THE DEMOGRAPHIC TRAJECTORY OF TWO CARNIVORE SPECIES USING A POPULATION GENOMICS APPROACH

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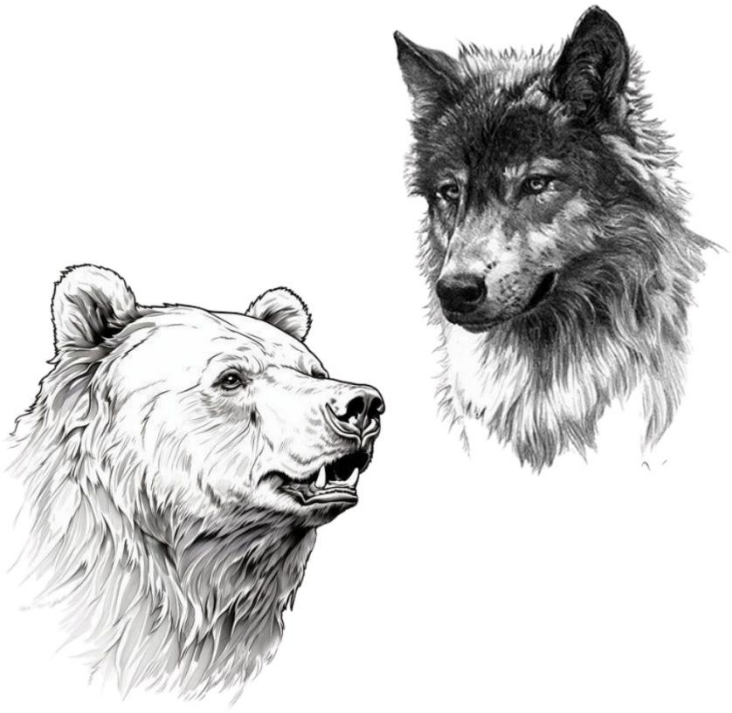

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Resumo

Durante a transição entre o Pleistoceno e o Holoceno, a Península Ibérica sofreu importantes alterações climáticas que afetaram diversas espécies que ocorriam nesta região durante o período glaciar. Mais recentemente, pressões antropogénicas têm impactado drasticamente a vida selvagem através da destruição de habitat e da redução de presas. Neste contexto, os grandes carnívoros têm sido especialmente afetados devido às suas exigências ecológicas e à sua baixa densidade populacional. Na Península Ibérica, duas espécies de grandes carnívoros, o lobo ibérico (*Canis lupus signatus*) e o urso-pardo cantábrico (*Ursus arctos*), sofreram acentuados declínios demográficos ao longo dos últimos milénios. No entanto, as dinâmicas demográficas históricas destas espécies, bem como os fatores que influenciaram o seu estado de conservação atual, permanecem pouco compreendidos. Este estudo utilizou uma abordagem genómica, com um mínimo de nove genomas de elevado *coverage* por espécie, para reconstruir as suas histórias demográficas. Foi dada especial atenção aos últimos 10.000 anos, com o objetivo de investigar os efeitos de fenómenos climáticos históricos e das atividades humanas nas tendências populacionais e na distribuição das espécies. Os nossos resultados revelaram trajetórias demográficas distintas ao longo dos períodos históricos, embora ambas as espécies tenham exibido um declínio semelhante durante o final do Pleistoceno até ao Último Máximo Glacial. Por volta de há 100 mil anos, estimámos tamanhos efetivos populacionais (N_e) entre 55.000–80.000 para o lobo ibérico e entre 43.000–52.000 para o urso pardo cantábrico. Em períodos mais recentes, as espécies apresentaram acentuados declínios populacionais entre os séculos XVIII e XX, provavelmente associados à intensa pressão antropogénica e à perseguição direta, com valores atuais de N_e bastante reduzidos: 292–318 para o lobo ibérico e 53–65 para o urso pardo. No caso da população do lobo ibérico, as nossas análises sugerem que a diferenciação genética em quatro principais grupos genéticos — previamente identificados — terá surgido no início do século XIX.

Este trabalho fornece contributos importantes para a compreensão dos efeitos combinados de fatores naturais e humanos na dinâmica populacional de grandes carnívoros na Península Ibérica, destacando também o seu papel ecológico essencial na manutenção do equilíbrio das cadeias tróficas

Palavras-chave: Grandes carnívoros, lobo ibérico (*Canis lupus signatus*), urso-pardo cantábrico (*Ursus arctos*), história demográfica, Península Ibérica

Abstract

During the Pleistocene transition to the Holocene, the Iberian Peninsula experienced major climatic shifts that affected many species harboured during the glacial era. In more recent times, anthropogenic pressures have drastically affected animal populations by destroying their habitat and reducing prey availability. In this context, large carnivores are especially affected due to their specific ecological requirements and low population densities. In the Iberian Peninsula, two iconic large carnivores, the Iberian wolf (*Canis lupus signatus*) and the Cantabrian brown bear (*Ursus arctos*), have undergone significant demographic declines over the past millennia. However, the historical demographic dynamics of these species and the drivers behind their current conservation status remain poorly understood. This study applied a population genomics approach using a minimum of nine high-coverage genomes per species to reconstruct their demographic histories. Special attention was given to the last 10,000 years in order to uncover the influence of human activity on population trends and species distribution. Our results revealed distinct demographic trajectories across historical periods, although both species exhibited a similar decline during the Late Pleistocene, leading up to the Last Glacial Maximum. Around 100,000 years ago, the Iberian wolf and Cantabrian brown bear had large effective population sizes (N_e) estimated between 55,000–80,000 and 43,000–52,000, respectively. In more recent times, both species experienced marked population declines between the 18th and 20th centuries, with current N_e estimates dropping to 292–318 for the Iberian wolf and 53–65 for the Cantabrian brown bear, likely reflecting intense anthropogenic pressure and persecution. Our analyses also suggest that the genetic differentiation of the Iberian wolf into four main groups—previously identified—likely emerged around the beginning of the 19th century.

Therefore, this study provides important insights regarding the impact of natural and anthropogenic factors on population dynamics of carnivores in the Iberian Peninsula, and also on the changes in our ecosystems, given that these animals play a crucial role in trophic chains.

Key words: large carnivores, Iberian wolf (*Canis lupus signatus*), Cantabrian brown bear (*Ursus arctos*), demographic history, Iberian Peninsula

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List of Abbreviations

N_e	EFFECTIVE POPULATION SIZE
N_C	CENSUS POPULATION SIZE
EBVS	ESSENTIAL BIODIVERSITY VARIABLES
WF	WRIGHT-FISHER
LD	LINKAGE DISEQUILIBRIUM
LGM	LAST GLACIAL MAXIMUM
IUCN	INTERNATIONAL UNION FOR CONSERVATION OF NATURAL RESOURCES
CITES	CONVENTION ON INTERNATIONAL TRADE IN ENDANGERED SPECIES OF WILD FAUNA AND FLORA
EU	EUROPEAN UNION
BP	BASE PAIRS
SNP	SINGLE NUCLEOTIDE POLYMORPHISMS
PSMC	PAIRWISE SEQUENTIAL MARKOVIAN COALESCENT
SMC++	SEQUENTIALLY MARKOVIAN COALESCENT ++
GONE	GENETIC ESTIMATION OF N_E
TMRCA	TIME TO THE MOST RECENT COMMON ANCESTOR
SFS	SITE FREQUENCY SPECTRUM
MQ	MAPPING QUALITY
BQ	BASE QUALITY
MAF	MINIMUM ALLELE FREQUENCY
SFS	SITE FREQUENCY SPECTRUM

1. Introduction

1.1. The concept of Effective Population Size, N_e

Effective population size (N_e) is a central parameter in evolutionary and conservation biology, providing essential insights into the dynamics of wild populations (Hoban et al., 2020; Waples, 2022). It plays a key role in species conservation, as it is fundamental to evaluate a population's evolutionary potential as well as its vulnerability to genetic risks, such as inbreeding depression, loss of genetic diversity, and genetic drift, especially in small populations (Santos-del-Blanco et al., 2022). N_e is one of the most important measures for assessing a population's short-term risk of extinction, not only due to inbreeding depression but also because populations with lower N_e have a reduced capacity to respond to demographic, environmental, and anthropogenic pressures (Hoban et al., 2020; Hoban et al., 2022). From a population genetics perspective, N_e represents the number of individuals that effectively contribute genetic material to the next generation, a value usually much lower than the census population size (N_c) (Frankham, 1995). In recognition of its significance, N_e has been identified as one of the genetic Essential Biodiversity Variables (EBVs), a set of standardized indicators used to monitor changes in biodiversity across spatial and temporal scales (Hoban et al., 2022).

The concept of effective population size was first introduced by Sewall Wright in 1931 (Wright, 1931). This parameter was used to quantify the rate of genetic change, such as allele frequency drift, in a real population by comparison with the Wright-Fisher (WF) idealized population model (Wang et al., 2016). A WF population is defined by a set of strict assumptions: equal sex ratio, constant population size across generations, non-overlapping generations, absence of natural or sexual selection, and no population structure. In such a model, genetic drift (alongside mutation) is the only force responsible for changes in allele frequencies between generations (Conner & Hartl, 2004). Therefore, in an ideal WF population, the variance in reproductive success would be equal to the mean reproductive success, since all individuals would have the same probability of mating, and the effective population size would correspond to the census size, that is, the total number of potential parents.

In real populations, however, these assumptions rarely hold. The variance in reproductive success is typically much higher, as not all individuals contribute equally to reproduction (Waples, 2022). As a result, the effective population size is often

substantially lower, on average an order of magnitude smaller, than the census size (Hoban et al., 2020). Thus, in its purest form, N_e reflects the variance in reproductive output relative to ideal conditions (Waples, 2022), and it is inversely proportional to the intensity of genetic drift acting on a population. In other words, smaller populations are more susceptible to stochastic processes such as genetic drift, which not only reduces genetic diversity over time but also creates non-random associations between alleles at different loci, a phenomenon known as linkage disequilibrium (LD) (Waples et al., 2016).

Different molecular methods have been developed to estimate ancient, historical, and contemporary N_e. Depending on the time scale, these methods usually rely on either mutation or recombination events (Santiago et al., 2020). In most species, mutations accumulate slowly over generations, making mutation-based methods, such as PSMC, MSMC, fastsimcoal2, and SMC++, the most common choice to infer ancient population sizes (Atkinson et al., 2008). In contrast, recombination-based methods – particularly those relying on LD patterns – are better suited to estimate more recent N_e (Santiago et al., 2020), as implemented in tools such as GONE, LDNe, and NeEstimator.

Given its importance for understanding population viability and evolutionary dynamics, accurately estimating N_e remains a fundamental objective in both evolutionary and conservation biology.

1.2. Human-Wildlife Conflict

Since the early stages of human expansion, increasing competition and overlap between human needs and those of wild species have led to persistent conflicts, as wildlife gradually loses space and resources (Nyhus, 2016). The recent growth in the human population during the last centuries has intensified these conflicts, exacerbating pressures on wild species, especially large carnivores. Consequently, anthropogenic activities have led to overall population declines and habitat fragmentation, resulting in a significant reduction in the genetic diversity of wild populations (Exposito-Alonso et al., 2022). These human-wildlife conflicts frequently arise from wild species' behaviours that interfere with human economic interests. Such behaviours include predation on livestock species by carnivores, crop damage, transmission of diseases to livestock, and direct competition for pastures by wild species, such as ungulates (Carpio et al., 2021; Acebes et al., 2024).

Large carnivores have been particularly vulnerable to anthropogenic pressures due to their life history traits and high potential for conflict with humans, mainly through livestock depredation (Boitani & Linnell, 2015; Ottolini et al., 2021). These pressures have led to serious consequences, including population declines, habitat fragmentation, extinction of populations or even species, disruption of social organization, inbreeding, and low genetic variation (Ripple et al., 2014). As a result, large carnivore populations suffered a general decrease worldwide over the past centuries, leading to widespread extinctions and range contractions (Ripple et al., 2014; Cimatti et al., 2021). In Europe, particularly in the Western and Central ranges, during the 19th and mid-20th centuries, large carnivore populations were reduced both in number and distribution (Chapron et al., 2014; Linnell et al., 2009). Nevertheless, large carnivores play a crucial role in ecosystem functioning as keystone species and apex predators (Hoeks et al., 2020). Their large body size and high energy demands make them particularly influential in regulating ecosystem processes (Ripple et al., 2014), and changes in their populations can lead to significant shifts in biodiversity through top-down effects (Ripple et al., 2014). Consequently, anthropogenic pressures might modify the ecological roles of large carnivores by interfering with their population size and behaviour (Kuijper et al., 2016).

Despite historical declines, since the mid-20th century, European conservation efforts, including regulatory and legislative changes, have contributed to the maintenance and restoration of wild carnivore populations (Linnell et al., 2010; Swenson et al., 1995). Currently, one-third of the total area of Europe (1,529,800 km²) is permanently occupied by at least one large carnivore species (Chapron et al., 2014).Notable examples include the grey wolf (Canis lupus) and the brown bear (Urs*u*s arctos), which have been recently expanding their ranges, including into areas where they were previously locally extinct (Chapron et al., 2014; Fernandez-Gil, 2018). Wolves have expanded their territory in the Italian and Iberian Peninsulas (López-Bao et al., 2018) and have re-colonized almost all the countries in Central Europe, as well as Scandinavia (Boitani & Linnell, 2015; Hindrikson et al., 2017). Brown bears have also been expanding its distribution in Scandinavia, where it was nearly extinct, and have been recovering in Norway and Finland (Hagen et al., 2015; Swenson et al., 1995).

Different factors have contributed to the expansion of wild carnivores in Europe, including ecological, societal, and cultural elements. After World War II, Europe underwent significant land-use changes, such as the abandonment of agricultural and pastoral lands, a decrease in natural grasslands, and an increase in forest cover due to reforestation and afforestation programs (Behr et al., 2017; Boitani & Linnell, 2015;

Fuchs et al., 2013). Additionally, rural depopulation and increasing urbanization, together with reduced human persecution of wild ungulates, have favoured prey recovery, benefiting large carnivores (Burbaitė & Csányi, 2009; 2010). Furthermore, changes in societal attitudes, coupled with conservation legislation, such as the Bern Convention led by the Council of Europe (1979) and the EU Habitats Directive (1992), led to restrictions on hunting and the banning of poisoned baits historically used in response to human–carnivore conflicts (Boitani, 1992; Díaz, 2010; Evans, 2012). However, countries where large carnivores were previously extinct face significant challenges in applying conservation measures, as traditional knowledge and practices for coexistence have often been lost (Chapron et al., 2014). In such contexts, the return of these species frequently triggers social conflict, which can lead to illegal practices such as poaching. This has been observed in rural Norway (Gangaas et al., 2013), limiting wolf recovery in Scandinavia (Liberg et al., 2012), and has even resulted in the extinction of a reintroduced bear population in Austria (Kruckenhauser et al., 2009).

1.3. Iberian Peninsula: Historical and Ecological Context

Located in the southwestern part of Europe and covering approximately 582,925 km², the Iberian Peninsula is characterized by diverse landscapes and climates, shaped by a complex geological and climatic history (Oliva et al., 2019).

During the Pleistocene (2.58 million to 11,700 years ago), Iberia served as one of the most significant glacial refugia within the European subcontinent (Hewitt, 1999). Consequently, the peninsula fostered the emergence of numerous endemic species (Ribera, 2000; García-Barros et al., 2002) through prolonged processes of adaptation, differentiation, and speciation. Throughout glacial–interglacial cycles, Iberia not only harboured high levels of genetic diversity (Vitales et al., 2016) but also acted as a key source of species that expanded into more northern latitudes following the end of the Pleistocene (Weiss & Ferrand, 2007). Among these species are mammals such as roe deer (*Capreolus capreolus*, Vernesi et al., 2002), woodmouse (*Apodemus sylvaticus*, Michaux et al., 2003), and field voles (*Microtus agrestis*, Jaarola & Searle 2002), birds (chaffinch, *Fringilla coelebs*, Griswold & Baker 2002), reptiles (pond turtle, *Emys orbicularis*, Lenk et al., 1999), amphibians (natterjack toad, *Bufo calamita*, Beebee & Rowe 2000), and plants (ivy, *Hedera ssp*., Grivet & Petit 2002).

