Universidade de Aveiro Departamento de Biologia

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Inês de Sousa Gregório

Estrutura genética, diversidade e fluxo genético numa população ameaçada de urso pardo (*Ursus arctos*) na Cantábria, Espanha

Genetic structure, diversity and gene flow on a threatened population of brown bear (*Ursus arctos*) in Cantabria, Spain

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica do Doutor Eduardo Manuel Silva Loureiro Ferreira, Professor Auxiliar Convidado do Departamento de Biologia da Universidade de Aveiro e coorientação da Doutora Tânia Sofia Queirós Barros, Investigadora em Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e do Prof. Doutor Carlos Manuel Martins Santos Fonseca, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro.

“Los osos también dejan huella en la vida.”
FAPAS

o júri

presidente

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palavras-chave

Ursus arctos, ADN mitocondrial, filogeografia, genética populacional, microssatélites, grandes carnívoros, conservação

resumo

Ao longo de vários séculos, a distribuição geográfica do urso pardo na Península Ibérica tem vindo a diminuir, estando de momento limitada ao norte de Espanha. A população de urso pardo da Cantábria é uma das mais pequenas da Europa e está dividida em duas subpopulações (Ocidental e Oriental), com conectividade limitada entre ambas. Para além disso, a perseguição, por parte das populações humanas, apresenta sérias ameaças à sobrevivência da população de urso pardo na Cantábria. Tendo em consideração a situação atual da população Cantábrica, é essencial ter uma imagem muito clara dos padrões genéticos da população. Foram usados três tipos de marcadores genéticos (ADN mitocondrial, microssatélites nucleares autossómicos e marcadores sexuais) para inferir a origem, estrutura e diversidade genética e fluxo genético da população. Os resultados aqui apresentados sugerem que a população Cantábrica está dividida em duas linhagens matrilineares distintas e que não é monofilética relativamente a outras populações europeias. Esta diferenciação, num eixo oriental-ocidental, poderá estar relacionada com eventos de colonização da cordilheira Cantábrica anteriores e contemporâneos ao último máximo glaciar. A população está estruturada em duas subpopulações com grande diferenciação genética entre as duas. Os resultados mostram fortes evidências de migração de ursos entre as duas subpopulações. Nomeadamente, encontramos evidências da existência de fluxo genético assimétrico e de maior fluxo recente de migrantes da subpopulação Oriental para a Ocidental. Contudo, os resultados sugerem uma maior introgressão recente em sentido contrário. Este estudo ajuda a clarificar as origens da população e fornece novo conhecimento sobre a condição genética e os padrões de migração e fluxo genético da população de urso pardo. Os resultados aqui apresentados irão ajudar na definição e implementação de novas estratégias de conservação relevantes para a subsistência de uma população de urso pardo viável na Cordilheira Cantábrica.

keywords *Ursus arctos*, mitochondrial DNA, phylogeography, populational genetics, microsatellites, large carnivores, conservation

abstract Over the centuries, the brown bear geographical distribution in the Iberian Peninsula has been decreasing, being currently limited to the North of Spain. The Cantabrian brown bear population is one of the smallest populations in Europe as is fragmented in two subpopulations (Western and Eastern), with limited connection between them. Additionally, human persecution represents serious threats to the survival of brown bear in Cantabria. Considering the current status of the Cantabrian population, it is essential to have a clear picture of the genetic patterns of the population. We used three molecular markers (mitochondrial DNA, autossomal and sex linked microsatellites) to assess the genetic origins, structure, diversity and gene flow of the Cantabrian brown bear population. Our results suggest that the Cantabrian population is divided in two distinct matrilineal lineages and is not monophyletic relative to other European populations. This differentiation, in an east-west axis might be related with colonization events of the Cantabrian mountains prior and contemporary to the last glacial maximum. The population is structured in two subpopulations with great genetic differentiation between them. The results also show strong evidences of migration between both subpopulations. Namely, we found evidence of asymmetrical gene flow and greater migrant flow from the Eastern to the Western subpopulation. However, results also suggest greater genetic admixture in the opposite way. This study reveals the origins and provides new insights on the genetic condition and migration patterns of the brown bear population. The results here presented will help in the definition of conservation strategies relevant for the maintenance of a viable brown bear population in the Cantabrian mountains.

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Chapter 1. GENERAL INTRODUCTION

The decrease of wildlife over the last decades is astonishing, with the loss of 58% of animal populations since the 1970's (WWF 2016). Anthropogenic causes such as habitat fragmentation for farming and logging, as well as poaching activities are among the main causes of the loss of wild populations.

Large carnivores are one of the most challenging group of species to preserve. During the human history, there has always been a significant hostility towards large carnivore species, which resulted in direct persecution and hunting, leading to a decrease in abundance and distribution of these populations. Additionally, large carnivores typically occur at low densities, have large vital areas and a great dispersal capability (Chapron et al. 2003). Therefore, it is crucial to improve the knowledge on these species to ensure that management and conservation strategies can be more effectively applied.

1.1 *Ursus arctos*. Ecology and Global Distribution

The brown bear (*Ursus arctos* Linnaeus, 1758) is a large carnivore included in the Ursidae family, which is composed by a total of eight species, divided in three subfamilies (Talbot and Shields 1996; Nyakatura and Bininda-Emonds 2012). Morphologically, the brown bear is characterized by its large head with prominent nose, small eyes, small rounded ears and short tail (Fig.1). Its body size depends greatly on habitat conditions and food availability, and it can range between 80kg and 600kg. The bigger specimens are found in coastal Alaska, where spawning salmon is abundant. The species exhibits sexual dimorphism, with adult males being considerably larger and heavier than adult females (Pasitschniak-Arts 1993; Swenson et al. 2000; Swenson et al. 2007).

Figure 1. Photography of a male brown bear (© FAPAS, 2015).

The brown bear is characterized as a generalist omnivorous, and its diet includes herbaceous plants, berries, fruits and nuts, carrion, small mammals, fish, insects and, sporadically, brown bears can prey on livestock (Pasitschniak-Arts 1993; Paralikidis et al. 2010; Ambarll 2016).

During the year, brown bears go through distinct physiological stages: hypophagia (low food intake) during spring, normal activity during summer, hyperphagia (high food intake) during the autumn and hibernation during colder months (Swenson et al. 2000).

The brown bear has a life span of 20 to 25 years in the wild and is a polygamous species, since both males and females have multiple partners during the mating season (Steyaert et al. 2013). Sexual maturation of individuals is late, with females becoming sexually mature at approximately 3 years old and males at 5.5 years old. Females have a reproductive cycle of 2 to 4 years and don't reproduce during all weaning period and until their cubs are completely independent (Pasitschniak-Arts 1993). Brown bears are non-territorial and solitary animals, meaning that social interactions between different individuals only occur during breeding season (Swenson et al. 2000). Chromosome number for this species is 2n=74 (Pasitschniak-Arts 1993).

The brown bear occupies the greatest diversity of habitats among all the bear species, reflecting its adaptive nature. It can be found in arctic tundra, boreal forests, mountains, coastal and desert habitats (Pasitschniak-Arts 1993; Servheen et al. 1999; Swenson et al. 2000). Historically, the brown bear was distributed across North America (including northern Mexico), Europe, North Africa, Middle East and Asia (McLellan et al. 2016). Currently, the species is

widely distributed across the northern hemisphere, from North America to Northeast Asia (Fig.2). Globally, the brown bear population is large (approximately 200.000 individuals), stable and may be increasing in certain areas.

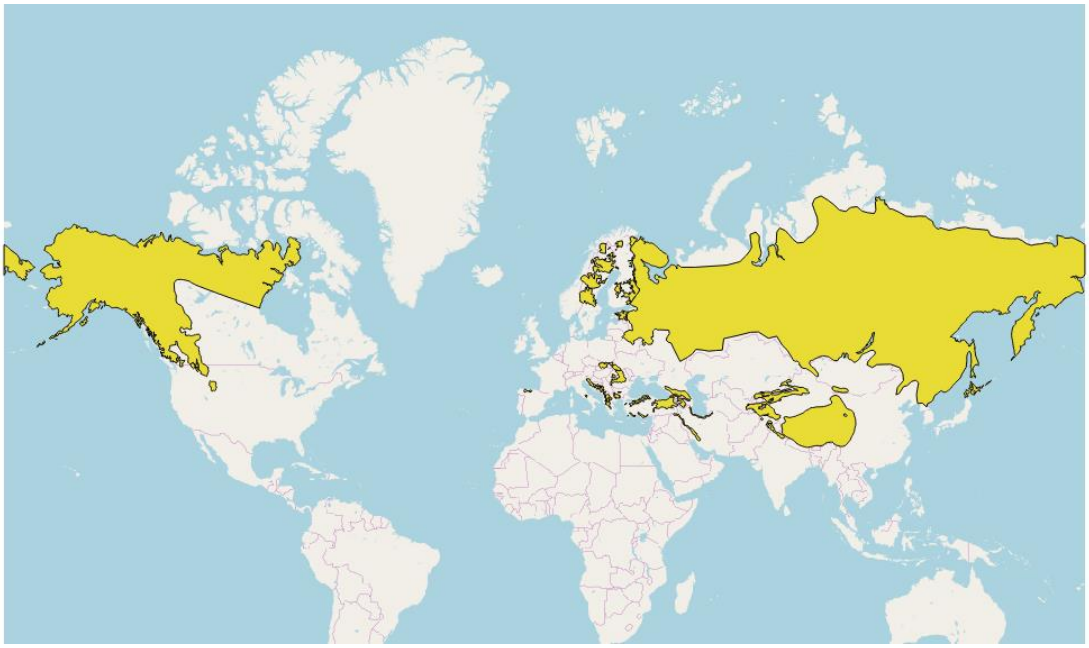

Figure 2. Current global distribution of *Ursus arctos*. Adapted from Mclellan et al. 2016

The brown bear is therefore listed as “Least Concern” by the IUCN Red List (McLellan et al. 2016). However, the species is not equally distributed across its range, with larger and more stable populations in its northern range and smaller fragmented populations in its southern range (Proctor et al. 2005; McLellan et al. 2016). This discrepancy in the distribution of its populations justified the need for IUCN to classify each brown bear population individually. Hence, some populations are classified as Least Concern, like the Kodiak Island population, while others are classified as Endangered or even Critically Endangered, as in the case of the Cantabrian and Alpine populations, respectively (McLellan et al. 2016).

1.2 Use of genetic markers in population studies

The arise of molecular tools contributed in a very significant way to the study of wildlife populations. Several questions concerning the evolution, ecology, conservation or management of a species can be addressed using genetic markers. One of the advantages in using genetic markers is that they provide better data for statistical analysis, as they can be quantified with much precision than other types of ecological measurements (Servheen et al. 1999; Beebee and Rowe 2008). The use of molecular markers can provide insight at: (i) the individual level, including sex determination, relatedness among individuals, probability of assignment to given populations, or even insights on the hybrid or migrant status of an individual; (ii) at population level, with the study of the demographic history, level of structure, diversity or inbreeding of a population; (iii) and at interspecific and community level, with the comparative analysis of phylogeographic patterns among different species (Miller and Waits 2003; DeYoung and Honeycutt 2005; Beebee and Rowe 2008).

The selection of a molecular marker is dependent of several factors. These include the molecular marker suitability to the research question being asked, availability as well as financial or logistic constraints. Genetic markers can be classified according to their genome location, inheritance and mutation rate (DeYoung and Honeycutt 2005). There are different DNA elements used as genetic markers, such as mitochondrial DNA (mtDNA) genes, nuclear microsatellites or single-nucleotide polymorphisms (SNP's) and even *loci* associated with the major histocompatibility complex (MHC) (DeYoung and Honeycutt 2005; Beebee and Rowe 2008). MtDNA is an extra-nuclear part of the genome and is composed by a noncoding control region, 13 protein-encoding genes, 22 transfer RNA (tRNA) genes and two ribosomal RNA (rRNA) genes. In mammals, mtDNA is maternally inherited, has a high mutation rate, when compared to nuclear genes, and is non-recombinant, making it a suitable genetic marker for evolutionary biology, conservation genetics and phylogeographic studies (Beebee and Rowe 2008; Montooth and Rand 2008; Hindrikson et al. 2016). In the case of studies concerning population genetics of brown bear,

mtDNA has been useful in studies of intraspecific phylogeography (e.g. Taberlet and Bouvet 1994; Waits et al. 1998; Salomashkina et al. 2014) and also on the assessment of the evolutionary processes driven by female lineages (Keis et al. 2013).

Microsatellites are autosomal and biparentally inherited markers, widely distributed in the nuclear genome of most eukaryotes and consisting in nucleotide short tandem repeats of 1 to 6 base pairs (Beebee and Rowe 2008; Guichoux et al. 2011). Microsatellites are abundant and have a high mutation rate (10^{-2} to 10^{-5} per generation) which generally results in high levels of polymorphism and high allelic richness (Jarne and Lagoda 1996). Therefore, they are a useful molecular marker to assess population genetics parameters, including genetic structure, inbreeding, gene flow, evidences of bottlenecks, genetic relatedness and genetic drift (DeYoung and Honeycutt 2005; Pérez et al. 2010; Xenikoudakis et al. 2015; Gonzalez et al. 2016). One of the limitations in the use of microsatellites are the strong methodological constraints to compare data between studies due to inconsistencies in allele size length of the different studies (Hindrikson et al. 2016; Torres et al. 2017).

