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RESEARCH

Unveiling population dynamics and diversity in two European brown bear (*Ursus arctos*) populations through non-invasive SNP genotyping

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Abstract
Non-invasive genetic analyses enable monitoring and understanding of population dynamics without disturbing wild animals. We present a non-invasive genetic method to identify and characterize the brown bear populations of Cantabrian and Pyrenean (of Slovenian origin). We selected an efficient 61-SNP panel to genotype more than 2,000 non-invasive samples from both populations. Results showed successful genotyping of 1,639 bear samples, revealing 400 distinct individuals. Genetic diversity was similar in both populations, and differentiation between populations was highly significant. The Pyrenean population did not show genetic substructuring despite the influence of the breeding male "Pyros". In contrast, two subpopulations were observed in the Cantabrian population. Furthermore, analyses indicated a sex ratio bias in the Cantabrian population, potentially influenced by male dispersal and landscape features. Overall, the study demonstrates the utility of non-invasive genetic methods for monitoring and understanding bear populations, highlighting differences between the Pyrenean and Cantabrian populations, and providing insights into their genetic diversity, structure, and demographic trends.

Keywords Brown bear · *Ursus arctos* · Non-invasive samples · SNPs · Genotyping

Introduction

Non-invasive techniques offer a significant advantage in that they allow the investigation of individuals without the need to interact with, disrupt, or visually observe the organisms (Taberlet et al. 1997; Bellemain and Taberlet 2004; Swanson et al. 2009; Beja-Pereira et al. 2009). Furthermore, non-invasive genetic analyses are useful to monitor and characterize wildlife populations, increasing the understanding of the ecology and genetic dynamics of populations. This includes identifying individuals to determine population size, sex ratio, and individual mobility, resolving wildlife forensic cases, and defining populations and their genetic parameters (including structure, gene flow, and demographic bottlenecks) (Bellemain and Taberlet 2004; Beja-Pereira et al. 2009; Swenson et al. 2011).

The first non-invasive genetic studies date back to the 1990s (Taberlet and Bouvet 1992; Höss et al. 1992; Kohn et al. 1995; Taberlet and Luikart 1999). Since then, an increasing number of non-invasive techniques have made it possible to obtain enough quantity and quality of DNA to address almost all inquiries that could previously only be studied using conventional high-quality samples such as tissue or blood (Kohn et al. 1999; Bellemain and Taberlet 2004; Waits and Paetkau 2005; Broquet et al. 2007; Sastre et al. 2009).

Various molecular markers have been used to characterize bear (*Ursus arctos*) populations from non-invasive samples. The use of microsatellite markers, or STRs (short tandem repeats), was widespread in the 1990s and early 2000s (Taberlet et al. 1997; Wasser et al. 1997; Taberlet and

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Luikart 1999; Pérez et al. 2009; Eiken et al. 2009; De Barba et al. 2010; Latham et al. 2012; Xenikoudakis et al. 2015; Gregório et al. 2020). STRs are DNA sequences that repeat in units of 2–6 base pairs, and the length of the fragment, rather than the primary sequence, determines the degree of polymorphism of microsatellites (Weber and Wong 1993; Xu et al. 2000; Harr and Schlötterer 2000; Brohede et al. 2002). However, the length of the fragment is the major disadvantage of STRs compared to single nucleotide polymorphisms (SNPs) when working with forensic samples (Gill 2001). Genome sequencing has enabled the discovery of millions of SNPs in species such as polar bears (*Ursus maritimus*), brown bears (*U. arctos*), and black bears (*U. americanus*) (Miller et al. 2012; Cronin et al. 2014; de Jong et al. 2023). Subsequently, bear samples began to be analysed using SNPs (Norman et al. 2013; Cronin et al. 2014; Viengkone et al. 2016; Giangregorio et al. 2019; López-Bao et al. 2020). SNPs are point mutations involving the substitution of one nucleotide for another. They are shorter, which favours the amplification of degraded DNA, and they are less likely to result in genotyping errors (Morin et al. 2004; Seddon et al. 2005; Anderson and Garza 2006). The most frequent error resulting from the stochastic effects of the PCR reaction is dropout, or allelic amplification failure (Pompanon et al. 2005; Giangregorio et al. 2019; López-Bao et al. 2020). Conducting a multiplex pre-amplification and increasing the number of sample replicates can reduce the dropout rate (Bellemain and Taberlet 2004; Sastre et al. 2009; Giangregorio et al. 2019). Moreover, some software can predict the dropout probability of an SNP panel a priori (Sastre et al. 2023). However, SNPs are less polymorphic than STRs, and therefore a greater number of SNPs is required to differentiate between individuals (Seddon et al. 2005; Hauser et al. 2011). Large SNP genotyping panels increase accuracy for individual assignments compared to smaller ones (Puckett and Eggert 2016). However, evidence suggests that relatedness can be reliably inferred using at least 60 SNPs (Krawczak 1999; Giangregorio et al. 2019). An informative SNP panel is characterized by each SNP maximizing allelic representation differences among individuals within a population in comparison to all other SNPs. Therefore, SNPs with higher minor allele frequencies (MAF) that are not linked to each other are more informative for identification purposes (Krawczak 1999; Norman et al. 2013).

The European brown bear (*Ursus arctos*) is widely distributed across Europe, encompassing multiple populations of varying sizes and geographic ranges (Swenson et al. 2020). The Pyrenees Mountains, located between southwestern France and north-eastern Spain, harbour one of the most endangered and smallest populations of the European brown bear. This population is classified as Critically Endangered according to the criteria of the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (McLellan et al. 2017) and exists due to translocations of several bears from Slovenia in 1996–1997, 2006 and 2016 (Chapron et al. 2009; Piédallu et al. 2019). The brown bear became extinct in Catalonia and the Central and Eastern Pyrenees in the 1990s, and from the Western Pyrenees in 2010 (Chapron et al. 2009). This means that the bears currently inhabiting the Pyrenees are of Slovenian origin. The Pyrenean population has grown from a dozen individuals to around 80 individuals (Vanpé et al. 2022; https://professionnels.ofb.fr/fr/doc/ours-infos-2023-rapport-annuel), primarily due to the reproductive success of the male bear "Pyros" (reintroduced to the Pyrenees in 1996–2017) https://pireslife.cat/wp-content/uploads/2020/03/ENG_layman_baixa.pdf.

