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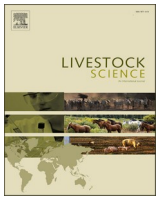

Removal of undesirable MC1R gene alleles from 'Berrenda en Negro', an endangered Spanish cattle breed, to enhance breed conservation programs

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HIGHLIGHTS

- Genetic analyses were performed in order to identify the Extensión locus genotypes
- Nine strategies for removal of animals carrying undesirable alleles were evaluated
- Results are very promising for management of *Berrenda en Negro* conservation program
- The most suitable strategy was to prioritise the elimination of E^De animals
- Coat markers might be used to differentiateproducts from others breeds ones

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ABSTRACT

'Berrenda en Negro' (BN) is an endangered Spanish cattle breed with substantial ecological and cultural value and meat quality. Phenotypically, it is differentiated by its black-sided coat pattern. The goal of this study were to analyse the presence of unwanted alleles at the *MC1R* locus (E^+ and *e*) and propose appropriate selective strategies for their eradication from the population. Both goals were based on the identification of genotypes of the *MC1R* locus using genetic markers and DNA microsatellites, to identify changes in the genetic variability of the resulting populations after applying selective strategies to decrease the frequency of red and wild-type alleles. Introgression and crossbreeding of BN with other Iberian cattle breeds in the past has left undesirable E^+ and *e* alleles, with frequencies of 7 and 12%, respectively. The extent to which the genetic variability of the BN population could be affected by selection strategies was quantified in terms of the number of affected animals and farms, effective population size (Ne) of the remaining genetic variability, and loss of gene diversity and allelic richness. The neutral genetic diversity found in BN suggested that selection strategies for the *MC1R* gene are possible. These strategies should be implemented progressively, in combination with extensive mating control. The best strategy to eliminate unwanted MC1R alleles in the short term would be to remove all cattle with the E^De genotype in the first phase, followed by a second phase, in which the E^DE^+ genotype would be eradicate after the recovery of the population to its effective size. The results of this study will assist the BN Breeders Association to managing the conservation program for the breed.

1. Introduction

In Spanish, the term 'berrendo' refers to the spotted coat colour of ruminants. There are two Spanish autochthonous spotted cattle breeds: the 'Berrenda en Negro' (BN) and the 'Berrenda en Colorado' (BC) with black- and red-sided coat patterns, respectively. Both 'berrendas' breeds are known for their rusticity and high maternal character, and they have been traditionally reared under extensive systems in the 'Dehesa'

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ecosystem. This ecosystem consists of a *Quercus* wooded pasture with high biodiversity maintained via human-livestock interactions in the Mediterranean forest. Most 'Dehesa' ecosystem are in the Natura 2000 network. Both breeds are recognised having high nature value (Ferraz et al., 2016). These two breeds play important ecological and environmental roles because they provide unique ecosystem services by producing meat with functional quality from 'Dehesa' pastures and have outstanding historical and cultural value. Oxen of these breeds were trained for managing Lidia cattle breeds on the countryside, the bull-fighting squares (Lidia shows), and popular bull runs (as the 'San Fermines Party' in Pamplona).- Additionally, they have been used for draught power during religious practices (such as the 'Virgen del Rocio' Pilgrimage in Andalusia). Both breeds are considered endangered. For the BN breed only 2,201 females and 84 sires are located in the 72 herds that remaining (2019 livestock census data from the Spanish Ministry of Agriculture, Fisheries and Food).

'Berrendas' breeds have long been considered a single-spotted coat breed with two colour variants, red and black, and have been mated together over an extended period (Rodero et al., 2007). Mitochondrial -DNA analyses have shown that both 'berrendas' breeds have different origins (Pedrosa Moro, 2006), and currently, they are officially considered two distinct breeds, despite their highly similar morphology (Rodero et al., 2012). The reproductive separation between the two breeds was established since the inception of their studbooks in 2005, being separately managed by the BN Cattle Breeders Association and BC Cattle Breeders Association [ANABE (Spanish acronym)]. Because of the morphological similarities between the two 'berrendas' breeds, decisions on founder animal records in the BN or BC studbooks were based solely on their red or black coat colours, respectively (Rodero et al., 2007). The coat phenotype is an important trait for the traditional uses of the 'berrendas' breeds. For example, pairs of yoked oxen or groups of five halters for driving fighting bulls must be as similar as possible. Accordingly, it is the aimed to fix the black coat-colour in BN, and consequently, its name will correspond not only to the phenotypic expression (Figure 1) but also to the genotype.

The *extension* (E) or *MC1R* locus, encoding the bovine melanocortin 1 receptor (MC1R), has a length of 1751 bp and is located on chromosome 18. The MC1R locus plays a vital role in determining coat colour by regulating red pigment synthesis against the black (NCBI Reference Sequence: NC_037345).

The evolutionary pattern of the MC1R locus has been linked to the natural and artificial selection of coat-colour phenotypes; consequently it has been useful in studying the formation process of the different livestock breeds by tracing their molecular evolution throughout history. They have been identified as a genetic signature for selection (Xu et al., 2015; Gurgula et al., 2020). The main changes in cattle occurred from the late Iron Age to the Middle Age, followed by intensive selection for coat colour by humans (Cieslak et al., 2011; Niemi et al., 2016).

Genes affecting the morphological characteristics and coat colour, such as MC1R, have proven to be useful as molecular markers for the identification of local breeds, detection of introgression or interbreeding with other breeds (Hulsman Hanna et al., 2014), and traceability and verification of the origin of their products (Maudet and Taberlet, 2002; Russo et al., 2007; Gan et al., 2007).