Single nucleotide polymorphisms (SNP's) are a relatively new class of molecular markers and have been recently more common in population genetics studies. SNP's are the most frequent type of variation in the genome and represent a substitution in a single nucleotide (A, T, C or G) (Brumfield et al. 2003; DeYoung and Honeycutt 2005). They have a relatively low mutation rate (10^{-8} - 10^{-9}) and have simpler mutation patterns when compared to microsatellites (Hindrikson et al. 2016). Additionally, SNP's could have a larger statistical power since they allow the simultaneous typing of thousands of *loci*. An advantage in the use of SNP's is that, depending on the screening method, the data generated by single nucleotide polymorphisms are universally comparable. Although the use of SNP's can be very useful in genome-wide association studies, they are not necessarily more powerful in population genetics studies. When addressing questions related to genetic structure or linkage disequilibrium, microsatellites have more informative power than SNP's. For instance, in genetic structure studies, 12 SNP's have the same informative power as four microsatellites, and only five microsatellites are needed to obtain the same genetic information as 20 SNP's, in linkage disequilibrium studies (Guichoux et al. 2011).

Genetic diversity can also be assessed by studying variations in the *loci* encoding proteins for the major histocompatibility complex (MHC). MHC consists of class I and class II genes related with immune response, having an important role in pathogen resistance and kin recognition (DeYoung and Honeycutt 2005; Sommer 2005). MHC diversity is believed to be maintained by pathogen-driven selection and can reflect evolutionary and adaptive processes that would be impossible to address using non-coding genetic markers (Sommer 2005; Hindrikson et al. 2016). MHC markers can be informative in studies of populations that could have suffered demographic bottlenecks or in phylogenetic studies (Wan et al. 2006; Kuduk et al. 2012)

Considering all the potential and applications of genetic markers, a great variety of research questions can be addressed, however, it is essential to consider the most suitable and effective marker for each research question.

1.3 Brown bear in the Iberian Peninsula

The brown bear population in the Iberian Peninsula is currently limited to the North of Spain (Fig.3). Over the centuries, the Iberian brown bear geographical distribution has been decreasing (Clevenger et al. 1999; García-Vázquez et al. 2015). Before the 17th century, the Cantabrian and Pyrenean brown bear ranges were connected, but suffered a separation between the 17th and 18th century, ceasing connectivity between the populations and further isolating both (Nores and Naves 1993). The Pyrenean population suffered a big decline in the 20th century mainly because of hunting, and was estimated to be of only 5 individuals in late 1990's (Taberlet et al. 1997; Arquilliere 1998). Aiming to protect and help the recovery of the Pyrenean brown bear population, a translocation plan was put into action. To guarantee its success, it would have been important to identify the brown bear population that was ecologically, genetically and ethologically closer to the Pyrenean population. However, the translocation action consisted in the release of three bears (two females and one male) from a Slovenian population, in the Pyrenees (Arquilliere 1998; Quenette et al. 2001; Clark et al. 2002).

The Cantabrian brown bear population is currently classified as Endangered by the IUCN Red List (McLellan et al. 2016). This is mainly justified by its isolation from other European brown bear populations, low population size and fragmented nature (Fig.3).

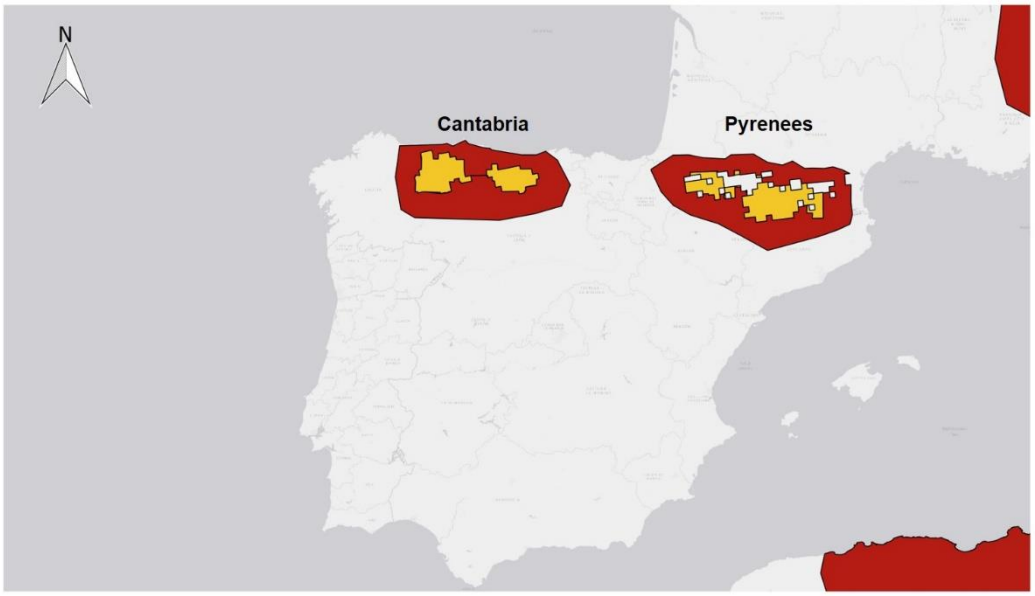

Figure 3. Historical (red) and current (yellow) distribution of brown bear in the Iberian Peninsula. Adapted from Mclellan et al. 2016

Brown bears in the Cantabrian mountains are smaller when compared with other European or Alaskan conspecifics (Swenson et al. 2007; Purroy 2017). Males and females weight on average 115kg and 85kg, respectively, which can be explained by the habitat conditions that can be found in the Cantabrian range, where shrublands and dense deciduous forest covers are predominant (Clevenger et al. 1992; Clevenger et al. 1997; Purroy 2017). The smaller size of the Cantabrian bears could also be related with them inhabiting a region with ancient and strong human presence (and direct bear persecution) such as the Iberian Peninsula (Roberto Hartasánchez, personal communication). In fact, Cantabrian bears are also shyer and less aggressive, which also may be due to a long history of human persecution and hunting (Wiegand et al. 1998; Swenson et al. 2000).

The Cantabrian population is divided in two subpopulations (Western and Eastern), separated by 50km of mountainous terrain and with limited inter-population connection (Mateo-Sánchez et al. 2014). Recent studies estimate

approximately 200 individuals in the western population and 19 individuals in the eastern population (Pérez et al. 2014). The lower number on the Eastern subpopulation could be explained by the fact that the habitat where the Eastern subpopulation resides is more fragmented and less suitable for brown bears when compared with the Western habitat conditions (Mateo-Sánchez et al. 2014).

Over the last years, several studies using genetic tools have been conducted focusing on the brown bear population in Cantabria. Their general aim was to assess genetic patterns, condition and population trends of the population (Pérez et al. 2009; Pérez et al. 2010; Ballesteros et al. 2014; Pérez et al. 2014; Gonzalez et al. 2016). According to these published studies, the genetic condition of the Cantabrian Brown bear population seems to be improving. The two subpopulations are thought to have been previously genetically isolated, without gene flow between them (Pérez et al. 2009). However, the connection between the subpopulations would have been recently established, with reported migration of males from the Western to the Eastern population (Pérez et al. 2010; Gonzalez et al. 2016). There is also evidence of gene flow between both subpopulations since genetically admixed individuals on both subpopulations have been identified (Pérez et al. 2010; Ballesteros et al. 2014; Gonzalez et al. 2016).

The Cantabrian brown bear population faces several threats to its viability and survival. Human persecution, hunting and unintentional killing (with poison aimed at Iberian wolfs, *Canis lupus signatus*, or snares aimed at wild boar, *Sus scrofa*) are major factors potentially affecting these populations. Additionally, the construction of roads and highways crossing brown bear's range can further isolate the two subpopulations (Zedrosser et al. 2001; Purroy 2017). The fragmented nature of these populations overexposes them to reduced gene flow, promoting genetic isolation. Moreover, the Cantabrian mountain range itself exerts a barrier effect towards population connectivity and gene flow (Swenson et al. 2000; Pérez et al. 2014).

General Objectives of this Thesis

The main goal of this study is to provide new insights to help inform the management and conservation strategies for Cantabrian brown bear population. Our approach is based on the analysis of molecular data and will allow us to assess the genetic structure, genetic diversity and gene flow in the Cantabrian brown bear population. In order to accomplish our main goal, we identified four specific objectives, further detailed in chapter 2:

(1) Identify the origins of the Cantabrian brown bear population and its affinities with other European populations;

(2) Confirm the existence of population structure and different subpopulations (in the sense of reproductive units) within the Cantabrian brown bear;

(3) Reassess the level of genetic health of the Cantabrian brown bear population, namely, its genetic diversity, endogamy, genetic structure and effective population size;

(4) Reevaluate the degree of connectivity between the western and eastern populations.

The results of this study will provide new information on the genetic health of this population and will further contribute to the effective management and conservation of the brown bear in Cantabria.

Chapter 2. New Insights on the origins and genetic condition of the Endangered Cantabrian brown bear population

2.1 Introduction

The global population of brown bear (*Ursus arctos*) is widely distributed across the northern hemisphere, with stable numbers and with an increasing trend in terms of population growth (McLellan et al. 2016). However, the southern range of the brown bear is mainly composed by small and fragmented populations that are locally endangered, which is the case of the brown bear population in Cantabria. The Cantabrian brown bear population is one of the smallest populations in Europe, with approximately 220 individuals (Pérez et al. 2014). This population is fragmented in two subpopulations (Western and Eastern) that are separated by a 50km mountain range (Zedrosser et al. 2001; Pérez et al. 2010). Human persecution and poaching represent serious threats to the brown bear population of Cantabria, especially in the Eastern subpopulation (Purroy 2017). Moreover, connectivity between both subpopulations is limited and the construction of roads and highways across brown bears' range can further isolate both subpopulations and, consequently, reduce connectivity and gene flow (Swenson et al. 2000; Pérez et al. 2014; Mateo-Sanchez et al. 2015). Considering the current status of the Cantabrian brown bear population, it is important to have a clear picture of the current genetic patterns of the population in order to infer about conservation needs and management strategies. To assess the genetic structure and diversity of the Cantabrian brown bear, we divided the present study in four main goals.

First, we considered it is pivotal to shed light on the origins and phylogeographic affinities of the Cantabrian brow bear. During the Last Glacial Maximum (LGM), the Iberian Peninsula was one of the three main Mediterranean glacial refuge areas that constituted the source for the postglacial recolonization of central and western Europe (Randi 2007). Several studies concerning the phylogeography of brown bear in Europe reported the existence of two main mitochondrial DNA lineages (namely Western and Eastern) (Randi et al. 1994;

Taberlet and Bouvet 1994; Kohn et al. 1995; Saarma et al. 2007). However, the details of the relations within the putative Cantabrian subpopulations and among these and other Iberian and European populations were not clarified.

Our second goal is to assess the genetic structure and diversity within the Cantabrian brown bear population. Assessing the genetic structure is a pivotal task, since it enables identification of discrete units within a population, that may be important for the demographic stability and genetic diversity of the population (Manel et al. 2005). Revealing the population structure will help to understand the population dynamics and it will constitute a solid first step to answer other questions such as the detection of migrants or gene flow patterns in a structured population (Waits et al. 2000; Kopatz et al. 2012; Xenikoudakis et al. 2015). Considering the existence of two subpopulations separated by a mountain range in the Cantabrian mountains, we expect to distinguish two population units (regardless the existence of phylogeographic differences within the Cantabrian population), corresponding to the Western and Eastern subpopulations (Pérez et al. 2009; Mateo-Sánchez et al. 2014; Gonzalez et al. 2016).

The third goal is to assess the genetic health of the brown bear population in Cantabria. Estimating effective population sizes (N_e), level of endogamy or detecting the occurrence of bottlenecks are important parameters when assessing the genetic health of a population since they influence the genetic diversity of the population. High genetic diversity is normally associated with higher population numbers while small populations are expected to show low genetic diversity (Swenson et al. 2011). The occurrence of a bottleneck can lead to significant declines in population size, making the population susceptible to genetic drift, inbreeding and, ultimately to low genetic diversity of the population (DeYoung and Honeycutt 2005; Beebee and Rowe 2008).

Finally, the fourth goal of our study is to determine at which degree the subpopulations of brown bear in the Cantabrian range are connected. Connectivity between populations and occurrence of gene flow contributes to prevent inbreeding and it ensures the maintenance of genetic diversity within a population (Waits et al. 2000; Kopatz et al. 2012; Xenikoudakis et al. 2015). The brown bear population in the Cantabrian range is supposed to be divided in two isolated subpopulations, with no connectivity between them (Pérez et al. 2009). Yet, it seems this scenario is changing and connectivity between both

subpopulations is being restored. Recent studies have reported the migration of individuals mainly from the Western to the Eastern subpopulation and evidences of gene flow were detected due to the presence of admixture individuals in the Eastern subpopulation (Pérez et al. 2010; Gonzalez et al. 2016). Therefore, we expect to find evidences of connectivity between both subpopulations as well as presence of gene flow.

We trust that the outcomes of this study will provide a broader picture of the genetic condition and health of the brown bear population in Cantabria. These results will aid on the implementation of management and conservation strategies that can guarantee the viability and survival of the Cantabrian brown bear population.

2.2 Materials and Methods

2.2.1 Study area. The Cantabrian mountains

The Cantabrian mountains are located along the Atlantic coast of northwestern Spain. The mountain range runs east to west between 4º-7º longitude west and 42º-43º latitude north, comprising the provinces of Asturias, Cantabria, León, Lugo and Palencia. It has a high geological and geomorphological heterogeneity and a complex topography, with altitudes ranging from sea level to 2647m (García et al. 2005; Mateo Sánchez et al. 2013). The proximity of the mountain range to the Atlantic Ocean results in abundant precipitation and humidity in the northern slope. The northern slope is mostly occupied by the Western brown bear subpopulation and is characterized by narrow and step valleys. Conversely, the southern slope of the Cantabrian mountains is occupied by the Eastern subpopulation and is characterized by wider valleys, with precipitation occurring mainly during winter. Giving its characteristics, the mountain range represents a transition zone between the Eurosiberian and Mediterranean phytogeographic regions (Moreno et al. 1990; Palomero et al. 1997). Forest coverage represents about 25% of the total area and is mainly characterized by beech (*Fagus sylvatica*), oaks (*Quercus pyrenaica*, *Quercus petraea, Quercus ilex*), birch (*Betula alba*), holly (*Ilex*

aquifolium), chestnut (*Castanea sativa*) and hazel (*Corylus avellana*) (García et al. 2005; García et al. 2007). At high altitudes (above 1700m), climatic conditions condition forest growth and the landscape is thus characterized by shrubland (*Juniperus communis*, *Vaccinium uliginosum*, *Vaccinium myrtillus*, *Arctostaphylos uva-ursi*) (García et al. 2005; García et al. 2007). Although the human population density in the Cantabrian mountains is low, human activities resulted in conversion of former forest cover into pasture lands and agricultural lands, which resulted in high fragmented forested areas (García et al. 2005). Brown bears prefer forest habitats for cover and protection, which means that forest fragmentation leads to fewer suitable areas for brown bears and increased vulnerability of bears when traveling between the patchy forested areas.

