact with domestic or feral dogs. This increased contact between wolves and dogs represents one of the potential factors responsible for the occurrence of hybridization. Throughout the years hybridization between wolves and dogs has been described worldwide, and the term “rare” should be understood as the number of individuals that undergo hybridization and not the number of populations. Studies have described the occurrence of wolf-dog

hybridization in Scandinavia (Vilà *et al.* 2003; Klütsch *et al.* 2011); Latvia (Andersone *et al.* 2002); Bulgaria (Randi *et al.* 2000); Israel (Vila & Wayne 1999); Georgia (Kopaliani *et al.* 2014); Italy (Randi & Lucchini 2002; Verardi *et al.* 2006; Caniglia *et al.* 2013); Iberian Peninsula (Godinho *et al.* 2011, 2014), among others. However, there is still no evidences for a significant level of domestic genes introgression in wild populations (Godinho *et al.* 2011, Kopaliani *et al.* 2014). Nevertheless, the possible consequences for the wild taxa of this type of introgression, (e.g. reduced fitness) raise a controversial debate in the scientific community (Blanco *et al.* 1992; Boitani & Ciucci 1993; Anderson *et al.* 2009; Randi 2011).

1.5. Detecting Hybridization in *Canis*

When studying hybridization is important to correctly identify the hybrid individuals and posteriorly their hybrids classes, in order to have a better understanding of this phenomenon, and consequently assess its conservation implications when applied. Nevertheless, when considering closely related taxa hybridizing, the correct identification of the cross-breeding events could be hampered by the morphological and genetic similarity of the two taxa, being the case of some hybridizing pairs in the *Canis* family, for instance, the wolf and dog.

Until the mid-1960, morphological traits was commonly used to detected admixture individuals (Allendorf *et al.* 2001). However, not all morphological variation has a genetic basis, and so the exclusive used of this traits is not viable, since hybrids not always present an intermediate phenotype between the two species, and rather an mosaic of parental phenotypes (Allendorf *et al.* 2001; Smith *et al.* 2003, Godinho *et al.* 2014). Additionally, morphological identification would not allow the distinction between hybrid generations (first generation hybrids (F1); backcrosses), which is crucial to a proper understanding of the implications of hybridization at the population level. In the particular case of wolf-dog hybridization there are some phenotypic traits described that can aid the distinction of hybrid from the wild species, as the coat colour/patterns or even the present of additional claws (Randi & Lucchini 2002; Vilà *et al.* 2003; Ciucci *et al.* 2003; Caniglia *et al.* 2013, Godinho *et al.* 2014). Nevertheless, the use of this traits should always be accompanied by genetic confirmation.

1.5.1. Using Molecular Markers to study wolf-dog hybridization

With the advent of genetics and the development of molecular markers for species and individual identification, the identification and assessment of hybridization events became an more accurate and efficient process. Nevertheless, the information obtained when analysing the genome depends on the analysed genomic region: different molecular markers have different modes of evolution, varying in the level of information and on possible subsequent interpretations.

The mitochondrial DNA was the first molecular marker to be applied in general when analysing DNA variation, since it is more abundant in the cell compared with nuclear DNA, its characterization is easer as well as its application, since it can be amplified through indirect (e.g. RFLP) or direct (e.g. sequencing) techniques. Its high mutation rate (in mammals this rate is three to five times higher than nuclear genes; Avise 2000) allow closely related species/populations to accumulate diagnostic mutations. Although, because it is only maternal inherited (Avise 1994), the isolate use of this marker in hybridization studies will provide us a gender-biased description of the gene flow between species/populations, therefore it should be used in combination with other type of markers (Roy *et al.* 1994). Nevertheless, mtDNA analysis can be important in hybridization studies since it enable the assessment of the direction of hybridization, once the mitochondrial lineage carried by first generation hybrids gives us information about the species of the mother (Godinho *et al.* 2011; Hindrikson *et al.* 2012). For instance, the presence of a wolf mitochondrial haplotype in an F1 wolf-dog hybrid tell us that it results from the crossbreeding between a female wolf and a male dog.

Nonetheless, the study of hybridization can only be solved using biparental molecular markers, like microsatellite loci, also known as Short Tandem Repeats (STRs). These are nuclear markers formed by motifs of one to six nucleotides, repeated a certain number of times, normally 100-200bp long, which simplifies its amplification by PCR (Polymerase Chain Reaction). STRs are typically codominant markers, with two alleles per locus/individual inherited from each parent, highly polymorphic, typical of high mutation rate, that this markers commonly present (Kelkar *et al.* 2010). Microsatellites have been highly used since the late eighties for many applications, including parentage analysis, genetic mapping or genetic structure (Ellegren 2004; Mittal & Dubey 2009), and were recognized as an important tool for hybridization analysis (Roy *et al.* 1994; Lu *et al.* 2001).

With the raising of new genome-wide approaches, other nuclear markers have been recognized as powerful population genetic tools. This is the case of single nucleotide polymorphisms (SNPs; Schlotterer 2004), which refers to a polymorphism in a single base pair position in genomic DNA. Although in theory this marker could be bi-, tri- or tetra-allelic, it is typically bi-allelic. It's a codominant marker, with the minor allele segregating at a frequency of at least 1% (Brookes 1999). In comparison to microsatellites, SNP present a less complex mutation pattern, as the number of possible alleles decrease from a high number in microsatellites to only two to four forms in SNPs.

One of the main drawbacks of microsatellites is the limited reproducibility among laboratories using different equipments or reagents (Guichoux *et al.* 2011). This is related with allele calling, because after amplification and sequencing, normally through capillary electrophoresis systems, genotypes need to be read, meaning that is necessary to identify peaks that correspond to alleles and assign its size. Although there are some commercial software that performed automatic corrections of common genotyping problems (*e.g.* stutter or saturated peaks or excessive baseline noise), allele calling often requires additional manual editing. The second step is allelic binning, *i.e*. the conversion of alleles from real-valued DNA fragments sizes into discrete units to which an integer label is assigned (Idury & Cardon 1997). This step is generally where errors occur. A comparative study by Weeks *et al.* (2002) reveal that 83% of the discrepancies between laboratories in scoring dinucleotide alleles were caused by arbitrary decisions in binning. In comparison, SNP do not have this limitation, since the allele calling is not subject to different interpretations, been easily compare across studies (Guichoux *et al.* 2011). On the other hand, one of the major drawback of SNPs is the ascertainment bias, *i.e.* the bias resulting from the choice of the initial panel of genotypes used to screen for polymorphisms (*e.g.* Li *et al.* 2008), which is considerably high comparing with microsatellites where it is practically non-existent.

Considering the mutation behaviour of both markers, and the analysis to be performed, both markers have advantages and drawbacks. For instance, when investigating parentage relationships, microsatellites require larger sample sizes in order to have an accurate estimation of allelic frequencies, also due to their higher mutation rate are more prone to spontaneous mutations within a pedigree which might hampered parentage reconstruction (Ellegren 2000; Phillips *et al.* 2007; Børsting *et al.* 2009). In contrast, the lower polymorphism in SNP should be compensated by an increase in the number of loci in order to achieve a similar high statistical power present in microsatellites. This increase depend on a set of factors, such as number of populations,

and allele frequencies per locus, and it can lead to an increase of two to six fold the number of markers required to have similar statistical power as microsatellites (Gärke *et al.* 2012). Throughout the literature wolf hybridization studies have been mainly relying on the combination of different molecular markers panels, from microsatellites (unlinked autosomal STRs - Randi 2008, 2011; Godinho *et al.* 2011; Hindrikson *et al.* 2012; recombinant linkage groups - Verardi *et al.* 2006) to uniparental markers as the hypervariable domain of the mtDNA control region and Y-linked STR haplotypes (Randi *et al.* 2000; Sundqvist *et al.* 2001; Vilà *et al.* 2003; Iacolina *et al.* 2010).