After the last glacial maximum (LGM, 26.5–19 kya; Clark et al., 2009), the Iberian Peninsula experienced a series of climatic fluctuations that profoundly shaped its

ecosystems and species dynamics (González et al., 2013). In fact, the low temperatures of the Younger Dryas (13-11.9 kya) and the Early to Mid-Holocene (11-5 kya) contributed to an overall unstable climatic regime in the region (Oliva et al., 2019). Additionally, abrupt cooling and aridification events, particularly those around 8.2 and 4.2 kya, further impacted the climate of the Iberian Peninsula and the broader Mediterranean basin (Bernal-Wormull et al., 2023).

Among the species present in the Iberian Peninsula, there is a rich assemblage of carnivores, including both native and introduced taxa (Bencatel et al., 2018), many of which have long coexisted with humans in increasingly modified landscapes (Blondel, 2010). Within the variety of large carnivores inhabiting the Iberian Peninsula, some stand out as particularly emblematic, including the Iberian wolf (*Canis lupus signatus*), the brown bear (*Ursus arctos*), and the Iberian lynx (*Lynx pardinus*).

Large carnivores are particularly vulnerable to habitat degradation and the expansion of human infrastructures, which not only fragments the landscape but also increases mortality through road collisions (Cañas, 2015). In fact, roadkill is among the most significant anthropogenic factors affecting wildlife communities in human-altered landscapes, in which Iberia is no exception (D'Amico et al., 2015). Furthermore, high human presence can increase species' stress levels, which can lead to reproductive disruption and immune system dysfunction (Barja et al. 2007).

Other major threats include the reduced trophic resources in some areas andintensification of agricultural and livestock practices, which often escalate into direct conflicts with humans (Cañas, 2015). In fact, large carnivores identify livestock as an easier target compared to wild species due to the lack of antipredator behaviour (Linnell et al., 1999). Losses resulting from livestock depredation by large carnivores are economically significant, for example, in 2020, more than 11,000 wolf attacks on livestock were reported in Spain, and more than 2 million euros were spent in compensation for farmers' losses (Boitani et al., 2022).

Such conflicts have resulted in strong human persecution of large carnivores in the Iberian Peninsula over the last two centuries. The increasing availability of firearms from the 17th century onwards (Martins, 2013) contributed significantly to this persecution. Additionally, the use of poison baits became a common practice during this period, as humans aimed to eradicate large carnivores such as wolves and bears due to predation on livestock and damage to crops (Nores & López-Bao, 2022).

Although census-based analyses have documented a decline in the number of individuals of both the Iberian wolf (Pimenta et al., 2023) and the Cantabrian brown bear (Gonzalez et al., 2016), the extent to which this decline has affected genetic diversity and structure remains largely unexplored. Molecular-based studies are therefore essential to assess the long-term genetic consequences of these demographic changes in the Iberian Peninsula.

1.4. The Iberian Wolf

The grey wolf is the second most common large carnivore in Europe, with more than 12,000 individuals distributed across 28 countries, occupying an area of approximately 798,300 km² (Chapron et al., 2014). Although the species is present across the Holarctic, it has experienced a widespread range contraction over time (Mech & Boitani, 2003). As a result, Central and Northern European populations experienced a sharp decline until their extinction in many countries (Delibes, 1990). In Eastern Europe, wolves have maintained a large and continuous population, with high connectivity between Russia and Asia (Mech & Boitani, 2003). In contrast, populations in Southern Europe began to diverge during the Pleistocene and have remained isolated for centuries (Fan et al., 2016). Isolated populations are more likely to be negatively affected by demographic and environmental stochasticity, as well as inbreeding (Palomares et al., 2012), which may contribute to increasing their risk of extinction (Benson et al., 2019). The Iberian wolf (Figure 1) became geographically and genetically isolated approximately 10 thousand years ago (abbreviated as 10 kya, where "kya" stands for "thousand years ago"), following the end of the last glaciation (Silva et al., 2020). Currently, in the Iberian Peninsula, the wolf population is restricted to the northwestern quadrant (Clavero et al., 2023; Nores & López-Bao, 2022) (Figure 2), forming the largest wolf population in southwestern Europe (Chapron et al., 2014).

Figure 1 – Iberian Wolf (*Canis lupus signatus*). Photo by Raquel Godinho.

Although Iberian wolves tend to prefer areas with dense forest cover and abundant prey (Llaneza et al., 2012), they are also capable of coexisting with humans in heavily modified landscapes (Blanco & Cortés, 2007; Llaneza et al., 2012; Sazatornil et al., 2016). However, such proximity often leads to significant conflict, primarily due to livestock depredation (Llaneza & López-Bao, 2015). Widespread poisoning campaigns during the 19th and 20th centuries (Nores & López-Bao, 2022; Cañas, 2015) contributed to a sharper population decline, which intensified between the 1950s and 1960s. This decline led to the lowest documented wolf population size in the 1970s, resulting in the extinction of almost all populations in the southern areas of the Iberian Peninsula (Figure 2) (Sastre et al., 2011; Valverde, 1971).

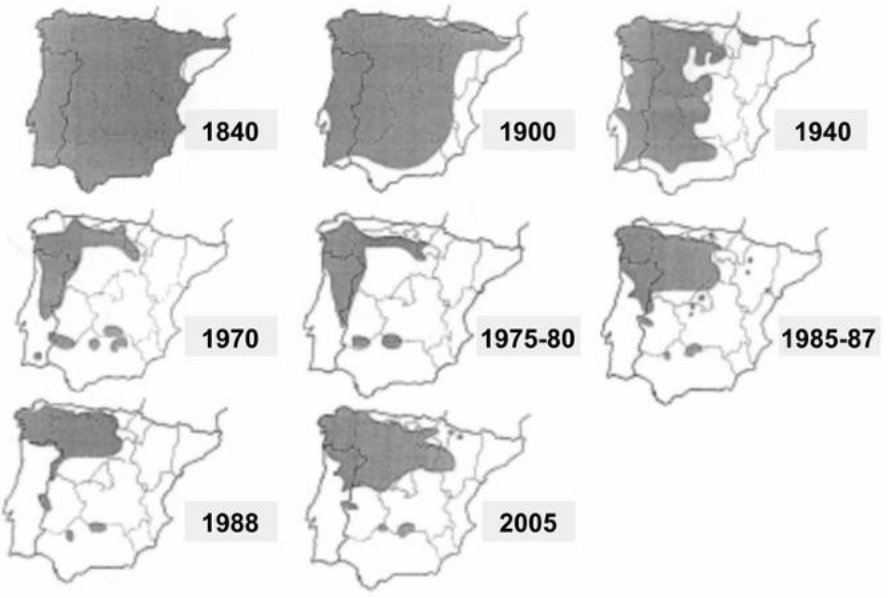

Figure 2 – Estimated Iberian wolf range between the 19th and 21st centuries. *Source: Sastre (2011), PhD dissertation.*

Other factors that actively contributed to the species' decline include the reduction in the abundance of wild prey, such as ungulates (Fernández & Ruiz de Azua, 2010), and the destruction and fragmentation of suitable habitat (Petrucci-Fonseca, 1990; Boitani, 2003).

Following this historical decline, conservation efforts and changes in societal attitudes towards large carnivores (Schwartz et al., 2003) have contributed to the partial recovery of wolves not only in Iberia but also across other European countries (Mech, 1995; Chapron et al., 2014). Additionally, increased forest cover and the subsequent rise in wild ungulate populations, along with land-use changes driven by widespread rural depopulation, have improved habitat suitability for the Iberian wolf (Cimatti et al., 2021).

Another key factor supporting the recovery of the Iberian wolf has been its protection under national and international legislation. The species is classified as Endangered (EN) in Portugal and Near Threatened (NT) in Spain (Cabral et al., 2005; Boitani et al., 2018), and is listed under various European agreements, including the Bern Convention, the Habitats Directive, and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). However, protection is not uniform across the Iberian Peninsula. Wolves south of the Douro River in Spain and throughout Portuguese territory are fully protected under Annexes II and IV of the European Union (EU) Habitats Directive 92/43/EEC (Council of the European Union, 1992), whereas those north of the

Douro in Spain are listed under Annex V, meaning that hunting is allowed under regulated quotas depending on regional jurisdiction.

Socially, wolves are organized into groups known as packs, which typically consist of a single reproductive pair and their offspring of that year (Mech & Boitani, 2003). Occasionally, subadult individuals from previous years may remain in the pack. Packs may also include unrelated individuals or immigrants (Jędrzejewski et al., 2005). In Portugal, the apparent male-to-female ratio was estimated to be approximately 2:1 (Godinho et al., 2007). Once juveniles reach adulthood, they often disperse to find mates and establish their own territories. However, both natural and anthropogenic barriers can constrain dispersal, limiting gene flow between subpopulations and, consequently, increasing the risk of inbreeding (Morales-González et al., 2022).

Compared to other wolf populations, the Iberian wolf exhibits unusually short long-distance dispersal, with an average dispersal distance of approximately 32 km (Blanco & Cortés, 2007; Silva et al., 2018). Such reduced dispersal rates may limit gene flow, which can lead to population structuring (Scandura et al., 2011). Indeed, a study by Silva et al. (2018) identified cryptic population structure in the Iberian wolf into four main genetic clusters, corresponding to Galicia (northwest Spain), northern Portugal, the western Cantabrian Mountains, and the plateau of Castilla y León (Figure 3).

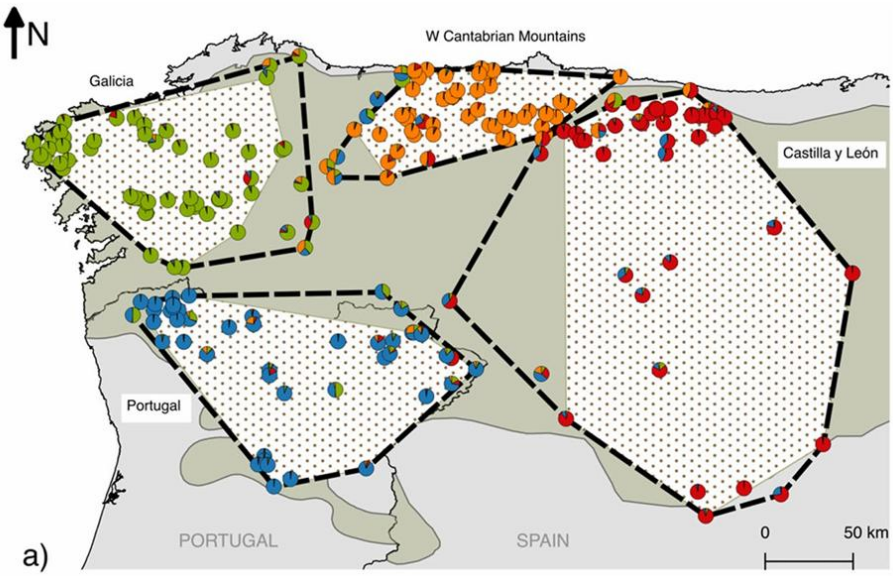

Figure 3 – Genetic clusters of the Iberian wolf. Distribution of genetic clusters for *K* = 4. Each individual is represented by a circle indicating the proportion of membership to each cluster. Dashed dark lines delimit the geographical boundaries of each cluster. The shaded green area represents the wolf's distribution in the northwestern Iberian Peninsula. *Source: Silva et al. (2018).*

1.5. The Cantabrian Brown Bear

The Eurasian brown bear (Figure 4) is a member of the Ursidae family (IUCN, 2023) and is considered the most abundant large carnivore in Europe (Chapron et al., 2014). Its population is estimated at around 17,000 individuals, distributed across 22 countries and occupying approximately 485,400 km² (Chapron et al., 2014).

Figure 4 – Brown bear (*Ursus arctos*). Photo by Thomas Wilken.

Although the species tends to avoid human-dominated landscapes, much of its European range overlaps with areas of high human density (19.0 ± 69.9 inhabitants/km²; Chapron et al., 2014), which has historically increased conflict between bears and humans. This persistent overlap has led to attacks on people or damage to property such as beehives and livestock, making coexistence increasingly difficult (Bautista et al., 2017; Bombieri et al., 2019). As a result, brown bears suffered a drastic decline in both Eastern and Northern Europe and were almost extirpated from Western Europe due to intense persecution during the 20th century (Wiegand et al., 1998; Zedrosser et al., 2011). In addition to direct persecution, other factors such as habitat fragmentation and destruction also contributed to the species' decline across Europe (Swenson et al., 2000).

The Cantabrian brown bear population, located in the Cantabrian Mountains in the northwestern Iberian Peninsula, represents the southwestern limit of the species' distribution in Europe (Figure 5) (Chapron et al., 2014; Díaz-Fernández et al., 2023). This small and isolated population is considered one of the most endangered bear populations in the world (Méndez et al., 2014; Penteriani et al., 2020). It is currently subdivided into two spatially segregated nuclei, the Western and Eastern (Valdiosera et al., 2008).

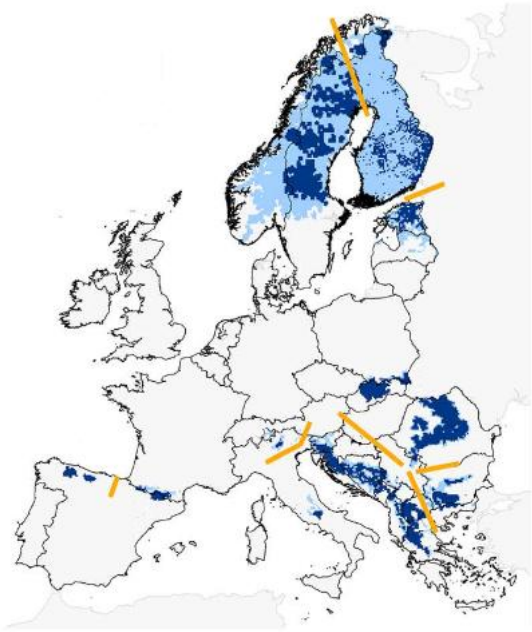

Figure 5 – Distribution of the brown bear in Europe (2011). Dark blue areas represent zones of permanent occurrence, light blue areas indicate sporadic occurrence, and orange lines delineate population boundaries. *Adapted from Chapron et al. (2014).*

Until the 14th century, brown bears inhabited most of the mountain ranges and forests in the Iberian Peninsula (Nores & Naves, 1993). At that time, their range was distributed across the Galaico-Portuguese massif to the forests in Andalusia until the Cantabrian and Pyrenees mountains, where the Iberian population maintained contact with other European populations, allowing the possibility of gene flow (Valdiosera et al., 2008). However, in the last four centuries, this region suffered many landscape modifications with hardwood forests being converted into pastures for livestock grazing (Clevenger et al. 1990). Later, following the same trend as in the rest of Europe, this species faced several threats during the first half of the 20th century including illegal poaching and

increased landscape fragmentation, which consequently led to reduced connectivity between the two subpopulations and increased genetic differentiation (Méndez et al. 2014; Penteriani et al. 2020). In 1973, this population began to be strictly protected; however, the numbers continued to decline (Clevenger et al., 1987). By 1990, the population had dropped to its all-time minimum, with fewer than 100 individuals remaining (Wiegand et al., 1998). In 1992, it was included in Annexes II and IV of the Habitats Directive 92/43/EEC (Council of the European Union, 1992). To reinforce the protection of this species, it was listed as Endangered (EN) by the Spanish Inventory of Endangered Species, and in the beginning of the 21st century, signs of recovering were noticed, with the number of females with cubs increasing especially in the western nuclei (Méndez et al. 2014; Gonzalez et al. 2016). Additionally, migration reports between the two subpopulations were also documented (Gonzalez et al. 2016; Gregório et al. 2020). In the last decades, the Cantabrian population has recovered from less than 100 individuals to approximately 330 in the present (López-Bao et al., 2021), being present in four autonomous regions of Spain, including Galicia, Asturias, Castilla y León, and Cantabria, occupying an area of almost 18,000 km² (Díaz-Fernández et al., 2023).