In northwest Spain, there is another isolated brown bear population located in the Cantabrian Mountains. The Cantabrian brown bear is also included in the IUCN Red List and classified as Endangered (McLellan et al. 2017). The population has recovered over the last three decades, growing from about 100 individuals in the 1990s to over 300 individuals today (Chapron et al. 2014; Gonzalez et al. 2016; https://comunicacion.jcyl.es/web/jcyl/Comunicacion/es/Plantilla100Detalle/1281372051501/NotaPrensa/1285242547188/Comunicacion; https://fundacionosopardo.org/wp-content/uploads/2023/03/ficha1_2023_ESP_Marzo.pdf). Genetic studies have demonstrated the existence of two connected subpopulations: a larger subpopulation in the western region (Galicia, Asturias, and northwest Castilla y León), and a smaller subpopulation in the eastern region (northeast Castilla y León and Cantabria) (Swenson et al. 2011; Gonzalez et al. 2016; Gregório et al. 2020; López-Bao et al. 2020; Díaz-Fernández et al. 2023). The subpopulations are connected by a strip of about 50 km and encompass seven and two municipalities in Castilla y León and the Principality of Asturias, respectively (http://fundacionosopardo.org/wp-content/uploads/2018/09/Layman-Report1.pdf).

In this study, we designed an efficient SNP panel to genotype non-invasive samples from the two bear populations of the Iberian Peninsula, with the aim of achieving individual identification and analysing the population structure.

Materials and methods

SNP panel selection, samples, and laboratory procedures

We have selected 61 SNPs, including a sexual marker, from the 180 SNPs described by Cronin et al. (2014) to genotype over 2,000 non-invasive samples of brown bears in the Iberian Peninsula.

This 61-SNP panel improves the efficiency of genetic analyses by reducing the time and cost required to identify

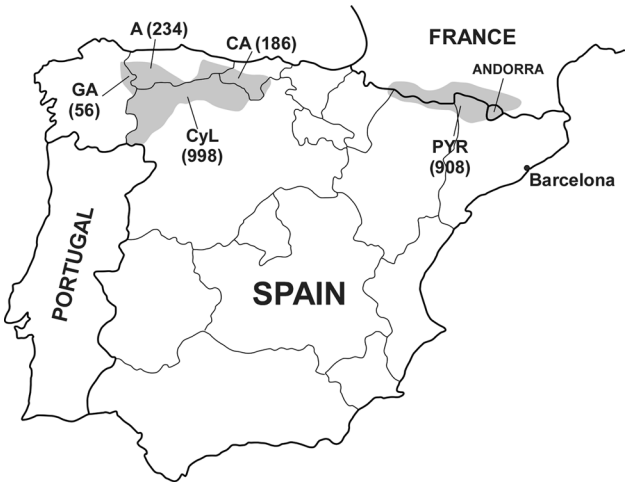

Fig. 1 Map showing the distribution (grey colour) of brown bear samples (number in brackets) collected in the Pyrenees (PYR) and the Cantabrian Mountains (*A* Asturias; *CA* Cantabria; *CyL* Castilla y León; *GA* Galicia). Note that the Pyrenees samples are not associated with a specific region

and characterize the two southernmost brown bear populations in Europe. The panel was designed for use on the OpenArray platform (Thermo Fisher Scientific, Waltham, US), which supports a configuration of 60 SNPs plus one quadruplicated SNP to address crosstalk errors (Thermo Fisher, personal communication).

To assess the informativeness of the final 61-SNP panel for individual identification, we first genotyped 16 brown bear individuals (11 and 5 from the Pyrenean and Cantabrian populations, respectively) using the 180-SNP panel described by Cronin et al. (2014). These samples, which had high-quality DNA, had previously been identified using STRs. Genotypes were obtained from Neogen/GeneSeek https://www.neogen.com/en-gb/. Then, we selected 61 SNPs with higher MAF and a maximum of seven SNPs per chromosome in the Pyrenean population.

Between 2009 and 2023, 2,382 brown bear samples were collected in the Pyrenees (Spain, France, and Andorra) and the Cantabrian Mountains (Castilla y León, Cantabria, Asturias, and Galicia) by field technicians. Samples were collected throughout the year, except during winter, for monitoring and control of both populations. Of these, 908 samples from Pyrenean bears were collected between 2009 and 2023, while 1,474 samples from Cantabrian bears were collected between 2018 and 2022 (Fig. 1). The analysed dataset included 1,276 and 1,094 non-invasive faecal and hair samples, and 6 muscular tissue and blood samples, respectively. Invasive samples were obtained from animals found dead or captured by the Competent Administration and subsequently released. All samples were stored at −20 °C until DNA extraction. DNA was isolated from faeces using the NucleoSpin DNA stool kit (Macherey–Nagel, Düren, Germany) following the manufacturer's instructions. DNA from hairs, blood, and tissue was automatically extracted using a Chemagic 360 instrument (PerkinElmer Inc., Waltham, US) according to the tissue sample protocols provided by the manufacturer. Blank DNA extractions and negative (UltraPureTM Distilled Water, Invitrogen, Waltham, US) PCR controls were included to detect exogenous DNA contamination. Sample extractions were performed on different days for each tissue type in a clean room dedicated to genomic samples. DNA preamplifications and reamplifications were prepared under a laminar flow hood. All samples were replicated four times to detect contamination, mistyping, and/or dropout errors. The preamplifications for OpenArray® assays were prepared in a 5 µl final volume reaction, following the manufacturer's instructions (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011116_TaqManOpenArrayGenotype_SamplePreamp_UB.pdf). Then, 3 µl of preamplified product, or 2 µl of DNA extraction from invasive samples, were used to genotype all the samples on a QuantStudio™ 12 K Flex system (Thermo Fisher Scientific, Waltham, US) coupled with OpenArray®technology (Thermo Fisher Scientific, Waltham, US), following the manufacturer's guidelines.