Three main alleles of the *extension* locus have been identified, regardless of those determining different levels of reddening intensity: i) dominant black E^D, a mutation typical of black animals when producing eumelanin; ii) wild-type E^+, typical of chestnut breeds, allowing the expression of the agouti locus and iii) recessive allele *e*, typical of blond or red breeds, which forms pheomelanin when it is homozygous. E^+ acts neutrally, in the following order of dominance $E^D > E^+ > e$ (Klungland et al., 1995; Seo et al., 2007). The current allele frequencies of the MC1R locus in bovines exhibit significant variation among breeds worldwide (Graphodatskaya et al., 2000; Rouzaud et al., 2000; Kantanen et al., 2000; Kriegesmann et al., 2001; Royo et al., 2003; Sasazaki et al., 2007; Gan et al., 2007; Russo et al., 2007; Carruthers et al., 2011; Nishinaki et al., 2016; Kasprzak-Filipeck et al., 2020), expressing different coat colours and showing that interaction of the E locus alleles with those of other loci is possible (Matsumoto et al., 2019).

In the case of the two Spanish 'berrendas' breeds, crossings between them or with other breeds before the establishment of their studbooks made it possible to frequently have offspring with a spotted black phenotype with genotypes containing *e* or E^+ recessive alleles at the MC1R locus, despite coming from BN parents. Additionally, calves born with brown (E^+E^+ or E^+e genotypes) or red (*ee* genotype) spots were negatively selected and not accepted into the BN breed studbook.

Fig. 1. Cattle and calf of the BN breed raised in the "Dehesa" system (rights owned by ANABE).

However, animals belonging to the BN breed with a black dominant genotype (E^De or E^DE^+) have been allowed to spread unwanted alleles (E^+or e) in the BN population. Failure to record the genotype of animals accepted into the BN Breed Studbook or the phenotypes of rejected offspring has made it difficult for breeders to predict the coat colour resulting from mattings and the potential for conservation of the BN population has been reduced.

For the past 8 years, the BN breed has been subjected to an official breeding program by ANABE to i) conserve the population using primarily *in vivo* and *in situ* methods, ii) monitor its neutral genetic variability using DNA microsatellite markers (FAO, 2008), iii) improve its morphology and phenotypical coat features, and iv) genetically evaluate the ability of cows to raise calves for meat in their natural habitat.

Regarding these goals, identifying E^+ and *e* alleles in the BN cattle breed by genotyping the *extension* locus would allow the implementation of negative selection policies to prevent the mating of unwanted carriers. However, adopting strategies for this purpose in the BN cattle breed could reduce the number of animals available for reproduction, thereby affecting the effective population size and the conservation of this endangered breed. In small populations, the selection of breeding animals by coat-colour traits could cause loss of genetic variability because of to genetic drift (Álvarez et al., 2011). Thus, it is necessary to devise strategies to maintain maximum genetic diversity while identifying marked traits and produced measures to reduce genetic drift and inbreeding (Williams, 2005; Leroy et al., 2011). The selective efficiency will depend on the initial frequency of the allele to be eliminated (Falconer, 1981); thus, if it is very high, its cost for breed conservation in terms of effective size could make it unfeasible. However, the availability of genomic data for the identification of different genotypes could help to achieve selective objectives of the BN cattle breeding program, leading to an increase in genetic progress while keeping consanguinity under control (Sonesson et al., 2012; Pryce et al., 2012; Cole, 2015; Xu et al., 2015; Upperman et al., 2019).

In order to meet the requests posed by ANABE regarding the removal of animals carrying undesirable recessive alleles (E^+ and e) of Extension locus (MC1R) from BN population and on the basis that the association was aware of the existence of some animals carrying them, the goals of this study were: a) to determine the prevalence of the different MC1R alleles in BN population; b) to determine the different variability genetic parameters in BN population; and c) to select the most appropriate strategy addressed to eradicate E^De or E^DE^+ genotypes from BN population, avoiding to the extent possible, both the genetic diversity loss and the decrease in the effective population size in this endangered breed. Currently, BN pedigree shows a lack of quality in their records; thus, changes in genetic diversity due to genetic selection measures might not be properly estimated by the optimal contribution selection method (OCS). As a consequence, genetic information provided by microsatellites analyses appears to be the most suitable approach to evaluate genetic diversity in BN population.

2. Materials and methods

2.1. Sample collection

A sample (*N1*) of 837 breeding BN cattle (626 females and 211 males), registered in the BN studbook by ANABE, was randomly and proportionally chosen for the census (14–20 animals per herd) of 66 herds in five geographical areas (10–21 farms by area), namely Andalusia, Castilla y León, Castilla La Mancha, Madrid, and Extremadura (Rodero et al., 2013). The selected cattle were genotyped for the *MC1R* or *extension* locus to determine the presence of different alleles. All the sampled females were breeding, whereas calf candidates for future breeding were included among the males. To avoid kinship relationships among the sampled animals, they were selected based on the pedigree data from the last two generations. Only black-spotted-coat animals are included in the studbook, and consequently, there was no possibility for the unwanted genotypes E^+E^+, E^+e, and *ee*, to be present in the sample. Further, to analyse the neutral genetic variability in the current BN population and evaluate the effects of possible selective actions for the *extension* locus on the breed, a smaller sample of animals was taken from *N1* (*N2* = 412). This second sample consisted of active breeding animals recorded in the BN studbook (305 females and 107 males) and was genotyped using 24 DNA microsatellite markers.