Brown bears are solitary and polygynous mammals, with both males and females mating with multiple partners (Steyaert et al., 2011). Their reproductive ecology is strongly influenced by environmental conditions, which affect both mating opportunities and reproductive success (Zedrosser et al., 2009). In the Cantabrian population, the observed male-to-female ratio is approximately 1.7:1, a pattern likely driven by the greater tendency of males to disperse over larger distances, as well as by landscape features that facilitate male movement and gene flow between subpopulations (Sastre et al., 2025).

1.6. Objectives

Studying ancient, historical, and contemporary demographic trends of emblematic large carnivore species, such as the Iberian wolf and the Cantabrian brown bear, is crucial to better understand the effects of natural and anthropogenic changes that have occurred in the Iberian Peninsula on the population size of these species.

Bearing this in mind, this thesis aims to unravel the demographic trajectories of both the Iberian wolf and the Cantabrian brown bear through a population genomics approach. We focus on the last 10,000 years to explore the effects of natural events and human activities on the effective population size of each species over time. In addition, we seek to identify convergent demographic patterns between the two large carnivores that may corroborate the occurrence of specific climatic, ecological, and/or anthropogenic events in their shared history.

We hypothesise that, during ancient periods, both species followed broadly similar demographic trajectories—particularly around the Last Glacial Maximum—due to shared exposure to climatic instability. However, some divergence is expected due to species-specific ecological and biological traits. In more recent periods, we expect parallel demographic declines shaped by comparable anthropogenic pressures, such as habitat loss, persecution, and fragmentation, affecting both species similarly across the Iberian Peninsula.

2. Methodology

2.1. Sample Selection

Raw short-read whole-genome data from 32 Iberian wolves were used in this study. These samples included individuals from all four major genetic clusters identified by Silva et al. (2018) as the most likely K for the population, also based on individual GPS movement analysis. The geographic distribution of these individuals is shown in Figure 6. Some samples originated from previous ECOGEN/BIOPOLIS studies, while others were obtained from publicly available datasets (Table S1).

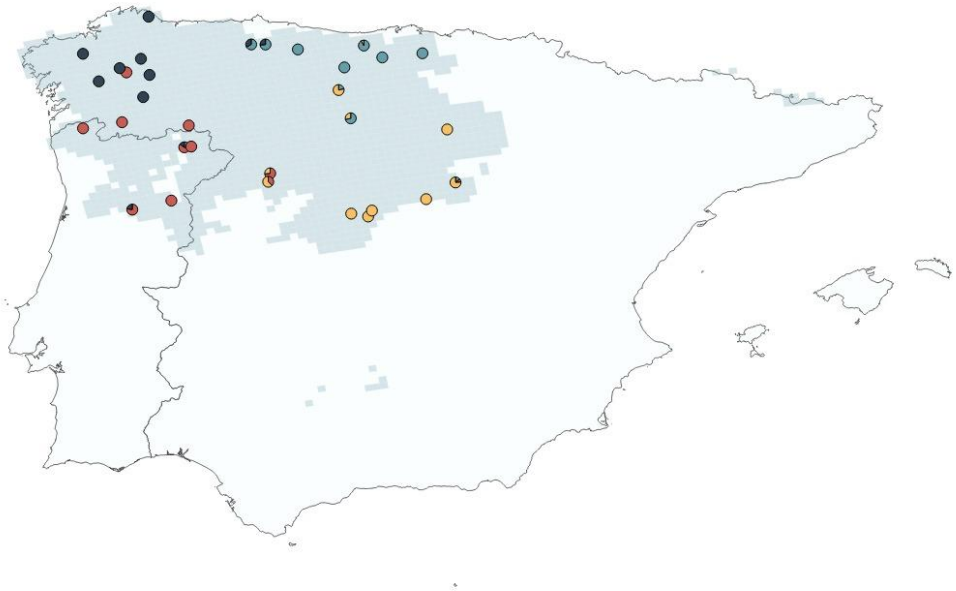

Figure 6 - Geographic location of 32 Iberian wolf samples across the Iberian Peninsula. Each sample is represented by a pie chart indicating the proportional genetic ancestry assigned to the four genetic clusters identified by Silva et al. (2018).

For the brown bear dataset, we selected 22 biological samples from a larger collection available at BIOPOLIS biobank. Samples were collected between 2000 and 2021 in the Cantabrian region by rangers from the Asturias Community, staff from the Fundación Oso Pardo, and other collaborators. The samples included both tissue and hair samples from individuals belonging to the Western (N=16) and Eastern (N=6) nuclei (Figure 7). Hair samples were collected non-invasively from barbed wire, trees, and vegetation,

without disturbing the animals. Sample selection was based on prior individual identification analyses, in which the probability of each bear belonging to either the Western or Eastern genetic nucleus was estimated using the software STRUCTURE (Porras-Hurtado et al., 2013) (Table S2).

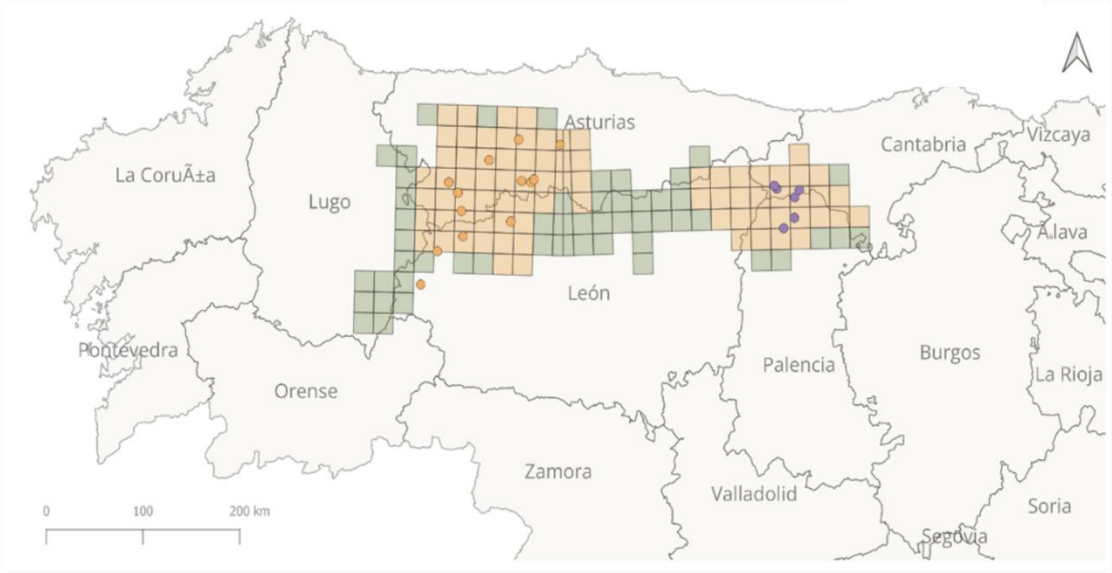

Figure 7 - Geographic distribution of Cantabrian brown bear samples assigned to the western and eastern subpopulations. Samples from the western subpopulation are shown in yellow, and samples from the eastern subpopulation are shown in purple. Orange areas represent the species' permanent range, while green areas indicate occasional presence.

2.2. Laboratory Procedures

2.2.1. DNA extraction

As wolf genomes were already available, laboratory procedures were only conducted for the 22 brown bear samples. Therefore, the DNA was extracted from 17 tissue samples and 5 hair samples. Hair samples were extracted using the QIAamp DNA Micro Kit (Qiagen, Carlsbad, CA, USA), a protocol designed to obtain high-quality DNA from limited amounts of biological material, such as hair. Some protocol adjustments were implemented, including extending the lysis period by incubating samples with Proteinase K at 56 °C overnight and reducing the elution buffer volume to increase the final DNA concentration. Tissue sample extractions were performed using the EZ-10 Spin Column Genomic DNA Minipreps Kit (Bio Basic Inc., Markham, Ontario, Canada), following all the manufacturer's instructions. Negative controls were included to monitor potential contamination during the extraction process.

DNA quality was assessed by electrophoresis on a 0.8% agarose gel to evaluate degradation and purity. High-molecular-weight DNA was expected to appear as a single band with minimal migration, indicating intact, non-degraded DNA. Finally, DNA concentration was quantified using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.2 Library preparation and whole-genome sequencing

PCR-free whole-genome libraries were outsourced to a sequencing company. All DNA samples underwent additional quality checks for DNA concentration and fragment size using the Agilent 5400. Subsequently, library construction involved several steps, including genomic DNA fragmentation, where the DNA was randomly sheared into shorter fragments. These fragments were then end-repaired, A-tailed, and ligated with P5 and P7 Illumina adapters. Next, libraries were quantified using both Qubit and qPCR, and the size distribution was assessed using a fragment analyzer. Finally, the quantified libraries were pooled and sequenced on an Illumina NovaSeq instrument, using 150 base pairs (bp) paired-end reads, to an average coverage of 20x per sample.

2.3 Bioinformatic Data Processing

Hereafter, all the bioinformatic procedures were implemented for both wolf and brown bear short-read data.

2.3.1 Quality Assessment, Mapping, and Processing of DNA Sequencing Data

Adapter sequences were trimmed, and ambiguous regions, low-quality reads (score Q< 20), and reads shorter than 30 bp were removed using AdapterRemoval v.2.3.1 (Schubert et al., 2016). Filtered wolf reads were mapped to both the grey wolf (*Canis lupus lupus*) reference genome (Gopalakrishnan et al., 2017) and the domestic dog (*Canis lupus familiaris*) reference genome (CanFam3.1; Lindblad-Toh et al., 2005), to minimize biases related to species divergence in specific demographic analyses. For initial alignments intended for Pairwise Sequentially Markovian Coalescent (PSMC; Li & Durbin, 2011) analysis, reads were aligned to the grey wolf reference genome, despite this assembly being at the scaffold level and lacking full chromosomal annotation. This approach was chosen to reduce potential artifacts caused by differences in rare or private variants between wolves and dogs, as PSMC can be sensitive to such variant

differences (Gopalakrishnan et al., 2017). For downstream demographic analyses performed with Genetic Estimation of N_e (GONE; Santiago et al., 2020) and Sequential Markovian Coalescent model (SMC++; Terhorst et al., 2017), reads were aligned to the domestic dog reference genome (CanFam3.1), which is annotated at the chromosome level. Brown bear reads were mapped against the brown bear (*Ursus arctos*) reference genome (Delisle & Strobeck, 2002). For both species, we used BWA v.2.2.1 (Vasimuddin et al., 2019) with the mem algorithm and kept default parameters for the alignment.

After mapping, Samtools v.1.12 (Li et al., 2009) was used to generate and index the sorted BAM files. The Read Group Tags were added, and duplicates were removed using Picard (Ebbert et al., 2016). Throughout each step, the number of reads was calculated and recorded using Samtools in order to verify how many reads were being filtered at each stage (Table S3 and Table S4). After all filtering steps, coverage was estimated using Samtools coverage. For the reads mapped against the wolf and brown bear reference genomes (assembled into scaffolds), only scaffolds longer than 100 kb were retained.

2.3.2. SNP calling and filtering

Genomic variants were called from the previously filtered BAM files using BCFtools v.1.15.1 (Danecek et al., 2021) mpileup/call -m tools, with minimum mapping quality (MQ) and base quality (BQ) scores set to 20. To ensure that only high-quality SNPs were retained, several filtering steps were applied using VCFtools v.0.1.16 (Danecek et al., 2011), including the removal of sites containing insertions or deletions (*--remove-indels*), filtering out low-frequency allelic variants (*--maf 0.01*), retaining only biallelic autosomal SNPs (*--max-alleles* 2) with less than 10% missing data (*--max-missing 0.9*). Variants with mean depth (DP) below 6 or above 50 were removed, and genotypes with individual depth below 6 *(--minDP 6*) were set as missing. Finally, individual-level missing data and depth statistics were estimated using VCFtools (Table S5 and Table S6).

2.4 Demographic Inference

2.4.1. Pairwise sequentially Markovian coalescent (PSMC)

To infer ancient demographic history, we employed the PSMC software. This method utilizes whole-genome data from a single individual to estimate population demographic trends (Beichman et al., 2017). PSMC is derived from the Sequentially Markovian

Coalescent (SMC) framework (McVean & Cardin, 2005) and focuses on comparing alleles across the two chromosomes of the same individual (Li & Durbin, 2011). Specifically, PSMC calculates the time to the most recent common ancestor (TMRCA) for different genomic segments, revealing changes in effective population size over time, indicative of historical expansions, contractions, and bottlenecks (Li & Durbin, 2011; Beichman et al., 2017). If many regions of the genome coalesce at the same time, this may indicate a population contraction, since during such events, many lineages go extinct and only a few survive to give rise to subsequent genetic lineages (Beichman et al., 2017).

While the PSMC model offers valuable insights into the historical demography of populations, its results must be interpreted with caution due to several limitations. The model has limited power to detect recent demographic events, particularly within the last 20,000 years, due to a reduced number of recombination events in recent times (Li & Durbin, 2011; Schiffels & Durbin, 2014). Moreover, PSMC tends to smooth abrupt demographic changes, which may cause sudden events, such as bottlenecks, to appear gradual (Li & Durbin, 2011). Lastly, factors such as balancing selection or false heterozygotes, often caused by genomic duplications, may artificially inflate TMRCA estimates and produce signals of population expansion in localized genomic regions (Li & Durbin, 2011).

To prepare the data for PSMC analysis, we chose nine individual genomes of each species. Only autosomal sequences of each individual were included. We converted each BAM file to a fasta-like consensus sequence using BCFtools mpileup+call -c tools to call variants and vcfutils.pl vcf2fq to convert the VCF file to FASTQ format. Variants were called using a filtering threshold similar to section 2.3.2, with a minimum mapping and base quality of 20. Only positions with a sequencing depth between 6× and 40× were retained to avoid low-confidence calls and repetitive regions.

Following this, the VCF files were converted into PSMC format using fq2psmcfa, in which sequences were split into 100 bp non-overlapping bins. Each bin was marked as *homozygous* ('0') if more than 10 bp were called and no heterozygous positions were present; *heterozygous* ('1') if more than 10 bp were called and at least one heterozygous position was detected; or *missing* ('.') if more than 90 bp positions were filtered or not called within the 100 bp bin.

Subsequently, PSMC was run for each sequence. Default parameters were used except for the time interval pattern division, which was set to -p "2+2+25*2+4+6" instead of the

default -p "4+25*2+4+6", as recommended by Hilgers et al. (2025). This adjustment prevented the occurrence of effective population size peaks followed by major population collapses around 10,000 years ago, as previously reported by the same authors.

The results were initially plotted using the psmc_plot.pl script from the PSMC package, applying a mutation rate of 4.5×10^{-9} (Koch et al., 2019) and a generation time of 4.5 years for wolves (Mech et al., 2016), and a mutation rate of 1×10^{-8} (Kumar et al., 2017) with a generation time of 10 years for brown bears (Tallmon et al., 2004). Final figure editing and formatting were performed using Adobe Illustrator 2020 (Adobe Inc., 2020).

2.4.2. Sequentially Markovian Coalescent (SMC++)

To reconstruct demographic history across both ancient and more recent timescales, we applied SMC++, which can produce highly accurate estimates of effective population size between 1,000 to 1,000,000 years ago (Terhorst et al., 2017).

By combining information from haplotype patterns and local linkage disequilibrium (captured implicitly through the coalescent with a recombination framework), SMC++ is capable of inferring historical demographic histories (Terhorst et al., 2017). Unlike other coalescent-based methods (such as PSMC or MSMC), SMC++ can estimate effective population size using hundreds of unphased genomes, which is a major advantage. In fact, incorporating data from multiple genomes allows for the detection of more recent demographic events, as more coalescent events can be observed (Terhorst et al., 2017). This makes SMC++ particularly advantageous compared to PSMC, which relies on a single diploid genome. Additionally, this method includes a new model, *cubic spline*, which tends to smooth the inferred demographic trajectories by reducing the variance of the estimates when compared to existing methods (Terhorst et al., 2017).