SNP genotyping

SNP consensus genotypes for the four replicates were assembled using the SNP+ software (Sastre et al. 2023). Heterozygous genotypes were confirmed when the same genotype was detected in two independent PCRs, while homozygous genotypes were confirmed when the genotype was consistently observed in three independent PCRs. Genotypes were classified in four ranges depending on their call rate: a value of call rate of 1 (≥ 95%) represents the highest sample quality (≤ 3 missing SNPs and/or dropout value ≤ 0.06), thereby identifying the bear. To be considered a new individual, a sample must have a call rate value of 1. Additionally, all new individuals must differ from others by at least six SNPs. A value of call rate of 2 indicates intermediate sample quality (call rate < 95% and ≥ 70%). Samples with ≤ 3 missing values but with a dropout value > 0.06 are also included in this range. In this case, the bear is identified as "probable" or "possible". The distinction between these categories depends on sample quality and whether it differs from another sample by more than six SNPs. A "possible" bear is unlikely to be a new individual (< 6 SNPs difference). A "probable" bear could be either a new individual or an existing individual in the population (> 6 SNPs difference). A value of 3 indicates low sample quality due to missing values (call rate < 70% and ≥ 20%). The sample cannot be identified but can still be confirmed as belonging to a bear. A value of 4 indicates very low-quality samples (call rate < 20%) that

are not amplifiable due to insufficient DNA quality and/or quantity. In such cases, it cannot be determined whether the sample corresponds to a bear.

To detect and consequently discard triallelics (cases of contamination from mixed DNA of two or more bears in a hair trap), hair samples identified once as a new individual were further analysed using a battery of nine hyper-variable microsatellite DNA markers (Mu23, Mu10, Mu51, UA06, LISTUA16, Mu59, Mu50, LIST11014, G10L), according to Taberlet et al. (1997) and De Barba et al. (2010). Triallelic samples and those with a call rate > 1 were excluded for further analyses. Y- and X-linked SNPs were used exclusively for sex identification.

SNP validation and population data analyses

Mean allelic dropout rates across loci and MAF were estimated using the SNP+ software (Sastre et al. 2023). The likelihood of sharing identical genotypes by chance (probability of identity [PID]) and the probability of identity for siblings (PID_{sibs}) were estimated using GENALEX (Peakall and Smouse 2012). Genetic variability statistics, such as observed (Ho) and expected (H_E) heterozygosities were estimated per locus and population using ARLEQUIN 3.5.2.2 (Excoffier et al. 2007). Deviations from Hardy–Weinberg equilibrium (HWE) were calculated from the inbreeding estimator F_{IS} (Guo and Thompson 1992) for loci and population using GENALEX. Differences in genetic variability between the two populations were evaluated using Wilcoxon *W* signed-rank tests. To test for genetic differentiation between and within populations, pairwise F_{ST} values were estimated using ARLEQUIN 3.5.2.2. To visualize the distribution of genetic variation across individuals, we carried out a factorial correspondence analysis (FCA) (Benzecri 1973) as implemented in GENETIX 4.05, and a Bayesian model-based clustering approach in STRUCTURE 2.3.4 (Hubisz et al. 2009). Ignoring prior population information and using an admixture model with independent allele frequencies, STRUCTURE assigns individuals to populations. To assess the partition in a varying number of genetic clusters (*K*), we ran 100,000 Markov chain Monte Carlo repetitions and a burn-in period of 10,000 iterations for $K = 1–6$, repeated 25 times. Following Evanno et al. (2005), we chose the optimal *K* based on the rate of change of the log-likelihood [ln Pr(X/K)] and its variance for $K = 1–6$.

Results and discussion

A total of 2,382 brown bear samples were collected in the Pyrenees and the Cantabrian Mountains by field technicians (Fig. 1). We analysed 1,276 faecal samples (53.5%), 1,094 hair samples (46.0%), and 12 invasive samples (0.5%). Samples were genotyped using a selected panel of 61 SNPs (Table 1). The relative frequencies of the dropout probability were estimated based on four replicates per sample with a call rate of 1 (1,627 non-invasive samples) and 2 (208 non-invasive samples) (Fig. 2). The dropout probability was low, with minimum and maximum values of 0.002 and 0.119, respectively. The unbiased Probability of Identity (PID) for the Cantabrian and Pyrenean bears were 8.5×10^{-15} and 1.9×10^{-19}, respectively, and the Probability of Identity for siblings (PID_{sibs}) was 6.00×10^{-8}, and 2.5×10^{-10}, respectively. Nine SNPs were needed to achieve the cutoff of PID = 0.0001, a threshold to identify individuals properly (López-Bao et al. 2020).

We obtained 1,639 bear genotypes (69% success) with a call rate = 1, including 1,030 genotypes from the Cantabrian population and 609 from the Pyrenean population. The technique's success rate was practically identical for faecal (68%, N = 871) and hair (69%; N = 756) samples.

Fresh fecal samples had a higher success rate (72%) compared to dry feces (58%), and the success rate was proportional to the number of hairs used for DNA extraction. Specifically, using more than 20 hairs resulted in an 82% success rate, 10–20 in 79%, 5–10 in 67%, and fewer than 5 hairs in only 34%. Invasive samples had a 100% success rate.

Genotypes from hair samples that appeared only once (N = 90) with a call rate = 1 were analysed using a set of nine microsatellites to discard triallelics. In total, 28 triallelic samples (31%) were identified and excluded from further analyses. Samples with a call rate = 2 (N = 208; 9%) were not used for variability and population structure analyses but were retained for ecological context, as they may provide valuable insights when linked to environmental data. Finally, 22% of the samples (N = 535) could not be identified, either due to the poor quality of the DNA or because they were not from a bear.