2.2. Genetic analyses, DNA extraction and purification for the genotyping of the MC1R locus

Genetic analyses were conducted by the MERAGEN Laboratory (University of Córdoba, Spain). The MC1R marker was amplified using conventional polymerase chain reaction PCR. Genomic DNA was isolated from aliquots of 200 μL of blood samples using a Higher Purity TM Blood Genomic DNA Extraction Kit (Canvax Biotech, S.L., Córdoba, Spain) according to the manufacturer's instructions. The quality and amount of DNA were measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

DNA was amplified with the forward 5′-CCATGAGTTGAGCAGGACCC-3′and reverse 5′- AGGCCAAAGCCCTGATGAAT-3 primers (766 bp) using the *Bos taurus* melanocortin 1 receptor as the target. PCR was performed in a 17.2 μL mixture containing 60 ng of template DNA, 2.5 μL of 10 × PCR Buffer, 1.5 mM MgCl2, 200 mM dNTPs, 10 pmol of each primer, and 2 U of Biotools DNA Polymerase (Biotools Biotechnological and Medical Laboratories SA, Madrid, Spain). PCR conditions were as follows: an initial denaturation at 96°C for 5 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 65°C for 70 s, and elongation at 72°C for 70 s. The final elongation step was at 72°C for 7 min. PCR was conducted using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR products were resolved using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) to determine the presence of different alleles.

Twenty-four microsatellites were successfully amplified and genotyped (BM1818, BM1824, BM2113, CSRM60, ETH10, ETH152, ETH185, ETH225, ETH3, HSC1, ILSTS006, ILSTS011, INRA005, INRA023, INRA32, INRA063, MGTG7, SPS115, TGLA122, TGLA126, TGLA227, TGLA48, TGLA53 and TGLA57). These markers are among those recommended by ISAG and FAO based on their polymorphisms.

All 24 microsatellites were amplified in four multiplex fluorescent PCR reactions, and fragment lengths were detected in a single semi-automated multiplex electrophoresis run using a 3500xL Dx Genetic Analyzer (Applied Biosystems) and GeneMapper™ Software (Applied Biosystems). Each reaction was performed in a total volume of 15 μL containing 50 ng of template DNA, 1 × Qiagen Multiplex PCR Master Mix, 1 × PCR Master Mix, primer mix, and nuclease-free water. Analysis of the amplified fragments was performed using the GeneScan Software V.3.7 by regression analysis to the standard size used, and the size of the fragments (alleles) was estimated and presented using GeneMapper™ Software 4.0.

2.3. Genetic parameters of variability

To obtain the allelic and genotypic frequencies of *MC1R* alleles and to analyse heterozygosity, the GENEPOP program v. 1.2 (Raymond and Rousset, 1995) was used. A χ^2 test was conducted to compare sex using Statistical 12.0 for Windows.

To better understand the contribution of each group to the genetic variability of BN cattle, the characteristics of the 24 microsatellite DNA marker panels and the neutral genetic variability in the BN population (*N2*) were analysed. The analysis considered the possibility that the BN population was substructured into subpopulations defined by their *MC1R* genotype and the following parameters: number of alleles per locus (Na), allelic richness (k) rarefracted for sample size, gene diversity (GD), observed (H_o) and expected (H_e) heterozygosity (Nei, 1978), and F Wright's statistic (F_{IS}, F_{IT}, and F_{ST}), determined using FSTAT 2.9.3.2,

GENETIX 4.05, and CERVUS 3.0.7 software. The mean effective molecular size (MN_e) was determined using NeEstimator v2.1., a software that applies Nomura's co-ancestry method, providing an effective size value for each allelic frequency and the mean for all effective size values. The Hardy-Weinberg equilibrium was tested using GENEPOP v. 1.2 software. To refine the data, the markers ETH152, HSC1 ILSTS011, INRA032, MGTG7, TGLA48 and TGLA57, which could only be genotyped in less than 78 animals indicated the lowest values in the number of alleles, were rejected, leaving only 17 markers for posterior analysis. To calculate the loss of genetic diversity that occurs when a subpopulation formed by carriers of red or wild-types alleles is eliminated from reproduction, Molking 3.0 software was used (Gutiérrez et al., 2005); consequently the importance of the eliminated subpopulation to genetic diversity (Caballero and Toro, 2002) or allelic richness (Petit et al., 1998) is elucidated. The contribution of the subpopulation to the total diversity (GD_T) is the sum of the contribution to the diversity within the subpopulation (GD_W) or molecular coancestrality within the subpopulation, and its contribution to the diversity between the subpopulations (GD_B) or Nei's minimum distance between subpopulations ($GD_T = GD_W + GD_B$) (Caballero and Toro, 2002). If GD_T has a positive value, it indicated that after removing a given subpopulation, the resulting set increases in its total genetic diversity, and, therefore, the subpopulation does not contribute substantially to the total diversity. In the approximation of Petit et al. (1998), if the contribution of the subpopulation to the total allelic richness is positive, it means that the set resulting after removing the subpopulation has a lower number of alleles than the original population and, therefore, the subpopulation must contribute substantially to the total allelic richness. The effective sizes (Ne) were calculated based on the reproducers remaining in the BN population after removing the proportion of sires or cows corresponding to each strategy, according to the classical equation of Wright (1931) under the assumption of random reproduction in the total population as follows:

$$Ne = \frac{(4Nm + 4Nf)}{(Nm + Nf)}$$

where *Nm* is the number of sires and *Nf* is the number of cows.

