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CONSERVATION OF ENDANGERED SPANISH CATTLE BREEDS USING MARKERS OF CANDIDATE GENES FOR MEAT QUALITY

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The aim was to analyze the allelic and genotypic frequencies for two genes associated with tenderness of meat* (CAPN1 *and* CAST) *and one with fat deposits* (DGAT1) *in three endangered Spanish cattle breeds: Berrenda en Colorado (BC), Berrenda en Negro (BN), and Cardena Andaluza (CA) to utility of their involvement in the selection of them and to help the adoption of conservation measurement. Seventy-five males and 298 females of those breeds were genotyped. Genotypic and allelic frequencies for each polymorphic locus were estimated. There were significant differences in the genotypic frequencies among breeds in* CAPN1 *and* DGTA1 *genes and in the case of the genic frequencies in* CAPN1, CAST, *and* DGAT1 *genes. The three breeds analyzed (BC, BN, and CA) presented high allelic frequencies for the favorable allele of the three markers (from 0.41 to 0.75). The association between the favorable allele and meat quality must be confirmed. In cases of association with differences in quality meat, the absence of differences in the genotypic and genic frequency distributions between the sexes is advantageous in mating planning because it implies that there is no handicap to be overcome for the conservation program and it would allow the use of sires to promote the increase in improvements within a short period of time.

Keywords: Cattle; Intramuscular fat; Minority breeds; Molecular markers; Tenderness

The optimal utilization of farm animal genetic resources is fundamental to sustain the contributions of the livestock system to the food supply, in both developed and developing countries (1).

Rare breeds are usually subjected to conservation programs whose main objective is to maintain or increase the population size, managing their genetic variability without losing their adaptation to particular environments, their resistance to parasites, the quality of their products, and other attributes such as traction or their usability for hobby or tourist activities.

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Assessing the value of rare breeds is one way to encourage breeders to keep them (2), but the preservation of their unique characteristics typically requires a greater range of genetic attributes (3).

Berrenda en Colorado (BC), Berrenda en Negro (BN), and Cardena Andaluza (CA) are three native cattle breeds that are reared in the southwest of Spain. These populations are characterized by their small census size. They were traditionally raised alongside fighting bulls and were used for draft and hobby activities. Currently, they are also used to produce meat under a traditional Spanish land management system, the Dehesa. The three native endangered cattle breeds from Spain are distinct from imported breeds in that they are particularly well adapted to their natural environment, and a better knowledge of these breeds may help promote their conservation as an animal genetic resource. The protection of these types of breeds is needed for their conservation, and the improvement of their efficiency will provide better meat performance with products of distinct quality. Since 1992, the European agricultural quality policy has used label systems that promote and protect valuable food names to ensure consumer safety and product quality. Recently, farmers of BC and BN breeds started working to obtain a quality label as a way to distinguish their product as characterized by singular properties. Spanish legislation (4) recommends the development of marker-assisted selection processes as a step toward receiving a specific product quality label for these breeds. Recent advances in identifying candidate genes linked to meat quality characteristics can help to achieve this aim. Previous analyses have identified μ-calpain (*CAPN1*), calpastatin (*CAST*), and diacylglycerol O-acyltransferase1 (*DGAT1*) as candidate genes for tenderness and intramuscular fat (IMF), respectively. These markers are associated with nonsynonymous changes, and one of the two alleles of each is associated with more tender meat [the *C* allele in *CAPN1* (5) and the *CAST* marker (6)] or higher intramuscular fat content [the *K* allele in the *DGAT1* marker (7)]. Tenderness is one of the major traits that consumers evaluate to define meat quality (together with flavor) (8). IMF represents an important beef quality trait because it contributes to the juiciness and flavor of cooked meat (9). Nevertheless, the association between markers and phenotypic traits is highly influenced by breed or population (10–12), management (11), and environmental conditions (12).