The recurrent used of microsatellites in these studies is many times associated with the use of non-invasive samples (scats and hairs), especially when focusing on rare and elusive species, such as the wolf, since it represents the only approach that allow a significant sample size of the population. Nevertheless, one of the main reasons for this association is the fact that these markers generally perform well with low quality DNA samples, mainly because of its short size fragments when performing the PCR amplification. Additionally, its high number of alleles allow a higher statistical power and resolution on subsequent analyses when comparing to SNPs, which would require a much higher number of loci to achieve the same statically power. The last would present an issue when dealing with noninvasive samples, since the quantity of DNA extracted from this type of samples is extremely low. Nonetheless, a recent study by Kraus *et al.* (2014) describe an efficient method to genotype a set of 100 SNP in non-invasive samples, arguing that it is faster and less expensive than genotype a number of microsatellites with an similar statistical power. However, the correct assessment of hybridization, especially among species with lower genetic divergence, needs a battery of carefully selected markers (Godinho *et al.* 2014) which may represent more than 100 highly diagnostic SNPs, as recently shown (VonHoldt *et al.* 2012). On the other hand, a similar study was able to achieve higher accuracy in identifying hybrids first and second generation hybrids, relaying on a set of 13 microsatellites chosen regarding the power of differentiation between wolves and dogs (Godinho *et al.* 2014), and so the use of microsatellites on hybridization studies may continue to be a trend.

1.6. Objectives

With this work we intend to improve the current knowledge on hybridization, allowing a better understanding of its extent at a population level and of the introgression of domestic genes in wild populations. Furthermore, having that knowledge, we aim to evaluate the effectiveness of the current management and conservation measures proposed to European wolf populations. In order to fulfill our goal, we will be focusing on a particular study case in nonwestern Iberia (Chapter 2).

Chapter 2 – Study Case: Assessing the spatial extent of wolf-dog hybridization in real-time and at population level using non-invasive DNA sampling

2.1. Introduction

Hybridization between wild species and their domestic forms is perceived as a threat by research and conservationist communities (Rhymer & Simberloff 1996; Randi 2008; Lescureux & Linnell 2014). The outcome of this cross-breeding may lead to the introgression of domestic alleles - shaped by artificial selection - into wild populations with potential negative consequences for species such as genetic homogenization and/or disruption of local adaptation (Rhymer & Simberloff 1996; Wolf *et al.* 2001; Allendorf *et al.* 2001; Verardi *et al.* 2006; Anderson *et al.* 2009; Oliveira *et al.* 2009; but see Musiani *et al.* 2007). On the other hand, the introgression of genetic novelties originated during domestication into natural populations may be particularly interesting since it augments the evolutionary potential in the wild. This would be especially interesting in small and/or endangered populations, thus having a potential positive impact when implementing conservation or management actions (Soulé 1985). In this context, introgressed genes could be the basis upon which natural selection can act and alter the evolutionary trajectory of hybrids parent taxa (Crandall *et al.* 2000; Placyk *et al.* 2012).

The management of wild x domestic hybrids is a controversial issue, mainly because its origin is only validated upon genetic information and clear genetic limits for considering an individual as an hybrid are still lacking, but also because the value and role of hybrids in the ecosystems and their legal status are not clear yet (Stronen & Paquet 2013; Lescureux & Linnell 2014; Trouwborst 2014). For example, in Europe the two major legal instruments governing biodiversity conservation, the Bern Convention and the EU Habitat Directive (92/43/EEC) do not contemplate any clear statement on the legal status of hybrids. Based on interpretations made by Trouwborst (2014), the prohibition of killing and capturing strictly protected species also includes free-ranging hybrids of the same threatened species living in the wild. Paradoxically, the same legal instruments impose to EU member states preventive and mitigation measures, leading to derogation of hybrids from strictly protected status.

In the last few years, the spatial evaluation of hybridization between wild species and their domestic counterparts has been well documented for most of wild/domesticated pairs (e.g. Geraldes *et al.* 2006; Oliveira *et al.* 2007; Godinho *et al.* 2011; Murakami *et*

al. 2014). However, this has been done mainly using opportunistically collected samples, leading to an evident lack of spatial systematization of sampling. More important, these studies have never made inferences about time, essentially hampered by difficulties of sampling the wild part in a large area in a generation time. In fact, most of the available literature on the subject has assessed hybridization using invasive samples (*i.e.* tissue or blood) collected from dead (*i.e.* roadkills, hunted) or capture animals throughout a temporal window that is large enough to allow a significant sample size across space. This generally embraces several generations and prevents a real-time assessment of the process. An alternative to overcome these constraints is the use of non-invasive samples (NIS) (*e.g.* faeces, hair; Long 2008). This was in fact done in a few occasions, although only for very restrictive areas. For example, both Caniglia *et al.* (2013) and Godinho *et al.* (2014) found wolf x dog hybrids when used NIS to study an area occupied by a single pack in Italy and Spain, respectively. Yet NIS present some limitations related with low DNA quality and/or quantity and PCR (Polymerase Chain Reaction) inhibitors (Beja-Pereira *et al.* 2009), they are of inevitable use for the study of a large area in a short time, especially when focusing on illusive or rare species. Another particularly important factor to consider in these studies is the appropriate selection of molecular markers because a rigorous selection of ancestry informative markers will perform significantly better than a random choose (Randi *et al.* 2014; Godinho *et al.* 2014).

A recent study in NW Iberia suggests a dynamic crossbreeding system between Iberian wolves and dogs (Godinho *et al.* 2014). Such evidence could represent a scenario of a widespread hybridization event, because packs in non-expanding populations are commonly formed by related individuals (Wayne *et al.* 1992; Lehman *et al.* 1992), and so authors stated that other non-observed individuals in their study might also exhibit admixed ancestries (Godinho *et al.* 2014). Thus, this situation represents an opportunity for assessing the spatial patterns of hybridization at population level and to advance our knowledge on the dynamics of wild x domesticated hybridization systems.

Here we present the real-time assessment of hybridization events in this wolf population at NW Iberian Peninsula. To do so, we rely on systematic sampling survey for the entire wolf distribution in the area and on a set of 18 ancestry informative markers (AIMs). Our main goals were i) to quantify the number and the proportion of admixed individuals in this wolf population; ii) to determine the spatial pattern of hybridization events in real-time for this population; iii) to identify the genetic composition of admixed individuals, inferring their hybrid class; iv) to assess the relatedness among admixed individuals, and between those and wolves; and v) to identify a probable pack of origin for hybrids.

2.2. Material and Methods

2.2.1. Study area and sample collection

Our study area comprises the wolf population of Costa da Morte and surroundings (Galicia, Spain), corresponding to ca. 5,000km^2 where 13 packs were estimated in the past decade (Llaneza *et al.* 2012; Fig .1). This is a human-dominated landscape mostly characterized by a patchy and heterogeneous landscape, with human settlements widely sparse (148 people/km^2; López-Bao *et al.* 2013). The land use is characterized by croplands, pastures, scrubs and forest plantations (*Eucalyptus* spp. and *Pinus* spp; Llaneza *et al.* 2012). Free-ranging domestic horses (*Equus caballus*) together with other domestic ungulates are the main food resource for wolves in the area (Cuesta *et al.* 1991; López-Bao *et al.* 2013). Recently, it has been detected the presence of a hybrid pack composed by nine hybrids together with two pure wolves (Godinho *et al.* 2014). Hybrids were assigned to first backcross to wolf or ambiguously assigned to different hybrid classes (Godinho *et al.* 2014). During spring and summer of 2013, 332 wolf-like feces were collected throughout all the study area (further referred as CM samples). In collaboration with rangers from Galicia administration (Xunta de Galicia), we searched for faeces along existing paths, forest trails and firebreaks, where the