2.2.2 Sample collection and DNA Extraction

A total of 98 samples (4 tissue and 94 hair samples) were collected in the Cantabrian mountain range, Spain. Samples were collected by experienced field technicians of the Spanish NGO Fondo para la Proteccion de los Animales Salvages (FAPAS), between the years 2010 and 2016. Hair samples were obtained using hair-traps monitored by camera-traps. Tissue samples were stored in ethanol 70% and hair samples were dried and preserved in paper envelopes at room temperature and in a dry environment until further analysis. DNA extraction was conducted using Qiagen® *DNeasy Blood and Tissue Kit*, following manufacturer's recommendations (protocol reference: DY04).

2.2.3 Mitochondrial DNA amplification and Sequencing

A 269bp fragment of mtDNA control region was selected and amplified using the reverse (5'CTCCACTATCAGCACCCAAAG-3') and forward (5'GGAGCGAGAAGAGGTACACGT-3') primers developed by Taberlet and Bouvet (1994). Amplification through polymerase chain reaction (PCR) was performed using Invitrogen® *Taq* DNA Polymerase kit, following the manufacturer's conditions. Reaction mixtures were initially denatured at 94ºC for 3min, followed by 45 amplification cycles (94ºC for 60s, annealing for 60s at 50ºC and extension for 90s at 72ºC) and a final extension step at 72ºC for 10min. PCR

products were visualized on 2% agarose gel and enzymatically purified with EXO-SapIT®. Purified samples were sequenced using a ABIPRISM® 3730-XL DNA Analyser from Applied Biosystems™. Sequences were aligned using MEGA version 7.0 (Kumar et al. 2015) with the CLULTALW algorithm (Thompson et al. 1994) and were manually edited posteriorly.

2.2.4 Microsatellite Amplification and Genotyping

A total of 16 autossomal and two sex linked microsatellite markers. Markers were arranged in four loci multiplexes with five (MU50, MU23, MU59, G10L, SRY), six (G10P, G10J, G1A, MU61, MU51, AMLX/Y), three (G10X, G1D, MU05) and four (G10C, MU64, MU09, MU10) *loci* used in previous studies (Paetkau and Strobeck 1994; Paetkau et al. 1995; Taberlet et al. 1997; Bellemain and Taberlet 2004; Pagès et al. 2009). DNA amplifications were performed using the QIAGEN® Multiplex amplification kit, following manufacturer's conditions. PCR amplifications consisted of denaturing at 95ºC for 10min followed by 38 amplification cycles (94ºC for 30s, annealing for 45s at 57ºC and extension for 90s at 72ºC) with a final extension step of 10 minutes at 72ºC. PCR products were visualized on 2% agarose gel and fragment analysis was performed using an ABIPRISM® 3730-XL DNA Analyser from Applied Biosystems™. Aiming to reduce the chance of mistype, each sample was independently amplified and genotyped a minimum of three times for each *loci*. Locus Mu64 (Taberlet et al. 1997) was excluded from analysis due to poor quality of the amplified products. Microsatellite genotyping was performed using Genemarker™ v2.4.1 (Holland and Parson 2011). Electrophoretograms were analysed using this software. However, allele calling was performed manually and carefully inspected. The identification of individual profiles was assessed only when at least 12 microsatellite markers were successfully amplified.

2.2.5 Data analyses

In order to simplify the understanding of the methodology and data analysis, we decided to divide the data analyses workflow in four different steps, each corresponding to each study aim.

Phylogeographic affinities

To contextualize the phylogeny and phylogeographic affinities of the Cantabrian brown bear population within the European population, 81 mtDNA control region haplotypes from different geographical regions were retrieved from GenBank (Taberlet and Bouvet 1994; Korsten et al. 2009; Kocijan et al. 2011; Salomashkina et al. 2014; Ashrafzadeh et al. 2016; Çilingir et al. 2016; see details in Appendix I) and combined with two haplotypes obtained in this study. Three additional sequences from Asia and North America were also retrieved from GenBank and used as outgroup for Bayesian inference. For each retrieved haplotype, the correspondent number of individuals per haplotype was obtained from the original publication. The defined geographical regions were: *Iberia*, *Apennines*, *Balkans*, *Carpathians*, *Scandinavia*, *Middle East* and *NW Russia, Baltic and Finland*.

A haplotype network was estimated using the software PopART (Leigh and Bryant 2015) using a median-joining algorithm (Bandelt et al. 1999), for reconstruction of possible evolutionary pathways among the different haplotypes. The median-joining network was constructed using equal weights for all mutations and setting the parameter ε to zero to restrict the choice of feasible links in the final network. Phylogenetic relations among brown bear haplotypes, within an European framework, were inferred using a Bayesian approach. A test for the best fitting model was conducted using MrModelTest (Posada and Crandall 2001). The Hasegawa-Kishino–Yano (HKY) model of nucleotide substitution, with a proportion of invariable sites equal to 0.630 and gamma distribution shape parameter equal to 0.667 for among-site variation in substitution rates, was the best fit for the dataset. These parameters were used

as priors in MrBayes 3.2 (Ronquist et al. 2012). Two independent runs of four Markov chain Monte Carlo (MCMC) permutations were performed for 1.000.000 generations, sampling every 100 generations. Tracer 1.6 (Rambaut et al. 2014) was used to summarize Bayesian analyses and to inspect the validity of the burn-in fraction applied. The first 25% of samples were discarded as burn-in, and 50% consensus trees were drawn using FigTree 1.4.0 (Rambaut and Drummond 2012).

Assessment of genetic patterns and structure units

A preliminary analysis of the dataset was made using Genalex 6.5 (Peakall and Smouse 2012) and matches between different samples were identified. The probability of identity ($P_{ID(SIBS)}$) was estimated using the same software, for a minimum of 12 *loci.* It was estimated using a conservative method, assuming a population of siblings, designed for wildlife populations by Waits et al. (2001). When matches between two different samples were detected (corresponding to the same individual), one of the samples was removed from the dataset. All the 15 used *loci* were tested for: deviations from Hardy-Weinberg equilibrium (HWE) using diveRsity R package (Keenan et al. 2013) using an exact Fisher's test; and presence of linkage disequilibrium (LD), using Arlequin version 3.5.1.2 (Excoffier and Lischer 2010). Bonferroni corrections were applied for all multiple tests.

Aiming to detect different structure units within the Cantabrian brown bear population, tests for evidences of genetic structure in the Cantabrian brown bear population were performed in STRUCTURE version 2.3.4 (Pritchard et al. 2000). This program implements a Bayesian algorithm to infer the number of distinct genetic clusters represented in a sampled dataset. We used the admixture model with correlated allele frequencies with no prior information about the original population of each individual. We ran the program for 2 000 000 iterations of the Markov Chain Monte Carlo, with a burn-in of 100 000 steps. The putative number of populations was simulated with K varying from 1 to 6. The analysis was run through 10 repetitions, obtaining a total of 10 replicates for each K. We used Structure Harvester (Earl and vonHoldt 2012) to summarize the results obtained

in STRUCTURE, and estimated the best K using the Evanno method (Evanno et al. 2005).

To assess the partition of the genetic variation among the identified subpopulations, a standard analysis of molecular variance (AMOVA) was calculated for the inferred clusters. Significance of the inferred genetic structure was assessed through pairwise F_{ST} (Wright 1951). All analyses were performed using Arlequin version 3.5.1.2, with 10 000 permutations.

Estimation of genetic and demographic parameters

We estimated number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient F_{IS} using diveRsity R package (Keenan et al. 2013). We tested for evidence of bottlenecks for each inferred cluster with two different softwares, Mratio (Garza and Williamson 2001) and Bottleneck version 1.2.02 (Cornuet and Luikart 1996). In Mratio, M is defined as the ratio between the number k of observed alleles of a given locus and the range r of the distribution of allele sizes for that microsatellite locus. The software calculates an average M value for stable theoretical populations as well as a critical M, above which 95% of the ratios for equilibrium populations are placed. Both average and critical M were calculated considering the same sample size of the studied subpopulations and given the parameters of the model: *ps* - proportion of mutations involving just one repeat unit; Δg - average size of mutations evolving more than one repeat unit; Θ - parameter based on effective population size previous to the bottleneck and mutation rate. A theoretical, conservative parameter values was simulated, with Δg=3.5 (Δg: mean size of larger mutations) and *ps*=0.9 (*ps*: mean % of mutations that add or delete only one repeat) (Garza and Williamson 2001). The parameter Θ was allowed to vary over several orders of magnitude (0.01; 0.1; 1 and 5) to account for a wide range of mutation rates and pre-bottleneck effective population sizes.

The method implemented in Bottleneck software is based on the detection of heterozygosity excess relative to the number of alleles, across all *loci*, that is expected to build after a bottleneck. It is expected that if a considerable number of *loci* presents a heterozygosity excess, the population may have suffered a

recent bottleneck. Simulations were made using a two-phased model (T.P.M), with 70% S.M.M., 20% variance and 1 000 replicates. Wilcoxon sign-rank tests were applied to determine significance of each model.

To estimate the effective population size (N_e) we used the linkage disequilibrium method (Waples and Do 2008) and the molecular co-ancestry method (Nomura 2008) to estimate the effective number of breeders (N_{eb}). Both methods were implemented in NeEstimator v2 software (Do et al. 2014). The 95% confidence intervals for both methods were obtained via Jackknife method and estimates for the linkage disequilibrium method excluded all alleles with a frequency of <0.05, to correct for known biases from rare alleles.

Connectivity and gene flow between subpopulations

An estimation of the likelihood of assignment of individual genotypes to both Western and Eastern subpopulations was made using Genalex 6.5. Detection of migrants and hybrids between subpopulations was performed based on the results of STRUCTURE version 2.3.4 and NEWHYBRIDS 1.0 (Anderson and Thompson 2002). Analysis with NEWHYBRIDS included all individuals from Cantabria, with no prior information about geographic origin or putative parent population. The analysis was ran considering two parental classes and four hybrid (F1, F2 and both backcrosses) classes. Three replicate runs were performed, with burn-in lengths of 50 000 and run lengths of 100 000 iterations. Results from individual posterior probabilities of assignment to each parental or hybrid class were tested for convergence among the different replicate runs. To estimate the level and the symmetry of gene flow among the western and eastern subpopulations, we estimated a relative migration network using the function *divMigrate* of diveRsity R package. This function implements a method described by Sundqvist et al. (2016) and plots the relative migration level between population samples, estimated from the microsatellite allele frequency data. The significant relative migration network was estimated based on a bootstrap procedure with 50 000 replicates.

2.3 Results

Success Rates

Of the 98 samples, 93 could be amplified for at least one of the genetic markers used in this study, resulting in a DNA isolation success rate of 95%. We obtained 78 mitochondrial DNA sequences (mitochondrial DNA amplification success rate of 80%) and 79 samples amplified for at least one microsatellite marker (microsatellite amplification rate of 81%). We obtained a reliable genotype, based on at least 12 microsatellite markers, for 65 of the samples, (genotyping success rate of 66%). Additionally, samples with matching unique genotypes were considered as recaptures and removed from the following analysis. A total of 7 samples from the western population were identified as recaptures. In the final dataset, we considered a total of 57 unique genotypes, corresponding to 43 and 14 samples from the Western and Eastern subpopulations, respectively. Out of these 57 genotypes, 56 were based on the information of at least 14 *loci*. The probability of identity, considering a siblings population, for the whole Cantabrian population, was 9.2×10^{-4}, for 12 *loci*, and 1.5×10^{-4}, for the whole set of 15 *loci*.

Phylogeographic affinities

A total of 78 new sequences were generated for the mtDNA control region, with 269bp in length (including recaptures). Among these 78 Cantabrian brown bear sequences, two haplotypes were identified (WeC and EaC) (Fig. 4b). Haplotype WeC was found only in samples collected in the Western subpopulation (n=57). The haplotype EaC was recovered in all samples collected in the Eastern subpopulation (n=14) as well as in other seven samples that were collected in the Western subpopulation.

In the median-joining network generated using both the newly generated sequences and the 81 haplotypes retrieved from Genbank (Fig. 4c), haplotype WeC corresponded to haplotype Can previously reported by Taberlet and Bouvet (1994). Haplotype EaC was recorded for the first time in this study and is more closely related to haplotype Pyr, from the Pyrenees, than to haplotype WeC,

separated by one and three mutational steps, respectively. All haplotypes from the Iberian Peninsula appear to be more related with those from southern Scandinavia, as previously reported in other studies, than to haplotypes from other southern European peninsulas (Taberlet & Bouvet 1994, Saarma et al. 2007). Brown bear haplotypes from Europe are divided in two groups: one corresponding to NorthEast Europe (NWRussia and Carpathians); and another to South and Western Europe (Iberian, Apennine, Balkans and southern Scandinavia). Both groups are connected through haplotypes from the Middle East (which includes sequences from Iran and Turkey). The relation between EaC and Pyr is strongly supported by Bayesian inference (Fig. 4a, complete phylogeny in Appendix II), with a posterior probability of 100%. Haplotypes from south and western Europe appear to be arranged in two major clades, as previously reported (Taberlet and Bouvet 1994), although the support for these clades is not significant. One of the clades includes haplotypes from the Iberian Peninsula and southern Scandinavia and other clade includes haplotypes from the Balkans and Apennine mountains.