The 1,611 successfully identified bear genotypes corresponded to 400 individuals, 110 animals from the Pyrenean bear population, and 290 from the Cantabrian bear population. Figure 3 shows the number of collected samples per genotype. In total, 175 genotypes (males = 104; females = 71) were unique, with the male and female with the highest number of captures (N = 72 and N = 24, respectively) belonging to the Pyrenean population. The total number of males detected (N = 236) was higher than females (N = 164). Within populations, the number of Cantabrian males detected (N = 183) was higher than Cantabrian females (N = 107), while the sex ratio was balanced in the Pyrenean bear population (males = 53; females = 57).

To study and compare both populations over a similar time frame (from 2018 to 2023), 11 individuals (7 females and 4 males) identified in the Pyrenean population between 2009 and 2017, were excluded from further analysis. The molecular marker set was analysed for MAF (Cantabrian

Table 1 GenBank dbSNP numbers of the 61 SNPs

Local_identifier	NCBI_ss
GL192338.1_3841069	538,974,401
GL192338.1_4743765	538,974,403
GL192338.1_5687446	538,974,682
GL192339.1_3051026	538,974,407
GL192340.1_2390776	538,974,416
GL192340.1_2739334	538,974,418
GL192340.1_28228	538,974,598
GL192340.1_3205674	538,974,420
GL192341.1_156225	538,974,429
GL192342.1_1196018	538,974,437
GL192342.1_85659	538,974,441
GL192343.1_1129410	538,974,442
GL192343.1_2056618	538,974,444
GL192343.1_3498523	538,974,446
GL192343.1_4101062	538,974,575
GL192344.1_56594	538,974,454
GL192345.1_1496056	538,974,458
GL192345.1_4386014	538,974,716
GL192345.1_4615261	538,974,460
GL192346.1_1662412	538,974,461
GL192346.1_1710151	538,974,463
GL192346.1_1818193	538,974,465
GL192346.1_2176302	538,974,467
GL192346.1_3785593	538,974,471
GL192347.1_149399	538,974,480
GL192347.1_2313568	538,974,482
GL192347.1_3195890	538,974,714
GL192347.1_5203431	538,974,709
GL192348.1_1569786	538,974,486
GL192348.1_4321454	538,974,490
GL192348.1_4540974	538,974,585
GL192349.1_137121	538,974,656
GL192349.1_3105684	538,974,498
GL192350.1_2165697	538,974,501
GL192350.1_233960	538,974,566
GL192350.1_4178367	538,974,505
GL192351.1_10113	538,974,507
GL192351.1_1775676	538,974,509
GL192352.1_1719564	538,974,512
GL192352.1_2509637	538,974,514
GL192353.1_139156	538,974,518
GL192353.1_2056307	538,974,520
GL192353.1_4003383	538,974,521
GL192354.1_506916	538,974,525
GL192355.1_3331547	538,974,532
GL192355.1_4355446	538,974,534
GL192356.1_1145900	538,974,617
GL192356.1_3095309	538,974,538
GL192356.1_4160080	538,974,540
GL192357.1_2738867	538,974,547
GL192436.1_163988	538,974,722
GL192458.1_314900	538,974,583
GL192567.1_546062	538,974,555
GL192818.1_522018	538,974,558
GL193388.1_310637	538,974,560
GL193990.1_102229	538,974,562
GL192338.1_3880	538,974,572
GL192339.1_4341286	538,974,409
GL192414.1_2044481	**538,974,553**
GL192628.1_441991	538,974,557

In bold, the sexual marker

mean = 0.27 out of 45 SNPs; Pyrenean mean = 0.27 out of 60 SNPs), H_E and H_O, HWD, F_{IS}, and F_{ST}. No monomorphic loci were found in the Pyrenean population compared to 15 in the Cantabrian population (Table 2). The 61-SNP panel was initially designed to genotype Pyrenean bears of Slovenian origin for individual identification regularly and was later applied to Cantabrian bears. This probably explains the lower monomorphism of the 61-SNP panel in the Pyrenean population compared to the Cantabrian population.

Tests of Hardy–Weinberg genotype proportions resulted in several loci that were significantly different from expected, likely due to genetic drift. This included 9 loci in the Pyrenean population, 13 loci in the Cantabrian population, and 3 in both populations (Table 2). Genetic diversity such as expected heterozygosity (H_E) was identical in Pyrenean ($H_E = 0.357$; 60 SNPs) and Cantabrian bears ($H_E = 0.357$; 45 SNPs). However, since the 61-SNP panel was originally designed to identify Pyrenean bears, genetic diversity in this population could be overestimated. H_E and H_O were not significantly different within populations (W test, $P > 0.05$). However, F_{IS} values were significantly different between populations (W test, $P < 0.05$), with significantly negative values in the Pyrenean population (Table 2). This result suggests that Pyrenean bears exhibit greater genetic diversity than expected under random mating conditions. Several factors could explain the excess of heterozygotes; in this case, we attribute it to non-random mating. The Pyrenean population has grown from a dozen individuals to around 80 individuals, primarily due to the reproductive success of the male bear "Pyros", who is related to 89% of the bears genotyped in this study (only 9 identified genotypes are unrelated, or 14 if considering genotypes before 2017). Although the "Pyros effect" is not currently detect in the population, future monitoring is recommended to assess potential excess of homozygotes over time. In contrast, despite being isolated for over a century, the Cantabrian population remains in genetic equilibrium. As expected for two geographically