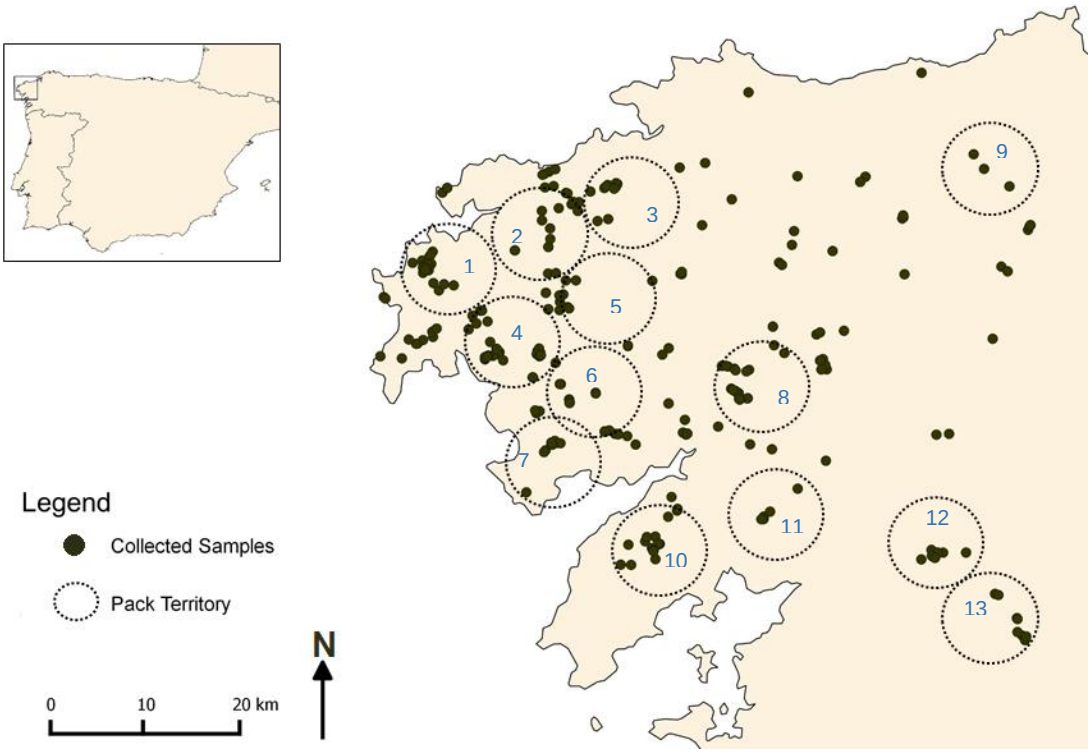

Figure 1 -Study area location in Iberia Peninsula (right). Distribution of collected samples (dark points) and of wolf packs territories (100km2 circles; Llaneza *et al.* 2012) in the study area. Each number represents a pack as follow: 1- Muxia; 2- Vimianzo; 3 - Passarela; 4 - Buxantes; 5- Baiñas; 6 – Ruña; 7 – Carnota; 8 – Negreira; 9 – Cerceda; 10 – Barbanza; 11 – Lousame; 12 – Xesteiras; 13 - Piorneiras . Location of the study area in Iberian Peninsula.

probability of wolf marks detection is maximized (Llaneza *et al.* 2014) and on a 10 x 10 km UTM cell grid. We invested a minimum of 10 km per cell and a total of ca. 750 km were surveyed. For each sample we took the spatial position with a GPS unit (see Fig.1) and all samples were preserved in ethanol 96%, at room temperature.

2.2.2. DNA extraction and quantification

Extraction and manipulation of DNA from fecal samples was confined to a dedicated laboratory with sterile conditions and positive air pressure, exclusively for low-quality DNA, in order to reduce possible contaminations. DNA was extracted from 333 samples following Frantz *et al.* (2003) protocol after the GuSCN/silica method (Boom *et al.* 1990). A final step for further removal of potential PCR inhibitors was performed using pre-rinsed Microcon® YM-30 centrifugal Filter Units (Millipore, Billerica, MA). Negative controls were included throughout the entire process to monitor for potential DNA contaminations. To assess the concentration of DNA, samples were quantified with Quant-iT^{TM} PicoGreen dsDNA Assay Kit (Invitrogen) method in VICTOR^3 Multilabel Plate Reader (PerkinElmer).

2.2.3. Markers and Genotyping

Species identification was performed based on the amplification of a fragment of the mitochondrial DNA control region (425bp), using universal primers Thr-L 15926 and DL-H 16340 and following lab procedures of Vilà *et al.* (1999). Successful amplifications were purified using enzymes exonuclease I and Shrimp alkaline phosphatase, and sequenced in a 3130XL genetic analyzer following the BigDye Terminator v3.1 Cycle sequencing protocol (Applied Biosystems). Sequences were visualized and aligned using SEQSCAPE 2.5 (Applied Biosystems). Sequences from other species rather than wolf or dog were excluded from the study.

The individual genetic profile of each sample was assessed based on 18 ancestry informative markers (AIMs). AIMs were selected from a panel of 52 markers available at our lab (Godinho *et al.* 2014) based on their F_{ST} values between wolves and dogs for 15 markers and their probability of identity for 3 markers. The set of markers included 17 microsatellites and a 5bp deletion at intron 3 of the KIT-ligand gene (KITLG; see Table1 for details on each marker). Additionally, a molecular sexing test was applied following Seddon (2005).

Table 1 - List of nuclear loci analysed in this study, along with repeat motif, allele range, multiplex in which it was genotyped and references.

Microsatellite	Repeat	Allelic Range	Multiplex	Reference
C09.474	Di	111-133	Mp2	Ostrander *et al.* 1995
CPH2	Di	87-113	Mp2	Fredholm & Winterø 1995
CPH9	Di	133-163	Mp2	Fredholm & Winterø 1995
Cfx30371	Tetra	125-161	Mp2	Godinho *et al.* 2014
FHC2010	Tetra	216-240	Mp4	Francisco *et al.* 1996
AHT103	Di	71-89	Mp3	Holmes *et al.* 1995
AHT111	Di	72-92	Mp3	Holmes *et al.* 1993
C20.253	Di	95-125	Mp3	Ostrander *et al.* 1993
C27.442	Di	158-172	Mp3	Ostrander *et al.* 1995
Dbar1	Di	183-274	Mp3	Kerns *et al.* 2004
AHT121	Di	74-118	Mp1	Holmes *et al.* 1995
AHTh171	Di	216-240	Mp4	Breen *et al.* 2001
AHTk211	Di	82-98	Mp1	Thomas *et al.* 1997
INU030	Di	136-156	Mp1	Finnzymes, Inc
REN162C04	Di	189-215	Mp1	Guyon *et al.* 2003
C09.173	Di	100-118	Mp4	Ostrander *et al.* 1993
C22.279	Di	108-132	Mp4	Ostrander *et al.* 1993
Indel	**Size**	**Sequence**	**Multiplex**	**Reference**
KITLG.indel	5bp	CAGCA	Mp2	Silva (2010)

All markers were amplified in a two-step PCR reaction using a preamplification protocol (Smith *et al.* 2011). Initially, a preamplification PCR (PCR1) was conducted to increase the available template DNA in subsequent genotyping reactions, overcoming the low amplification success associated with low concentrations of template common in NIS. Afterwards, products from PCR1 were used as template for a second PCR (PCR2). In both steps the 18 AIMs were divided into four multiplex (MP1, MP2, MP3 and MP4; see Table 1 for the allocation of loci to each multiplex), always using the Multiplex PCR Kit (QIAGEN) for a 10 *μ*l final volume reaction. In total each amplification for PCR1 and PCR2 was repeated 4 times (first 2 replicas followed by others 2, the threshold 4 was defined based on genotyping error estimations; see section 3.4.Data Analysis). For the preamplification PCR we used approximately 5 ng of DNA and a concentration of 0.2 *μ*M of unlabeled forward and reverse primers. Forward primers were M13-tailed to follow a fluorescent labelling protocol at PCR2 (Blacket *et al.* 2002). PCR conditions followed manufacturer's instructions, with an annealing temperature set to 57°C during 20 cycles (multiplex MP1, MP2 and MP4). For multiplex MP3 a touch down profile was used

decreasing from 60°C to 57°C in 7 cycles, followed by 13 cycles of constant annealing temperature set to 57°C. For PCR2, 1μl of undiluted product from PCR1 was used as template, and only reverse primers and respective M13 fluorescent tails were used Primer concentration depended on the locus. Thermo cycling used a touch down profile, decreasing from 60°C to 58°C in 5 cycles, followed by 37 cycles of constant annealing set to 58°C, for MP1, MP2 and MP4; and decreasing from 62°C to 58°C in 9 cycles, followed by 32 cycles of constant annealing temperature set to 58°C, for multiplex MP3. All amplifications were performed in Bio-Rad thermal cyclers (T100) always using negative controls to monitor possible contaminants. Initially, samples were screened four times for multiplex MP2, which was selected for its higher PCR success smaller fragment sizes amplified. Only samples presenting missing data under 40% for MP2 were selected and further amplified for the remaining 13 microsatellites. PCR products were separated by size on an ABI3100XL genetic analyzer using Genescan-500LIZ size standard (Applied Biosystems). Alleles were determined using GENEMAPPER 4.0 (Applied Biosystems) and checked manually.