2.4. Breeding strategies for elimination of E^+ or e alleles

To simulate the results obtained when applying policies to eliminate the recessive alleles *e* and E^+ in the current population of the BN breed, nine possible strategies were established for the removal of animals with E^De or E^DE^+ genotypes, depending on whether the strategy acts on one or both genotypes, as well as on cows, bulls or both sexes: strategy S1, eliminate all carrier animals of the red (*e*) or wild-type (E^+) allele; strategy S2, eliminate carrier males and females of the red allele (*e*) and male carriers of the wild-type allele (E^+); strategy S3, eliminate male and female carriers of the red allele (*e*) and female carriers of the wild-type allele (E^+); strategy S4, eliminate all carriers of the wild-type allele (E^+) and male carriers of the red allele (*e*); strategy S5, eliminate all carriers of the wild-type allele (E^+) and female carriers of the red allele (*e*); strategy S6, eliminate male carriers of the wild-type (E^+) or red alleles (*e*), and strategy S7, eliminate female carriers of the wild-type (E^+) or red alleles (*e*). The possibility of working by eliminating a single allele, red or wild-type (strategies S8 and S9, respectively), was also considered.

3. Results and Discussion

3.1. Prevalence and variability of recessive red and wild-type alleles on the MC1R locus in the BN breed

The allelic and genotypic frequencies of the *MC1R* locus in the BN breed are shown in Table 1. In both sexes, unwanted alleles (E^+ and *e*) were found, with the recessive allele *e* having a higher than E^+. Phenotypic selection in the BN breed, since the creation of its studbook, against non-black coats, is probably why the allelic frequencies of *e* and E^+ are relatively low (0.1213 and 0.0771, respectively). The percentage of animals with the E^De genotype was approximately 24% in both sexes, and E^DE^+ was present in 17% of males and 15% of females. The observed heterozygosity of the *MC1R* locus was higher in males (0.4123) than in females (0.3914), but not significantly ($P > 0.05$). The presence of E^+ allele was consistent with the ancestral origin of most Iberian cattle breeds (Cañón et al., 2011) since the E^+ wild-type allele is present in several traditional European breeds (Kantanen et al., 2000). Accordingly, crossing with the wild-coated "Pajuna" breed, which has shown a phylogenetic relationship with BN, could have resulted in the presence of the E^+ allele (Luque et al., 2006). The presence of the *e* allele in the BN breed could also be a consequence of crossbreeding with the BC, prior to their separate management. It could also be a consequence of uncontrolled crossbreeding with the F1 progeny of Limousine or Charolais breeds typically used for industrial crossing with the BN breed (Rodero et al., 2007). The homozygous *MC1R* gene is typically lower in crossbreeds (Hartatik, 2017), but in our case, the heterozygote excess (F_{IS} = -0.177) was chiefly explained by the rejection of the wild-type and red phenotypes in the studbook; therefore, homozygotic E^+E^+ or *ee* were not possible.

Similar situations have been experienced with other breeds because of the decision of breeders to fix the coat colour of their animals. Thus, the Swedish Red and Swedish Mountain breeds with red and black coat colours, respectively, were managed to have different genotypes at the *MC1R* locus. The E^D allele was fixed in the Swedish Mountain breed, in the same manner that the recessive *e* allele was fixed in the Swedish Red breed (Kantanen et al., 2000). Although in the Friesian breed from Canada and the USA, the selection to fix the black coat colour has been practised for decades, in 1969, they allowed the registration of red-coated breeders in the herd book because the sole objective of selection shifted to the improvement of milk yield (Leduc, 2006).

3.2. Characterisation of genetic variability in the population of BN cattle breed

Parameters characterising the genetic variability of microsatellite markers used are presented in Table 2, including 214 alleles across24 loci. The number of alleles per locus ranged from three at TGLA48 to 23 at TGLA122 and, averaged 8.29 and 7.17across all loci for the number of alleles and allelic richness, respectively.

Despite the phenotypic selection against red or chestnut animals (*ee*, E^+e, and E^+E^+ genotypes) practised in the BN breed for 20 years, levels of neutral genetic variability remained high. The observed heterozygosity varied from 0.43 at INRA063 to 0.77 at BM2113, with an average of 0.63 across all loci indicating moderate GD in the BN cattle breed (0.712). F_{IS} mean values revealed a slight excess of heterozygotes at the HSC1 and TGLA57 loci. Fourteen of 24 analysed loci were in Hardy-

Table 1
Allelic and genotypic frequencies, observed heterozygosity (H_o), expected heterozygosity (H_e), and F_{IS} values of the *MC1R* locus in the BN cattle breed (Sampled Size *N1* = 837). Number of animals in brackets.

	e	E^D	E^+	E^De	E^DE^+	E^DE^D	H_o	H_e	F_{IS}
Total	0.12	0.80	0.08	0.24 (203)	0.15 (129)	0.60 (505)	0.40	0.34	-0.18
Females	0.122	0.80	0.07	0.24 (153)	0.15 (92)	0.61 (381)	0.39	0.33	-0.17
Males	0.12	0.79	0.09	0.24 (50)	0.17 (37)	0.59 (124)	0.41	0.35	-0.18

Table 2
Characteristics of the 24 DNA microsatellite markers used and genetic variability in the BN cattle breed: analysed population (*N2* = 412), number of animals (*N*), numbers of alleles per marker (Na), allelic richness (*k*), chromosome location (Chr), gene diversity (GD), observed (H_o) and expected (H_e) heterozygosities, F_{IS} values and deviation from the Hardy-Weinberg equilibrium test (HW) per locus and average over total of loci.