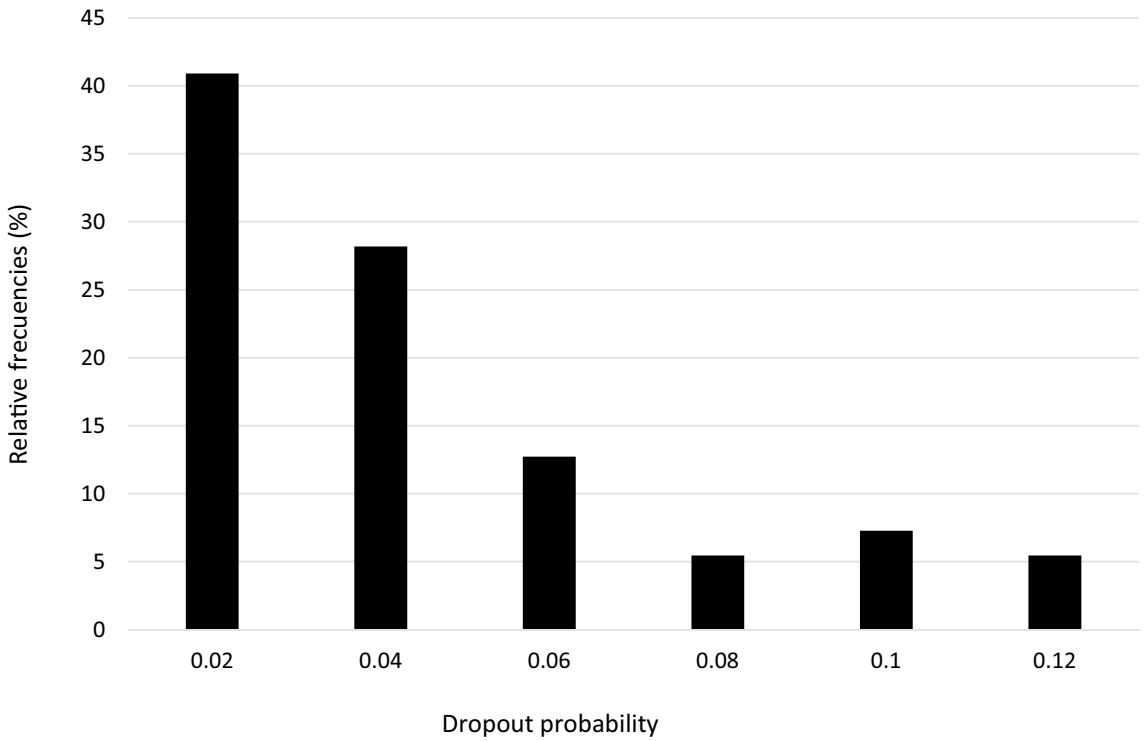

Fig. 2 Histogram showing the relative frequencies (%) of dropout probability using the SNP+ software (Sastre et al. 2023). A total of 1,835 non-invasive samples (×4 replicates) from brown bears were used to calculate the dropout probability of the panel

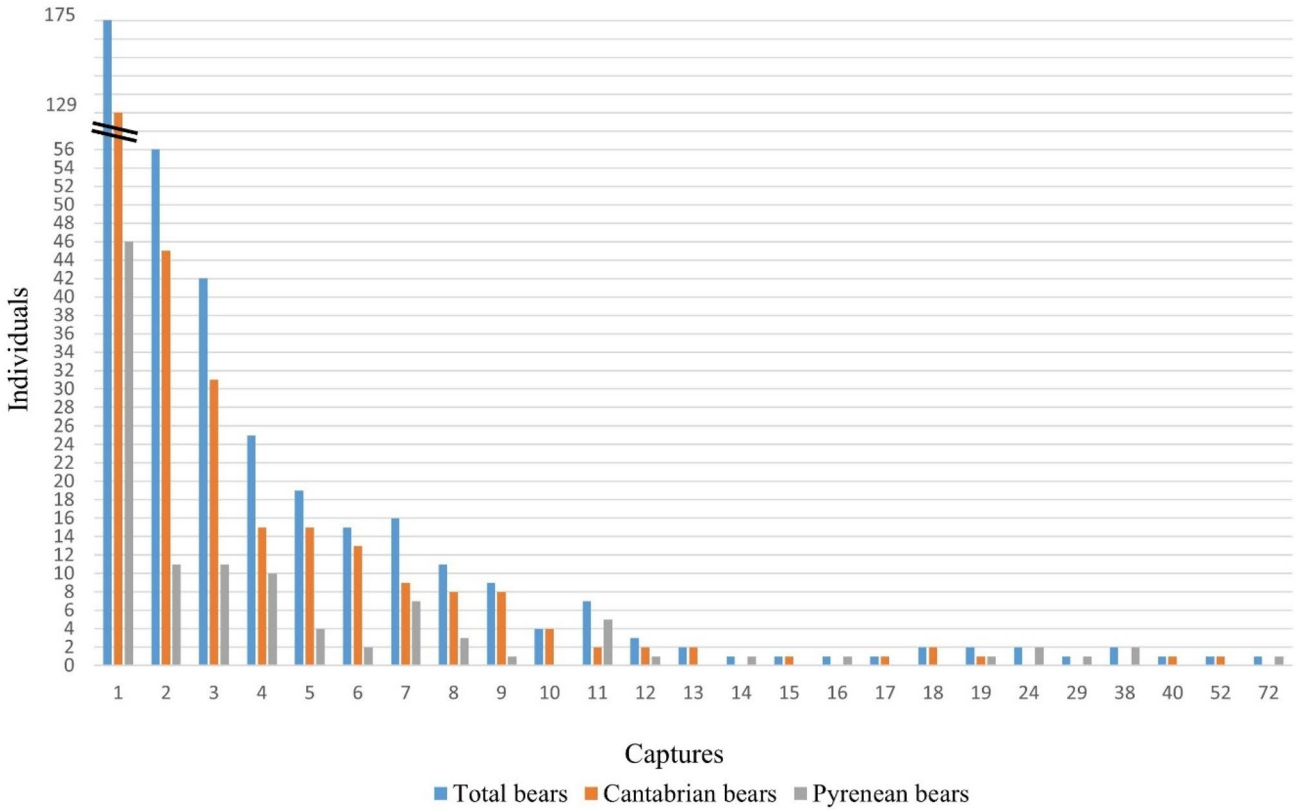

Fig. 3 Distribution of 1,611 brown bear samples from Northern Spain based on the number of laboratory identifications (with call rate = 1) per individual (captures). The orange bars represent the Cantabrian population (N = 290), the grey bars represent the Pyrenean population (N = 110), and the blue bars represent the total number of bears identified (N = 400)