Avilés et al. (13) sequenced a fragment of the *CAPN1* gene in nonminority Spanish native cattle breeds (Retinta, Avileña N-I, and Morucha) and identified five novel single nucleotide polymorphisms (SNPs) in addition to the well-known *CAPN1*, which is widely studied in the vast majority of commercial breeds.

In the current study, the *CAPN1* marker was analyzed in Spanish native endangered breeds that are reared in the Dehesa (the same ecosystem as previous breeds) and had never been genotyped for this marker; two other markers (*CAST* and *DGAT1*) were also analyzed. The estimation of allelic and genotypic frequencies of these breeds will provide an overview of the current situation as a starting point for improved meat quality in this population.

The aim of this study was to analyze the allelic and genotypic frequencies of two genes associated with meat tenderness (*CAPN1* and *CAST*) and of one gene associated with fat deposits (*DGAT1*) in three Spanish cattle breeds (BC, BN, and CA) to evaluate their utility for the selection of these minority breeds and for promoting the establishment of conservation measures.

MATERIALS AND METHODS

The breeds tested in this study have a specific geographical distribution in the southwest of Spain; the BC and the BN breeds are the two most dispersed. Table 1 shows the census of each breed divided by sex and by number of herds. The sampling procedure followed is also shown in the Table 1.

Blood samples (5 mL) were collected from the caudal veins of 75 males and 298 females belonging to three breeds (BC, BN, and CA) from different herds using a Vacutainer system. Genomic DNA was isolated from blood samples using a commercial kit (Dominion, MBL). The SNP in the μ-calpain gene (identified as *CAPN1*) was reported by Page et al. (5) and is mapped to BTA29. The single nucleotide polymorphism (SNP) referred to as *CAST* is located on BTA7 and was reported by Schenkel et al. (6). The SNP identified by Winter et al. (14), referred to as *DGAT1*, is linked to a quantitative trait locus situated on BTA14.

The amplification and genotyping of the three genes were carried out using a TaqMan allelic discrimination assay. Primers and ABI fluorogenic probes were designed to amplify each polymorphism to target the two alleles of each SNP. Samples were screened on an ABI PRISM 7500 FAST Real Time system (Applied Biosystems) from the genomics facility of the central research support service of the University of Cordoba (Spain) following the manufacturer's protocol.

Deviations from Hardy-Weinberg equilibrium were tested by running the Genepop version 4.1 statistical package (15). The allelic and genotypic frequencies for each marker and group studied (breed and sex) and pairwise tests between breeds for genotypic frequencies were calculated. The Genetix v 4.02 software was used to calculate the genotypic frequency analyses by comparison of the expected and observed heterozygosities considering Hardy-Weinberg equilibrium. A chi-squared maximum likelihood test was carried out to compare the frequencies of the three candidate genes between breed and sex. The threshold for significance was $P < 0.05$. Statistical analysis was carried out using the software Statistica for Windows 7.0 (Statsoft, Inc. Tulsa, OK, USA).

RESULTS

Genotype and allele frequencies for each polymorphic locus were estimated for the three breeds tested (Table 2). There were significant differences in the genotypic

Table 1 Total population and number of animals sampled for each cattle breed and sex

Animal Breed	Female Population[a]	Male Population[1]	Herds[a]	Sampled Animals		Sampled herds
				Females	Males	
Berrenda en Colorado	3205	589	149	85	32	17
Berrenda en Negro	2151	244	104	100	30	23
Cardena Andaluza	861	9	14	113	13[b]	6

[a]Data obtained from Spanish Ministry of Agriculture on December 31, 2011.

[b]The sample size of the Cardena Andaluza breed exceeds the current census of sires because several of the animals have died in the last two years.