2.2.4. Data Analysis

The two initial replicated of each genotype was used to assess genotyping error in the software PEDANT 1.0 (Johnson & Haydon 2007). This estimations were then used to determine the minimum number of repetitions needed in order to minimize the genotyping error of the combination samples/loci, performed in the software GEMINI 1.3.0 (Valière & Berthier 2002).

Consensus genotypes were constructed using GIMLET 1.3.3 (Valière 2002) and then checked manually. Heterozygous genotypes were accepted if the same genotype was observed in two independent PCRs and homozygous genotypes were accepted if the genotype was observed in three independent PCRs (following Godinho *et al.* 2014). All samples with a percentage of missing data higher than 20% were removed from the analysis, in order to reduce possible bias. Identical genotypes were identified using the software GIMLET 1.3.3 (Valière 2002) and were discarded from further analysis. The same software was used to evaluate mean allelic dropout and false allele amplification rates across loci.

Microsatellites diversity was evaluated for wolves and dogs separately (hybrid individuals were excluded) based on the allele frequencies, mean number of alleles (na), private alleles (na private) per locus, and observed (Ho) and expected (He)

heterozygosities for each locus using ARLEQUIN 3.5. (Excoffier & Lischer 2010). The same software was used for testing deviation from Hardy–Weinberg equilibrium and significance of association between genotypes at pairs of loci in each population (LD). The level of statistical significance was adjusted using strict Bonferroni corrections. The probability of identity for each locus and overall loci, was assessed using GIMLET 1.3.3 (Valière 2002). Population differentiation was assessed by Fisher's exact test, analogues of pairwise mean F_{ST} (Cockerham & Weir 1984) and analysis of molecular variance (AMOVA, (Michalakis & Excoffier 1996) using also ARLEQUIN 3.5.

Bayesian clustering analysis implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007; Hubisz *et al.* 2009) was used to quantify admixture and estimate ancestry in our sample in relation to two reference populations (K=2), by assessing the average membership proportion (*Qi*) for wolf and dog reference populations and the individual membership proportions (*qi*) to the inferred clusters and their 90% credible intervals (CI). Each reference population was composed by previously validated Iberian wolves and dogs (250 and 230 individuals, respectively; Godinho *et al.* 2014). All the individuals from the reference populations were previously analyzed in the software STRUCTURE 2.3.4, with a K=2 and no prior information, having all presented a *qi* > 98% to their putative population. The dog reference population comprises a set of autochthonous dog breeds predominant in rural areas of Iberian Peninsula, and another of mongrel dogs from NW Iberia (our dataset is similar to the one used by Godinho *et al.* 2014 but additionanlly includes 18 Can de Palleiro samples, the authoctonous herding breed from Galicia). Bayesian clustering analysis was carried out using the admixture model with correlated allele frequencies (Usepopinfo activated, with 1 in reference samples and zero in the CM samples), in 10 independent runs each with 10^6 MCMC iterations following a burn-in period of 10^5 iterations, in order to guarantee the achievement of similar posterior probabilities of the data in each run. Afterwards, a similar analysis was performed using the ancestry model "*use population information for testing for migration*", assuming that every sample belongs to one of two clusters (wolves or dogs) previously assigned in the admixture model analysis (Usepopinfo=1). Admixed individuals were considered in the population with higher *qi* value. This model assesses the posterior probability (*q*) of each individual belonging to the a priori assigned cluster and its ancestry in the other clustersfor the present and past generations. This analysis was performed using the same parameters, except for the ancestry model where the defaults settings were used with GENBACK= 2.

The appropriate threshold *qi* value to identify hybrids for our panel of 18 AIMs was defined based on the power of admixture analysis to correctly identify individuals

with prior known ancestry using simulations. We used the reference wolf and dog samples to generate 100 simulated genotypes for each of the following classes: parental, first (F1) and second (F2) generations hybrids, and first (BxW, BxD) and second (BxW2, BxD2) generation backcrosses with wolves and dogs, respectively, using HYBRIDLAB 1.0 (Nielsen *et al.* 2006). Simulated genotypes were analyzed in STRUCTURE 2.3.4, with the *admixture model* and correlated allele frequencies without any prior population information. The proportion of individuals correctly assigned to each class allowed the definition of the appropriate threshold value for our panel of markers.

To further explore the genetic structure in our dataset a Factorial Correspondence Analysis (FCA) was also performed using the software GENETIX 4.2 (Belkhir *et al.* 2004) under default settings.

Calculation of pairwise genetic relatedness (*r*) was performed using dyadic maximum-likelihood estimator (Milligan 2003) implemented in software COANCESTRY 1.0 (Wang 2011), and without taking into account inbreeding. The selection of the estimator was done based on results obtained after simulations. For that, we generated 100 genotypes for five possible kinship groups i) parent/offspring, ii) full siblings, iii) half siblings, iv) grandparent-grandchild and v) unrelated individuals, using COANCESTRY 1.0 and assessing *r* with four different relatedness estimators. The comparison was done with two likelihood estimators, the triadic likelihood estimator (TrioML; Wang 2007) and the dyadic likelihood estimator (DyadML; Milligan 2003), and two moment estimators, the Lynch and Ritland estimator (LynchRd; Lynch & Ritland 1999) and the Queller and Goodnight estimator (QuellerGt; (Queller & Goodnight 1989). Success rate of each estimator in correctly infer the *r* in each kinship group was calculated. Hereafter we will only focus on the method that best suite our data, and so our inferences will be only based on the selected estimator. Relatedness estimations were carried out using pure wolves and hybrids detected in our set of CM samples, previously identified in the aforementioned admixture analysis. We excluded dogs due to limitation of the estimator in considering two different biological entities, leading to bias in *r* estimations. Frist, pairwise relatedness was estimated for all pairs of individuals in the population. Secondly we compared pairwise relatedness within hybrid individuals. Finally, we compared relatedness between each detected hybrid and each pack. For that we cluster together all the wolf samples collected within the estimated packs territory (100Km^2 area centered on the *rendezvous site* of each pack), and calculated and calculating the mean, maximum and minimum values of *r* between all cluster individuals and each hybrid.

2.3. Results

2.3.1. Genotyping and Individual Identification

MtDNA sequences were successfully obtained for 304 NIS (91%), from which 172 (57%) matched with the Iberian wolf haplotype W7 (Valière *et al.* 2003), 101 (33%) matched with different dog haplotypes, 28 (9%) were classified as red fox (*Vulpes vulpes*), and three (1%) as wild board *(Sus scrofa)* likely due to amplification of prey DNA. Samples carrying wolf or dog mtDNA (273) were further screened for a single multiplex in which 91 samples were filtered out because of poor DNA quality. Thus, 182 samples were further analyzed for the remaining markers. In the following process, fourteen samples were also excluded from subsequent analysis due to missing data (over 20% of all genotype). Finally, consensus genotypes at 18 nuclear markers were obtained for 168 NIS (51% of the 332 collected samples), with an allelic dropout rate of 0.026 on average among loci, and no evidence in any loci of false alleles. These are optimum values under the average expected for non-invasive genetic studies (Broquet *et al.* 2006), and also lower than values observed in other similar studies (e.g. Caniglia *et al.* 2013; Godinho *et al.* 2014).