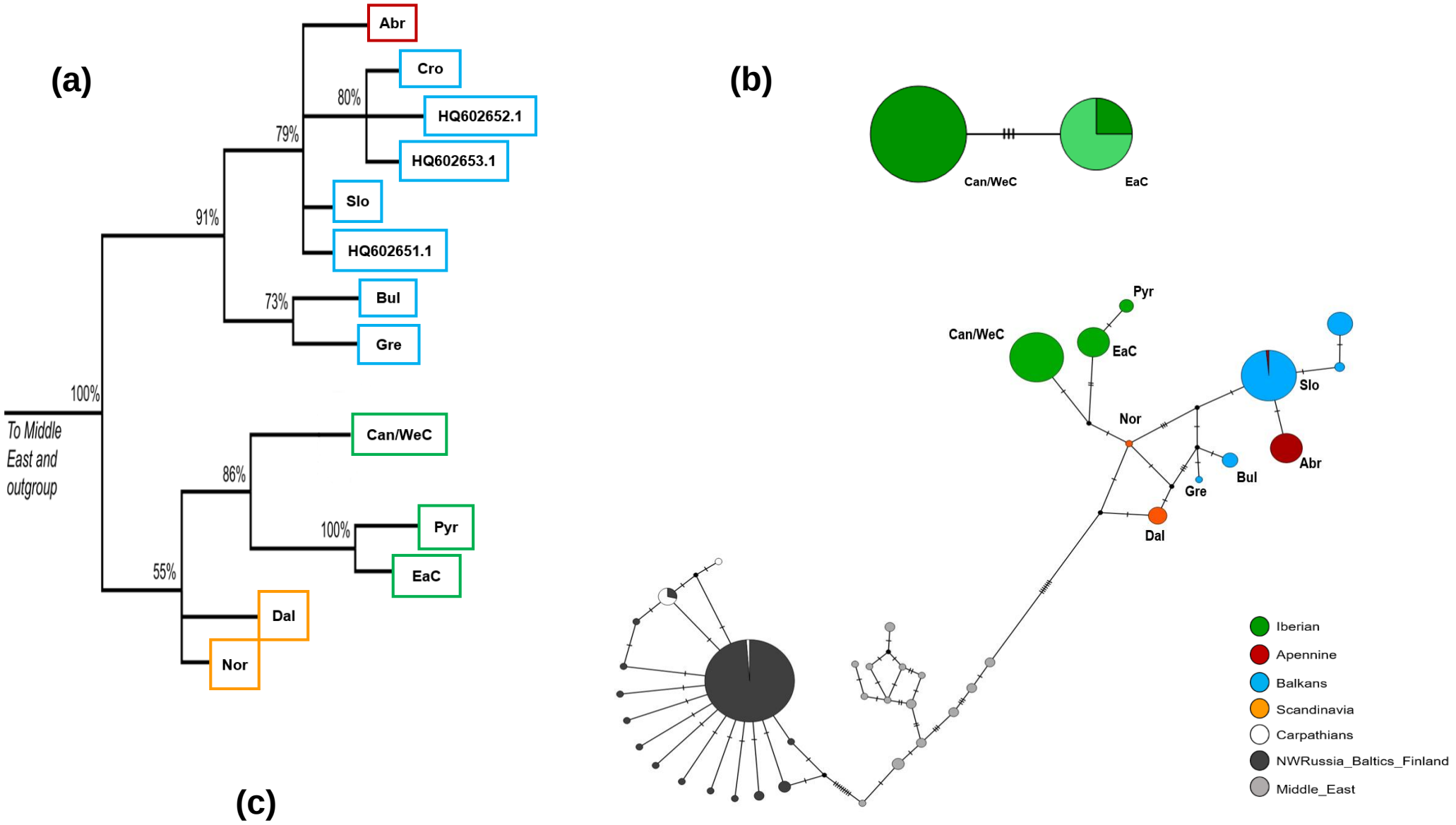

Figure 4. Phylogenetic and phylogeographic affinities of the Cantabrian brown bear, within European brown bear populations. **(a)** Detail of the Bayesian inference tree based on 83 brown bear haplotypes from Europe and Middle East. The scale bars indicate expected number of changes by site. Values at nodes are posterior probabilities. Haplotypes are colour-coded according to the geographic origin. **(b)** Median-joining network of the two mtDNA haplotypes detected in the Cantabrian population. Dark green corresponds to samples collected in the western subpopulation and light green corresponds to samples collected in the eastern subpopulation. **(c)** Median-joining network of 83 brown bear mtDNA haplotypes from Europe and Middle East. Haplotypes are colour-coded according to geographic origin, in agreement with the nomenclature given by Taberlet & Bouvet (1994). Iberian haplotypes were named “WeC” and “EaC” according to the region of origin in Cantabria. Mutational steps between haplotypes, in median-joining networks, are represented by dashes.

Genetic structure

When considering the Cantabrian population as a whole, three *loci* showed departure from Hardy-Weinberg equilibrium (HWE) conditions and 21 out of 105 pairwise *loci* combinations showed linkage disequilibrium (Table 2), after Bonferroni correction. When both West and East subpopulations were analyzed separately, deviations to HWE and linkage disequilibrium were substantially reduced: 1 and 0 loci showed departure from HWE, respectively; in both subpopulations, 2 out of 105 pairs of *loci* showed significant linkage disequilibrium, after Bonferroni correction (Table 2).

The Cantabrian population was consistently divided in two distinct genetic clusters (K=2), based on the 10 replicate runs for each K, performed with STRUCTURE (Fig. 5), suggesting the existence of two gene pools in the Cantabrian brown bear population. The *Q* proportions of the individual genotypes assigned to each of the inferred genetic clusters were also highly convergent among replicate runs. There was a strong agreement among the inferred genetic clusters and the geographic origin of sampled individuals (West and East Cantabria). Therefore, each genetic cluster was nominated West and East, corresponding to both sampling areas and known subpopulations. Individual genotypes were mostly assigned to the genetic cluster corresponding to the subpopulation where the individuals were sampled. However, 6 individuals (8OC, 14OC, 71OC, 77OC, 92OC and 93OC) sampled in the Western subpopulation were assigned (<95%) to the Eastern genetic cluster. These individuals also presented the Eastern subpopulation haplotype (EaC).

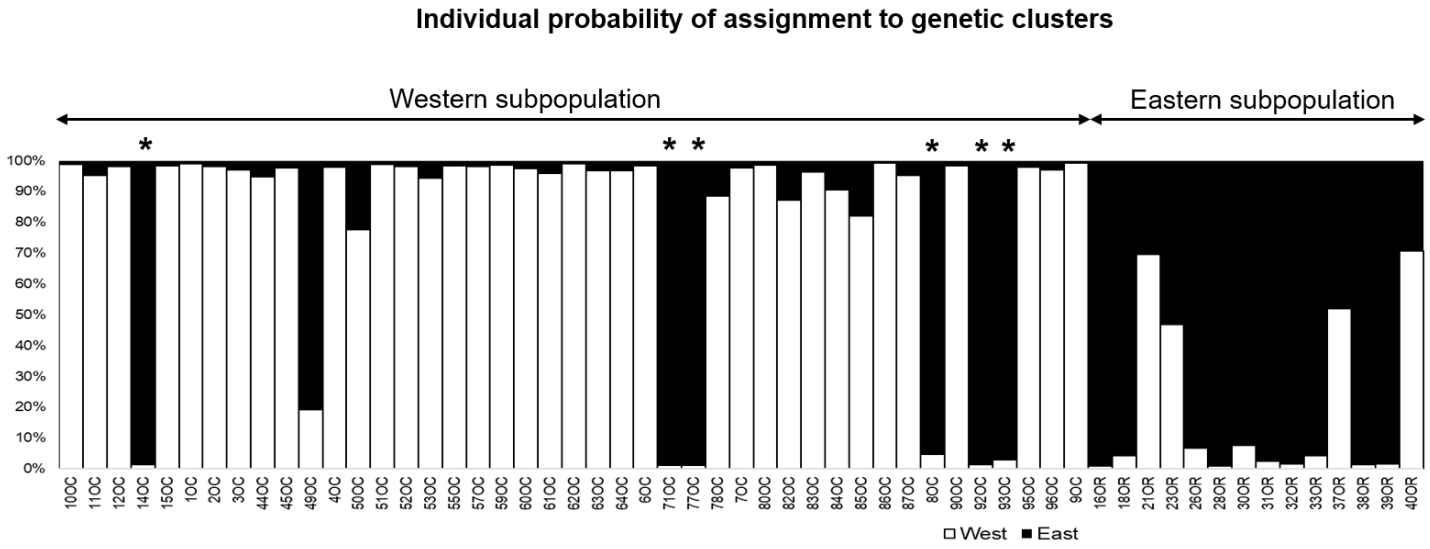

Figure 5. Proportion of each individual genotypes assigned to each genetic cluster (West – white; East – black) inferred in STRUCTURE (for best K=2). Individuals identified as migrants are marked with an asterisk.

Genetic distance (F_{ST}) (Table 1) between Western and Eastern subpopulations was significant ($p<0.001$), with a value of 0.175 (when confirmed recent migrants were excluded from analysis) and 0.167 (when migrants were included in Eastern subpopulation). According to Wright (1978), these values indicate a great genetic differentiation between both subpopulations. In either case, structuration of the Cantabrian population in Western and Eastern subpopulations was significant ($p<0.001$). When migrants were removed from the analysis, 85.6% of the total genetic differentiation was attributed to differences within individuals and 17.5% to differences among subpopulations. When migrants were included in the Eastern subpopulation, 87.9% of the total genetic differentiation is attributed to differences within individuals and 16.7% to differences among populations.

Table 1. Genetic differentiation of the two Cantabrian subpopulations

		SubPopulation	
		Western vs Eastern	***Western vs Eastern with Migrants***
AMOVA	F_{ST}	*0.175*	*0.167*
	Variation within individuals	85.6%	87.9%
	Variation among pops	17.5%	16.7%

Estimation of genetic and demographic parameters

The average number of alleles was higher in the Western subpopulation (3.06) than in the Eastern subpopulation, either excluding (2.73) or including (2.87) migrants sampled in the Western Cantabria. When considering the total Cantabrian population, the average number of alleles was higher (3.53) (Table 2). Rarefied allelic richness was also higher in the Western subpopulation (2.76) than in the Eastern subpopulation with (2.63) or without migrants (2.56) (Table 2). The expected heterozygosity (H_E) was higher in the Western subpopulation (0.470) than in the Eastern subpopulation, that presented the same value either excluding or including migrants (0.460). The observed heterozygosity (H_O) was equal (0.500) in the Western and Eastern (including migrants) subpopulations. The total Cantabrian population exhibits a significant heterozygosity deficit (H_E>H_O), most likely related with the presence of structure. The inbreeding coefficients were slightly negative in the Western subpopulation (-0.065) and in the Eastern subpopulation including migrants (-0.071). The Eastern subpopulation without the migrants has a small and positive, but not significant, inbreeding coefficient (0.010) (Table 2).

Estimations of effective population size (N_e) for the total Cantabrian population were not considered since population structure can affect LD and, consequently, N_e estimations using the Linkage Disequilibrium method. Effective population size estimations varied from 2.0 in the East subpopulation and 24.8 in the West population. Effective number of breeders (N_{eb}) ranged from 2.8 and 11.5 in the total population and East with migrants, respectively (Table 2).

Significant evidences of a bottleneck (M value of sample significantly lower than critical M_c value) was found for the total Cantabrian brown bear population and all the considered subpopulations. The excess of heterozygosity that is expected in bottlenecked populations (Cornuet and Luikart 1996) was observed in all the subpopulations and in the total Cantabrian population, considering both sign and Wilcoxon tests (Table 2). The excess was significant ($p<0.05$) in all cases for the Wilcoxon test, and significant ($p<0.05$; Western subpopulation) or marginally significant ($p<0.1$; all other cases) for the sign test.

Table 2. General genetic diversity indices for 2 brown bear subpopulations, based on 15 microsatellite markers. Number of loci or pairs of loci with significant deviations to HW and linkage equilibrium conditions, after Bonferroni correction are indicated. Significant values in italics.

		Population or sub-population			
		Cantabria n=57	***West n=37***	***East n=14***	***East with Migrants n=20***
Structure	Loci in HWD	3/15	1/15	0/13*	1/13*
	LD (pair of loci in LD)	21	2	2	5
Genetic Diversity	A	3.53	3.06	2.73	2.87
	A_r	3.04	2.76	2.56	2.63
	Gene Diversity	0.534	0.481	0.485	-
	H_E	0.520	0.470	0.460	0.460
	H_O	0.500	0.500	0.460	0.500
Endogamy	F_{IS}	0.046	-0.065	0.010	-0.071
Effective Population Sizes	N_e (95% CI)	-	24.8 (13.8-53.8)	2.0 (1.5-2.6)	2.7 (2.1–4.0)
	N_{eb} (95% CI)	2.8 (2.0-3.7)	9.0 (2.2-20.5)	5.3 (2.1-9.9)	11.5 (1.4–32.0)
Bottlenecks	Mratio	*0.599*	*0.658*	*0.643*	*0.638*
	Heterozygosity Excess** (p values)	*0.008/<u>0.001</u>*	0.089/*<u>0.015</u>*	0.061/*<u>0.001</u>*	0.058/*<u>0.002</u>*

Abbreviations: HWD, Hardy-Weinberg disequilibrium; LD, Linkage disequilibrium; A, Number of alleles; A_r, Allele richness (rarefied); H_E, expected heterozygosity; H_O, observed heterozygosity; F_{IS}, inbreeding coefficient; Ne, effective population size; Neb, effective number of breeders;

* - two monomorphic loci
**- Significance of excess: p values of Sign/<u>Wilcoxon test</u> under two phase model (TPM)

Connectivity and gene flow between subpopulations

Assignment of individuals to their putative source subpopulations was has expected, with some exceptions. Seven individuals (8OC, 14OC, 49OC, 71OC, 77OC, 92OC, 93OC) sampled in the Western subpopulation territory where assigned to the Eastern subpopulation (Fig. 6). One individual (40OR) captured in the Eastern population territory, was assigned to the Western subpopulation (Fig. 6), while other two (21OR and 23OR) had very close assignment probabilities for both populations. Since there is some difference in the sampling sizes of the Western and Eastern subpopulations, assignment tests were repeated for rarefied samples of the Western subpopulations. The same pattern of assignment was obtained in the assignment tests using rarefied samples.