Table 2 Comparison of the genotypic and allelic frequencies for each marker and breed

		CAPN1					CAST					DGAT1				
Breed	N	CC	CG	GG	C	G	CC	CG	GG	C	G	AA	AK	KK	A	K
BC	117	0.42	0.24	0.34[a]	0.54	0.46	0.45	0.48	0.07[a]	0.69	0.31	0.11	0.35	0.54[a]	0.29	0.71
BN	130	0.38	0.18	0.44[a]	0.47	0.53	0.56	0.37	0.07[a]	0.75	0.25	0.33	0.29	0.38[b]	0.48	0.52
CA	126	0.15	0.52	0.33[b]	0.41	0.59	0.47	0.42	0.11[a]	0.68	0.32	0.06	0.40	0.54[a]	0.26	0.74
	χ^2	47.60***			7.72*		5.19 n. s.			7.72*		36.09***			30.86***	

Abbreviations: BC, Berrenda en Colorado; BN, Berrenda en Negro; CA, Cárdena Andaluza.

[a,b]Genotypic frequencies within columns with different superscript letters are significantly different ($P < 0.05$).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n. s.: no significant differences.

($P < 0.001$) and genic ($P < 0.05$) frequencies among breeds for the *CAPN1* marker. Pair-wise tests for genotypic and genic differentiation indicated that the frequency distributions for the BC and the BN breeds were quite homogenous, while the CA breed was significantly different ($P < 0.05$). Regarding the *CAST* SNP, there were no significant differences ($P \geq 0.05$) among the populations tested. The *DGAT1* locus exhibited significant differences ($P < 0.001$) in both genotypic and genic frequencies among breeds. Pair-wise tests showed that the BN breed was significantly differentiated ($P < 0.001$) from BC and CA, which presented similar genotypic and genic frequency distributions.

The obtained genotype and allele frequencies demonstrated different behaviors in the three breeds, with greater differences found in the *CAPN1* marker.

Table 3 shows the genetic equilibria of the three markers in the three breeds. Significant departures ($P < 0.001$) from Hardy-Weinberg equilibrium were identified for the *CAPN1* marker in the BC and BN breeds and for the *DGAT1* gene in the BN population. A lower than expected number of heterozygotes for these loci was found. None of the three genes was found to be in Hardy-Weinberg disequilibrium in the CA breed, and the *CAST* gene was in equilibrium in all three populations.

The results for the three markers and the three breeds are shown in the Table 4, divided by sex. The *GG* genotype frequency for the *CAPN1* marker was the highest in both sexes of the BC breed, while the *CG* genotype was most frequent in the CA

Table 3 Observed and expected heterozygosities for each marker and breed

	CAPN1			CAST			DGAT1		
Breed	Ho	He	HW[a]	Ho	He	HW[a]	Ho	He	HW[a]
BC	0.2393	0.4968	***	0.4786	0.4278	n. s.	0.3504	0.4118	n. s.
BN	0.1769	0.4982	***	0.3692	0.3750	n. s.	0.2923	0.4992	***
CA	0.5238	0.4838	n. s.	0.4206	0.4352	n. s.	0.3968	0.3848	n. s.

Abbreviations: Ho, Observed heterozygosity; He, Expected heterozygosity; BC, Berrenda en Colorado; BN, Berrenda en Negro; CA, Cárdena Andaluza.

[a]Estimated P-value associated with the null hypothesis of Hardy-Weinberg equilibrium.

*** $P < 0.001$; n. s.: no significant differences.

Table 4 Genotypic and allelic frequencies by sex and comparison test between sexes for each marker and breed