Locus	N	Na	*k*	Chr	GD	H_o	F_{IS}	HW[1]
BM1818	342	8	7	23	0.754	0.669	0.092	***
BM1824	391	8	7	1	0.767	0.663	0.142	***
BM2113	412	12	12	2	0.866	0.771	0.117	***
CSRM60	363	8	7	10	0.719	0.674	0.124	n.s.
ETH10	410	8	8	5	0.710	0.652	0.083	***
ETH152	73	5	5	5	0.490	0.360	0.281	n.s.
ETH185	213	11	11	17	0.717	0.626	0.143	n.s.
ETH225	399	8	7	9	0.717	0.627	0.131	***
ETH3	409	10	10	19	0.772	0.686	0.138	***
HSC1	77	8	7	20	0.691	0.685	-0.041	n.s.
ILSTS006	363	9	9	7	0.807	0.741	0.073	n.s.
ILSTS011	77	5	4	14	0.625	0.559	0.097	n.s.
INRA005	359	4	4	12	0.637	0.566	0.100	n.s.
INRA023	410	11	10	3	0.762	0.655	0.173	***
INRA32	77	9	7	11	0.666	0.58	0.110	***
INRA063	363	4	4	18	0.491	0.433	0.140	*
MGTG7	73	6	7	23	0.598	0.560	0.026	n.s.
SPS115	407	8	6	15	0.680	0.590	0.125	***
TGLA122	412	23	21	21	0.799	0.701	0.115	***
TGLA126	397	7	7	20	0.722	0.657	0.117	*
TGLA227	375	14	12	18	0.836	0.710	0.150	***
TGLA48	73	3	3	7	0.595	0.547	0.057	n.s.
TGLA53	388	19	18	16	0.884	0.779	0.081	***
TGLA57	73	6	6	1	0.537	0.560	-0.039	n.s.
Total Mean (Std.Dev.)		8.29 (4.60)	7.17					
(4.49)		0.712 (0.108)		0.627 (0.098)	0.106 (0.065)			

[1] * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s.: not significantly different.

Table 3
Mean number of alleles (MNA) and allelic richness (*k*) per locus, observed (H_o) and expected (H_e) heterozygosity, Hardy-Weinberg test (HW) and F_{IS} value within groups of genotypes by sex and in the whole BN population analysed (*N2* = 412) for 17 microsatellite markers. Mean contributions (in percentage) to the genetic diversity and allelic richness of BN cattle breed subpopulations established by genotype when one of them was removed from the population of active sires and cows (*N2* Sample Size = 412).

Subpopulation	N	MNA	*k*	H_o	H_e	HW	F_{IS}	GD	GD_W	GD_B[a]	GD_T Loss/ Gain	C_T	C_W	C_B
E^DE^D Subpopulation														
E^DE^D Males	62	7.24	6.82	0.67±0.11	0.74±0.08	***	0.097±0.06	0.7543	-1.5818	0.8382	- 0.7436	-0.19703	- 0.6125	0.4155
E^DE^D Females	169	8.24	7.71	0.63±0.09	0.74±0.09	***	0.148±0.04	0.7519	-2.5787	1.5131	- 1.0655	-1.7484	-0.87427	-0.87422
Total E^DE^D [b] Animals	231	8.35	7.97	0.64±0.09	0.74±0.09	***	0.134±0.04	0.7529	0.1884	0.3843	0.5727	-3.6774	-2.7576	-0.9197
E^De Subpopulation														
E^De Males	26	6.00	5.45	0.69±0.12	0.73±0.09	***	0.054±0.11	0.7539	-1.5910	0.7881	- 0.8028	-0.2178	- 1.3645	1.1466
E^De Females	93	7.88	7.58	0.68±0.10	0.74±0.09	*	0.077±0.07	0.7564	-1.3888	0.9117	- 0.4771	-0.5330	- 0.8706	0.3376
Total E^De [b] Animals	119	7.94	7.64	0.68±0.09	0.74±0.08	***	0.073±0.06	0.7533	0.6117	0.0174	0.6292	-0.9859	-2.7962	1.8102
E^DE^+ Subpopulation														
E^DE^+ Males	19	6.59	6.15	0.63±0.17	0.77±0.14	***	0.186±0.18	0.7507	-1.8911	0.6647	- 1.2264	1.0609	2.7420	-1.6811
E^DE^+ Females	43	7.29	4.83	0.65±0.12	0.75±0.11	***	0.134±0.12	0.7497	-1.9710	0.6208	- 1.3502	1.1124	0.9798	0.1325
Total E^DE^+ [b] Animals	62	8.12	6.26	0.65±0.10	0.76±0.12	***	0.145±0.09	0.7398	-0.6755	-0.5085	- 1.1840	3.4691	5.5539	-2.0847
Mean over total	412	9.47	8.94	0.65±0.08	0.74±0.09	***	0.117±0.03							

GD: Genetic diversity of subpopulation. GD_T or loss/gain, contribution to total GD; GD_W, contribution to within-population GD; GD_B, contribution to between-populations GD; C_W, contribution to within-population allelic richness; C_B, contribution to between-populations allelic richness; C_T, total contribution to allelic richness.

[a] Corresponding to Nei's minimum distance.

[b] Contributions of the three subpopulations without differentiating sexes.

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

Weinberg disequilibrium. This can be expecting considering that the BN breed is endangered and subject to genetic drift or the Wahlund effect.

3.3. Genetic variability of different subpopulations of the BN cattle breed

The obtained values of genetic variability using the panel of 17 DNA microsatellite markers for different subpopulations of*MC1R* genotypes showed that animals with the desirable genotype (E^DE^D) presented the highest allelic richness (7.97). In contrast, females with the E^DE^+ genotype had the lowest *k* value (4.83) (Table 3).