As input data to SMC++, we used the entire SNP dataset for wolves (N = 32), comprising 2,918,644 SNPs (filtered as described in Section 2.3.2). For brown bears, only individuals from the Western nuclei (N=10; individuals were selected based on previous ancestry assignment values and genome-wide coverage), as we did not have a sufficient sample size for the Eastern nuclei. A total of 8,353,650 SNPs were included in the analysis.

The VCF file was converted into SMC format using the *vcf2smc* command for each dataset. An SMC file was generated for each chromosome/scaffold and each individual

separately, allowing SMC++ to capture individual-specific signals and compare with the rest of the population, producing more robust demographic inferences.

Demographic inferences were performed using the *estimate* model with a fixed mutation rate of 4.5×10^{-9} mutations per bp per generation for wolves and 1×10^{-8} for brown bears. Additionally, the *cubic spline* model was applied to smooth the population size trajectories. To visualize the demographic trajectories, the *plot* option was used, applying a generation time of 4.5 years for wolves and 10 years for brown bears.

To introduce confidence intervals, a Python script provided by the developers of SMC++ was used to generate 20 bootstrap replicates for each estimate. This script reads the compressed SMC genomic data files (one per chromosome per individual) and partitions them into fixed-size regions (5,000,000 bp). For each bootstrap replicate, 38 simulated chromosomes were created by randomly sampling and concatenating these chunks.

To explore the effect of genetic structure within the Iberian wolf population in SMC++ estimates, we repeated the analysis separately for each of the four main clusters known in the Iberian Peninsula (Silva et al., 2018). To confirm individual ancestry assignments to each cluster, we ran ADMIXTURE v.1.3 (Alexander et al., 2009) for K=4, in 200 iterations and a 10-fold cross-validation procedure (Alexander & Lange, 2011) (Table S7). We then selected five individuals with the highest assignment probabilities to each genetic cluster ($q > 0.90$) for demographic analysis.

The final estimates were plotted using R v.4.4.0 (R Core Team, 2024) in RStudio v.2025.05.0 (RStudio Team, 2025).

2.4.3. Genetic Optimization for N_e Estimation (GONE)

Models that infer demographic history based on mutation rates are limited in their ability to estimate recent population sizes (Santiago et al., 2020). As a result, new approaches based on recombination rates and linkage disequilibrium (LD) between loci have emerged, enabling the inference of more recent demographic events (Santiago et al., 2020). The variation in LD between SNPs at different genetic distances can provide information about a population's N_e, since LD tends to decay over time due to recombination. In fact, LD between closely linked loci can retain signals of both recent and ancient demographic events. In contrast, LD between more distant loci is predominantly shaped by recent genetic drift, since historical signals are more likely to have been broken down by recombination over time (Hayes, 2003). GONE is a

population-based approach that estimates recent Ne using patterns of LD, allowing the detection of demographic events within the last 200 generations that are not well captured by coalescent models.

Although GONE offers a powerful alternative to coalescent-based models, such as PSMC and SMC++, its performance is subject to certain limitations. According to Santiago et al. (2020), the accuracy of GONE estimations declines for events further in the past, as drastic demographic events such as population collapses or rapid expansions partially erase the LD footprint of older events. Therefore, the authors suggest analyzing the estimations until generation 200, taking into account that demographic trends are more accurate within the last 100 generations. This method is also sensitive to certain sampling and demographic conditions. In species with overlapping generations, recent N_e may be underestimated (Waples et al., 2014; Santiago et al., 2020) because individuals from different generations with origins from distinct breeders may present allele frequency changes. This bias would be similar to including individuals from different genetic clusters in a structured population (Kardos & Waples, 2024). GONE can be robust to population structure when there is high migration rates, providing estimates at the metapopulation level; however, in the presence of low connectivity, estimates tend to be unrealistic in the most recent generations (Waples & England, 2011).

As input data for wolves, we used the same VCF file employed in the SMC++ analyses, containing genotypes from 32 Iberian wolves. This file was then converted to .ped and .map format using PLINK v1.9 (Chang et al., 2015).

GONE was configured using the following options: the phase of genotypes was set to “unknown” (*PHASE=2*), which is recommended for non-model species; a recombination rate of 0.97 centiMorgans per megabase (cM/Mb), commonly used for canid species (Payseur, 2024), was applied; and the Haldane model was selected for recombination correction. The number of generations and generation bins were left at their default values (2000 and 400, respectively), although only the most recent 200 generations were considered in the analysis, as recommended. A minimum allele frequency (*maf*) threshold of 0.01 was applied, and missing genotypes were allowed to prevent data loss. The number of SNPs per chromosome (N=50,000) and the maximum recombination rate (0.05) were also set to the default, as suggested by the developers.

We used a custom Python script to estimate confidence intervals by resampling SNPs. Specifically, we generated 20 replicate datasets by randomly resampling 90% of the total

SNPs, without replacement, from the original .ped and .map files. These replicate datasets were subsequently used as input for GONE to compute N_e estimates and derive 95% confidence intervals across generations. The final estimates were plotted using the R software.

We performed the same analysis for each of the four genetic clusters in Iberia, using the same five individuals that were used in SMC++ analyses.

For the Cantabrian brown bear, the same 10 individuals used in the SMC++ analysis were analyzed. Input parameters followed those previously described for wolves, except for the recombination rate, which was set to 1 centiMorgan per megabase (cM/Mb) as recommended (Dumont & Payseur, 2008). Due to computational limitations, only four replicate datasets were generated for bootstrap confidence interval estimation.

3. Results

3.1. Data quality assessment

Regarding the samples selected for PSMC analysis, the proportion of retained reads after mapping to the respective reference genome and applying all the filtering procedures was higher for wolf samples, with an average of 89.09%, and 65.22% for brown bears (Figure 8). Read retention was generally consistent within each species, except for one bear hair sample (OSO945_950), which had a lower value (53%) compared to the others. The higher read retention in wolves may reflect better DNA quality in these samples relative to the bears. This is supported by the observation that brown bear samples presented a higher proportion of duplicate reads (average of 23.8%) than grey wolf samples (average of 4.14%). Regarding reads lost after filtering for scaffolds >100 kb, brown bear samples showed an average loss of 2.90%, while grey wolf samples had a slightly lower average of 1.85%, indicating minimal data loss during this filtering step.

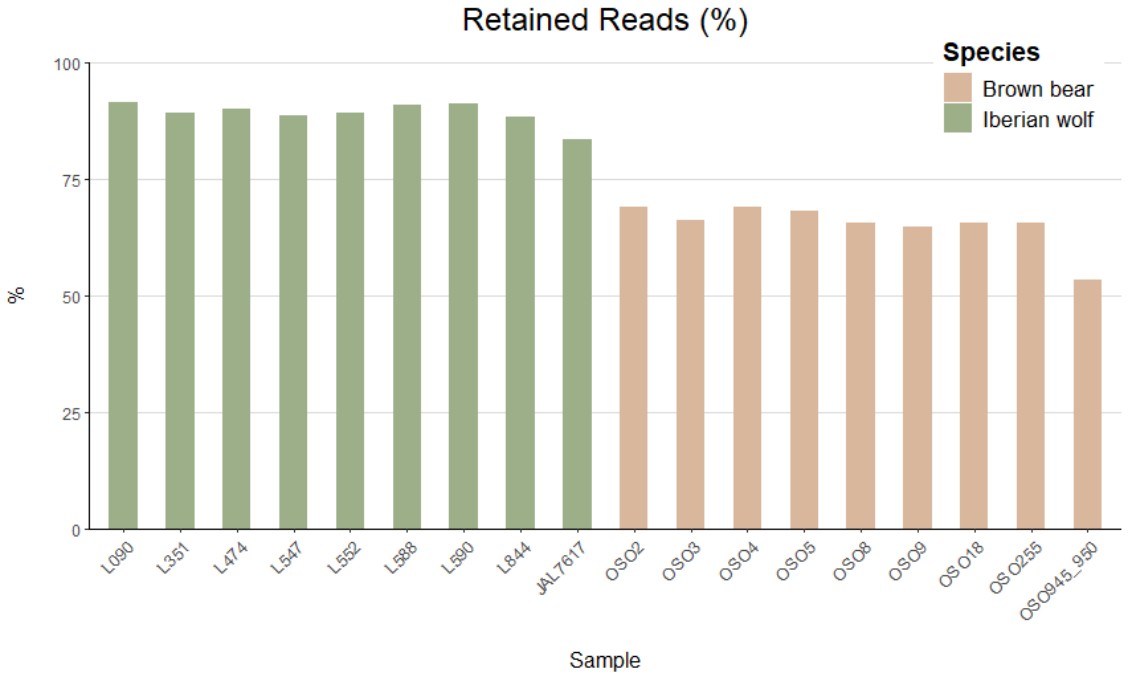

Figure 8 – Percentage of retained reads. Proportion of reads retained after quality filtering and mapping to the reference genome, relative to the number of trimmed reads, for wolf samples (green) and brown bear samples (brown) used in subsequent PSMC analyses.

Overall, the brown bear samples showed higher coverage levels (16x to 26x) compared to the Iberian wolf samples (10x to 20x), although the coverage values among the wolf individuals were more consistent (Figure 9). One bear sample (OSO2) had notably higher coverage (40x).

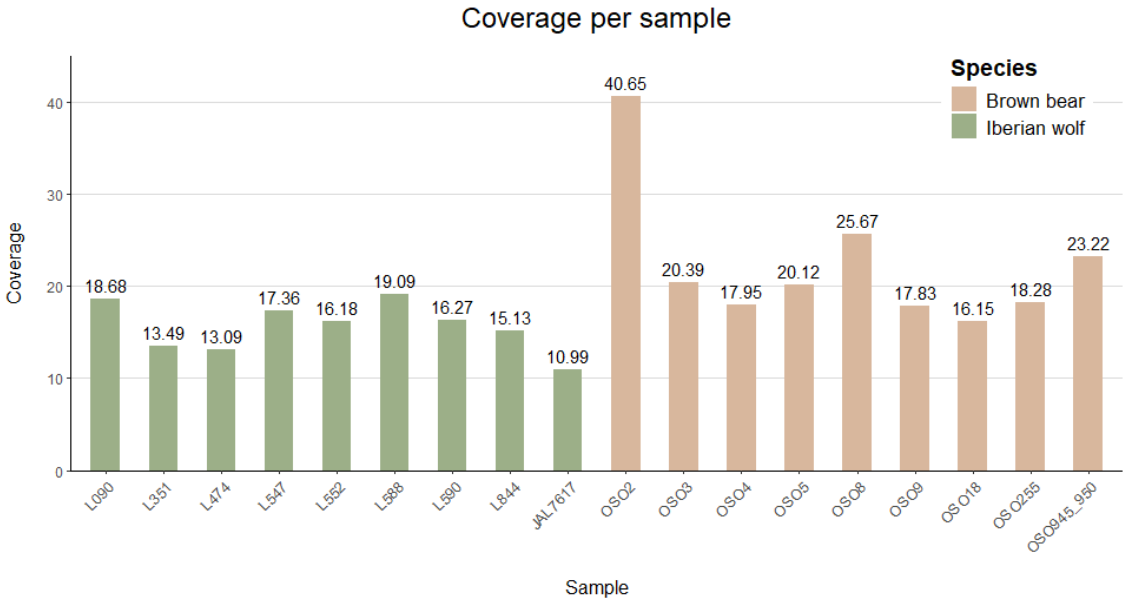

Figure 9 – Mean coverage(X) per individual. Average sequencing coverage across scaffolds longer than 100 kb for wolf samples (green) and brown bear samples (brown) used in subsequent PSMC analysis.

Similarly, for the SNP dataset used in the SMC++ and GONE analyses, Iberian wolf samples exhibited lower average depth compared to brown bear samples, with mean values of 14.92x and 20.30x, respectively (Figure 10). The mean depth for wolf SNPs ranged from 9x to 30x, whereas bear SNPs ranged from 12x to 49x, with only two samples (OSO2 and OSO17) exceeding 30x. Rigorous quality filters were applied to the SNP dataset to ensure reliability, including the exclusion of low-confidence and rare variants, as well as loci with excessive missing data or depth values. As a result, the final dataset of 42 individuals had no missing data, supporting robust downstream demographic inferences.

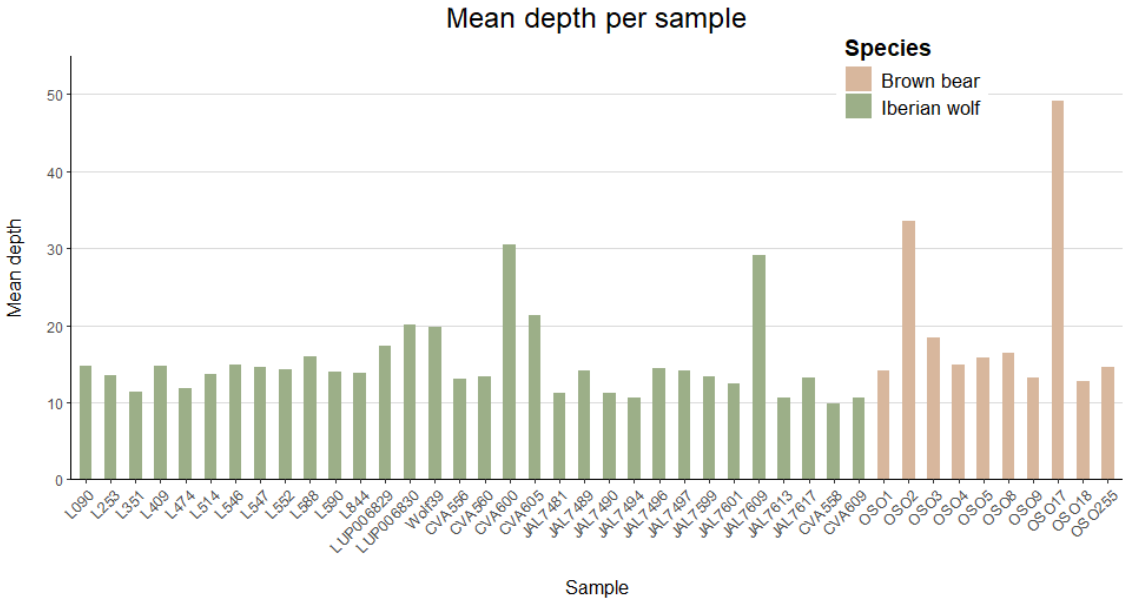

Figure 10 – Mean depth (X) of SNPs per individual. Average sequencing depth of SNPs across wolf samples (green) and brown bear samples (brown) used in subsequent SMC++ and GONE analyses.

3.2. Demographic analyses

3.2.1. Ancient demography

All the wolves showed similar demographic trajectories until approximately 20 kya, beyond which PSMC results become less reliable due to reduced resolution in more recent periods (Figure 11).

The analysis revealed two major periods of population decline, flanking a period of relatively stable N_e between approximately 1 million years ago (hereafter abbreviated as Mya) and 100 kya. The first decline began around 4 Mya and led to a reduction in N_e by nearly half (from 160,000 to 70,000). The second decline began around 100 kya and lasted until approximately 20 kya, reducing the N_e from 70,000 to 15,000.

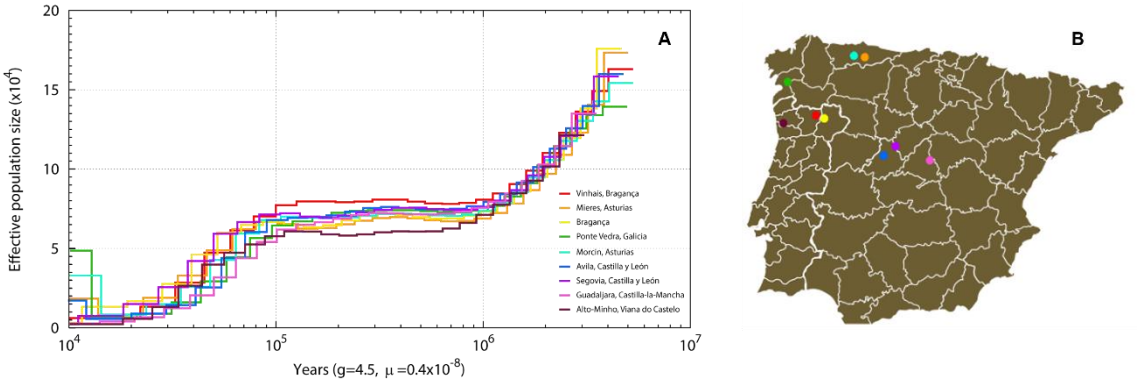

Figure 11 – PSMC trajectories and sampling locations for wolf individuals. (A) Demographic histories inferred using PSMC for nine wolf samples, each represented by a separate line. (B) Geographic location of the individuals included in the analyses.