One-hundred and forty unique genotypes were identified out of 168 NIS. This dataset had a PIDsibs = 8.15×10^{-7}, and an expected number of individuals sharing the same genotype of PID × sample size = 0.000114, meaning that a “shadow effect” was very unlikely. Seventy-three per cent of individual genotypes were only detected once, and the maximum number of recaptures was five for wolves and two for dogs.

2.3.2. Detecting Hybridization

Bayesian clustering on simulated genotypes revealed high average assignments to the correct parental class for wolves and dogs (qi_{wolf} = 0.959, minimum qi_{wolf} = 0.934; qi_{dog} = 0.943, minimum qi_{dog} = 0.916). Thus, based on these results we used a threshold value of qi = 0.90 in the assignment procedures. In further Bayesian cluster admixture analysis performed with CM samples plus reference individuals, all reference wolves were assigned to one cluster with average QWolf=0.998 (90% CI: 0.994 – 1.00), and all reference dogs were assigned to the other cluster with QDog=0.993 (90% CI: 0.978 – 1.00). All reference genotypes (dogs and wolves) were assigned to their respective cluster with qi > 0.95. From the 140 genotypes identified in CM, 96% were assigned to

one of the parental species, 78 individuals assigned to wolf cluster with average *qi* of 0.981, and 58 assigned to dog cluster with average *qi* of 0.979 (Tables 2 and 3, see Fig.3 for the spatial distribution of the individuals).

Table 2 - Individual assignment (qi) and inferred ancestry of wolves and dogs, and of the 5 individuals from Costa da Morte wolf population that present signals of admixture. Qi values were calculated in structure considering two clusters (K=2). 90% confidence intervals are presented in brackets. F: female. M: male.

Sample	Sex	MtDNA	Wolf		Dog	
			qi	CI 90%	qi	CI 90%
Reference Wolf	-	-	0.998	(0.994,1.000)	0.002	(0.000,0.007)
Reference Dog	-	-	0.007	(0.000,0.022)	0.993	(0.978,1.000)
CM Wolfs	-	-	0.981	(0.919,1.000)	0.020	(0.000,0.082)
CM Dogs	-	-	0.021	(0.000,0.086)	0.979	(0.824,1.000)
CM 13	F	Dog	0.324	(0.080,0.555)	0.676	(0.445,0.920)
CM 50	M	Wolf	0.825	(0.651,0.970)	0.175	(0.030,0.349)
CM 203	M	Wolf	0.711	(0.536,0.862)	0.289	(0.138,0.464)
CM 228	M	Dog	0.220	(0.000,0.463)	0.780	(0.537,1.000)

Table 3 - Total number of individual genotypes obtain (Ne Total), effective number of samples assigned to each cluster in the STRUCTURE analysis, and percentage of samples assigned to each cluster in relation to the total number of samples analysed. Density of each cluster in the study area (5000km^2) assess with the effective number of samples assigned to each clusters.

NeTotal	Ne Structure Assigment		NeStructure/ NeTotal (%)	Density (individuals/100km^2)
140	Wolf	78	56%	1.54
	Dog	58	41%	1.16
	Hybrid	4	3%	0.1

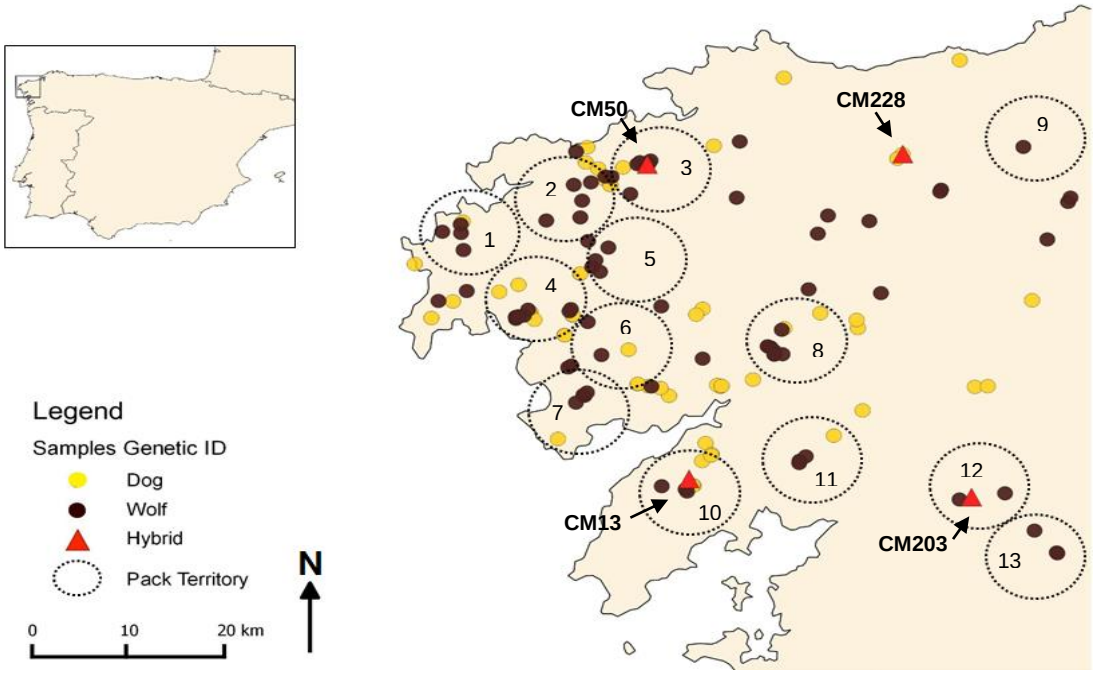

Figure 2 - Location of samples assigned to wolf and dog clusters, and to individuals with admixed ancestry in the Bayesian analysis performed in STRUCTURE. Wolf pack territories are marked. Each number represents a pack as follow: 1- Muxia; 2- Vimianzo; 3 - Passarela; 4 - Buxantes; 5- Baiñas; 6 – Ruña; 7 – Carnota; 8 – Negreira; 9 – Cerceda; 10 – Barbanza; 11 – Lousame; 12 – Xesteiras; 13 - Piorneiras . Location of the study area in Iberian Peninsula.

2.3.2. Nuclear Diversity

All loci were polymorphic, showing between 2 to 13 alleles per locus for CM samples and values of expected heterozygosity ranging from 0.182 (KitLG) to 0.782 (C09.173; Table 4). Iberian wolves showed lower genetic diversity in comparison with dogs The mean number of alleles per locus, excluding the low frequency alleles ($p \leq 0.05$) was Nawolf = 2.4 (± 0.24) and Nadog = 4.6 (± 0.36), whereas the mean expected heterozygosity was Hewolf = 0.40 (± 0.06) and He dog = 0.70 (± 0.03) (Table 4; same patterns are described in Verardi *et al.* 2006 and Godinho *et al.* 2011).

When testing for linkage disequilibrium (LD) for all pairs of loci, we found six significant pairwise combinations in dogs and four in wolves (significance probability level $p<0.05$, Bonferroni corrected for 306 comparisons), although none of these comparisons were common to both wolves and dogs. Likewise, some of the loci used deviated from Hardy-Weinberg expectations (four loci in dogs and one in wolves, Table 4), although none were common to both populations either.

Table 4 - Number of alleles per locus (NA). Number of alleles per locus (Na), number of private alleles per locus (PA) and expected heterozygosity (He) in each population analyzed; the * in the He values mark loci that deviated from Hardy-Weinberg equilibrium. Fst, Pid and Pid sib values for each locus in all analyzed samples. All values present were estimated regarding only CM samples. (#) refers to loci included on the dataset for increasing power of individual identification.