The three subpopulations defined by their MC1R genotype, were relatively close (mean $F_{ST} = 0.004$), and the differences among them did not indicate that the neutral genetic diversity within the BN breed to be substructured by genotypes of the *MC1R* locus; most of the total variability was caused by individual differences ($F_{IT} = 0.121$). An F_{ST} value of 0.00049 between the E^DE^D and E^De subpopulations, indicated them to be the nearest genetically.

A slight decrease in the positive values of F_{IS} statistic, was noted for heterozygotes in all cases, especially the E^DE^+ male population ($F_{IS} = 0.186$).

The greater heterozygosity of microsatellites found in the E^De subpopulation could be explained by uncontrolled crossbreeding with BC, Limousine or Charolais breeds which are carriers of the *e* allele (Ginja et al., 2013).

The positive values of Petit approximations suggest that the E^DE^+ subpopulation contributes substantially to the total allelic richness of the BN breed ($C_T = 3.469$), especially the allelic richness within subpopulations ($C_W = 5.5539$). C_B values suggest that moving the E^DE^+ population, mainly the males (C_B=-1.6811), would increase the differences between the remaining subpopulations. (Table 3).

When the contribution to genetic diversity was analysed using the Caballero and Toro (2002) coefficient, a small difference among the six subgroups was revealed because the removal of any of the remaining subgroups, except the E^DE^+ genotype, resulted in a loss of the between-genotype diversity in the whole population. This could be explained by the low estimated genetic distance between the E^DE^D and E^De groups. E^DE^D females (GD_T = -1.0655) exhibited a higher contribution to total diversity than E^DE^D males (GD_T = -0.7436), which was primarily determined by their contribution to within-subgroup diversity (GD_W = -2.5787).

If we focused on the contribution to the between-subpopulation diversity (GD_B) and total diversity (GD_T), or we wanted to maintain the contribution to the total allelic richness (C_T), our priority would be to maintain the E^DE^+ female subpopulation. However, the highest contribution to within-subpopulation allelic richness appeared in the E^DE^+ male subpopulation ($C_W = 2.7420$).

We analysed the global breed neutral diversity using DNA microsatellites to simulate the loss of genetic variability following selective decisions under the hypothesis that existence of BN subpopulations was based on the various genotypes at the *MC1R* locus. Here, wet did not consider other possible subdivisions or related comparisons, such as herds or lineages. Because information from paternity test analysis was used, microsatellites are an economical and suitable solution for analysing heterozygosity and allelic richness; however, the use of dense panels of single nucleotide polymorphism (SNP) markers could provide greater precision to the calculations performed for analysing the genetic architecture of the BN breed and the genetic variability within subpopulations (Lenstra et al., 2012).

3.4. Loss of genetic variability because of the application of strategies for the eradication of wild-type and red alleles

The E^D allele at the *MC1R* locus in the BN breed exhibited a very high frequency, close to 80% in both sires and breeding cows. Thus, to achieve a higher short-term fixation, it is possible to utilise strategies based on molecular diagnosis to remove of breeding animals carrying the unwanted E^+ and *e* alleles without excessive risk to general genetic diversity. This could contribute to debugging possible introgression with other non-black coat breeds and increase the possibility of the progeny of the current population to be enlisted in the studbook (Stachurska et al., 2012). The loss of genetic variability could lead to the loss of desirable rare alleles in some of the genes of high interest that are unrelated to coat colour, for the BN cattle breed population. Consequently, it could reduce their ability to adapt to future environmental changes (Allendorf et al., 2010; Bruford et al., 2015) or the genetic progress in selection for meat performance (Molina et al., 2006).

The likely number of animals to be eliminated from the *N1* sample will differ depending on the adopted strategy, impacting the population differently (Table 4). Logically, strategy S1, which proposes the simultaneous removal of carriers from both sexes and both genotypes have the highest cost in terms of numeric loss of breeding animals, which could affect approximately 40% of the BN population, in 49 herds. At the other extreme, the strategy S6 would eliminate males carrying both genotypes, representing 10.39% of the population. However, the latter option would provide the worst results in terms of Ne in the remaining population ($Ne_{S6} = 199.1$) and affect a larger number of herds (40 herds) than other strategies. Strategy S9, which focuses only on the E^DE^+ genotype, involves the elimination of only 15% of the breeding animals (37 males and 92 females), affecting a lower number of herds (32 herds); nevertheless, strategy S7 appeared to be the most favourable strategy for Ne (Ne_{S7}=316.17 vs Ne_{S9}=269.70).

In terms of retaining the genetic variability in the BN breed, according to the MN_e and *k* values, strategy S6 could be the better option (*k* =8.74±3.96 and $MN_e = 31.95±15.59$). Additionally, strategy S6 is the most suitable when accounting analytical cost because we would not have to perform genotyping of females for the *MC1R* locus. However, strategy S6 would result in a low Ne value.

If the intention is to shorten the time to achieve total elimination of the E^+ and *e* alleles in the BN cattle, a strategy rejecting carriers of both sexes would be appropriate; in this case, the best options, in terms of *k* and MN_e values, are the strategies S8 ($k = 8.53±3.57$) and S4 ($MN_e = 29.33±12.49$).Considering the negative values obtained for the contribution to the total genetic diversity (GD_T), a loss of the overall genetic diversity would occur using any of the proposed strategies (Table 5). The contribution to the internal diversity (GD_W = -1.9710) of females carrying the E^DE^+ genotype, would be removed by applying strategies S1, S4, S5, or S9. Of these four strategies, strategy S9 (GD_T = -1.8294 and GD_W = -2.0826), will be the most damaging to the total genetic diversity and the genetic diversity within subpopulations, whereas However, t strategy S8, which proposes eliminating only animals (cows and sires) carrying the E^De genotype, appears to be the best option (GD_T = -0.3997 and GD_W = -1.0770).