Table 2 Genetic diversity for each SNP in two bear populations

Locus	Pyrenean bear (n = 99)				Cantabrian bear (n = 290)			
	N	H_O	H_E	F_{IS}	N	H_O	H_E	F_{IS}
1	99	0.485	0.447	−0.091	290	0.507	0.495	−0.026
2	99	0.061	0.078	0.218	290	0.269	0.387	0.305***
3	99	0.232	0.222	−0.053	288	0.361	0.322	−0.123*
4	99	0.475	0.486	0.018	290	0.197	0.189	−0.044
5	98	0.357	0.501	0.284**	290	0.472	0.422	−0.121
6	99	0.384	0.347	−0.110	This locus is monomorphic: no test done			
7	98	0.449	0.435	−0.038	289	0.433	0.492	0.120*
8	99	0.354	0.305	−0.163	290	0.507	0.462	−0.100
9	99	0.576	0.497	−0.163	290	0.5	0.501	−0.001
10	99	0.404	0.359	−0.133	This locus is monomorphic: no test done			
11	99	0.545	0.416	−0.317*	289	0.412	0.336	−0.229***
12	99	0.515	0.502	−0.031	288	0.333	0.392	0.149*
13	97	0.412	0.329	−0.260*	289	0.118	0.117	−0.007
14	99	0.455	0.456	−0.001	This locus is monomorphic: no test done			
15	99	0.465	0.47	0.007	This locus is monomorphic: no test done			
16	99	0.141	0.199	0.284*	290	0.283	0.325	0.128*
17	99	0.586	0.48	−0.227*	This locus is monomorphic: no test done			
18	99	0.434	0.482	0.094	290	0.303	0.312	0.026
19	98	0.01	0.01	−0.005	290	0.079	0.089	0.107
20	99	0.556	0.495	−0.128	290	0.507	0.479	−0.061
21	99	0.343	0.347	0.006	290	0.386	0.421	0.080
22	99	0.545	0.416	−0.317**	290	0.303	0.299	−0.016
23	99	0.434	0.412	−0.059	290	0.152	0.14	−0.082
24	99	0.535	0.499	−0.078	290	0.141	0.143	0.012
25	99	0.444	0.416	−0.073	290	0.003	0.003	−0.002
26	98	0.408	0.327	−0.256*	290	0.124	0.135	0.076
27	99	0.414	0.394	−0.056	This locus is monomorphic: no test done			
28	98	0.531	0.455	−0.171	290	0.541	0.499	−0.087
29	99	0.515	0.489	−0.058	This locus is monomorphic: no test done			
30	99	0.202	0.199	−0.023	This locus is monomorphic: no test done			
31	98	0.439	0.431	−0.023	This locus is monomorphic: no test done			
32	99	0.253	0.279	0.091	290	0.441	0.423	−0.044
33	99	0.131	0.123	−0.070	This locus is monomorphic: no test done			
34	99	0.515	0.501	−0.034	This locus is monomorphic: no test done			
35	98	0.49	0.401	−0.227*	290	0.521	0.466	−0.120*
36	99	0.182	0.166	−0.100	This locus is monomorphic: no test done			
37	99	0.455	0.456	−0.001	289	0.325	0.313	−0.041
38	99	0.273	0.251	−0.091	290	0.493	0.447	−0.105
39	99	0.273	0.237	−0.158	286	0.385	0.5	0.230***
40	99	0.485	0.48	−0.015	290	0.321	0.498	0.355***
41	99	0.455	0.473	0.034	290	0.2	0.238	0.159*
42	99	0.364	0.312	−0.172	290	0.476	0.465	−0.026
43	99	0.495	0.482	−0.032	289	0.356	0.37	0.034
44	99	0.313	0.293	−0.076	285	0.526	0.492	−0.071
45	99	0.212	0.191	−0.119	This locus is monomorphic: no test done			
46	99	0.111	0.105	−0.059	289	0.024	0.024	−0.012
47	99	0.444	0.47	0.051	290	0.414	0.387	−0.070
48	99	0.121	0.114	−0.065	290	0.279	0.27	−0.037
49	99	0.343	0.324	−0.065	290	0.49	0.495	0.009

Table 2 (continued)

Locus	Pyrenean bear (n = 99)				Cantabrian bear (n = 290)			
	N	H_O	H_E	F_{IS}	N	H_O	H_E	F_{IS}
50	99	0.495	0.462	−0.076	290	0.379	0.37	−0.026
51	98	0.571	0.501	−0.147	290	0.424	0.414	−0.027
52	99	0.01	0.01	−0.005	289	0.349	0.45	0.222***
53	99	0.576	0.478	−0.211	290	0.507	0.469	−0.082
54	99	0.343	0.347	0.006	290	0.538	0.5	−0.078
55	99	0.576	0.468	−0.237*	This locus is monomorphic: no test done			
56	99	0.495	0.468	−0.063	289	0.526	0.492	−0.071
57	99	0.455	0.436	−0.048	288	0.278	0.274	−0.017
58	99	0.323	0.299	−0.086	This locus is monomorphic: no test done			
59	99	0.384	0.336	−0.148	290	0.321	0.386	0.167**
60	80	0.038	0.037	−0.019	287	0.408	0.345	−0.183**
Total		0.381	0.357	−0.068*		0.354	0.357	0.006

Deviations from Hardy–Weinberg equilibrium were assessed from the fixation index F_{IS} for each locus

H_O, observed heterozygosity; H_E, expected heterozygosity

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

separate populations, F_{ST} showed significant differentiation between the two bear populations ($F_{ST} = 0.309$; $P < 0.05$). The Bayesian model-based clustering method showed a peak in ΔK at K = 2 (Fig. 4a, b; Evanno et al. 2005), clearly distinguishing the two populations (Fig. 4c). Factorial correspondence analysis (FCA) also indicated genetic differentiation, with Pyrenean bears forming a distinct cluster on the first factorial axis (FA-I), which explained 18.61% of the total genetic diversity (Fig. 5). We have created five clusters based on the available ecological information: 1 (in yellow on the FCA)—samples collected in the Western area of the Cantabrian Mountains; 2 (in blue on the FCA)—samples collected in the Eastern area of the Cantabrian Mountains; 3 (in grey on the FCA)—samples collected in both areas of the Cantabrian Mountains; 4 (in white on the FCA)—genotypes unrelated to the bear "Pyros" in the Pyrenees; 5 (in pink on the FCA)—genotypes related to the bear "Pyros" in the Pyrenees (Figs. 4c, 5). Although the Bayesian analysis suggested K = 2, our 61-SNP panel effectively differentiated between the western and eastern Cantabrian subpopulations (Figs. 4, 5).