Regarding the historical demography of the brown bear, all genomes displayed a similar decreasing trend in N_e, with higher values around 1 Mya and lower values in more recent times, around 10 kya. Two sharp population declines were detected, flanking a period of population expansion lasting approximately 200 kya (between ~300 kya and ~100 kya). The first population decline occurred 1 Mya and resulted in a decrease in N_e from around 62,000 to approximately 35,000. During the subsequent expansion phase, N_e increased to an average of 47,000, followed by a second population decline that persisted until 10 kya, reducing N_e to approximately 3,000 (Figure 12).

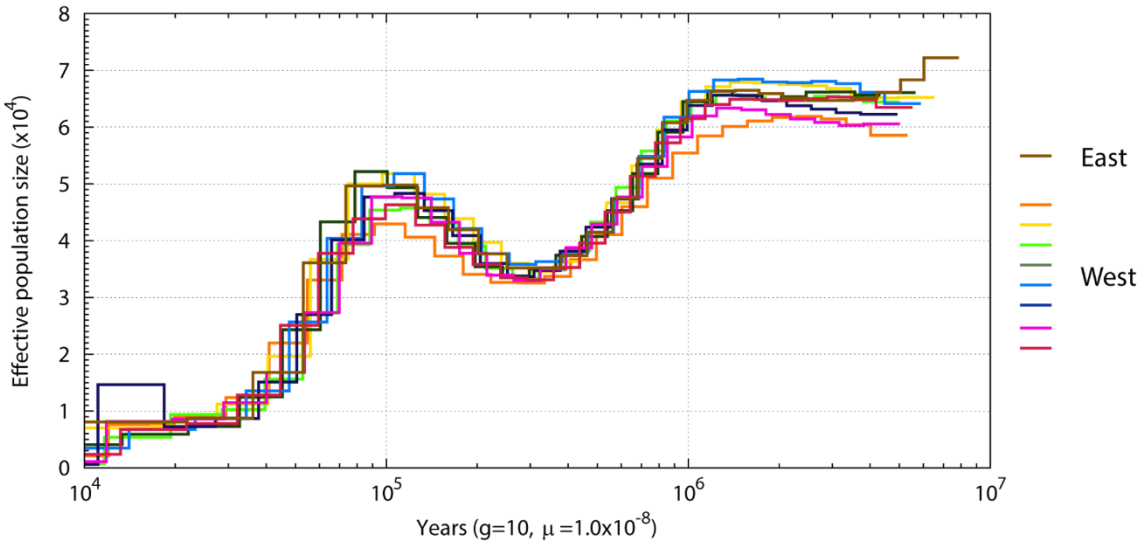

Figure 12 – PSMC trajectories for bear individuals. Demographic histories inferred using PSMC for nine bear samples, each represented by a separate line.

3.2.2. Historical demography

For the entire Iberian wolf population (Figure 13), we found a continuous general decline from approximately 100 kya until 10 kya, as previously observed in PSMC analysis. At 10 kya, the N_e estimates appeared to be concordant between both methods at approximately 7,000 individuals. The long-term decline appeared to reverse around 3 kya when the population seemed to experience an increase in N_e and went from 3,500 up to almost 5,000. From 1 kya onwards, the population appears to continue declining in N_e up to the present day. However, the period more recent than 1 kya lies outside the optimal temporal range of the method.

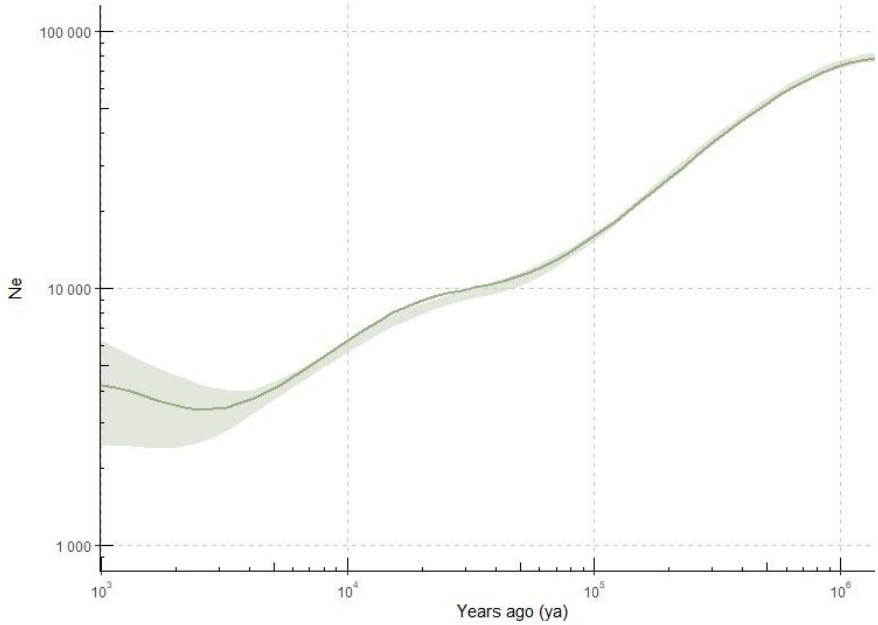

Figure 13 – SMC++ trajectory for the Iberian wolf population. Demographic history inferred using SMC++ based on 32 Iberian wolf individuals. The shaded green area represents the 95% confidence interval.

Regarding the N_e inference for each Iberian wolf genetic cluster, the analyses revealed an overall decreasing trend consistent with the findings at the metapopulation level (Figure 14). However, differences emerged in the timing and value of minimum N_e, as well as in the different intensities of N_e decline observed over the last 10 kya .

All four genetic clusters showed a broadly similar pattern of decline in effective population size (N_e) between approximately 10 kya and 1 kya. The Portugal and Galicia clusters reached their lowest N_e around 2 kya, with N_e values close to 2,000 and 3,000, respectively. For the W Cantabrian Mountains and Castilla y León clusters, the timing of minimum N_e falls outside the optimal resolution of SMC++ (Figure 14C and 14D).

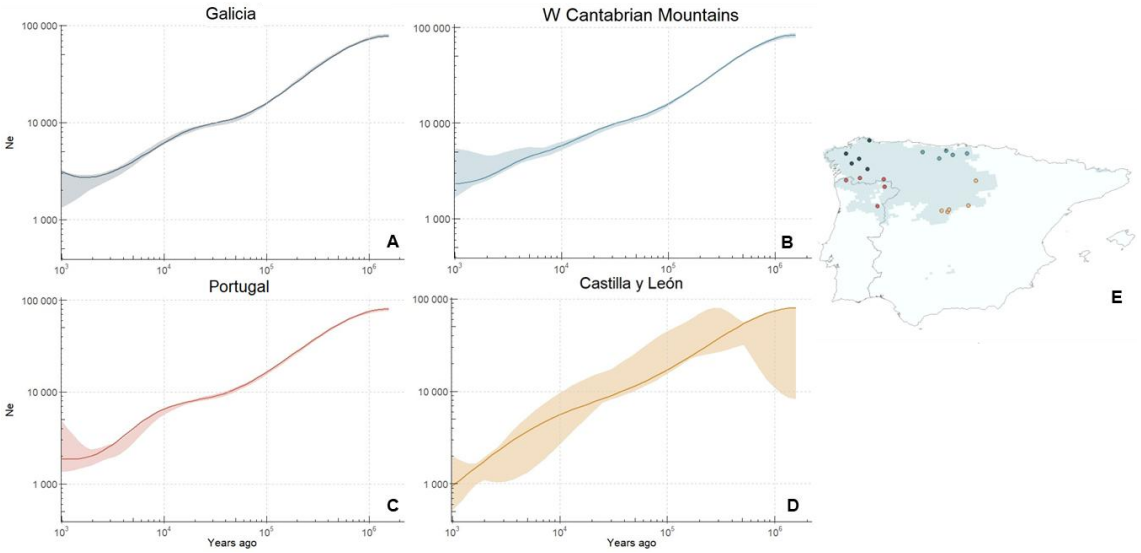

Figure 14 – SMC++ trajectories for Iberian wolf clusters. Demographic history inferred using SMC++ for each cluster in the Iberian wolf population, based on 5 individuals per cluster. A: Galicia genetic cluster, B: W Cantabrian Mountains genetic cluster, C: Portugal genetic cluster, D: Castilla y León genetic cluster; E: Geographic location of the individuals included in the analyses, with individual ancestry proportions inferred using the ADMIXTURE software for K = 4. The shaded coloured areas represent the 95% confidence interval.

The brown bear demographic analyses for this period also showed a decreasing trend, which became more pronounced since 100 kya and led to a minimum N_e of 2,000 at 10 kya. After this point, the Cantabrian brown bear population experienced an increase until 1 kya, with N_e reaching approximately 5,000. This growth appears gradual and continuous over several millennia (Figure 15).

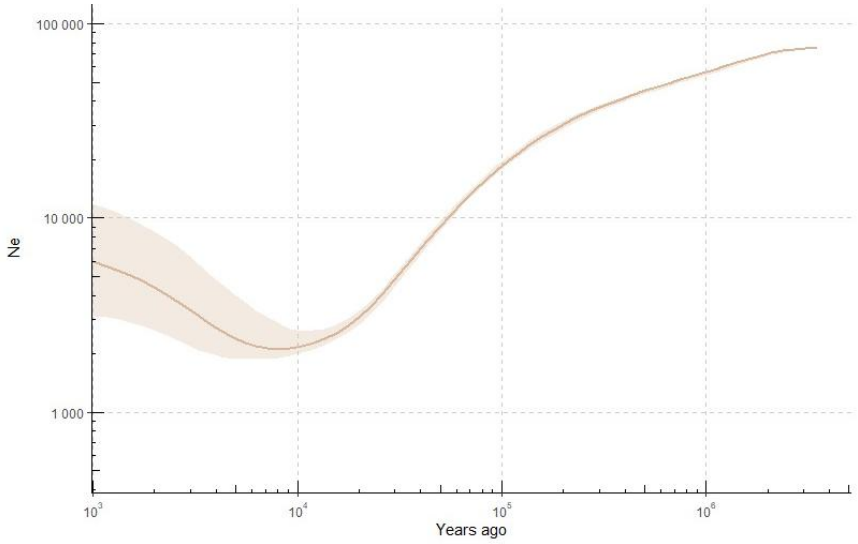

Figure 15 – SMC++ trajectory for the Cantabrian brown bear population. Demographic history inferred using SMC++ based on 10 brown bear individuals. The brown shaded area represents the 95% confidence interval. The shaded grey area indicates the resolution limit of the method.

3.2.3. Recent demography

For the Iberian wolf population, GONE was able to perform strong and supported estimations of N_e up to 900 years ago (200 generations). In general, the analyses indicated a continuous decreasing trajectory for the entire population, consistent with the decline that began around 1,000 years ago as inferred by SMC++ (Figure 16). The decline was more pronounced around 350 years ago and continued until 250 years ago, when the wolf population in Iberia was reduced to an N_e of 350. After that, a relatively stable period lasted for about 150 years, until roughly 100 years ago, during which a slight increase was detected. However, the population experienced another bottleneck, with N_e decreasing from approximately 350 to just over 100.

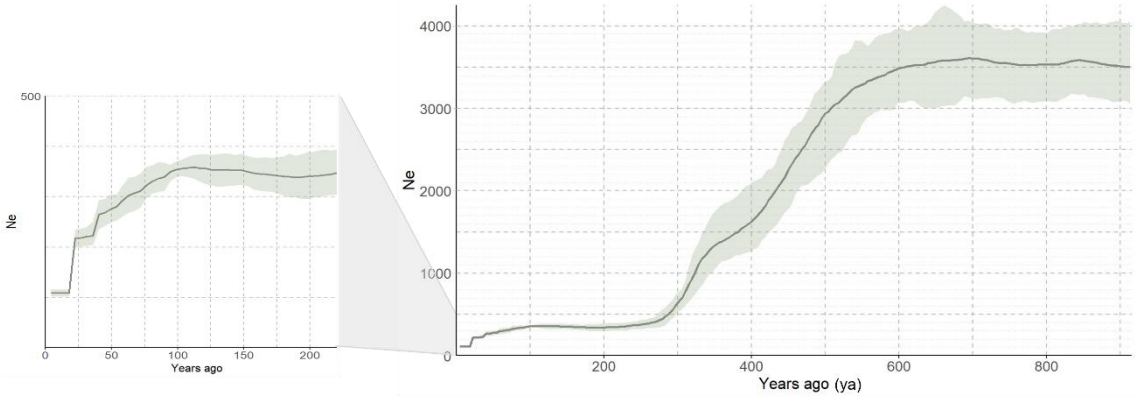

Figure 16 – GONE trajectory for the Iberian wolf population. Demographic history inferred using GONE based on 32 Iberian wolf individuals, with a zoom-in view over the last 200 years. The shaded green areas represent the 95% confidence interval.

When analysing the recent demography within each genetic cluster, we observed differences in the effective population size among clusters and in the timing of their demographic events (Figure 17).

Around 700 years ago, the four genetic clusters appeared to be in a stable period, with few variations observed in N_e. The exception to the general stability was the W Cantabrian Mountains cluster, which showed a slight decrease in N_e between approximately 880 and 450 years ago, from around 1,100 to 900, followed by an increase back to 1,100 by around 200 years ago. However, despite the overall stability, the estimated N_e varied considerably between clusters during this period, from approximately 1,500 in the Galicia cluster to as few as 50 in Castilla y León, with the Portuguese cluster showing intermediate values around 800.

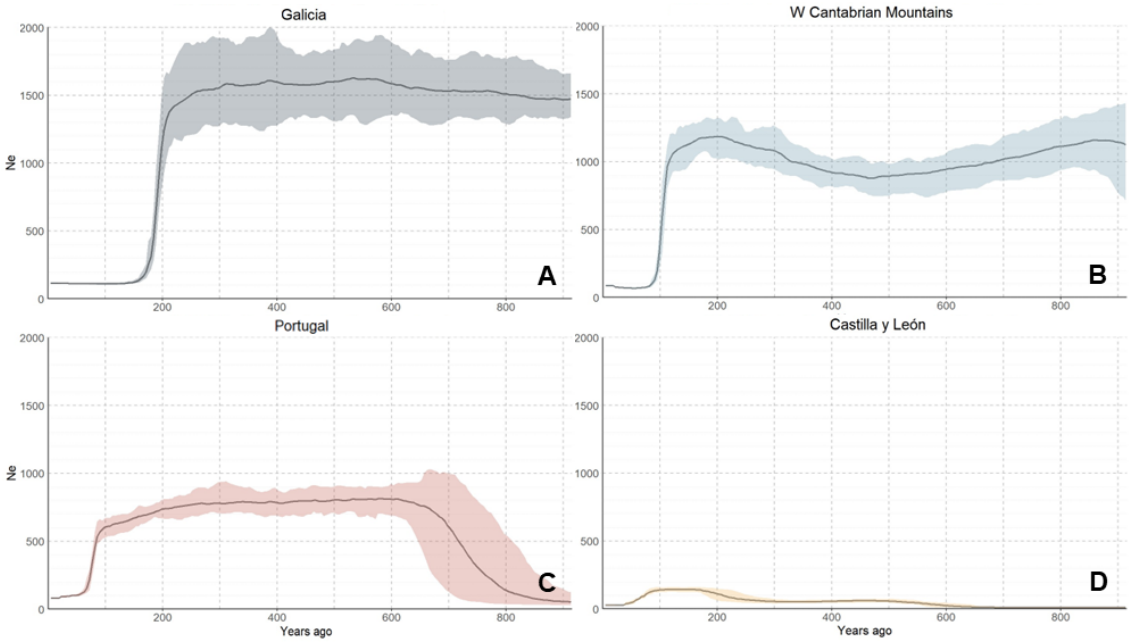

Figure 17 – GONE trajectories for Iberian wolf clusters. Demographic history inferred using GONE for each cluster in the Iberian wolf population, based on 5 individuals per cluster. A: Galicia genetic cluster, B: W Cantabrian Mountains genetic cluster, C: Portugal genetic cluster, D: Castilla y León genetic cluster. The coloured shaded areas represent the 95% confidence interval.