None of the proposed strategies, except S1 strategy, would increase the existing differences in genetic diversity among the remaining groups (GD_B) established by genotype and sex. This could be explained by the low genetic distances between the subpopulations, according to F_{ST} values.

To eradicate an undesirable allele, the best approach could be to start with a mild strategy and then progress toward a moderate or severe strategy (Windig et al., 2004). If the goal is to maintain diversity in the short term, maintaining the levels of neutral heterozygosity would be preferable, which in our case, was especially high in the E^De male population. However, if the goal is to maintain the population long term, emphasis should be placed on maintaining allelic richness (Medugorac et al., 2009); in this case, the main contribution would be from the E^DE^+ female population ($C_T = 1.1124$).

Based on our results, we propose to start by applying the strategy S8. This would immediately eliminate the *e* allele from the population, removing every cow and sire carrying the E^De genotype, with a moderate impact on the effective size and low impact on allelic richness. Selection against the E^DE^+ genotypes would be put into practice in a second phase, following the recovery of the effective size of the BN population;

Table 4
Proposed strategies for eradicating genotypes carrying the red and wild alleles at the *MC1R* locus in the BN cattle breed (*N1* Sample Size = 837). Ne = Effective size remaining after application of strategy

Strategies	Carriers of genotypes to be removed		N° of animals and percentage of N1 to be removed				
	E^DE^+	E^De	Sires	Cows	Total	% of N1 (herds)	Ne Remaining
S1	Males and females	Males and females	87	245	332	39.67 (49)	196.27
S2	Only males	Males and females	87	153	240	29.15 (46)	197.67
S3	Only females	Males and females	50	245	295	35.24 (45)	247.95
S4	Males and females	Only males	87	92	179	21.39 (46)	198.35
S5	Males and females	Only females	37	245	282	33.69 (39)	265.87
S6	Only males	Only males	87	0	87	10.39 (40)	199.12
S7	Only females	Only females	0	245	245	29.27 (31)	316.17
S8		Males and females	50	153	183	24.25 (39)	250.20
S9	Males and females		37	92	129	15.41 (32)	269.70

Table 5
Mean number of alleles (MNA), Allelic richness per locus (*k*), Observed heterozygosity (H_o), Expected heterozygosity (H_e), Hardy-Weinberg test (HW), F_{IS} value (F_{IS}) and Mean molecular effective size (MN_e), obtained from analysing 17 microsatellites markers in the BN population, after applying each one of the selection strategies over the *MC1R* locus. Percentage contribution to genetic diversity of the different BN subpopulations obtained from the nine removal strategies of genotypes carrying red or wild alleles at the *MC1R* locus and percentage loss or gain of the total genetic diversity in the BN cattle breed when one of the subpopulations was removed from the current breeding population (*N2* Size Sample = 412).

Applied Strategy	N	MNA	k	H_o	H_e	HW[1]	F_{IS}	MN_e	GD	GD_W	GD_B[a]	GD_T
Without acting	412	9.47	8.94	0.65±0.06	0.74±0.08	***	0.117±0.027	33.48				
S1	231	8.35	7.97	0.64±0.07	0.74±0.08	***	0.134±0.036	23.93	0.7478	-1.3113	-0.2933	-1.6047
S2	274	8.94	8.33	0.64±0.07	0.74±0.07	***	0.133±0.035	27.30	0.7533	-1.1576	0.2796	-0.8779
S3	250	8.70	8.24	0.64±0.07	0.74±0.08	***	0.139±0.035	25.88	0.7529	-1.2052	0.2802	-0.9250
S4	324	8.82	8.47	0.65±0.06	0.74±0.08	***	0.117±0.029	29.33	0.7461	-1.8563	0.0313	-1.8250
S5	257	8.41	8.01	0.64±0.07	0.74±0.08	***	0.126±0.037	25.00	0.7477	-1.6746	0.0600	-1.6146
S6	367	9.23	8.74	0.65±0.07	0.74±0.08	***	0.118±0.027	31.95	0.7510	-1.6777	0.4898	-1.1879
S7	276	8.76	8.28	0.64±0.07	0.74±0.08	***	0.130±0.035	26.65	0.7525	-1.5535	0.5668	-0.9866
S8	293	9.18	8.53	0.64±0.08	0.74±0.09	***	0.137±0.034	28.50	0.7569	-1.0770	0.6773	-0.3997
S9	350	8.88	8.51	0.65±0.09	0.74±0.07	***	0.113±0.030	29.55	0.7461	-2.0826	0.2532	-1.8294

GD: genetic diversity of the subpopulation; GD_T or loss/gain, contribution to total GD; GD_W, contribution to within-population GD; GD_B, contribution to between-subpopulations GD.
[a] Corresponding to Nei's minimum distance.
[1] * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

concurrently, pedigree would require monitoring to conserve genetic diversity and minimise between-animal relationships simultaneously.