Locus	Na	He	Dog			Iberian Wolf			Fst	Pid	PidSib
			Na	Pa	He	Na	Pa	He			
C09.474	9	0.675	9	6	0.711	3	0	0.366	0.386	0.143	0.45
CPH2	8	0.656	8	5	0.69	3	0	0.327	0.419	0.157	0.462
CPH9	10	0.711	8	5	0.706	5	2	0.549	0.234	0.13	0.428
Cfx30371	3	0.392	2	1	0.465	2	0	0.019	0.644	0.566	0.755
KITLG.indel	2	0.182	2	0	0.351	2	0	0.013	0.223	0.687	0.831
AHT103	6	0.521	6	4	0.741	2	0	0.013	0.544	0.267	0.557
AHT111	10	0.766	10	6	0.679	4	0	0.596	0.299	0.096	0.393
C20.253(#)	8	0.684	7	3	0.414	5	1	0.633	0.355	0.154	0.448
C27.442	10	0.778	9	5	0.743	5	1	0.552	0.313	0.084	0.384
Dbar1	10	0.63	10	6	0.805*	4	0	0.052	0.574	0.165	0.477
AHT121(#)	14	0.843	13	6	0.801	8	1	0.766*	0.137	0.045	0.342
AHTh171	13	0.682	13	8	0.878*	5	0	0.364	0.276	0.126	0.442
AHTk211	8	0.569	7	3	0.794*	5	1	0.15	0.404	0.216	0.521
INU030(#)	9	0.779	8	4	0.767	5	1	0.595	0.251	0.081	0.382
REN162C04	10	0.695	9	5	0.751*	5	1	0.366	0.409	0.136	0.438
C09.173	9	0.782	9	6	0.805	3	0	0.633	0.183	0.083	0.381
C22.279	8	0.824	8	3	0.797	5	0	0.729	0.15	0.057	0.354
FHC2010	7	0.669	7	4	0.716	3	0	0.526	0.18	0.17	0.459
								Overall loci	0.326	6.60E-16	8.15E-07

2.3.3. Population Genetic Differentiation

Although the analysis of population differentiation (AMOVA) revealed that the highest genetic variation was found among individuals considering both populations (56%), following by genetic variation among populations (32.6%), and finally, the lowest variation is found among individuals within populations (11.4%), results reflect that Iberian wolf and dog are two well-distinct genetic identities. Overall loci, a significant differentiation was achieved between wolves and dog (F_{ST} = 0.326; p <0.003). The loci that contributed more for this differentiation were Cfx30371, Dbar1 and AHT103 showing F_{ST} values of 0.644, 0.574 and 0.544, respectively (Table 2). FCA scores for all individuals were graphically showed in a dimensional plot defined by two principal axes that explain, cumulatively, 15.9% of the total genetic variability (Fig. 2). This result reinforces the clear genetic differentiation between wolves and dogs.

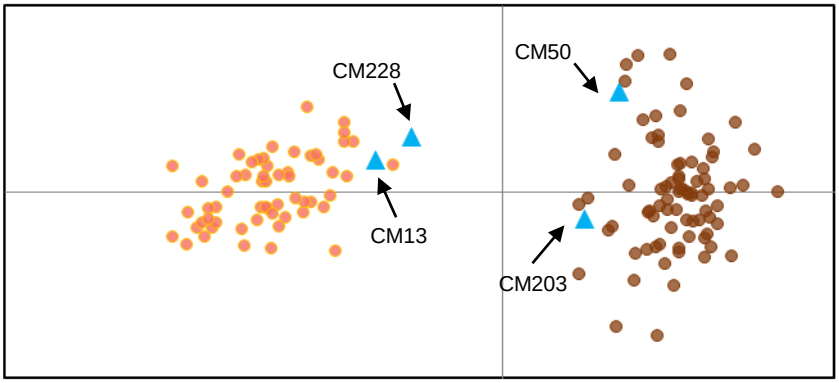

Figure 3 - Plot of individuals scores of the first two synthetic variables of a Factorial Correspondence Analysis (first factor on X and second factor on Y axis) using the 18 AIMs panel. Orange circles indicate dog samples, brown circles indicated wolf samples, and triangles indicate hybrids identified by Bayesian analysis.

2.3.4. Hybrid identification in Costa da Morte

Average *qi* of simulated genotypes was 0.50 for F1 (highest qi_{F1} = 0.709), and 0.50 for F2 (highest qi_{F2} = 0.817), 0.737 for BxWolf and 0.725 for BxDog. For second-generation backcrosses, the average of qi_{Bx2W} and qi_{Bx2D} was 0.866 and 0.841, respectively. Therefore, using the threshold value defined from the observed *qi* values of parental simulated individuals (0.90), our set of 18 AIMs allowed the identification of 100% of F1 individuals, and 98% and 76% of first generation backcross individuals to wolf and dog, respectively. For the second-generation backcrosses, the power identification achieved by this set of loci was 64% and 31% to backcross to wolf and dog, respectively.

Additionally, four genotypes were partially assigned to both clusters (Table 2; see Fig.3 for geographic location of admixed individuals), with *qi* values greatly shifted towards one or the other cluster, without showing intermediate *qi* values, expected for F1 or F2 hybrid classes. Noteworthy is that these individuals showed a wide 90% CI values overlapping with the defined threshold, whereas in pure individuals, both the simulated parental and the reference genotypes showed always a narrow and non-overlapping 90% CI values (Table 2). Thus, we observe that the existence of wider 90% CI values associated with a *qi* value under the defined threshold may be a good indicator of admixture, as also stated by Randi (2008). Pooling wolves and hybrids together we achieve to a value of 5% of individuals that were hybrids. The geographic location of hybrids in Costa da Morte is scattered, meaning that hybrids were not concentrated in space. We observed individuals with admixed ancestry in at least three pack territories (23% of total number of estimated packs in the area), plus another hybrid individual outside pack territories or their surroundings (Fig. 2).

Bayesian analysis using the "*population information for testing for migration*" ancestry model, provided us with some insights on the ancestry history of hybrids. Results revealed that three out of four hybrids showed a posterior probability assignment over $q > 0.116$ of having an ancestry origin in the other population two generations ago (Table 5). The two admixed individuals closer to Dog cluster (CM13 and CM228) showed a posterior probability of q_{CM13}=0.522 and q_{CM228}=0.529 of being correctly assigned to the dog population and a q_{CM13}=0.478 and q_{CM228}=0.471, of having an ancestry two generations back in the wolf population (Table 5). On the other hand, the two admixed individuals belonging to the wolf cluster, showed extremely low probability of being correctly assigned to the wolf population (q_{CM50} = 0.116 and q_{CM203}=0.000,), and a posterior probability of having an ancestry two generations back in the dog population of q_{CM50} = 0.823 and q_{CM203} = 0.999 (Table 5).

Table 5 - Population assignment and inferred ancestry of individuals estimated using structure. Estimations of the posterior probabilities of each individual belonging to the assigned population (P Prior pop). Estimations of the posterior probability of each individuals to have ancestry either in the sampled (Gen 0), first (Gen 1) or second (Gen2) past generations (q-value computed with prior migration rate =0.05).

Samples	Prior Pop	*q* *prior pop*	*q*			
			Pop	Gen 0	Gen 1	Gen 2
CM13	Dog	0.522	Wolf	0.000	0.000	0.478
CM50	Wolf	0.116	Dog	0.060	0.000	0.823
CM203	Wolf	0.000	Dog	0.000	0.000	0.999
CM228	Dog	0.529	Wolf	0.000	0.000	0.471

2.3.5. Relatedness Analysis

Simulations showed that the dyadic maximum likelihood estimator had the highest success rate in estimating correctly *r* for the different kinship groups (Table 6), considering the combination of allele frequencies and distribution in our dataset. So, we analyzed CM individuals using this model. Since it is a maximum likelihood estimators, *r* values range from 0 to 1. With zero representing complete unrelated relationship and one fully-related, a value of 0.5 will represent the relation of parent-offspring or full-sibling, and a value of 0.25 a relation of half-siblings or grandparent/grandson.