			CAPN1					CAST					DGAT1				
Breed	Sex	N	CC	CG	GG	C	G	CC	CG	GG	C	G	AA	AK	KK	A	K
BC	Female	85	0.36	0.28	0.35	0.51	0.49	0.38	0.54	0.08	0.65	0.35	0.11	0.32	0.58	0.26	0.74
	Male	32	0.56	0.13	0.31	0.63	0.38	0.66	0.31	0.03	0.81	0.19	0.13	0.44	0.44	0.34	0.66
	χ^2		4.90 n. s.			2.68 n. s.		7.53*			6.35*		1.85 n. s.			1.39 n. s.	
BN	Female	100	0.39	0.17	0.44	0.48	0.53	0.55	0.40	0.05	0.75	0.25	0.36	0.24	0.40	0.48	0.52
	Male	30	0.37	0.20	0.43	0.47	0.53	0.60	0.27	0.13	0.73	0.27	0.23	0.47	0.30	0.47	0.53
	χ^2		0.15 n. s.			0.01 n. s.		3.29 n. s.			0.07 n. s.		5.49 n. s.			0.03 n. s.	
CA	Female	113	0.15	0.52	0.33	0.41	0.59	0.45	0.43	0.12	0.67	0.33	0.07	0.41	0.52	0.27	0.73
	Male	13	0.15	0.54	0.31	0.42	0.58	0.62	0.31	0.08	0.77	0.23	0.00	0.31	0.69	0.15	0.85
	χ^2		0.02 n. s.			0.01 n. s.		1.26 n. s.			1.15 n. s.		2.63 n. s.			0.93 n. s.	

Abbreviations: BC, Berrenda en Colorado; BN, Berrenda en Negro; CA, Cardena Andaluza.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s.: no significant differences.

population. However, the *C* allele and *CC* genotype frequencies were higher in both sexes in the BC population.

For the *CAST* marker, the *C* allele and the *CC* genotype had the highest frequencies in the whole population. Only females from the BC breed showed a higher frequency of the CG genotype. Significant differences ($P < 0.05$) between sexes were found for the BC breed. The *C* allele frequency in females was lower than that in males; moreover, the *CG* genotype was more frequent in females, while the *CC* genotype was the most common in males from the BC population.

For the *DGAT1* locus, the *K* allele showed higher frequencies in both sexes for all three breeds. Although males belonging to the BC and BN breeds showed the same or higher *AK* genotype frequency, no significant differences ($P \geq 0.05$) were observed between sexes. No male animal belonging to the CA breed showed the *AA* genotype.

DISCUSSION

The breed is the classification unit of diversity or "primary focus" of diversity in Animal Genetic Resources (AnGR) (16). The amount of diversity of AnGR is directly related to the capacity of livestock populations to adapt to future changes in environmental and market conditions. Therefore, breed conservation and use deserve attention and merit the application of adequate knowledge and skills. Within a species, the percentage of the genetic variation between breeds relative to the total genetic variation in the species varies between 0.25 and 0.66, depending on the trait (17).

As already was said in the Interlaken Declaration the continuing erosion and loss of animal genetic resources for food and agriculture could compromise efforts to achieve food security, improve human nutritional status and enhance rural development. We acknowledge that efforts to further conserve, develop, improve and sustainably use animal genetic resources should be enhanced. *In situ* conservation is

expected to facilitate breed evolution and adaptation to the environment, give insight into breed characteristics, help maintain the indigenous knowledge of livestock keepers and create possibilities for sustainable development in rural areas, and it can be financially self-sustainable (18).

The association between genotypic and allelic frequencies and meat tenderness and intramuscular fat is important for deducing how these frequencies impact the meat characteristics of animals with different genetic makeup (19, 6, 7).

Curi et al. (10) reported low levels and even the absence of the C allele of the *CAPN1* marker in Nellore breed and some *B. indicus* crosses. On the other hand, in a previous study of nonendangered native Spanish breeds (Retinta, Avileña N-I, and Morucha), Avilés et al. (13) detected lower allelic frequencies for the *CAPN1* marker than were observed in our study, reporting a *C* allele frequency of 0.18. Even the traditional British beef breeds reached frequencies of just 0.30 for the same allele, and the Continental and European dual purpose breeds exhibited frequencies of 0.05 (19). Thus, the BC, the BN and the CA breeds presented the highest frequencies (from 0.41 to 0.54) for the favorable allele for tenderness. This provides a potential to animals of this breed that controlling preslaughter factors (handle, gender and age) may eventually lead to differentiated quality meat products. Alderson (20) proposed a common origin for the BC and the BN breeds and the British White Park breed, although this hypothesis has never been confirmed with any phylogenetic procedure.