After the stable period, all the clusters, except Castilla y León, experienced a severe bottleneck, which substantially reduced their effective population size (Figure 18). The Galicia cluster was the one where this bottleneck firstly occurred and was more pronounced. In fact, this event occurred 200 years ago and resulted in a reduction of N_e from 1400 to around 110. The W Cantabrian Mountains and Portugal cluster experienced slight N_e reduction prior to this extreme bottleneck, which occurred around 110 and 75 years ago, respectively. In the Portugal cluster, N_e dropped from 600 to near 100 and in the W Cantabrian Mountains cluster, from 1000 to 100. The Castilla y León cluster presented a different scenario. This cluster also showed a past bottleneck around 70 years ago, which led to a reduction of the N_e from 150 to just 20; however, since its population size was already very small, this event was not so pronounced as it was on the other clusters.

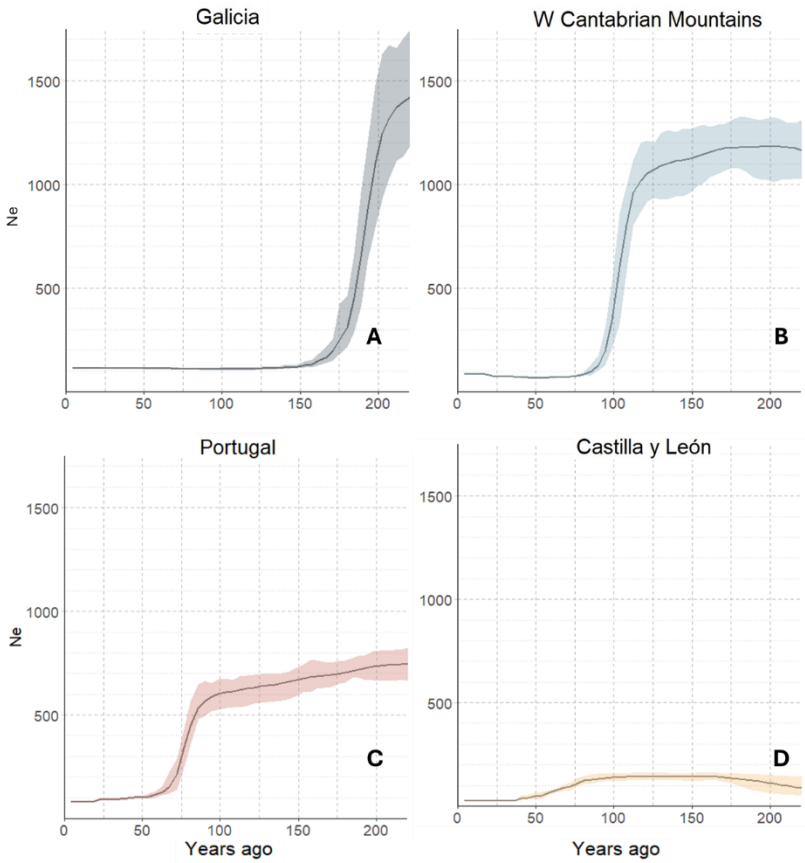

Figure 18 – GONE trajectories for Iberian wolf clusters over the last 200 years. Demographic history inferred using GONE for each cluster in the Iberian wolf population, based on 5 individuals per cluster. A: Galicia genetic cluster, B: W Cantabrian Mountains genetic cluster, C: Portugal genetic cluster, D: Castilla y León genetic cluster. The coloured shaded area represents the 95% confidence interval.

For the Cantabrian brown bear population, GONE was able to perform strong and supported estimations of N_e up to 1500 years ago (150 generations). The demographic trajectory of this species differed from that of the Iberian wolf until around 400 years ago. However, after this period, both species experienced a marked population decline. Specifically, for the brown bear, we identified a prolonged period of N_e increase extending from 1400 until 450 years ago. After this extended period, the brown bear population underwent a severe population contraction. Between 300 and 200 years ago, this decline became particularly pronounced, with N_e dropping sharply from around 2,300 to less than 100. More recently, we were able to detect a slight demographic recovery around 50 years ago that fixed N_e in approximately 60 (Figure 19).

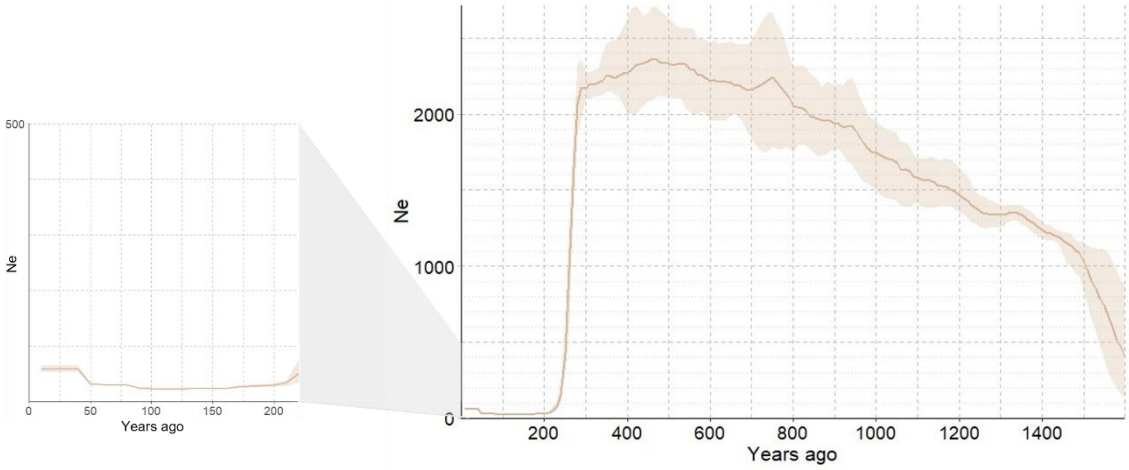

Figure 19 – GONE trajectory for the Cantabrian brown bear population. Demographic history inferred using GONE based on 10 Cantabrian brown bear individuals, with a zoom-in view on the last 200 years. The shaded brown area represents the 95% confidence interval.

To help interpret and compare the demographic history of both species, we summarised the estimated effective population sizes (*Ne*) at three key time points (ancient, historical, and recent) in Table 1. The results highlight the pronounced demographic decline experienced by both the Iberian wolf and the Cantabrian brown bear, particularly in recent times.

Table 1 – Summary of estimated effective population sizes at three time points (Ancient, Historical, and Recent) for the Iberian wolf and the Cantabrian brown bear.

	Ancient (100 kya)	**Historical (10 kya)**	**Recent (current)**
Iberian Wolf	55 000 - 80 000	5 400 - 6 000	292 - 318
Cantabrian Brown bear	43 000 - 52 000	2 000 - 2 800	53 - 65

4. Discussion

This study focused on unravelling the demographic history of two emblematic large carnivores inhabiting the Iberian Peninsula: the Iberian wolf and the Cantabrian brown bear. Whole-genome data enable us to infer the demographic trajectories of both species using three complementary approaches, each capturing the genetic signals of population size changes at different temporal resolutions.

4.1. Data quality assessment

As a first step, we quantified the amount of data retained after the bioinformatic pipeline to assess both sample quality and the impact of the filtering procedures. A higher proportion of reads was retained in the Iberian wolf samples (89.09%) compared to the Cantabrian brown bear (65.22%). This difference may be partially explained by the lower DNA quality of bear samples, although only one sample was derived from hair (OSO945_950), precisely the one with the smallest number of retained reads (53%). Another contributing factor could be the removal of duplicate reads, as brown bear samples showed a much higher percentage of duplicates removed (23.8%) than grey wolf samples (4.14%). On the other hand, filtering reads based on scaffold size (>100 kb) did not seem to account for the difference in read retention between species, since only a small proportion of reads were removed at this step (1.85% and 2.90% for wolves and bears, respectively).

Despite these differences, the filtered genomic data provided sufficient quality and coverage to support reliable demographic inferences for both species. The three methods used (PSMC, SMC++, and GONE) enable us to reconstruct from ancient to recent demographic trends, giving insight into how large carnivores have responded to potential past environmental changes and more recent human pressures in the Iberian Peninsula.

4.2 Demographic Inference

To contextualise demographic trends more recent than 10 kya, we first estimated the demographic history of both species prior to that timeframe. The demographic inferences obtained during ancient times up to 10 kya are not exclusive to the Iberian Peninsula,

since during the Pleistocene, Iberian populations of grey wolves and brown bears were not yet genetically or geographically isolated from other European populations. Therefore, the results for this timeframe likely reflect broader continental-scale demographic patterns.

Our demographic analyses reveal distinct historical trajectories for the grey wolf and the brown bear European populations during the Early and Middle Pleistocene, extending back to at least 100 kya (Figure 20). These periods were characterised by cyclic climatic oscillations, ranging from warm and humid interglacials with mild winters during the Early Pleistocene to increasingly severe and prolonged glacial periods with cold winters in the Middle Pleistocene (López-García et al., 2021). This climatic instability likely affected differently each species, shaping their demographic responses up to 100 kya.

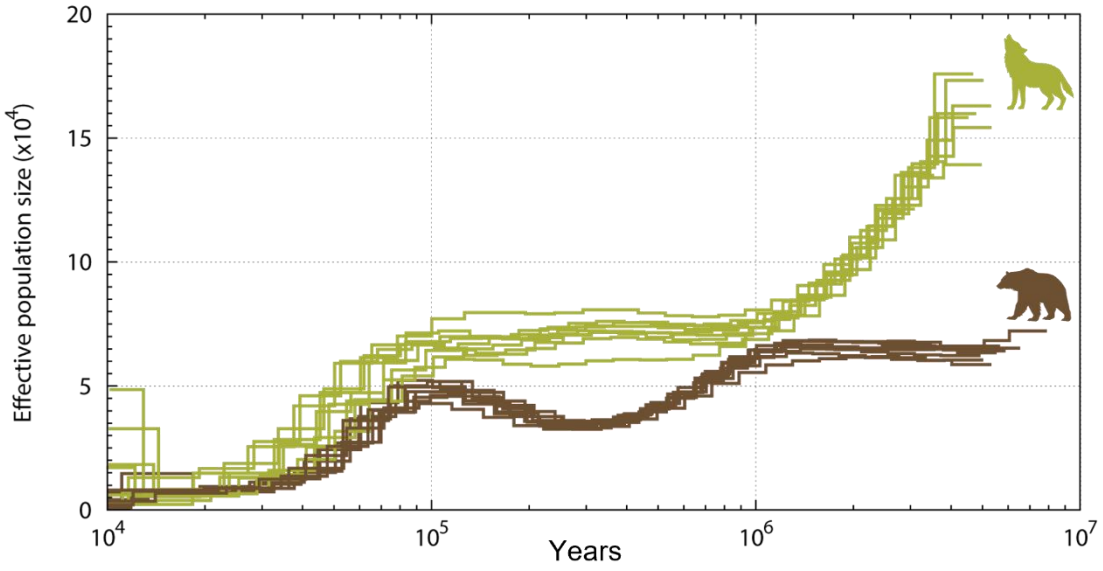

Figure 20 – Comparative PSMC trajectories of wolves and bears. PSMC-inferred demographic histories for nine wolf and nine bear individuals. Each line represents a single sample, with wolf trajectories shown in green and bear trajectories in brown.

Since the Late Pleistocene (130 to 11,7 kya), both species exhibited a simultaneous decline in effective population size, particularly during the period leading up to and including the Last Glacial Maximum (26.5–19 kya). Nevertheless, these climatic oscillations may not have been the main drivers of demographic change in the two carnivore species, as our results show no evidence of consistent cyclical fluctuations matching climate cycles but instead reveal a continuous decline throughout the Late Pleistocene. Therefore, other factors could have impacted the presence of these two species, such as the arrival of modern humans to Europe around 60 kya (Hublin, 2012).

For grey wolves, our ancient demographic inferences are in line with those reported by Hennelly et al. (2024) based on over a hundred wolf whole genomes across Eurasia. The only notable difference is the slight increase in N_e between 1 Mya and 100 kya in Hennelly et al., leading to a higher effective population size at the time of the bottleneck, which also occurs slightly later (around 80 kya), whereas we observed a stable N_e during that period. Fan et al. (2016) inferred a more recent bottleneck (around 11 kya) using a higher mutation rate. However, when considering mutation rates similar to ours, their results also indicate a decline starting close to 100 kya, which aligns with our findings. This highlights the need for caution when comparing demographic inferences across studies using different parameters. Notably, this period of decline may coincide with the disappearance of several ancient and divergent wolf lineages during the Late Pleistocene, as reported by Bergström et al. (2022).

Regarding the brown bear's historical demography, our findings are consistent with demographic trajectories reported for other brown bear populations across Eurasia (Endo et al., 2021; Tumendemberel et al., 2023). The demographic curves of brown bears studied across Italy, Slovakia, and central Asia show a similar increase in N_e before 100 kya to that observed in our work, followed by a steep bottleneck. These similar results reflect a shared evolutionary history, including demography and genetic connectivity between brown bears across Eurasia over the last hundreds of thousands of years (Nadachowska- Brzyska et al., 2016).

The historical demographic trends after 10 kya show differences for wolves and brown bears in the Iberian Peninsula (Figure 21). The demographic trajectory of the Iberian wolf reveals a declining trend until approximately 3 kya, which partially overlaps with a period described as relatively warm and moist between 9.4 and 4.8 kya, though interspersed with cold and dry episodes (Morellón et al., 2009; Olivia M. et al., 2019). More recently, from 6 to 2.5 kya, climate conditions in the Iberian Peninsula were predominantly cold and dry (Bernal-Wormull et al., 2023), which may have further influenced demographic decline. During the Late-Holocene (4.8 - 1.2 kya), the Iberian Peninsula experienced extreme arid conditions until around 4.0 kya (Morellón et al., 2009; Bernal-Wormull et al., 2023), closely preceding the point at which the Iberian wolf N_e ceased its population decline and began to recover, reversing the decreasing N_e trend. In contrast, the Iberian brown bear population does not appear to have been affected by the unstable ecological conditions during the Mid and Late Holocene, as its N_e increased steadily from 2,000 to 6,000 between 10 kya to 1 kya.