We found only two hybrids (CM 228 and CM13) with *r* value significantly over zero (*r* = 0.280), indicating that they might be a second degree relative, as between half-siblings. Regarding the kinship between hybrids and the individuals belonging to the different packs: i) the hybrid CM228 did not show any evidence of being related with any pack, ii) in the case of hybrid CM13 results show that it has a distant relative in Pasarela pack (*r* = 0.169), however this pack is located 40 km apart from the location where the samples was collected (Barbanza Pack), suggesting a potential migratory movement, and so it is not possible to say with certainty that this Passarela is the home-pack of this hybrid; iii) the hybrid CM203 is probably from Xesteiras pack given the location of the sample and the *r* value to the pack. However, it showed slightly lower r values with two wolves in another pack (Vimianzo pack) ca. 50 km apart, and iv) the hybrid CM50 belonged to the pack where it was found (Passarela pack) (Table 7).

Table 6 - Success rate of each estimator in correctly infer r within each kinship group, assessed with a set of simulated genotypes in the software COANCESTRY.

Estimator / Kinship Group	TrioML	DyadML	LynchRd	QuellerGt
Parent/offspring	85%	100%	55%	80%
Full siblings	87%	100%	49%	76%
Half siblings	40%	100%	18%	50%
Unrelated	90%	100%	90%	80%

Table 7 - Relatedness estimation according to dyadic maximum likelihood estimator. Mean, maximum a minimum values of r were calculated for each pack. Only packs showing r values over zero for at least one individual are showed here (8 out of 13). See Fig. 3 for details on the spatial position of packs.

			Relatedness estimation (*r*)			
Pack	**N Indiv.**		CM228	CM13	CM203	CM50
Carnota	7	Média	0.000	0.000	0.000	0.017
		Max.	0.000	0.000	0.000	0.119
		Min.	0.000	0.000	0.000	0.000
Negreira	11	Mean	0.007	0.000	0.007	0.028
		Max.	0.069	0.000	0.047	0.122
		Min.	0.000	0.000	0.000	0.000
Passarela	12	Mean	0.023	0.047	0.006	**0.213**
		Max.	0.064	**0.169**	0.066	**0.523**
		Min.	0.000	0.000	0.000	0.000
Ruña	3	Mean	0.000	0.000	0.000	0.076
		Max.	0.000	0.000	0.000	**0.227**
		Min.	0.000	0.000	0.000	0.000
Sardiñeiro	2	Mean	0.000	0.000	0.066	0.045
		Max.	0.000	0.000	0.132	0.090
		Min.	0.000	0.000	0.000	0.000
Vimianzo	4	Mean	0.000	0.009	0.085	0.004
		Max.	0.000	0.034	**0.172**	0.016
		Min.	0.000	0.000	0.000	0.000
Xesteiras	5	Mean	0.028	0.000	0.109	0.007
		Max.	0.048	0.000	**0.191**	0.034
		Min.	0.000	0.000	0.000	0.000

2.4. Discussion

In this study, we carried out for the first time a real-time assessment of hybridization between wolves and dogs at a population level. We achieved a good compromise between our sampling, based on NIS, and the molecular approach used, based on previously selected AIMs (Godinho *et al.* 2014). When working with NIS, one of the first constrains to be faced regards the number of markers to be used and its performance to address the proposed objectives. In this context, we selected our 18 markers panel out of 52 molecular markers available at our laboratory (Godinho *et al.* 2011), which provide the most informative set of markers differentiating wolves and dogs

in our population. With this panel, we were able to achieve not only high differentiation values (F_{ST} = 0.326), but also high success rates in the identification of hybrid classes further than F1s, with a threshold value of 0.90 to identify hybrids established with simulated genotypes. Similar results were obtained with the 13 AIMs panel used by Godinho *et al.* (2014), contrasting with similar studies addressing wolf-dog hybridization (Randi & Lucchini 2002; Vilà *et al.* 2003; Verardi *et al.* 2006; Hindrikson *et al.* 2012) where differentiation rates and success identifying hybrid class were lower. We could easily increase the number of loci used in this study, comparing to the average on other studies (Andersone *et al.* 2002; Vilà *et al.* 2003; Verardi *et al.* 2006) because the methodological procedure used here using a two-step PCR amplification allowed us to increase available DNA, as well as significantly reduce the genotyping errors characteristic of NIS. The allelic dropout and the false allele rates were comparable with studies using invasive samples (i.e. tissue or blood; Caniglia *et al.* 2013; Hindrikson *et al.* 2012), which not only brings more confidence to our results, but also allows for a better comparison between results from invasive and NIS. In conclusion, our approach overcomes with the limitation associate with low quality of DNA typical of NIS.

Despite our sampling protocol was focused on collecting wolf-like faeces, surveying areas distant from human settlements (Llaneza *et al.* 2014), we detected 78 wolves and 58 dogs (Table 3). Thus, dogs accounted for a significant number of individuals found in CM. Although we believe that dogs were underestimated in our dataset in comparison to wolves because of the sampling collection protocol, the fact that we found very similar wolf and dog densities (1.54 and 1.16, respectively, Table 4; Fig. 2), reflects a scenario in NW Iberia where the probability to have an encounter with dogs by wolves is expected to be high, increasing the chances for hybridization events (Petrucci-Fonseca 1982; Blanco *et al.* 1992, Godinho *et al.* 2011).

Hybridization at Costa da Morte wolf population

Bayesian clustering analysis revealed the presence of four hybrids in the study area, representing 5% of our sampling if considering wolf samples and hybrids. Similar values were also found in other wolf-dog hybridization studies (Verardi *et al.* 2006; Godinho *et al.* 2011; Hindrikson *et al.* 2012), although these studies were based on invasive sampling and larger temporal scales. This similarity among studies may suggest that lower values of hybridization might be a pattern across European wolf populations, independently of spatial and temporal scales used to survey a population. Interestingly, regardless of hybridization events, all studies aforementioned including the present one

found that wolves and dogs represent two distinct genetic units, indicating no significant introgression of dog genes into wolf populations. Pairwise differentiation values found in this study were high, F_{ST} = 0.326, and similar to the ones found in other studies (Verardi *et al.* 2006; Godinho *et al.* 2011; Caniglia *et al.* 2013). Additionally, our values of genetic diversity are comparable to other studies for isolated population, both in the Iberian Peninsula (Godinho *et al.* 2011) and Europe (Randi & Lucchini 2002; Caniglia *et al.* 2013).

Regarding ancestry of admixed individuals, we were not able to correctly assign each individual to a hybrid class. As for other studies, our panel of selected AIMs exhibited a high efficiency when considering first generation hybrids and first generation backcrosses, although the efficiency reduces when identifying second generation backcrosses. Consequently, the lack of definition in the four detected hybrids could be an indication of an older hybrid class, further than a first generation backcross. Notwithstanding, we could estimate that admixed individuals had significant ancestry in the other population in the past second generation (Table 5). Individuals CM50 and CM203 showed ancestry in the dog population (q = 0.823 and 0.999, respectively), whereas individuals CM13 and CM228 showed ancestry in the wolf population (q = 0.478 and 0.471, respectively). Therefore, we consider plausible to conclude that the most recent hybridization events in this area regarding the four hybrids detected occurred at least two generations ago and they were backcrosses to both species. Similarly, evidence of the absence of F1 hybrids was found by Godinho *et al.* (2014) in one of the packs included in the present study (Barbanza pack). Because hybrids with higher assignment values to wolf population (CM50 and CM203) showed wolf mtDNA haplotypes and hybrids with higher assignment values to dogs (CM13 and CM228) showed dog mtDNA haplotypes, our results suggest that hybridization events in CM were both between female wolf and a male dog (the most common pattern; Vilà *et al.* 2003) and also between a female dog and a male wolf (similar patterns were found by Hindrikson *et al.* 2012).

Gene flow between wolves and dogs is commonly described as spatially restricted, confined to peripheral areas of wolf distributions, and occurring in areas of recent colonization (Vila & Wayne 1999; Blanco & Cortés 2002; Randi & Lucchini 2002; Verardi *et al.* 2006). However, admixed individuals found in this study weren't geographical clustered, taking into account the size of the sampling area, and although peripheral, wolf has been traditional present in this area, not corresponding to an area of recent colonization where population is expanding (Núñez-Quirós *et al.* 2007).