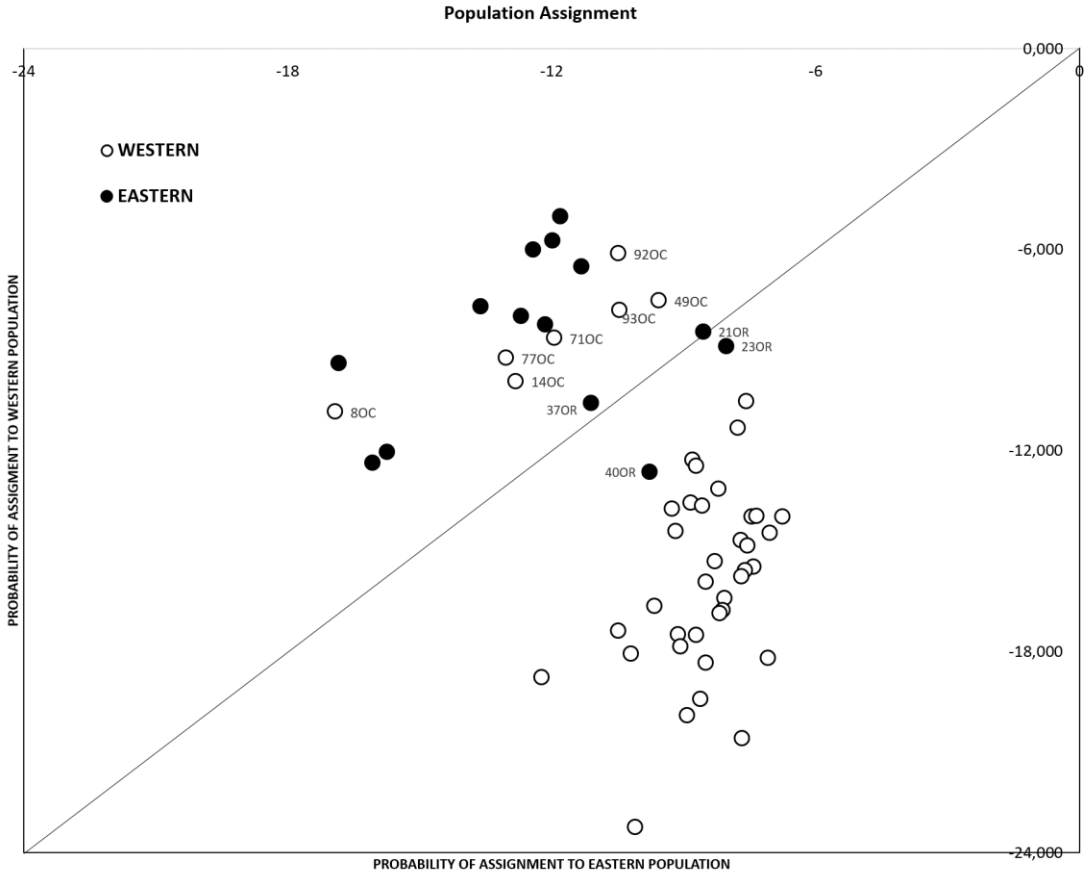

Figure 6. Population assignment for Western and Eastern subpopulations.

Most of the individuals were assigned to their putative parental subpopulation, with some exceptions. Six individuals sampled in the Western subpopulation (8OC, 14OC, 71OC, 77OC, 92OC, 93OC), but bearing the EaC mtDNA haplotype, were assigned with high probability (>95%) to the East parental class (Fig. 7). Another individual bearing the EaC (49OC) was not clearly assigned to the West parental class, being assigned to the East parental class (62%), or to hybrid classes (32%). Two individuals (21OR, 40OR) sampled in the territory of Eastern subpopulation (and with haplotype EaC) were assigned with high probability (> 95%) to the West parental class (21OR: 63%; 40OR: 58%) or to one of the hybrid classes (21OR:33%; 40OR: 40%). Another two individuals (23OR and 37OR) revealed the same pattern, but probability of assignment to other class, rather their putative parental class, was bellow 95%.

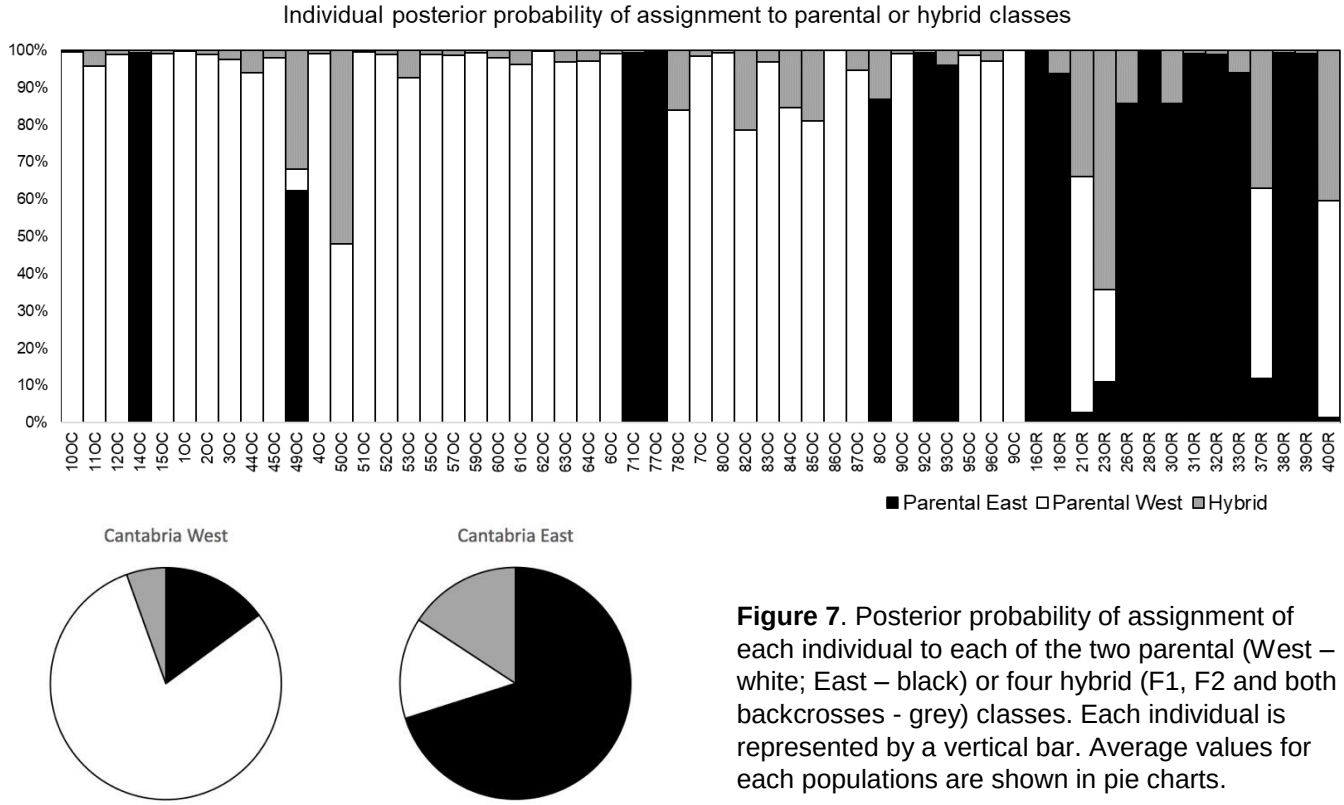

Figure 7. Posterior probability of assignment of each individual to each of the two parental (West – white; East – black) or four hybrid (F1, F2 and both backcrosses - grey) classes. Each individual is represented by a vertical bar. Average values for each populations are shown in pie charts.

The analysis of migration dynamics revealed the same patterns, regardless of the differentiation statistic. There are relative migration flows between the Western and Eastern subpopulations. However, the relative migration is asymmetric since its only significant when occurs from the Eastern to the Western subpopulation (Fig. 8).

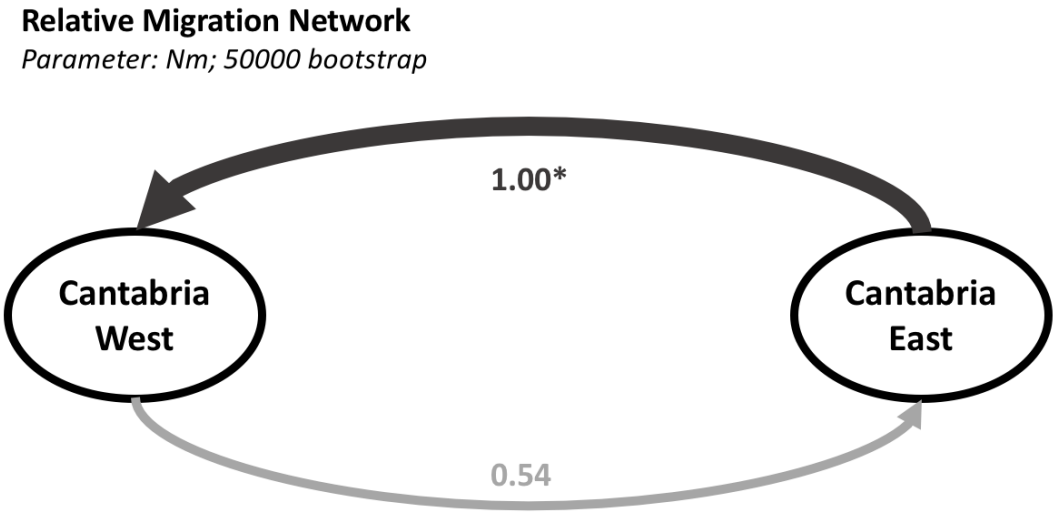

Figure 8. Relative migration network between the western and eastern subpopulations.

Discussion

Origins and phylogeographic affinities

The results here presented help to clarify the phylogeographic relations within the putative Cantabrian subpopulations and with other Iberian populations. Previous studies reported the existence of two mitochondrial DNA lineages in Europe, corresponding to Western and Eastern lineages. In those studies, the Cantabrian brown bear population was included in the Western lineage, closely related to the Pyrenean population (Randi et al. 1994; Taberlet and Bouvet 1994; Kohn et al. 1995; Saarma et al. 2007). Although, the relations within the putative Cantabrian subpopulations were not clarified.

According to the mtDNA analysis, the Cantabrian brown bear population is divided in two distinct lineages, one corresponding to the haplotype Can/WeC and other corresponding to haplotype EaC. Haplotype EaC is more related to haplotype Pyr, previously reported in Taberlet & Bouvet (1994), than to Can/WeC, which means that the Eastern subpopulation is more closely related with the historical brown bear population of the Pyrenees. The current Pyrenean population resulted from the translocation of individuals from Slovenia in 1995 and, currently, there is no evidence that the original Pyrenean population has persisted after the translocation. It is likely that the current Pyrenean brown bear population is genetically more similar to the Slovenian population (Taberlet et al. 1997; Arquilliere 1998; Quenette et al. 2001), and the closest population to historical Pyrenean bear is actually the Eastern Cantabrian population.

During the Last Glacial Maximum (LGM), several mammal species found refuge in southern European peninsulas (Randi 2007). In some species, mtDNA phylogenetic patterns show a differentiation within peninsulas, with some populations being more related to central and north European populations than to other peninsular populations, namely in Iberian Peninsula (wild boar: Veličković et al. 2015; Veličković et al. 2016; roe deer: Randi et al, 2004; Royo et al, 2010). For this species, as for brown bear, an east-west differentiation axis is found in northwestern Iberia. The phylogeographic patterns are consistent with

the entrance, in the peninsulas, from populations fleding from northern regions, during the last glacial maximum (LGM), that pushed the pre-LGM populations into the peninsulas (Veličković et al., 2015). Since these populations persisted in the peninsulas, it is possible today to observe the existence of phylogenetic lineages with different affinities. Similarly, it is possible that the differences within the Cantabrian brown bear population could result from identical population dynamics occurred before and during the LGM. In this sense, Western Cantabrian population (represented by the haplotype WeC) should represent the remnant of the pre-LGM Cantabrian populations (pushed westward during the LGM). The Eastern population (represented by EaC) should descend of bears colonizing the Cantabrian mountains secondarily, coming from the Pyrenees. It is important to notice that despite being closer to the Pyr haplotype, the EaC differs from this by one mutational step, again consistent with the pattern observed in wild boar (Veličković et al., 2015). Despite the distinct origins of both Cantabrian subpopulations, this scenario does not invalidate the possibility of past gene flow between both subpopulations, that in brown bears is mediated by male dispersal and should not influence the pattern of matrilineal (mtDNA) lineages.

Genetic structure, diversity and health

The results showed that the Cantabrian brown bear population is structured in two genetic clusters, corresponding to Western and Eastern putative subpopulations, with great genetic differentiation between both. This is consistent with previous results obtained in other studies and can be explained by the division of the Cantabrian population into two subpopulations with limited connection, occurred nearly a century ago (Nores and Naves 1993; Pérez et al. 2010; Mateo-Sánchez et al. 2014; Gonzalez et al. 2016).