Furthermore, the presence of migrants demonstrates the connectivity between the two subpopulations. This alignment between ecological data (sample collection location) and genetic data (allelic frequencies) highlights the differentiation of two distinct clusters within the Cantabrian population. Connectivity of the territory in small and isolated populations is crucial to avoid the negative effects of inbreeding and genetic drift (Frankham 1998; Pérez et al. 2010; Sawaya et al. 2014). The presence of migrants in the Cantabrian bear population and the lack of substructure in the Pyrenean population based on kinship with the bear "Pyros" are good long-term survival indicators for two endangered populations. However, another crucial factor for population viability is the sex ratio. The population's sex ratio has the potential to influence individual behaviours, for instance, competition among males, male coaction, and female mate selection (Clutton-brock and Parker 1995; Jirotkul 1999; Bellemain et al. 2006; Ancona et al. 2017). Therefore, a sex ratio bias can significantly affect demographic parameters such as reproductive success and survival, ultimately influencing long-term population viability (Dale 2001; Grayson et al. 2014; Heinsohn et al. 2019). The Pyrenean population (N = 99) exhibited an expected sex ratio (0.51 for females and 0.49 for males), consistent with other bear populations, such as the Scandinavian brown bear and Beaufort Sea polar bear (Regehr et al. 2006; Bellemain et al. 2006; Cronin et al. 2009). However, a male-biased sex ratio was observed in the Cantabrian population. The number of males detected (N = 183) was about twofold compared to females (N = 107). Within subpopulations and based on the location of the faeces, the numbers of males and females detected in the east was slightly significant toward males χ^2 ((1, N = 113) = 4.68, P = 0.03), and strongly significant in the west χ^2 ((1, N = 177) = 15.87, P = 6.78E−05). In brown bears, dispersal populations could be sex-biased since males cover long distances while females remain philopatric (Shirane et al. 2019). This would explain why all genotyped individuals from the extreme northwest of the Iberian Peninsula were males. However, this alone does not explain the observed sex bias, as only 7 of 115 Western males were from Galicia. Another explanation could be that if the technique lacks specific, the Y chromosome of the prey might be amplified, mistakenly identifying a female sample as male (Murphy et al. 2003; Sastre et al. 2009). However, the sexual primes were specific and in the eastern and Pyrenean