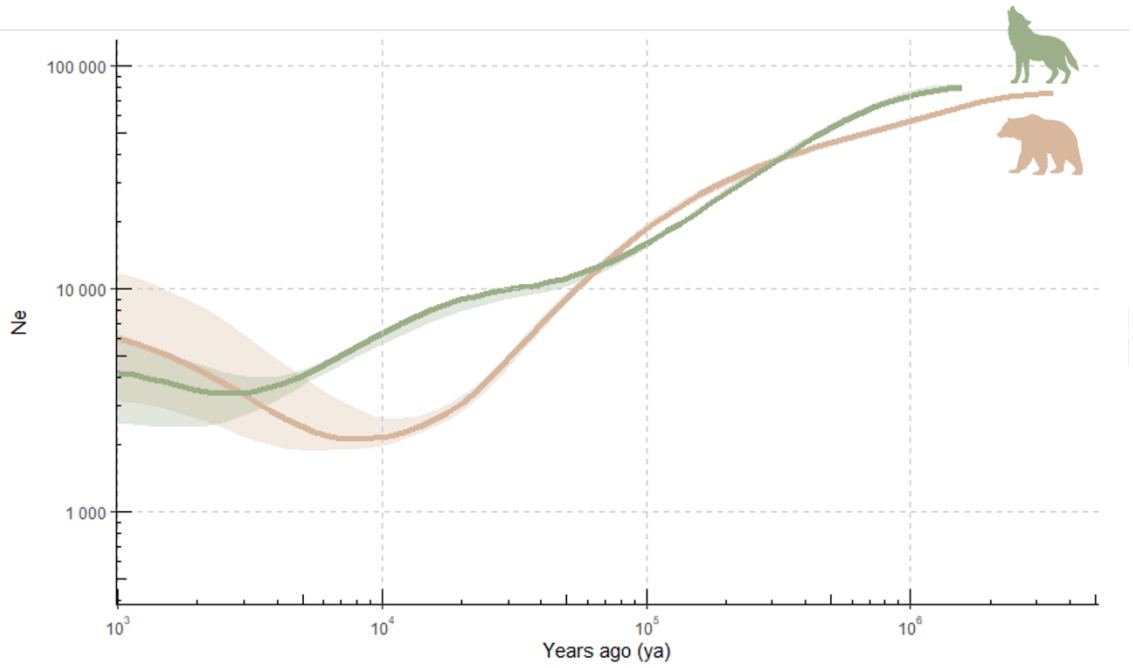

Figure 21 – Comparative SMC++ trajectories of wolves and bears.SMC++-inferred demographic histories for wolves and bears. Each line represents a species-wide trajectory, with the wolf trajectory shown in green and the bear trajectory in brown. The shaded areas represent the 95% confidence interval.

The demographic trend differences observed across Holocene phases may reflect the distinct ecological niches of the two species, particularly in their dietary breadth. The grey wolf is a highly adaptable carnivore with an opportunistic feeding behaviour, capable of surviving in a wide range of environments (Newsome et al., 2016) and feeding on wild ungulates, livestock in human-modified landscapes, and carrion (Barja, 2009; Newsome et al., 2016). In contrast, the brown bear has an omnivorous diet that includes not only wild ungulates and livestock but also invertebrates, fruits, and seeds (Naves et al., 2006). This greater dietary flexibility may have offered brown bears an advantage over wolves during periods when ungulate populations likely declined due to climatic instability across the Iberian Peninsula. Additionally, brown bears are known to scavenge wolf kills; however, the reciprocal behaviour is not usual by wolves. The presence of bears can also reduce wolf kill rates, which suggests asymmetric interactions and competition (Ordiz et al., 2020; Tallian et al., 2017), potentially influencing the demographic trends of these two large carnivores between 10 kya and 1 kya.

According to our analyses, the effective population size of wolves has declined markedly over the past millennium, from over 3,500 to just a few hundred. This decline is consistent

with a reported increase in human-wolf conflict, primarily driven by livestock depredation (Grande del Brío, 1984; Boitani, 1995). The conflict was further amplified by cultural negative perceptions about this species, many of which were propagated by religious groups that held substantial influence over societal attitudes at the time (Boitani, 1995). As a result, monarchs across Europe created specific legislation encouraging the general population to hunt wolves. These laws offered monetary rewards, and those who failed to comply were punished (Grande del Brío, 1984; Boitani, 1995; Domingues, 2005).

Our results suggest that the Iberian wolf decline over the past millennium became more pronounced around 350 years ago, resulting in a bottleneck that decreased the population N_e from 1300 to 340. This period coincides with the introduction of strychnine, a poison derived from *Strychnos nux-vomica*, into Europe by Portuguese and Spanish sailors during the 17th century (Valverde & Teruelo, 2001). In the following years, strychnine was widely used to poison wolves and, in general, other animals considered harmful for human activities (Gutiérrez, 2006).

Around 250 years ago, our demographic analysis revealed a period of N_e stabilization, which coincides with a lack of wolf hunting reports (Fernández & Ruiz de Azua, 2010). Specifically, no records of wolf kills were found in the provincial or municipal council archives of northern Spain prior to 1813 and during the period 1835 -1837. This absence may be attributed to administrative instability caused by ongoing armed conflicts in the region, including the War of Independence (1808–1814) against France, and the First Carlist War (1833–1839), a major civil conflict. During these war periods, monetary bounties for wolf hunting were no longer offered, and firearms were banned, which likely contributed to a temporary recovery, or at least stabilisation, of the Iberian wolf population (Grande del Brío, 1984; Gutiérrez, 2006).

More recently, around 50 years ago, we detected another bottleneck. This N_e reduction was less severe than the one identified in the 17th century, with an estimated decrease of approximately 60% (from about 300 to 100 individuals), compared to 72% during the earlier event. As expected, given its recent occurrence, this latter bottleneck identified by the demographic analysis is well documented in the literature (Petrucci-Fonseca & Álvares, 1997; Sastre et al., 2011; Valverde, 1971). At the beginning of the 20th century, wolves were still widely distributed across the Iberian Peninsula (Grande del Brio, 1984; Petrucci-Fonseca, 1990). Their distribution, however, was discontinuous, with some areas lacking stable populations (Nores & López-Bao, 2022). The persecution re-initiated in the 19th century and intensified throughout the 20th century, leading to the identified bottleneck of the 1970s. This decline led to the extirpation of wolves from

central and southern Iberia, as evidenced by the historical distribution data of the Iberian wolf population (Nores & López-Bao, 2022; Clavero et al., 2023). According to Clavero et al. (2023), the species has lost approximately 68% of its historical range in the Iberian Peninsula since the mid-19th century, further illustrating the impact of this demographic contraction. This recent bottleneck is also known to have contributed to the genetic diversity exhibited today in the Iberian wolf population (Lobo et al., 2023).

Regarding the brown bear's recent demographic history, our estimates reveal an increasing N_e trend 1400 years ago, with the Iberian population reaching its maximum 450 years ago, with a N_e of 2500 (Figure 22). However, we were not able to directly compare these results with other studies, as the earliest demographic records for the Cantabrian brown bear population date back only to the 19th century. By that time, brown bears were already confined to the north of the Iberian Peninsula, with their presence reported in the provinces of Asturias, Lugo, León, Cantabria, Zamora, and Palencia (Madoz, 1843). Our results reveal a sharp bottleneck beginning around 300 years ago and lasting for approximately a century, which aligns with historical records of intensive bear hunting in Spain. During the 18th and 19th centuries, dozens of bears were reportedly killed each year in Asturias (Noval, 1976), numbers that nearly exceeded the estimated population of the species in the region in the years that followed. This suggests that bear numbers were considerably higher during the 18th century (Clevenger, 1987). Additional factors contributing to the decline of brown bears in Iberia during the 19th and 20th centuries include mining activities in northern Spain, the use of non-selective poison to kill wolves responsible for livestock depredation, and the reduction and fragmentation of habitat (Clevenger, 1987). In fact, forest cover decreased due to human-related factors, including livestock grazing, which contributed to the reduction of brown bear habitat. Among other reasons for habitat loss were the lack of forest protection policies, the series of warfare in Spain where fire was used as a weapon, and deforestation for the construction of boats for the Spanish Armada (Clevenger, 1987).

As a response to the severe population decline, the Spanish government declared the brown bear a protected species in 1973. Since the 1990s, the Cantabrian population has shown signs of recovery and continues to increase to the present day (Palmoero et al., 2007; Pérez et al., 2014), which is also suggested by our demographic results by the slight increase in the N_e in recent years.

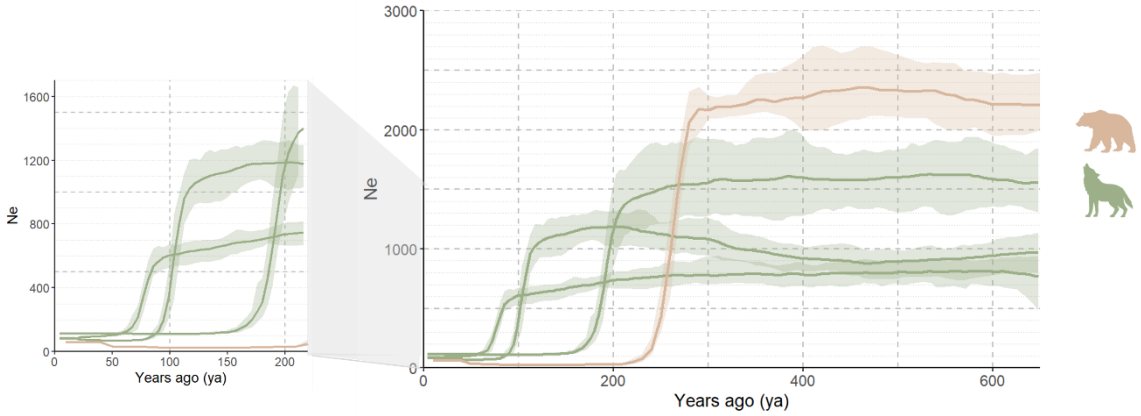

Figure 22 – Comparative GONE trajectories of wolves and bears. GONE-inferred recent effective population size estimates. The three green lines represent wolf clusters from Galicia, the western Cantabrian Mountains, and Portugal, while the brown line corresponds to the bear population. On the left is a zoom-in view of the last 200 years. The shaded areas represent the 95% confidence interval.

4.3 Effect of Genetic Structure on N_e estimations

The Iberian wolf population is currently divided into genetic clusters with reduced gene flow, as documented by Silva et al. (2018). Studies across Europe have shown that genetic structure can impact effective population size estimates in wolf populations (Mergeay et al., 2024). To assess the potential impact of population structure on our previous estimates, we performed LD-based analyses separately for the four main genetic clusters in Iberia identified by Silva et al. (2018).

Our results showed distinct patterns within the four genetic clusters of the Iberian wolf compared to the entire Iberian wolf population. In general, all clusters revealed a period of stable N_e lasting until 200 years ago, followed by a recent bottleneck occurring at different times in the past (Figure 22). In contrast, the entire Iberian population showed a continuous population decline over the same period. This discrepancy may reflect the known sensitivity of LD-based methods to population structure (Mergeay et al., 2024). Indeed, LD-based methods detect population size declines through increases in linkage disequilibrium patterns (Waples & Do, 2008). However, linkage disequilibrium can increase not only due to inbreeding, but also as a result of non-random mating driven by spatial structure (Néel et al., 2013). Therefore, the asynchronous decline signals observed across the four clusters may reflect reduced gene flow and the emergence of population structure. Moreover, the social organization of wolves into packs, along with isolation by distance, likely contributes to non-random mating patterns that can elevate LD and mimic demographic bottlenecks.

According to our estimates, genetic isolation appears to have occurred earlier in the northern clusters. Specifically, wolves from Galicia and the W Cantabrian Mountains genetic clusters became isolated earlier, approximately 200 and 100 years ago, respectively, whereas the genetic cluster in Portugal appears to have experienced a decline around 75 years ago. Regarding the Castilla y León cluster, its low N_e values and distinct demographic trend may reflect a founder effect, as the five individuals included in our analyses were located on the southern edge of the cluster's geographic distribution. Furthermore, wolves currently occupying the Castilla y León plateau originated from a post-1970s expansion from the southeastern Cantabrian Mountains (Asturias), which acted as a refugium during the population bottleneck of that period (Silva et al., 2018; Valverde, 1971). This recent colonization likely explains the distinct demographic signals observed in the Castilla y León genetic cluster.

Mergeay et al. (2024), using an LD approach, estimated N_e for the Iberian Peninsula both as a metapopulation and for the four main clusters, as well as for a total of eleven clusters identified by Silva et al. (2018). Considering the metapopulation analysis, our results inferred a N_e of approximately 107, while Mergeay et al. (2024) reported an N_e of around 75. Their N_e estimates for the four main clusters (Galicia: 47.1, Castilla y León: 28.5, Portugal: 27.2, and W Cantabrian Mountains: 24.3) summed to 127.1, whereas in our study, the sum of the cluster-specific N_e values reached 316. Additionally, when Mergeay et al. (2024) summed the N_e estimates across all eleven clusters, the total reached 234, still notably lower than the value obtained in our study using four clusters. However, in both cases, the summed N_e across clusters was considerably higher than the N_e estimated for the Iberian population as a whole, which indicates an underestimation of effective population size when population structure is not considered. Additionally, the detailed estimates of Mergeay et al. (2024) suggest that the number of clusters can influence demographic estimations.

4.4 Limitations of demographic model inference

Consistent with findings by Beichman et al. (2017) in human populations, we also observed discrepancies between PSMC and SMC++ estimates during ancient periods in our case studies. Specifically, between 1 Mya and 100 kya, the PSMC reconstruction for wolves suggests a relatively stable effective population size of approximately 70,000. In contrast, SMC++ indicates a continuous decline in N_e over the same period, reaching a N_e around 20,000 by 100 kya. This could likely be a result of the data used in each

method. While PSMC uses whole genome data from a single individual, SMC++ follows a population approach by incorporating whole genome data of multiple individuals, which may provide finer resolution both for recent (Patton et al., 2019) and ancient demographic events. Moreover, these differences may also reflect the distinct inference frameworks employed: PSMC relies solely on pairwise coalescent times from a single diploid genome, while SMC++ combines coalescent modelling with allele frequency information from the site frequency spectrum (SFS) from multiple genomes, allowing it to detect gradual demographic trends that PSMC might overlook or oversimplify (Terhorst et al., 2017).

Regarding the differences observed in brown bear N_e estimates around 1,000 years ago, SMC++ inferred a N_e of approximately 5,000, while GONE estimated a lower value of about 1,750. This discrepancy is likely due to methodological differences: GONE, which is based on patterns of linkage disequilibrium, provides higher resolution for recent demographic events (Santiago et al., 2020). In contrast, SMC++, although integrating information from the Site Frequency Spectrum, still relies on coalescent theory and may have lower sensitivity in detecting very recent changes (Terhorst et al., 2017).

LD-based approaches also have their limitations, as they rely on several assumptions about population parameters. In particular, they can be sensitive to temporal sampling across multiple breeding windows, which may introduce biases similar to those caused by population structure (Néel et al., 2013). This occurs because sampling individuals from different generations can capture heterogeneous LD patterns, and potentially lead to underestimation of N_e over time (Waples et al., 2014; Santiago et al., 2020). In this study, samples were collected between 2004 and 2017 and between 2000 and 2021 for wolves and brown bears, respectively. However, these intervals only represent about three wolf generations and two bear generations. Therefore, while this temporal spread should be acknowledged, it is unlikely to have introduced substantial bias in the LD patterns, given the relatively small number of generations involved and the expected limited variation in LD structure across them.

Additionally, our LD-based analyses for each Iberian wolf genetic cluster were based on only five individuals per group. A small sample size provides limited information about LD patterns, and when the number of sampled individuals is much smaller than the actual N_e, LD-based methods may overestimate N_e (Santiago et al., 2020; Wang, 2023; Waples, 2023). This limitation may have affected our estimates, particularly those obtained with SMC++, although the most recent time points fall outside the reliable estimation range of the method.

To improve the reliability of future demographic inferences, increasing the number of sampled individuals would be beneficial, particularly for refining the timing of isolation among Iberian wolf clusters. Additionally, a larger sample size would capture a broader representation of the population's genetic variation and allow for more precise sampling of specific periods, which could further minimize potential biases related to temporal sampling.

4.5 Implications for management and conservation

Effective population size is a key parameter in both evolutionary and conservation biology, as it provides insights into a species' demographic history, reflects the risk of inbreeding, and informs the status of a population (Hoban et al. 2020). Our results demonstrate that the Iberian wolf and the Cantabrian brown bear populations maintained reduced N_e values over the past 200 years, potentially increasing their vulnerability to genetic drift and inbreeding depression (Newman & Pilson, 1997). These risks are particularly pronounced in isolated populations, as is the case for both large carnivores in Iberia.

Since 2020, the Convention on Biological Diversity has recognized an N_e threshold of >500 as a benchmark to evaluate a population's capacity to maintain high levels of genetic diversity. This threshold is crucial for enabling species to adapt to environmental changes, such as climate change, pollution, habitat change, and emerging pests and diseases, as indicated in Annex A-4 of the Global Biodiversity Framework (Hoban et al., 2023; CBD Indicator A-4).