The genetic diversity of both Cantabrian brown bear subpopulations appears to have been increasing over the years (Table 3). However, the observed diversity is low, when compared with other European populations, such as the Scandinavian brown bear population (H_o=0.82) (Kopatz et al. 2014).

Evidences of bottleneck were detected in the Cantabrian brown bear population, which can explain the observed low genetic diversity. Higher genetic diversity is normally associated with stable populations, with higher population numbers, as the ones observed in the Scandinavian brown bear population (Waits et al. 2000; Xenikoudakis et al. 2015). Therefore, the low genetic diversity observed in the Cantabrian population can be related with its isolation from other European brown bear populations and fragmented nature (McLellan et al. 2016). Moreover, the low population numbers observed in the Cantabrian population can contribute to lower genetic diversity. Recent studies estimate approximately 200 individuals in the western population and 19 individuals in the eastern population (Pérez et al. 2014). We identified a minimum number of 37 individuals in the Western population and a minimum number of 14 individuals in the Eastern population (20 individuals, if East-West migrants are considered). Among other causes of decline, it is possible that Eastern population is losing migrants to the Western population. Our estimates show a large difference also in the effective population sizes of Western (N_e=24.8) and Eastern (N_e=2.0) subpopulations. Notwithstanding, we suggest that these results should be cautiously interpreted. There are several methods for the estimation of effective population sizes with different time scales and initial assumptions (Wang 2005). A violation on the initial assumptions of the method can biases greatly N_e estimations, possibly leading to under or overestimations of effective population sizes.

Table 3. Summary of the genetic diversity and endogamy levels of the Cantabrian brown bear subpopulations obtained in past studies and this study.

	Period of study (years)	*No. of genotypes used*	H_o	F_{IS}	*Reference*
Western subpopulation	2006-2008	31	0.44	-	Pérez et al. 2009
	2010-2016	43	0.50	-0.065	This study
	2013-2014	12	0.49	0.026	Gonzalez et al. 2016
Eastern Subpopulation	2006-2008	9	0.28	-	Pérez et al. 2009
	2010-2016	14	0.50	-0.071	This study
	2013-2014	26	0.54	0.038	Gonzalez et al. 2016

Gene Flow and dispersal of individuals

The results show solid proof of migration between Western and Eastern subpopulations. There is evidence of migration of bears from the Eastern to Western subpopulation, since six individuals sampled in the Western subpopulation were assigned with high probability to the Eastern subpopulation. All migrant were males (see Appendix III) and they all presented haplotype EaC, corresponding to the Eastern matrilineal lineage identified in the Cantabrian population. However, our results also show higher level of hybridization in the Eastern subpopulation, suggesting migration of potentially mating individuals from the western to the eastern subpopulation. Distribution of allelic frequencies suggests long-term asymmetrical gene flow from the Eastern to the Western subpopulation, contradicting previous studies that reported gene flow from the Western to the Eastern subpopulation (Pérez et al. 2010; Gonzalez et al. 2016). These results, considered together, support the idea that movement of individuals from one subpopulation to another, does not necessarily reflect gene flow.

The Western population is considerably larger than the Eastern subpopulation, meaning that the previously recorded Western-Eastern gene flow would allow the recovery of the Eastern subpopulation, with the entrance and reproduction of individuals from the Western subpopulation. However, the results here obtained showed strong evidences of migration of males from the Eastern to the Western subpopulation, opposing the gradient of population density.

From the ecological point of view, this result could seem contradictory, as it would be assumed that populations more stable and with higher number of individuals (Western) function as a source population and populations less stable and more fragmented (Eastern) would work as sink population. Nevertheless, we present three alternative and not mutually exclusive hypothesis that could explain the migration of bears from Eastern to Western subpopulations. 1) Since we detected only males in the Eastern subpopulation (see Appendix III & IV), the sex ratio is clearly more favorable to males in the Western subpopulation (9 females: 25 males), which may lead to the dispersal of males to Western territories, were the number of females is higher; 2) Habitat conditions may be asymmetrical in Western and Eastern areas. If habitat is more suitable in the Western area, carrying capacity may be higher in this area, which may justify the movement and settlement of individuals, both males and females, in the Western subpopulation; 3) If human disturbance and poaching activities are more intense in the Eastern area, it is reasonable that individuals from the Eastern subpopulation disperse towards the Western areas, escaping from human persecution and searching for habitats with less human interference. These hypotheses show that the corridor promoting geneflow between both subpopulations may be functioning in the inverse direction to what was expected, leading to the movement of brown bears from the Eastern subpopulation to Western areas. These outcomes may justify the rethinking of conservation measurements applied in the Cantabrian brown bear population. Additional to the creation of ecological corridors between both subpopulations, it is necessary to restore habitat conditions, control poaching activities, consequently improving the sex ratio and the settlement of individuals in the Eastern subpopulation.

The results from this study revealed the origins and provided new insights on the genetic condition and migration patterns in the Cantabrian brown bear population. This will further help on the evaluation of conservation strategies implemented for the brown bear population in Cantabria and in the definition of new strategies relevant for the maintenance of a viable brown bear population in the region.

Chapter 3. Final Considerations

As mentioned in the previous chapter, this study provided new insights on historical and current population dynamics of the brown bear in Cantabria. The relations within the putative Cantabrian subpopulations were clarified, with the identification of two distinct matrilineal lineages that may have been separated due to population dynamics before and during the Last Glacial Maximum. The low genetic diversity observed in the Cantabrian population may be explained by the occurrence of bottlenecks and low population numbers, in addition to the complete isolation of the Cantabrian population from other European brown bear populations. But the most striking result must be the detection of asymmetrical gene flow against the population density gradient, which contradicts previous studies (Pérez et al. 2010; Gonzalez et al. 2016). This result allowed the formulation of new hypothesis that should be adressed and clarified in future studies. Are the Cantabrian brown bear recent migration patterns different from historical ones? If there is, in fact, a shift on the asymetry of migration flow, what are the drivers of this shift? Is it mainly driven by sex ratio? Or is this migration pattern driven by differences in habitat suitability and carrying capacity or direct human persecution? An increase in the number of genotyped individuals, with a particular focus on the Eastern subpopulation will help answering these questions. Additionally, complementary approaches as linking the patterns of bear and gene flow with landscape features, will help clarify the detected patterns.

Efforts for the conservation of the brown bear in the Cantabrian mountains are being made by several organizations, including FAPAS (Fondo para la Protección de los Animales Salvages). In the particular case of FAPAS, this NGO is working on the conservation of brown bears for 35 years and have built an impressive amount of information and knowledge on the demographics, population dynamics and behaviour of the Cantabrian brown bear population. We expect the results obtained in this study, together with this comprehensive field knowledge, will allow a more accurate and insightful evaluation of current implemented conservation strategies. Surely it has raised several new questions relevant for the effective management of the Cantabrian brown bear population.

References

Ambarlı, H. (2016). Litter size and basic diet of brown bears (*Ursus arctos*, Carnivora) in northeastern Turkey. *Mammalia*, *80*(2), 235-240.

Anderson, E. C., & Thompson, E. A. (2002). A model-based method for identifying species hybrids using multilocus genetic data. *Genetics*, *160*(3), 1217-1229.

Arquillière, A. (1998). Experimental reintroduction of brown bears in the French Pyrénées. *Oryx*, *32*(1), 8-10.

Ashrafzadeh, M. R., Kaboli, M., & Naghavi, M. R. (2016). Mitochondrial DNA analysis of Iranian brown bears (*Ursus arctos*) reveals new phylogeographic lineage. *Mammalian Biology-Zeitschrift für Säugetierkunde*, *81*(1), 1-9.

Ballesteros, F., Blanco, J., Alcaraz, L. (2014). Estudio genetico del oso pardo en el corredor interpoblacional y en la subpoblacion oriental cantabrica. *Technical report*.

Bandelt, H. J., Forster, P., & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular biology and evolution*, *16*(1), 37-48.

Bellemain, E., & Taberlet, P. (2004). Improved noninvasive genotyping method: application to brown bear (*Ursus arctos*) faeces. *Molecular Ecology Resources*, *4*(3), 519-522.

Brumfield, R. T., Beerli, P., Nickerson, D. A., & Edwards, S. V. (2003). The utility of single nucleotide polymorphisms in inferences of population history. *Trends in Ecology & Evolution*, *18*(5), 249-256.

Chapron, G., Quenette, P. Y., Legendre, S., & Clobert, J. (2003). Which future for the French Pyrenean brown bear (*Ursus arctos*) population? An approach using stage-structured deterministic and stochastic models. *Comptes rendus biologies*, *326*, 174-182.

Çilingir, F. G., Akın Pekşen, Ç., Ambarlı, H., Beerli, P., & Bilgin, C. C. (2016). Exceptional maternal lineage diversity in brown bears (*Ursus arctos*) from Turkey. *Zoological Journal of the Linnean Society*, *176*(2), 463-477.

Clark, J. D., Huber, D., & Servheen, C. (2002). Bear reintroductions: lessons and challenges. *Ursus*, 335-345.

Clevenger, A. P., Purroy, F. J., & Pelton, M. R. (1992). Brown bear (*Ursus arctos* L.) habitat use in the Cantabrian Mountains, Spain. *Mammalia*, *56*(2), 203-214.

Clevenger, A. P., Purroy, F. J., Cienfuegos, J. N., & Quesada, C. N. (1999). Status and management of the brown bear in eastern and western Cantabria, Spain. *C. Servheen, S. Herrero, and B. Peyton, compilers. Bears. Status survey and conservation action plan. IUCN/SSC Bear and Polar Bear Specialist Groups. IUCN, Gland, Switzerland and Cambridge, UK*, 100-110.

Clevenger, A. P., Purroy, F. J., & Campos, M. A. (1997). Habitat assessment of a relict brown bear *Ursus arctos* population in northern Spain. *Biological Conservation*, *80*(1), 17-22.

Cornuet, J. M., & Luikart, G. (1996). Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, *144*(4), 2001-2014.

DeYoung, R. W., & Honeycutt, R. L. (2005). The molecular toolbox: genetic techniques in wildlife ecology and management. *Journal of Wildlife Management*, *69*(4), 1362-1384.

Do, C., Waples, R. S., Peel, D., Macbeth, G. M., Tillett, B. J., & Ovenden, J. R. (2014). NeEstimator v2: re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. *Molecular Ecology Resources*, *14*(1), 209-214.

Earl, D. A. (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation genetics resources*, *4*(2), 359-361.

Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular ecology resources*, *10*(3), 564-567.

García, D., Quevedo, M., Obeso, J. R., & Abajo, A. (2005). Fragmentation patterns and protection of montane forest in the Cantabrian range (NW Spain). *Forest Ecology and Management*, *208*(1), 29-43.

García, P., Lastra, J., Marquínez, J., & Nores, C. (2007). Detailed model of shelter areas for the Cantabrian brown bear. *Ecological informatics*, *2*(4), 297-307.

García-Vázquez, A., Pinto Llona, A.C., González-Fortes, G.M., Grandal-D'anglade, A., 2015. Distribution and chronology of brownbear (*Ursus arctos*) in the Iberian peninsula during Upper Pleistocene and Holocene. *Spanish Journal of Paleontology*, 30(1), pp. 161.183.

Garza, J. C., & Williamson, E. G. (2001). Detection of reduction in population size using data from microsatellite loci. *Molecular ecology*, *10*(2), 305-318.

Gonzalez, E. G., Blanco, J. C., Ballesteros, F., Alcaraz, L., Palomero, G., & Doadrio, I. (2016). Genetic and demographic recovery of an isolated population of brown bear *Ursus arctos* L., 1758. *PeerJ*, *4*, e1928.

Guichoux, E., Lagache, L., Wagner, S., Chaumeil, P., Léger, P., Lepais, O., ... & Petit, R. J. (2011). Current trends in microsatellite genotyping. *Molecular ecology resources*, *11*(4), 591-611.

Hindrikson, M., Remm, J., Pilot, M., Godinho, R., Stronen, A. V., Baltrūnaitė, L., ... & Åkesson, M. (2017). Wolf population genetics in Europe: A systematic review, meta-analysis and suggestions for conservation and management. *Biological Reviews*, *92*(3), 1601-1629.

Holland, M. M., & Parson, W. (2011). GeneMarker® HID: A reliable software tool for the analysis of forensic STR data. *Journal of forensic sciences*, *56*(1), 29-35.

Jarne, P., & Lagoda, P. J. (1996). Microsatellites, from molecules to populations and back. *Trends in ecology & evolution*, *11*(10), 424-429.

Keenan, K., McGinnity, P., Cross, T. F., Crozier, W. W., & Prodöhl, P. A. (2013). diveRsity: An R package for the estimation and exploration of population genetics parameters and their associated errors. *Methods in Ecology and Evolution*, *4*(8), 782-788.

Keis, M., Remm, J., Ho, S. Y., Davison, J., Tammeleht, E., Tumanov, I. L., ... & Margus, T. (2013). Complete mitochondrial genomes and a novel spatial genetic method reveal cryptic phylogeographical structure and migration patterns among brown bears in north-western Eurasia. *Journal of Biogeography*, *40*(5), 915-927.

Kocijan, I., Galov, A., Ćetković, H., Kusak, J., Gomerčić, T., & Huber, Đ. (2011). Genetic diversity of Dinaric brown bears (*Ursus arctos*) in Croatia with implications for bear conservation in Europe. *Mammalian Biology-Zeitschrift für Säugetierkunde*, *76*(5), 615-621.

Kohn, M., Knauer, F., Stoffella, A., Schröder, W., & Pääbo, S. (1995). Conservation genetics of the European brown bear - a study using excremental PCR of nuclear and mitochondrial sequences. *Molecular Ecology*, *4*(1), 95-104.