We detected hybrids in three pack territories (23% of total number of packs) plus another hybrid outside pack territories or their surroundings (Fig. 3). Accordingly, relatedness analysis allowed us to increase our understanding of the dynamics of hybridization at population level. Our results support that the presence of hybrids in this wolf population cannot be a result of a single hybridization event associated to posterior migration movements of the descendants. The fact that each hybrid appeared related with a different pack (or any) supports this idea. Therefore, we conclude that instead of a single hybridization event in the study area, the most plausible scenario is the occurrence of multiple hybridization events both in space and time. In our particular case, since we did not find any F1 individual, hybridization in this area seems to be and old process to which we are able to go at least two generations back. Furthermore, is important to notice that one of the hybrid individuals found in this study was sampled within the territory of Barbanza pack, where evidence from 2011 reveal the presence of hybrids in the territory of this pack (Godinho *et al.* 2014), further supporting the suggestion that hybridization is a not an isolated event in time and in this population has been occurring through the past year.

Additionally, the lack of relatedness with any of the pack of one of the hybrids (CM228), might not directly mean that such is not integrated in a pack. In the admixture analysis this individual was identify as backcross to dog, with the more recent ancestral in the wolf population appearing two generations ago, which suggests that is related with at least one individuals in the wolf population. Although, since we use a non-invasive approach to survey the population, it would be expected that not all individuals were sampled, which probably happen in this case preventing us to infer the source pack of this hybrid. Moreover, is important to mention that since we are dealing with fecal samples the allocation of each individual to a pack are prone to a certain error, since the sample location could represent a dispersal movement.

Therefore, our results not only confirmed the spatial extension of hybridization at this population, but also show a new prespective of hybridization patterns in European wolf populations, particular in areas deeply humanized as the one sampled in this study.

Management Implications

Although hybridization is considered one of the most important conservation problems facing fragmented and isolated wolf populations in Europe (Boitani 2003) the legal status of wolf-dog hybrids is not clear (Trouwborst 2014), and other important information in relation to hybridization is very limited yet. Current management guidelines

for wolf-dog hybrids state that everything practically possible should be done to remove obvious hybrids from the wild once an event of hybridization has been detected (Boitani 2000). However, the efficiency of removing hybrids remains very uncertain without clear assessments of hybridization at a population level. So, the elaboration of simple, fast and efficient methods for assessing real-time hybridization events at a population level is essential for evaluating the efficiency of current management guidelines, and if necessary, to the proposal of other suitable conservation interventions to mitigate this problem. For example, there are no clear evidence that interventions aimed to remove hybrids from the wild are effective in reducing hybridization level in the population. On the contrary, a recent study showed that only 44% of the wolf-dog hybrids present in a pack subjected to this intervention were removed (Godinho *et al.* 2014). Interestingly, the same pack studied by Godinho *et al.* (2014) in 2011 has been considered here (Barbanza pack) and our results confirmed that hybridization was still present in this pack two years later (Fig. 3).

Although the implementation of such interventions is costly, requiring a significant effort for identifying the individuals and removing them, it is important to mention that the implementation of mitigation measures, such as the removal or capturing of hybrids from the wild, cannot rely only on visual identification (Godinho *et al.* 2014). Despite that some studies have associated some phenotypic traits with hybridization such as black coat color (Godinho *et al.* 2011; Caniglia *et al.* 2013) or the presence of dewclaws (Ciucci *et al.* 2003), the phenotypic traits present in hybrids can be quit diverse (see Hindrikson *et al.* (2012) for an example), and in some cases distinguishing between hybrids and pure wolves might be difficult. Therefore, the previous genetic identification of the individuals would be important to enhance the efficiency of this approach, since the accidental application of mitigation measures (lethal or not) in pure wolves might occur and have negative consequences in the wild population. Further in case were hybrids are disperse throughout the population, the persecution and capturing of such individuals would require an intensive “on the ground” survey in order to identify hybrids and then to implement the intervention, which most of the times is not doable due to the high logistical and economical investment required.

The similarity between the low hybridization rates found in this real-time study with other studies throughout Europe (Randi *et al.* 2000; Andersone *et al.* 2002; Vilà *et al.* 2003; Verardi *et al.* 2006; Godinho *et al.* 2011), along with the maintenance of the genetic identity of wolves and dogs in each case, might be an indication that wolf populations can be resilient to a certain small amount of hybridization. Which is remarkably less worrying compared to other species, for instance the wildcat that not

only present high values of hybridization with their domestic form across European populations (13% on average), but in particular contexts, such as in Hungary, the wild population is highly endangered due to introgressive hybridization, with an approximately degree of hybridization between 25% and 31% according to different studies (Randi 2008; Witzenberger & Hochkirch 2014).

The reduced rates of hybridization found across wolf populations raise further evidence of the need to increase our knowledge on other aspects of hybridization beyond genetics, such as the behavioral or ecological effects of hybridization and its legal framework, in order to understand the evolutionary, ecological, conservation and management implications of wolf-dog hybridization. Until such knowledge will not be available, other mechanisms should be taken into consideration for dealing with wolf-dog hybridization, since interventions aimed to the removal of hybrids might not be efficient (Godinho *et al.* 2014). In this regard, an alternative strategy to mitigate wolf-dog hybridization might be the implementation of interventions aiming to control and limit the number of free-ranging and feral dogs (Caniglia *et al.* 2013).

Chapter 3 – Final Remarks and future perspectives

Hybridization raises a lot of questions, concerns and divergence of opinions. By one hand, it's a powerful evolutionary force able to shape local adaptation and speciation (Arnold 1997) whereas, on the other hand, it can blend species boundaries leading to genetic homogenization or disruption of local adaptation (Allendorf *et al.* 2001). However, in the case of hybridization between wild species and their domestic counterparts, general opinions become more homogeneous considering wild-domestic hybridization events as an important threat to the wild populations. The paradigmatic case of hybridization between wolves and dogs is a good example, where wolf-dog hybridization is considered as an important threat for wolf population in Europe. To face this potential conservation problem, proposed management interventions in Europe state that everything possible should be done in order to eliminate hybrids from the wild (Boitani 2000). However, recently it has been showed the ineffectiveness of this action (Godinho *et al.* 2014), illustrating the need to properly evaluate the efficiency of these types of mitigation measures. When establishing conservation measures to mitigate hybridization, it is crucial to understand the temporal and spatial patterns of hybridization at the population level, but also other aspects of the problem such as the interactions between wolves and dogs, the potential ecological role that hybrids can play in some contexts, or even the legal status of hybrids.

In this study, through the combination of non-invasive sampling (feces) and a panel of carefully selected molecular AIMs, it was possible to assess the extent of hybridization in real-time at the population level. In our study area (≈5000km^2) we were able to identify 140 different individual genotypes, corresponding to 78 wolfs, 58 dogs and 4 hybrids. Two hybrids were backcross to wolf and the other two backcross to dog, and all of them present high posterior probability of having an ancestry two generations back in the other population. Also our findings show that hybridization in this particular population is patchy distributed, being the result of multiple hybridization events over time. Moreover, it was possible to conclude that the application of mitigation measures, as described above, would be challenging and probably inefficient. The application of this approach to other wolf populations in Europe showing wolf-dog hybridization events, is a pressing need to get a general understanding of this process at population level and its evolutionary, ecological and conservation implications.

On the other hand, we consider urgent to gain insights into the potential ecological value of hybrids in wolf populations and ecosystems. For instance, if a hybrid is integrated in a pack, its elimination might have consequences in the social structure of the same pack. Finally, the role that hybridization may have had enhancing species adaptation to highly humanized areas deserved further investigation.

Concluding, future paths should lead us to a better understanding the real consequences of hybridization in wolf populations. On one hand, a behavioral and ecological approach of hybridization will influence our understanding of repercussion of this process at individual, population and ecosystem levels. On the other hand, genetic studies focused on functional traits in wolf populations with hybridization would help to increase our knowledge on the role that hybridization has played in the adaptation of wolves to highly humanized areas. However, to reach these goals in the future, more multidisciplinary approaches are needed in the study of hybridization between dogs and wolves in order to make a conscious decision during the implementation of potential conservation actions.

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