Recent studies have tried to establish reliable ratios between N_e and N_c to facilitate the inference of the census size from genetic estimates. For Iberian wolves, Mergeay et al. (2024) proposed a N_e/N_c ratio around 0.12. Applying this ratio to our summed cluster-specific N_e estimate (316) suggests a total N_c around 2600 individuals across Iberia, which is consistent with the estimate of approximately 2500 individuals by Boitani et al. (2022). Similarly, for brown bear, Pérez et al. (2014) inferred an N_e/N_c ratio of 0.22. Applying this value to our estimates yields a census size around 264 bears, which is in line with the estimates of Pérez et al. (2014) that reported 223 individuals for the whole Cantabrian population and around 200 for the western nuclei.

In the case of the grey wolf, N_e can also serve as a reliable proxy for the number of breeding pairs, as suggested by Mergeay et al. (2024). In our study, the estimated current N_e corresponds to approximately 316 packs, closely matching the 350 packs reported by Boitani et al. (2022) for the Iberian wolf population. These results support the reliability of N_e as a tool for conservation and management strategies, not only for the Iberian wolf but also for other carnivore populations.

Our results provide important insights into the recent demographic trajectories of two threatened Iberian carnivores and their implications for conservation. The increase in effective population size observed for the Cantabrian brown bear over recent generations suggests that the conservation measures implemented over the past decades have been effective in promoting the genetic recovery of this small and isolated population. This aligns with observed census increases and highlights the value of strict legal protection and active management. In contrast, the Iberian wolf does not show clear evidence of recent genetic recovery, with N_e remaining relatively stable over the past five decades despite some local population growth. This discrepancy may reflect the fragmented and inconsistent conservation status of the species across the Iberian Peninsula. While wolves are fully protected in Portugal and in some parts of Spain south of the Douro River (Annexes II and IV of the EU Habitats Directive), they are listed under Annex V in northern Spain, where they are subject to varying management regimes, including legal hunting in some autonomous regions. These contrasting trajectories underscore the need for harmonized and science-based conservation strategies across political boundaries, particularly for species with high ecological importance like the Iberian wolf.

Nonetheless, while our results suggest a recent increase in effective population size for the Cantabrian brown bear and apparent stability for the Iberian wolf, they also imply that much of the genetic diversity lost in the past has not been recovered. The persistently low effective population sizes indicate that strong anthropogenic pressures may leave lasting genomic impacts, with reduced N_e leading to a loss of genetic variation that can be difficult or impossible to restore. These findings highlight the importance of preventing sharp declines, as their genetic consequences may persist even after demographic recovery.

5. Conclusion

In this thesis, we reconstructed the demographic history of two emblematic large carnivore species currently present in the Iberian Peninsula: the Iberian wolf and the Cantabrian brown bear. To achieve this, we employed three complementary genomic approaches (PSMC, SMC++, and GONE) which together enable the inference of demographic trends across ancient, historic, and recent timescales. To our knowledge, this is the first study to combine such comprehensive information to characterize the demographic trajectories of these two species over both historical and contemporary periods.

Our analyses revealed distinct demographic trajectories over historical times, likely reflecting differences in ecological strategies and species-specific resilience to past climate oscillations. In contrast, recent trends appear more similar between the two species, probably driven by shared anthropogenic pressures, including habitat fragmentation and, most notably, direct persecution.

The comparison between methods also highlighted the strengths and limitations of each approach. In fact, including different tools contributes to a more robust understanding of demographic trends across different timescales. Consistent with previous studies, we found that methods incorporating data from multiple genomes, such as SMC++ and GONE, were more suitable for recent demographic estimates, whereas PSMC provided broader insights into more ancient periods.

In future research, increasing the number of genomes analysed would help minimize limitations related to sample size. Additionally, incorporating genomic data from individuals originating from extinct or currently unrepresented areas of Iberia could improve our understanding of population isolation events and provide a more comprehensive picture of the species' demographic history across the region. Furthermore, analysing genomic data from other ecologically linked species – including prey species such as ungulates and scavengers – could offer valuable insights into the broader ecological context of carnivore populations, including the effects of climate and dietary changes.

Overall, this study contributes to a better understanding of the demographic evolution of large carnivores in Iberia and highlights the importance of combining genomic data, ecological information, and historical records to inform the development of effective conservation strategies for these species.

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Supplementary Information

Table S1 - Detailed information about Iberian wolf sample localities, collection dates, sex, and study.

Sample ID	Locality	Collection Date	Sex	Study
L090	Bragança	2004	M	ECOGEN data
L253	Galicia	2005	M	ECOGEN data
L351	Asturias	2007	F	ECOGEN data
L409	Galicia	2009	F	ECOGEN data
L474	Bragança	2011	F	ECOGEN data
L514	Galicia	2010	M	ECOGEN data
L546	Galicia	2012	F	ECOGEN data
L547	Galicia	2012	F	ECOGEN data
L552	Asturias	2010	F	ECOGEN data
L588	Castilla y León	2009	M	ECOGEN data
L590	Castilla y León	2012	F	ECOGEN data
L844	Castilla-la-Mancha	2017	F	ECOGEN data
LUP006829	NA			NCBI data
LUP006830	NA			NCBI data
Wolf39 (spw)	Spain		F	Fan et al., 2016
CVA556	Castilla y León, Palencia	2009	F	Sarabia et al., 2025
CVA560	Castilla y León, Zamora	2012	M	Sarabia et al., 2025
CVA600	Castilla y León, Salamanca		M	Sarabia et al., 2025
CVA605	Castilla y León, Segovia		M	Sarabia et al., 2025
JAL7481	Castilla-la-Mancha	2016	M	Sarabia et al., 2025
JAL7489	Castilla y León, Burgos	2020	M	Sarabia et al., 2025
JAL7490	Galicia, A Coruna	2019	M	Sarabia et al., 2025
JAL7494	Galicia, Ourense	2020	M	Sarabia et al., 2025
JAL7496	Galicia, Lugo	2020	F	Sarabia et al., 2025
JAL7497	Galicia, Lugo	2020	M	Sarabia et al., 2025
JAL7599	Cantabria	2020	M	Sarabia et al., 2025
JAL7601	Cantabria	2020	M	Sarabia et al., 2025
JAL7609	Asturias	2019	F	Sarabia et al., 2025
JAL7613	Castilla y León, Burgos	2021	M	Sarabia et al., 2025
JAL7617	Viana do Castelo	2020	M	Sarabia et al., 2025
CVA558 (M2)	Castilla y León, Palencia		M	Salado et al., 2024
CVA609 (M3)	Castilla y León, Salamanca		M	Salado et al., 2024

Table S2 - Detailed information about Brown bear sample type, collection date, sex, and nuclei genetic assignment (ECOGEN data).

Sample ID	Type	Collection Date	Sex	Nucleus	Pop assign (K2) Eastern	Pop assign (K2) Western
OSO1	muscle	2000	M	west	0.011	0.989
OSO2	muscle	2012	M	west	0.005	0.995
OSO3	muscle	2015	F	west	0.012	0.988
OSO4	muscle	2016	M	west	0.006	0.994
OSO5	muscle	2017	M	west	0.015	0.985
OSO6	muscle	2012	F	east	0.995	0.005
OSO7	muscle	2014	M	west	0.015	0.985
OSO8	muscle	2006	M	west	0.004	0.996
OSO9	muscle	2008	M	west	0.010	0.990
OSO11	muscle	2015	F	west	0.005	0.995
OSO23	hair	2016	F	east	0.996	0.004
OSO28	hair	2016	M	east	0.996	0.004
OSO17	muscle	2017	M	west	0.004	0.996
OSO18	muscle	2017	M	west	0.006	0.994
OSO19	muscle	2017	M	west	0.025	0.975
OSO197	hair	2017	F	east	0.995	0.005
OSO198	hair	2017	M	east	0.960	0.040
OSO254	muscle	2019	M	west	0.012	0.988
OSO255	muscle	2019	F	west	0.008	0.992
OSO873	muscle	2021	M	west	0.004	0.996
OSO874	muscle	2021	M	west	0.005	0.995
OSO945_950	hair	2021	F	east	0.845	0.155

Table S3 – Iberian wolf's sequencing data information for each of the ten individuals used for PSMC analysis: number of read pairs, total reads, trimmed reads, mapped reads, mapped and unique reads (without duplicates), mapped, unique, and MQ > 20.

Sample	Raw read pairs	Trimmed Reads	Mapped Reads	Mapped and Unique	Mapped, unique and MQ >20	Mapped, unique and MQ >20 (Scaffolds >100kb)
L090	165343489	330685540	329286665	320946862	308186849	302502119
L351	125957986	251915150	250670217	239239541	228851648	224405985
L474	119993596	239985804	238536319	230262474	220099823	215689366
L547	162417549	324832400	322926771	306167377	293322040	287729794
L552	148135457	296268922	294910896	282781492	269776020	263996277
L588	168168994	336336108	334743516	323704927	311090491	305233480
L590	148093692	296185508	294753943	286441860	274955996	270046511
L844	140777128	281553004	278384616	265088375	253621418	248604099
JAL7617	108732608	194040222	191904735	178897185	166906996	161920277

Table S4 – Cantabrian brown bear's sequencing data information for each of the nine individuals used for PSMC analysis: number of read pairs, total reads, trimmed reads, mapped reads, mapped and unique reads (without duplicates), mapped, unique, and MQ > 20.

Sample	Raw read pairs	Trimed Reads	Mapped Reads	Mapped and Unique	Mapped, unique and MQ >20	Mapped, unique and MQ >20 (Scaffolds >100kb)
OSO2	376502529	752971331	745075142	576720156	535114684	518851864
OSO3	212742374	425458952	420904697	318399814	296668244	281448876
OSO4	166215152	332409039	327921605	255540794	236823703	229367055
OSO5	178781060	357540988	349530896	273257548	251694583	243773092
OSO8	194845244	389657882	386390786	294864195	266341965	255429856
OSO9	160030409	320033947	314500515	236066790	215132105	207423614
OSO18	149928704	299831306	297138646	220286195	202894025	196391346
OSO255	171062909	342111345	339641346	258970961	240498900	223802698
OSO945_950	218254711	436480911	419258708	269078123	245722610	233252752

Table S5 – Summary of missing data and sequencing coverage for the nine Iberian wolf and nine brown bear samples used in the PSMC analysis.

Sample	Species	Missing genotype rate	Coverage
L090	*Canis lupus signatus*	0.00953783	15.37
L351	*Canis lupus signatus*	0.0272999	12.03
L474	*Canis lupus signatus*	0.0280379	11.71
L547	*Canis lupus signatus*	0.00570509	15.08
L552	*Canis lupus signatus*	0.00658398	14.46
L588	*Canis lupus signatus*	0.00388641	16.45
L590	*Canis lupus signatus*	0.00850922	14.28
L844	*Canis lupus signatus*	0.00727168	13.93
JAL7617	*Canis lupus signatus*	0.233216	9.64
OSO2	*Ursus arctos*	0.000111448	33.50
OSO3	*Ursus arctos*	0.00129177	18.36
OSO4	*Ursus arctos*	0.0085316	14.90
OSO5	*Ursus arctos*	0.00679296	15.84
OSO8	*Ursus arctos*	0.00391637	16.45
OSO9	*Ursus arctos*	0.0179715	13.25
OSO18	*Ursus arctos*	0.0201062	12.80
OSO255	*Ursus arctos*	0.0271547	14.53
OSO945_950	*Ursus arctos*	0.00767186	15.38

Table S6 – Summary of missing data and sequencing coverage for the 32 Iberian wolf and 10 western brown bear samples used in the SMC++ and GONE analysis.

Sample	Species	Missing genotype rate	Coverage
L090	Canis lupus signatus	0	14,70
L253	Canis lupus signatus	0	13,49
L351	Canis lupus signatus	0	11,33
L409	Canis lupus signatus	0	14,70
L474	Canis lupus signatus	0	11,81
L514	Canis lupus signatus	0	13,74
L546	Canis lupus signatus	0	14,93
L547	Canis lupus signatus	0	14,56
L552	Canis lupus signatus	0	14,26
L588	Canis lupus signatus	0	16,04
L590	Canis lupus signatus	0	13,93
L844	Canis lupus signatus	0	13,83
LUP006829	Canis lupus signatus	0	17,31
LUP006830	Canis lupus signatus	0	20,08

Wolf39	Canis lupus signatus	0	19,76
CVA556	Canis lupus signatus	0	13,08
CVA560	Canis lupus signatus	0	13,38
CVA600	Canis lupus signatus	0	30,50
CVA605	Canis lupus signatus	0	21,32
JAL7481	Canis lupus signatus	0	11,16
JAL7489	Canis lupus signatus	0	14,11
JAL7490	Canis lupus signatus	0	11,23
JAL7494	Canis lupus signatus	0	10,60
JAL7496	Canis lupus signatus	0	14,45
JAL7497	Canis lupus signatus	0	14,15
JAL7599	Canis lupus signatus	0	13,30
JAL7601	Canis lupus signatus	0	12,39
JAL7609	Canis lupus signatus	0	29,07
JAL7613	Canis lupus signatus	0	10,58
JAL7617	Canis lupus signatus	0	13,15
CVA558	Canis lupus signatus	0	9,86
CVA609	Canis lupus signatus	0	10,59
OSO1	Ursus arctos	0.0106952	14,16
OSO2	Ursus arctos	0.000114713	33,50
OSO3	Ursus arctos	0.00128964	18,36
OSO4	Ursus arctos	0.00928644	14,90
OSO5	Ursus arctos	0.00741354	15,84
OSO8	Ursus arctos	0.00452904	16,45
OSO9	Ursus arctos	0.019473	13,25
OSO17	Ursus arctos	4.93148e-05	49,19
OSO18	Ursus arctos	0.0217846	12,80
OSO255	Ursus arctos	0.0269191	14,53

Table S7 – Proportion of ancestry for each Iberian wolf individual assigned to each inferred genetic cluster.

Sample ID	Locality	K1	K2	K3	K4
JAL7496	Galicia, Lugo	0,000	0,000	1,000	0,000
LUP006830	Portugal?	0,000	0,776	0,224	0,000
LUP006829	Portugal?	0,000	1,000	0,000	0,000
Wolf39	Castilla y Leon	0,216	0,000	0,000	0,784
JAL7490	Galicia, A Coruna	0,000	0,000	1,000	0,000
CVA600	Castilla y Leon, Salamanca	0,000	0,733	0,000	0,267
JAL7609	Asturias	1,000	0,000	0,000	0,000
CVA556	Castilla y León, Palencia	1,000	0,000	0,000	0,000
JAL7601	Cantabria	1,000	0,000	0,000	0,000
JAL7481	Castilla-la-Mancha	0,141	0,102	0,000	0,757
CVA560	Castilla y León, Zamora	0,000	1,000	0,000	0,000
JAL7613	Castilla y Leon, Burgos	0,000	0,000	0,000	1,000
JAL7599	Cantabria	0,916	0,000	0,000	0,084
JAL7494	Galicia, Ourense	0,000	1,000	0,000	0,000
CVA605	Castilla y Leon, Segovia	0,000	0,000	0,000	1,000
JAL7617	Viana do Castelo	0,000	1,000	0,000	0,000
CVA558	Castilla y León, Palencia	0,716	0,000	0,000	0,284
JAL7489	Castilla y Leon, Burgos	1,000	0,000	0,000	0,000
JAL7497	Galicia, Lugo	0,000	0,000	1,000	0,000
CVA609	Castilla y Leon, Salamanca	0,000	0,371	0,000	0,629
L090	Bragança	0,304	0,545	0,151	0,000
L253	Galicia	0,000	0,000	1,000	0,000
L351	Asturias	0,734	0,000	0,266	0,000
L409	Galicia	0,000	0,000	1,000	0,000
L474	Bragança	0,000	1,000	0,000	0,000
L514	Galicia	0,000	1,000	0,000	0,000
L546	Galicia	0,000	0,000	1,000	0,000
L547	Galicia	0,000	0,000	1,000	0,000
L552	Asturias	0,668	0,000	0,332	0,000
L588	Castilla y León	0,000	0,000	0,000	1,000
L590	Castilla y León	0,000	0,000	0,000	1,000
L844	Castilla-la-Mancha	0,000	0,000	0,000	1,000