Kopatz, A., Eiken, H. G., Hagen, S. B., Ruokonen, M., Esparza-Salas, R., Schregel, J., ... & Wikan, S. (2012). Connectivity and population subdivision at the fringe of a large brown bear (*Ursus arctos*) population in North Western Europe. *Conservation Genetics*, *13*(3), 681-692.

Kopatz, A., Eiken, H. G., Aspi, J., Kojola, I., Tobiassen, C., Tirronen, K. F., ... & Hagen, S. B. (2014). Admixture and gene flow from Russia in the recovering Northern European brown bear (Ursus arctos). *PLoS One*, *9*(5), e97558.

Korsten, M., Ho, S. Y., Davison, J., PÄHN, B., Vulla, E., Roht, M., ... & Pilot, M. (2009). Sudden expansion of a single brown bear maternal lineage across northern continental Eurasia after the last ice age: a general demographic model for mammals?. *Molecular Ecology*, *18*(9), 1963-1979.

Kuduk, K., Babik, W., Bojarska, K., Sliwinska, E. B., Kindberg, J., Taberlet, P., ... & Radwan, J. (2012). Evolution of major histocompatibility complex class I and class II genes in the brown bear. *BMC Evol Biol*, *12*(1), 197.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, *33*(7), 1870-1874.

Manel, S., Gaggiotti, O. E., & Waples, R. S. (2005). Assignment methods: matching biological questions with appropriate techniques. *Trends in Ecology & Evolution*, *20*(3), 136-142.

Mateo-Sanchez, M. C., Balkenhol, N., Cushman, S., Perez, T., Dominguez, A., & Saura, S. (2015). Estimating effective landscape distances and movement corridors: comparison of habitat and genetic data. *Ecosphere*, *6*(4), 1-16.

Mateo-Sánchez, M. C., Cushman, S. A., & Saura, S. (2014). Connecting endangered brown bear subpopulations in the Cantabrian Range (north-western Spain). *Animal conservation*, *17*(5), 430-440.

Mateo Sanchez, M. C., Cushman, S. A., & Saura, S. (2014). Scale dependence in habitat selection: the case of the endangered brown bear (*Ursus arctos*) in the Cantabrian Range (NW Spain). *International Journal of Geographical Information Science*, *28*(8), 1531-1546.

McLellan, B.N., Proctor, M.F., Huber, D. & Michel, S. (2016). *Ursus arctos.* The IUCN Red List of Threatened Species 2017: e.T41688A121229971. Downloaded on 03 October 2017.

Miller, C. R., & Waits, L. P. (2003). The history of effective population size and genetic diversity in the Yellowstone grizzly (Ursus arctos): implications for conservation. *Proceedings of the National Academy of Sciences*, *100*(7), 4334-4339.

Montooth, K. L., & Rand, D. M. (2008). The spectrum of mitochondrial mutation differs across species. *PLoS biology*, *6*(8), e213.

Moreno, J. M., Pineda, F. D., & Rivas-Martinez, S. (1990). Climate and vegetation at the Eurosiberian-Mediterranean boundary in the Iberian Peninsula. *Journal of Vegetation Science*, *1*(2), 233-244.

Nomura, T. (2008). Estimation of effective number of breeders from molecular coancestry of single cohort sample. *Evolutionary Applications*, *1*(3), 462-474.

Nores, C., & Naves, J. (1993). Distribución histórica del oso pardo en la Península Ibérica. *El oso pardo*, 13-33.

Nyakatura, K., & Bininda-Emonds, O. R. (2012). Updating the evolutionary history of Carnivora (Mammalia): a new species-level supertree complete with divergence time estimates. *BMC biology*, *10*(1), 12.

Paetkau, D., Calvert, W., Stirling, I., & Strobeck, C. (1995). Microsatellite analysis of population structure in Canadian polar bears. *Molecular ecology*, *4*(3), 347-354.

Paetkau, D., & Strobeck, C. (1994). Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, *3*(5), 489-495.

Pages, M., Maudet, C., Bellemain, E., Taberlet, P., Hughes, S., & Hänni, C. (2009). A system for sex determination from degraded DNA: a useful tool for palaeogenetics and conservation genetics of ursids. *Conservation Genetics*, *10*(4), 897-907.

Palomero, G., Fernandez, A., & Naves, J. (1997). Reproductive rates of brown bears in the Cantabrian Mountains, Spain. *Bears: their biology and*

management, 129-132.

Paralikidis, N. P., Papageorgiou, N. K., Kontsiotis, V. J., & Tsiompanoudis, A. C. (2010). The dietary habits of the brown bear (Ursus arctos) in western Greece. *Mammalian Biology-Zeitschrift für Säugetierkunde*, *75*(1), 29-35.

Pasitschniak-Arts, M., (1993). Ursus arctos. *Mammalian Species*, (439), pp.1-10.

Peakall, R. O. D., & Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Resources*, *6*(1), 288-295.

Pérez, T., Naves, J., Vázquez, J. F., Seijas, J., Corao, A., Albornoz, J., & Domínguez, A. (2010). Evidence for improved connectivity between Cantabrian brown bear subpopulations. *Ursus*, *21*(1), 104-108.

Pérez, T., Naves, J., Vázquez, J. F., Fernández-Gil, A., Seijas, J., Albornoz, J., ... & Domínguez, A. (2014). Estimating the population size of the endangered Cantabrian brown bear through genetic sampling. *Wildlife biology*, *20*(5), 300-309.

Pérez, T., Vázquez, F., Naves, J., Fernández, A., Corao, A., Albornoz, J., & Domínguez, A. (2009). Non-invasive genetic study of the endangered Cantabrian brown bear (Ursus arctos). *Conservation genetics*, *10*(2), 291-301.

Posada, D., & Crandall, K. A. (2001). Selecting the best-fit model of nucleotide substitution. *Systematic Biology*, *50*(4), 580-601.

Proctor, M. F., McLellan, B. N., Strobeck, C., & Barclay, R. M. (2005). Genetic analysis reveals demographic fragmentation of grizzly bears yielding vulnerably small populations. *Proceedings of the Royal Society of London B: Biological Sciences*, *272*(1579), 2409-2416.

Purroy, F. (2017) Oso pardo (Ursus arctos Linnaeus, 1758). *Mamíferos España (Volume I).* Insectívoros, Quirópteros, Primates y Carnívoros la Península ibérica, Balear y Canar. 260–269.

Quenette, P. Y., Alonso, M., Chayron, L., Cluzel, P., Dubarry, E., Dubreuil, D., ... & Pomarol, M. (2001). Preliminary results of the first transplantation of brown bears in the French Pyrenees. *Ursus*, 115-120.

Rambaut, A., & Drummond, A. J. (2012). FigTree. Version 1.4. 0. Available at h ttp. *tree. bio. ed. ac. uk/software/figtree*.

Rambaut, A., Suchard, M., Xie, D., & Drummond, A. (2014). Tracer v1. 6

http://beast. bio. ed. ac. uk. *Tracer>(Online 2015, May 29)*.

Randi, E. (2007). Phylogeography of south European mammals. In *Phylogeography of southern European refugia* (pp. 101-126). Springer Netherlands.

Randi, E., Alves, P.C., Carranza, J., Milosevic-Zlatanovic, S., Sfougaris, A. & Mucci, N. (2004). Phylogeography of roe deer (*Capreolus capreolus*) populations: the effects of historical genetic subdivisions and recent nonequilibrium dynamics. *Molecular Ecology*, 13: 3071–3083.

Randi, E., Huber, D., Boscagli, G., Roth, H. U., & Gentile, L. (1994). Mitochondrial DNA sequence divergence among some west European brown bear (*Ursus arctos* L.) populations. Lessons for conservation. *Heredity*, *73*(5), 480.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic biology*, *61*(3), 539-542.

Rowe, G., Sweet, M., & Beebee, T. (2017). *An introduction to molecular ecology*. Oxford University Press.

Royo, L.J., Pajares, G., Álvarez, I., Fernández, I. & Goyache, F. (2007). Genetic variability and differentiation in Spanish roe deer (*Capreolus capreolus*): A phylogeographic reassessment within the European framework. *Molecular Phylogenetics and Evolution*, 42: 47–61.

Saarma, U., Ho, S. Y., Pybus, O. G., Kaljuste, M., Tumanov, I. L., Kojola, I., ... & Lyapunova, E. A. (2007). Mitogenetic structure of brown bears (Ursus arctos L.) in northeastern Europe and a new time frame for the formation of European brown bear lineages. *Molecular Ecology*, *16*(2), 401-413.

Salomashkina, V. V., Kholodova, M. V., Tuten'kov, O. Y., Moskvitina, N. S., & Erokhin, N. G. (2014). New data on the phylogeography and genetic diversity of the brown bear Ursus arctos Linnaeus, 1758 of Northeastern Eurasia (mtDNA control region polymorphism analysis). *Biology Bulletin*, *41*(1), 38-46.

Servheen C, Herrero S, Peyton B (1999) Status, survey and conservation action plan: Bears. IUCN/SSC Bear Spec Group 321pp 1–310.

Sommer, S. (2005). The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Frontiers in zoology*, *2*(1), 16.

Steyaert, S. M., Kindberg, J., Swenson, J. E., & Zedrosser, A. (2013). Male reproductive strategy explains spatiotemporal segregation in brown bears. *Journal of Animal Ecology*, *82*(4), 836-845.

Sundqvist, L., Keenan, K., Zackrisson, M., Prodöhl, P., & Kleinhans, D. (2016). Directional genetic differentiation and relative migration. *Ecology and evolution*, *6*(11), 3461-3475.

Swenson, J. E., Adamič, M., Huber, D., & Stokke, S. (2007). Brown bear body mass and growth in northern and southern Europe. *Oecologia*, *153*(1), 37-47.

Swenson, J. E. (2000). *Action plan for the conservation of the brown bear in Europe (Ursus arctos)* (No. 18-114). Council of Europe.

Taberlet, P., & Bouvet, J. (1994). Mitochondrial DNA polymorphism, phylogeography, and conservation genetics of the brown bear Ursus arctos in Europe. *Proceedings of the Royal Society of London B: Biological Sciences*, *255*(1344), 195-200.

Taberlet, P., Camarra, J. J., Griffin, S., Uhres, E., Hanotte, O., Waits, L. P., ... & Bouvet, J. (1997). Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology*, *6*(9), 869-876.

Talbot, S. L., & Shields, G. F. (1996). Phylogeography of brown bears (Ursus arctos) of Alaska and paraphyly within the Ursidae. *Molecular phylogenetics and evolution*, *5*(3), 477-494.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research*, *22*(22), 4673-4680.

Torres, R. T., Ferreira, E., Rocha, R. G., & Fonseca, C. (2017). Hybridization between wolf and domestic dog: First evidence from an endangered population in central Portugal. *Mammalian Biology-Zeitschrift für Säugetierkunde*, *86*, 70-74.

Veličković, N., Djan, M., Ferreira, E., Stergar, M., Obreht, D., Maletić, V., & Fonseca, C. (2015). From north to south and back: the role of the Balkans and other southern peninsulas in the recolonization of Europe by wild boar. *Journal of Biogeography*, *42*(4), 716-728.

Veličković, N., Ferreira, E., Djan, M., Ernst, M., Vidaković, D. O., Monaco, A., & Fonseca, C. (2016). Demographic history, current expansion and future management challenges of wild boar populations in the Balkans and Europe. *Heredity*, *117*(5), 348.

Waits, L. P., Luikart, G., & Taberlet, P. (2001). Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Molecular ecology*, *10*(1), 249-256.

Waits, L., Taberlet, P., Swenson, J. E., Sandegren, F., & Franzen, R. (2000). Nuclear DNA microsatellite analysis of genetic diversity and gene flow in the Scandinavian brown bear (Ursus arctos). *Molecular ecology*, *9*(4), 421-431.

Waits, L. P., Talbot, S. L., Ward, R. H., & Shields, G. F. (1998). Mitochondrial DNA phylogeography of the North American brown bear and implications for conservation. *Conservation Biology*, *12*(2), 408-417.

WAN, Q. H., Zhu, L., Wu, H. U. A., & FANG, S. G. (2006). Major histocompatibility complex class II variation in the giant panda (Ailuropoda melanoleuca). *Molecular Ecology*, *15*(9), 2441-2450.

Wang, J. (2005). Estimation of effective population sizes from data on genetic markers. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *360*(1459), 1395-1409.

Waples, R. S., & Do, C. H. I. (2008). LDNE: a program for estimating effective population size from data on linkage disequilibrium. *Molecular ecology resources*, *8*(4), 753-756.

Wiegand, T., Naves, J., Stephan, T., & Fernandez, A. (1998). Assessing the risk of extinction for the brown bear (Ursus arctos) in the Cordillera Cantabrica, Spain. *Ecological Monographs*, *68*(4), 539-570.

Wright S (1978) Evolution and the Genetics of Population, Variability Within and Among Natural Populations. Chicago

WWF (2016) Living Planet Report.

Xenikoudakis, G., Ersmark, E., Tison, J. L., Waits, L., Kindberg, J., Swenson, J. E., & Dalén, L. (2015). Consequences of a demographic bottleneck on genetic structure and variation in the Scandinavian brown bear. *Molecular ecology*, *24*(13), 3441-3454.

Zedrosser, A., Dahle, B., Swenson, J. E., & Gerstl, N. (2001). Status and management of the brown bear in Europe. *Ursus*, 9-20.

Appendix I. Details on mitochondrial DNA sequences used in the phylogeographic and phylogenetic analysis.

Table continues in the next three pages.