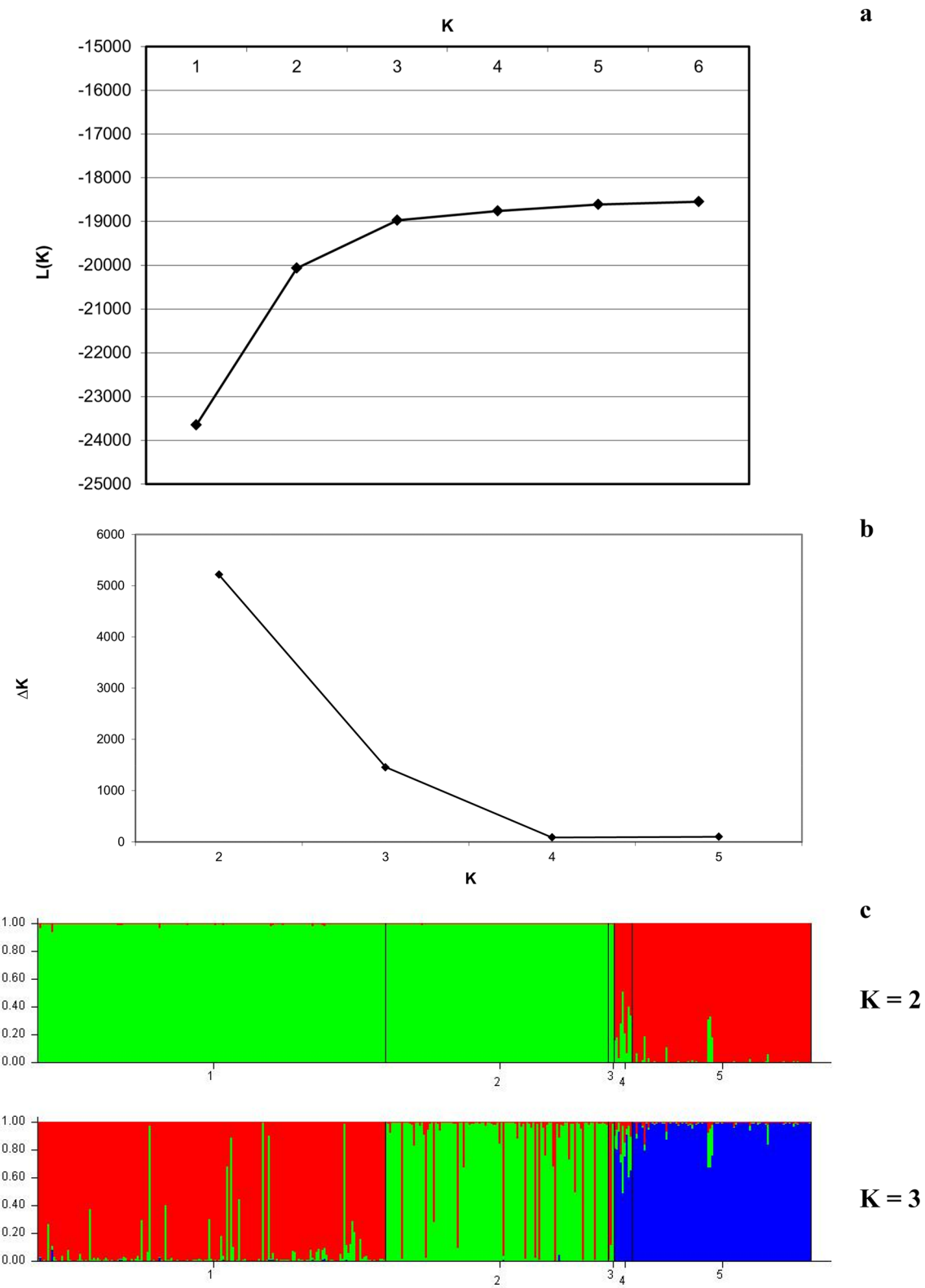

Fig. 4 Bayesian clustering approach for two bear populations. **a)** Mean likelihood L(K) over 25 runs assuming K clusters (K = 1–6). **b)** ΔK following Evano et al. (2005), where the modal value of the distribution is the highest level of structuring. **c)** Individual assignment using K = 2 and K = 3 clusters. Each individual is represented as a vertical bar sectioned into K colored segments, whose length is proportional to the likelihood of assignment to the K cluster. Cluster 1—samples collected in the western area of the Cantabrian Mountains; cluster 2—samples collected in the eastern area of the Cantabrian Mountains; cluster 3—samples collected in both areas of the Cantabrian Mountains; cluster 4—genotypes unrelated to the bear "Pyros" in the Pyrenees; cluster 5—genotypes related to the bear "Pyros" in the Pyrenees

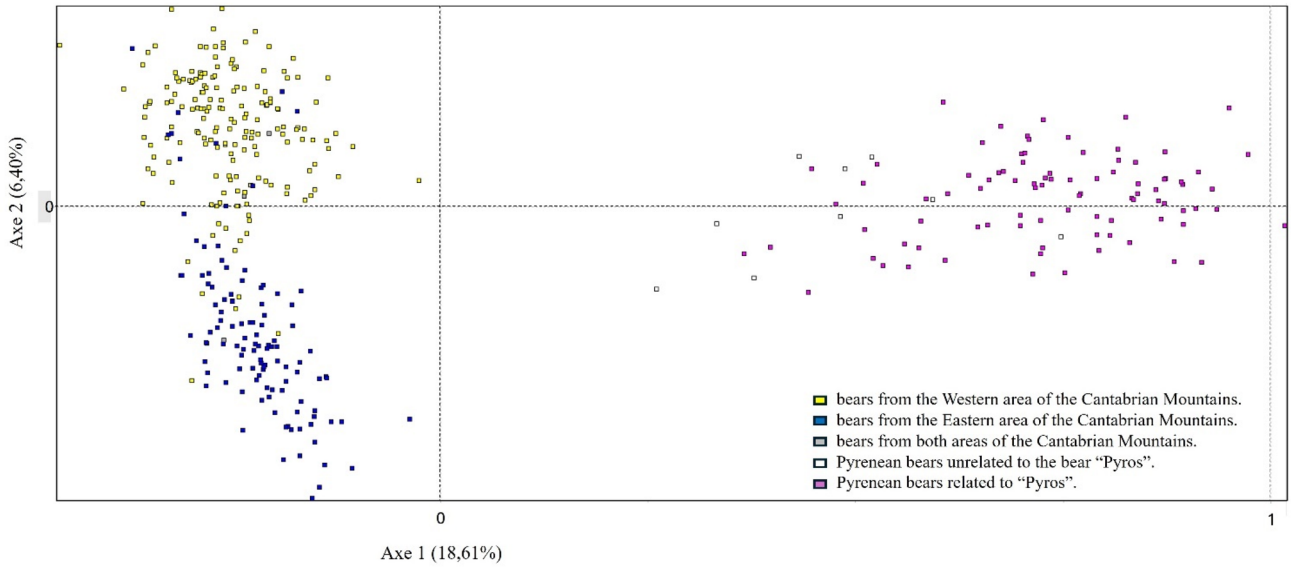

Fig. 5 The factorial correspondence analysis for two bear populations. The Pyrenean bears were distinct from the Cantabrian bears on the first factorial axis, FA-I, explaining 18.61% of the total genetic diversity. In yellow—samples collected in the Western area of the Cantabrian Mountains; in blue—samples collected in the Eastern area of the Cantabrian Mountains; in grey—samples collected in both areas of the Cantabrian Mountains; in white—genotypes unrelated to the bear "Pyros" in the Pyrenees; in pink—genotypes related to the bear "Pyros" in the Pyrenees

populations, the sexual ratio was nearly as expected. Moreover, there is no evidence in the literature suggesting that females have a higher mortality rate than males. Gonzalez et al. (2016) found a sex bias in the Western subpopulation, which they attributed to greater scent marking on trees by males. This was confirmed in the Pyrenean population, where hair samples showed a male-biased sex ratio, though fecal samples did not. However, in the Cantabrian population, the sex bias was significant regardless of sample type and subpopulation. Therefore, a plausible explanation could be the terrain's orography and the difficulty of finding samples from females, who are less dispersed throughout the territory, especially pregnant females and those with cubs.

To summarize, our methodology of DNA extraction, the preliminary step of preamplification, the subsequent amplification and the strict filters established to identify an individual allowed us to reliably identify about 70% of non-invasive brown bear samples from the Iberian Peninsula. Our 61-SNP panel effectively distinguishes individuals and populations, helping to unveil the population dynamics and diversity in Pyrenean and Cantabrian brown bear populations through non-invasive sampling. Monitoring and managing these two endangered populations are essential to controlling genetic diversity and promoting their adaptation to changing environmental conditions. This crucial non-invasive genetic data can be used to develop effective conservation strategies without disturbing wild animals.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics statement Animals were not killed for the purpose of this manuscript.

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