The afore mentioned results should be considered preliminary, owing the unavailability of genealogical information with sufficient quality for applying an optimal selection strategy as an acceptable compromise to maintain both objectives of maximising E^D allele frequency and diversity in the breeding population (Eynard et al., 2018). Further simulation studies on the evolution of genetic diversity are necessary after the implementing the strategies in each of the phases. Because of the crucial contribution of the BN breed to genetic diversity among Spanish bovine breeds Cañón et al. (2011), the selection of the BN population favouring the genotype E^DE^D and the control of non-native contributions via elimination of the *e* and E^+ alleles, would contribute to the conservation of the differentiation and the intraspecific variation of the BN population (Boettcher et al., 2010; Lenstra et al., 2012), making it more interesting in terms of uniqueness with respect to other races. This strategy has the potential to improve the conservation of uncompetitive breeds, such as the BN, which are extensively raised not for their productivity, but for their phenotypic traits, cultural values, and role in the ecosystem (Wang et al., 2019). However, favouring intra-breed diversity and preserving a high level of allelic diversity in neutral genes is important because these act as a proxy for genetic diversity at selective (unknown) loci.

The assisted selection of the *MC1R* locus and its relationship with quality meat traits could offer favourable possibilities without slaughtering the animals (Nishimaki et al., 2016). In Canadian beef cattle, an association between the selection for the *MC1R* locus and carcass quality has been reported. Accordingly, animals carrying the E^DE^D or E^De genotypes showed a greater increase in backfat and faster fattening than the red-coated ones; however, animals carrying the *ee* genotype displayed greater longissimus muscle lengths and higher carcass weights (McLean and Schmutz, 2009).

However, BN meat products are integrated under the quality label '100% Raza Autóctona' (100% autochthonous breed) established by the Spanish Ministry of Agriculture to safeguard Spanish endogenous breeds, by guaranteeing breed purity. To use coat molecular markers for the differentiation and traceability of BN products, it would be essential to eliminate the recessive *e* and E^+ alleles of the *MC1R* locus. These recessive alleles have been reported in other breeds reared in the same locale as the BN breed such as 'Berrenda en Colorado', 'Pajuna' (Royo et al., 2003), 'Retinta', 'Lidia' (González-Rodríguez et al., 2017), Charolais, and Limousine or their crosses (Rouzard et al., 2000). BN and BC are the only spot-sided coloured Spanish bovine breeds. Therefore integrating MC1R information with other genes affecting the sided-spotted coats in cattle breeds, such as the coding variant of the KIT gene (Durkin et al., 2012) and the haplotype variability of the MITF gene (Fontanesi et al., 2012 and Edea et al., 2017), the authentication of mono-breed BN products from those of other breeds with solid-black or roan phenotypes ('Avileña-Negra Ibérica', 'Lidia', 'Negra Andaluza', 'Negra Serrana', 'Cárdena'), carrying the E^D allele.

An example of the use of coat-colour gene markers in local breeds has been reported by Tinarelli et al. (2021). The implementation of strategies to exclude animals from Herd book (removing 4% of the animals) of the local population of the Mora Romagnola Pig Breed by fixing the MC1R and NR6A1 alleles proved successful to distinguishing mono-breed products and did not greatly affect the conservation of the genetic variability of the population.

To refine the usage of the *MC1R* locus as a specific molecular marker in the identifying of the BN breed, future studies should incorporate SNP markers in more complex panels for exploring differences in the coding

region of the *MC1R* locus (Yang et al., 2014). The maintenance of genetic diversity of in the case of the BN breed is one aspect for the conservation of this endangered breed, with other aspects pertaining to the protection of singular coat colour of animals of this breed (because they are a part of the unique landscape and ecosystem of the 'Dehesa') and to usage of the BN breed in expression of social values and performance of cultural and religious traditions

4. Conclusions

The phenotypic selection, carried out by farmers, to eliminate non-black coats in the BN breed since the establishment of the BN studbook has led to low frequencies and low homozygosity levels, which are associated with undesirable E^+ and *e* alleles of the *MC1R* locus; moreover, the presence of these alleles is explained by introgression and crossbreeding with other Iberian breeds in the past.

The endangered BN breed has remarkable genetic diversity and allelic richness, making it ideal for selection strategies associated with the *MC1R* locus. However, these strategies should be conducted progressively and concurrently with extensive control of mattings, owing to the imbalance in the Hardy-Weinberg equilibrium produced by the effects of genetic drift in the population.

The neutral genetic diversity found in the BN breed is primarily attributable to individual differences rather than those existing between subpopulations, according to the genotypes at the *MC1R* locus. Animals carrying the unwanted E^DE^+ genotype appear to contribute more to the allelic richness.

To improve the quality and identification of BN meat, it is essential to eliminate the recessive *e* and E^+ alleles from the population, allowing traceability protocols to distinguish mono-breed products, based on genetic markers associated with the *MC1R, KIT* and *MITF* loci.

These results could affect the decisions made to fix the E^D allele in the current breeding program developed by BN breeders. We demonstrated that the best strategy to eliminate unwanted *MC1R* alleles in the short term, based on the contributions of the population, would be applying strategy S8, as per which every E^De male and female would be remove, followed by a second phase focused on eradicating the E^+ allele. The second phase should commence after sufficient recovery of the effective size of the populations and concurrent with extensive mating using pedigree information.

Author contributions

Conceptualisation, R.G., A.G., and E.R.; Data curation, A. G., E.M.M., and ER; Formal analysis, A.G, E.M.M., and ER; Funding acquisition, E.R. and P.V.; Investigation, A.G., R.G., and E.R.; Methodology, A.G. and E. R.; Project administration, R.G. and E.R.; Software, A.G. and E.R.; Supervision, E.R.; Validation, A.G. and E.R.; All authors have been involved in developing, writing, commenting, editing, and reviewing the manuscript. All authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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