GenBank Acession No.	*Location*	*Reference*
X75862.1	Abruzzo, Italy	Taberlet & Bouvet 1994
X75864.1	Bulgary	Taberlet & Bouvet 1994
X75865.1	Cantabria, Spain	Taberlet & Bouvet 1994
X75866.1	Cantabria, Spain	Taberlet & Bouvet 1994
X75867.1	Croatia	Taberlet & Bouvet 1994
X75868.1	Sweden	Taberlet & Bouvet 1994
X75869.1	Estonia	Taberlet & Bouvet 1994
X75870.1	Greece	Taberlet & Bouvet 1994
X75871.1	Norway	Taberlet & Bouvet 1994
X75872.1	Romania	Taberlet & Bouvet 1994
X75873.1	Romania	Taberlet & Bouvet 1994
X75874.1	Estonia, Sweden, Finland, Russia	Taberlet & Bouvet 1994
X75875.1	Slovakia	Taberlet & Bouvet 1994
X75876.1	Slovakia	Taberlet & Bouvet 1994
X75877.1	Trentino, Italy	Taberlet & Bouvet 1994
X75878.1	Pyrenees, France	Taberlet & Bouvet 1994
EU526765.2	Estonia, Finland, European Russia	Korsten et al. 2009
EU526766.2	European Russia	Korsten et al. 2009
EU526767.2	Finland	Korsten et al. 2009
EU526768.2	European Russia	Korsten et al. 2009
EU526769.2	European Russia	Korsten et al. 2009

(Continued from previous page)		
EU526770.2	Finland, European Russia	Korsten et al. 2009
EU526771.2	European Russia	Korsten et al. 2009
EU526772.2	European Russia	Korsten et al. 2009
EU526773.2	Finland	Korsten et al. 2009
EU526774.2	European Russia	Korsten et al. 2009
EU526776.2	European Russia	Korsten et al. 2009
EU526777.2	Finland, European Russia	Korsten et al. 2009
EU526778.2	Finland	Korsten et al. 2009
EU526779.2	Finland	Korsten et al. 2009
EU526780.2	Finland	Korsten et al. 2009
EU526781.2	European Russia	Korsten et al. 2009
EU526782.2	European Russia	Korsten et al. 2009
EU526783.2	European Russia	Korsten et al. 2009
EU526784.2	Estonia	Korsten et al. 2009
EU526785.2	Estonia, European Russia	Korsten et al. 2009
EU526786.2	European Russia	Korsten et al. 2009
EU526787.2	European Russia	Korsten et al. 2009
EU526788.2	European Russia	Korsten et al. 2009
EU526789.2	European Russia	Korsten et al. 2009
EU526791.2	European Russia	Korsten et al. 2009
EU526792.2	Finland	Korsten et al. 2009
EU526793.2	Finland, European Russia	Korsten et al. 2009
EU526799.2	Finland	Korsten et al. 2009
EU526800.2	Russia	Korsten et al. 2009
EU526801.2	Estonia	Korsten et al. 2009

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EU526802.2	Estonia	Korsten et al. 2009
EU526808.2	Russia	Korsten et al. 2009
EU526809.2	Russia	Korsten et al. 2009
EU526810.2	Russia	Korsten et al. 2009
HQ602651.1	Croatia	Kocijan et al. 2011
HQ602652.1	Croatia	Kocijan et al. 2011
HQ602653.1	Croatia	Kocijan et al. 2011
KF545627.1	Russia	Salomishkina et al. 2014
KF545628.1	Russia	Salomishkina et al. 2014
KF545636.1	Russia	Salomishkina et al. 2014
KF545637.1	Russia	Salomishkina et al. 2014
KF545638.1	Russia	Salomishkina et al. 2014
KF545643.1	Russia	Salomishkina et al. 2014
KF563083.1	Russia	Salomishkina et al. 2014
KF563086.1	Russia	Salomishkina et al. 2014
KF563087.1	Russia	Salomishkina et al. 2014
KP668987.1	Iran	Ashrafzadeh et al. 2016
KP668986.1	Iran	Ashrafzadeh et al. 2016
KP668985.1	Iran	Ashrafzadeh et al. 2016
KP668984.1	Iran	Ashrafzadeh et al. 2016
KP668981.1	Iran	Ashrafzadeh et al. 2016
KP668980.1	Iran	Ashrafzadeh et al. 2016
KP668978.1	Iran	Ashrafzadeh et al. 2016
KP668977.1	Iran	Ashrafzadeh et al. 2016
KP668976.1	Iran	Ashrafzadeh et al. 2016
KP668975.1	Iran	Ashrafzadeh et al. 2016

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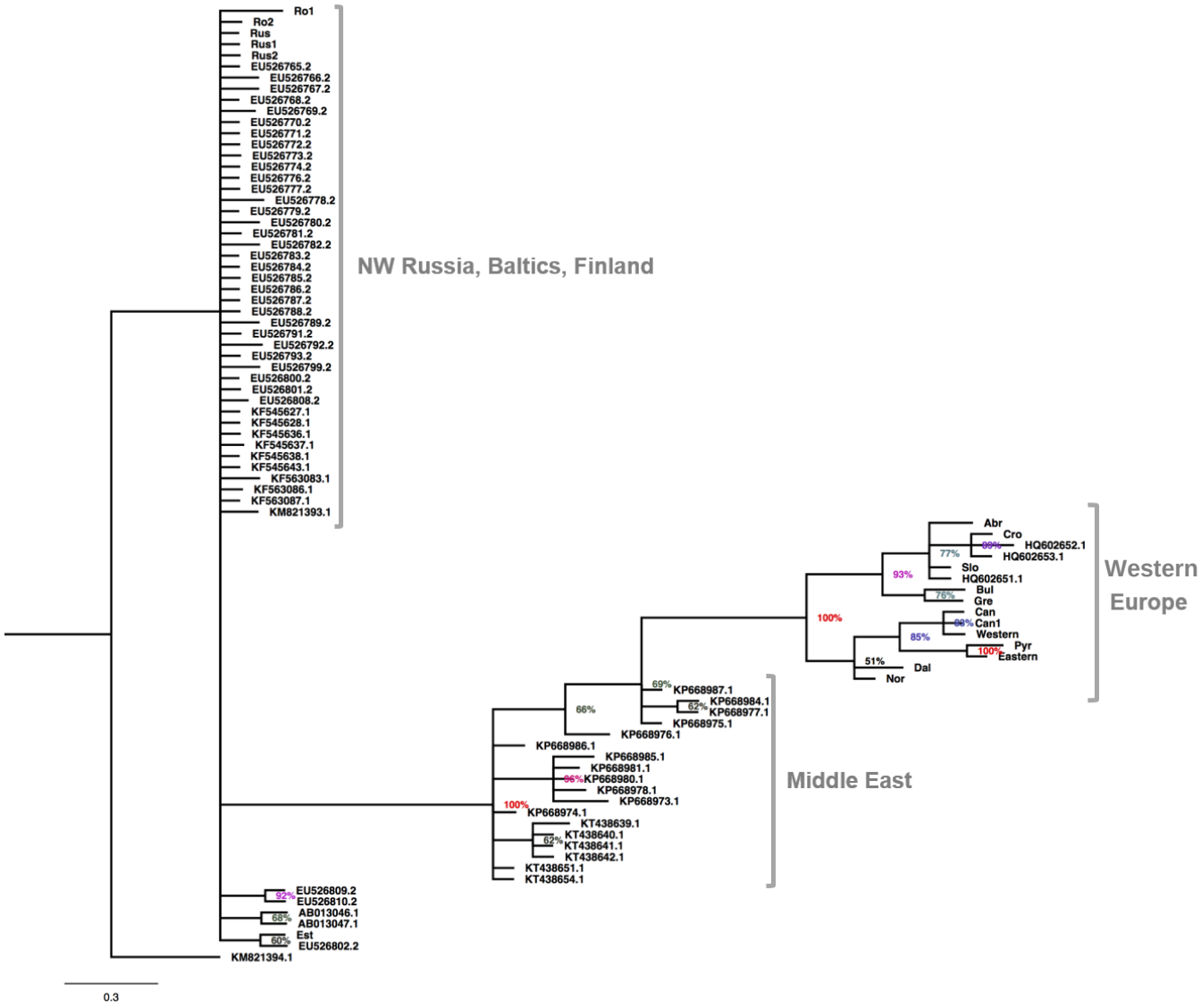
KP668974.1	Iran	Ashrafzadeh et al. 2016
KP668973.1	Iran	Ashrafzadeh et al. 2016
KT438639.1	Turkey	Cilingir et al. 2016
KT438640.1	Turkey	Cilingir et al. 2016
KT438641.1	Turkey	Cilingir et al. 2016
KT438642.1	Turkey	Cilingir et al. 2016
KT438651.1	Turkey	Cilingir et al. 2016
KT438654.1	Turkey	Cilingir et al. 2016
AB013046.1*****	Japan	Matsuhashi et al. 1999
AB013047.1*****	Japan	Matsuhashi et al. 1999
KM821394.1*	Alaska	Talbot et al. (unpublished)

*****- Sequences used as outgroup for Bayesian Inference

Appendix II. Complete Bayesian Inference tree

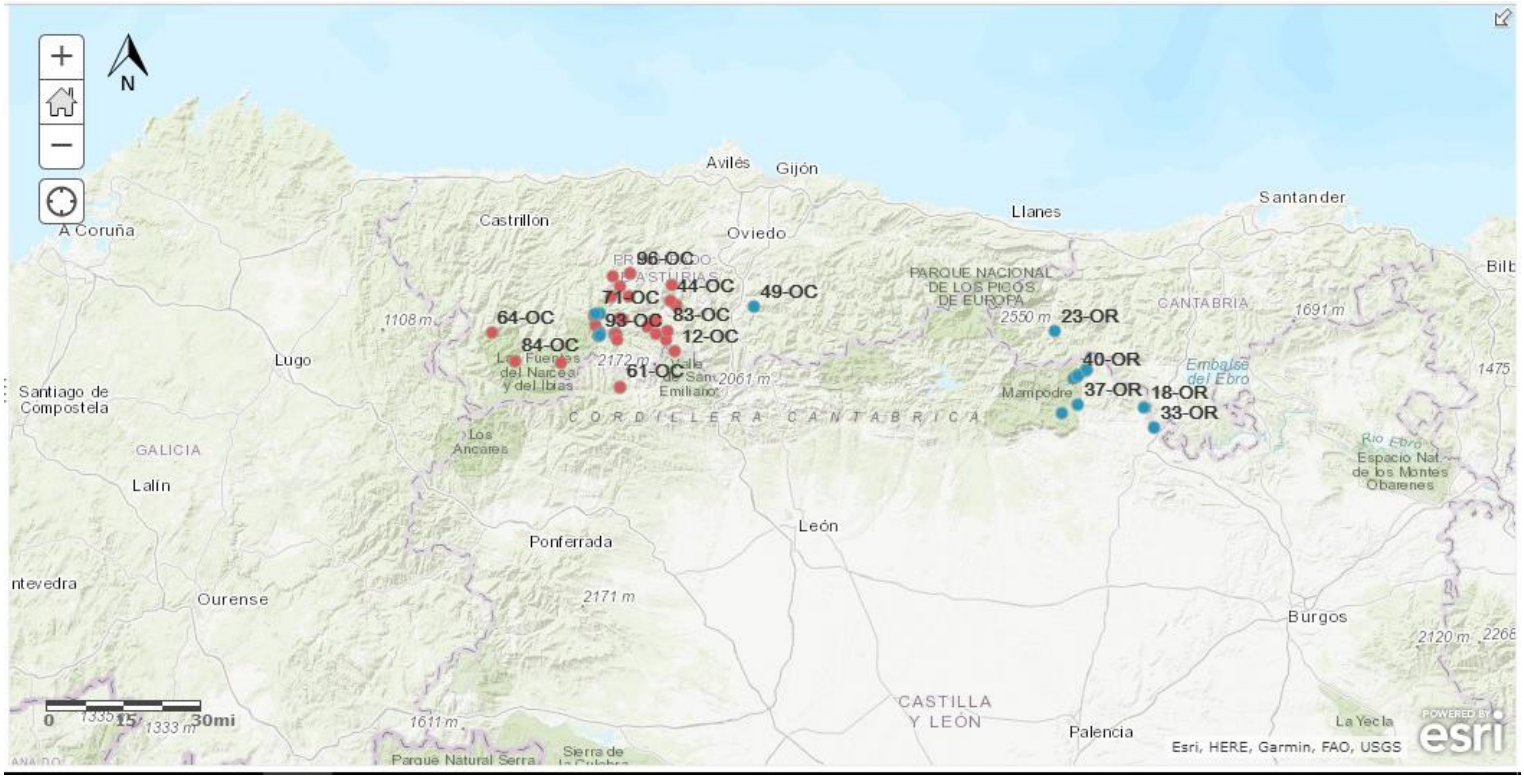


Appendix III. Molecular sex determination of the sampled individuals

WESTERN SUBPOPULATION			
1-OC	XX	62-OC	XY
2-OC	XY	63-OC	XX
3-OC	XY	64-OC	XY
4-OC	XX	71-OC	XY
7-OC	XY	72-OC	XY
8-OC	XY	74-OC	XX
9-OC	XY	77-OC	XY
12-OC	XY	78-OC	XY
14-OC	XY	80-OC	XX
15-OC	XX	82-OC	XY
44-OC	XY	83-OC	XX
45-OC	XY	84-OC	XY
47-OC	XY	85-OC	XY
49-OC	XY	86-OC	XY
50-OC	XY	87-OC	XY
52-OC	XX	89-OC	XX
53-OC	XY	90-OC	XY
54-OC	XY	91-OC	XY
55-OC	XY	92-OC	XY
56-OC	XY	93-OC	XY
57-OC	XY	94-OC	XY
59-OC	XY	95-OC	XY
60-OC	XY	96-OC	XY

EASTERN SUBPOPULATION	
16-OR	XY
18-OR	XY
21-OR	XY
23-OR	XY
26-OR	XY
28-OR	XY
30-OR	XY
31-OR	XY
32-OR	XY
33-OR	XY
37-OR	XY
38-OR	XY
39-OR	XY
40-OR	XY

Appendix IV. Geographical location of the sampled individuals



- Red and blue dots correspond to Western and Eastern putative subpopulations, respectively