The allelic frequencies for the *CAPN1* marker in the Charolais and the Limousine breeds were 0.05 and 0.08, respectively (19). These two highly specialized in meat production breeds were introduced in Spain as a way to improve native breeds by the exploitation of the F1 generation. The frequency distribution of the *CAPN1* gene is remarkable in that in most of the experiments carried out in different breeds and crosses, the G allele was observed at much higher frequencies than the C allele, while in our sample, the frequencies of the two alleles are similar, with values close to 0.50.

These divergences are unlikely to be justified by the effect of selection for tenderness (assuming that the C allele is the favorable one) because these breeds have long been selected for handling herds of fighting bulls or as draft animals, and more recently as maternal breeds to be crossed with imported improved breeds. They are raised in complex production systems and presented low performances with few favorable features for meat production.

Only ten years have elapsed since the initiation of a selection program to improve growth and meat quality of the BC and the BN breeds; this may be the reason underlying the observed genetic disequilibrium. The complete isolation of both breeds was achieved only ten years ago by the establishment of separate Herdbooks, which required that only pure animals be raised in both breeds and prohibited crossing between these breeds. That is why, although a slow gene flow occurred between these two breeds, this event should have been intra-herd and not due to animal interchanges or artificial methodologies. This may be an explanation for the observed heterozygote deficiency despite the random selection of individuals from the sample.

However, breed improvement by selecting animals to carry alleles linked to the meat quality can either strengthen a conservation program or lead points of tension that depend on the frequency at which these alleles are present and selection strategies that maintain these frequencies (2).

The results cannot be explained by the effective size, which is quite small, or by pro-homozygous selection or an increase in inbreeding. Paradoxically, the heterozygosity determined by neutral molecular markers (microsatellites) as genetic variability indicators presented high values (Ho = 0.668 for BC; Ho = 0.653 for BN; and Ho = 0.678 for CA) (21). Therefore, the effect of sire and dam selection and its use in favor of this marker must be carefully monitored and accurately estimated in our endangered breeds.

The improvement in the performance records of the carcasses belonging to this type of cross makes them more attractive for the meat industry. If these breeds are considered useful as tools to improve adaptation to the environment while maintaining good meat quality through the contribution of the desirable genotype for the *CAPN1* marker, added value can be assigned to the final product.

Schenkel et al. (6) found a positive effect of the *C* allele of the *CAST* marker on tenderness. These authors reported *C* allele frequencies of 0.69 for the Charolais breed and 0.73 for the Limousin breed. Those frequencies were quite similar to those measured for the BC, the BN, and the CA breed (0.69, 0.75 and 0.68, respectively). The similarities in the frequency distributions of the marker in the different classical commercial crosses (Continental breed × Endangered breed) indicate that the *CAST* marker may not satisfy the requirements for use for meat tenderness improvement.

Although the three breeds analyzed are at a good starting point with regard to these two markers, meat tenderness depends on many other genes and is greatly affected by other factors such as carcass management and aging time (22). An association analysis is necessary to check the relationship between the markers and meat of animal belonging to each breed to assess and confirm their use to make selection decisions.

The majority of European breeds showed low frequencies (23, 24) of the favorable allele (*K*) of the marker linked to the IMF (*DGAT1* gene), first described by Winter et al. (14) in dairy breeds. Thaller et al. (24) found a *K* allele frequencies of 0.45 in German Holsteins and 0.11 in the Charolais breed. However, Pannier et al. (23) reported higher frequencies in Continental beef breeds varying from 0.12 in the Limousine breed to 0.18 in the Charolais breed. The *K* allele frequencies in our three endangered breeds are considerably higher (from 0.52 to 0.74). Although previous authors were not able to confirm the association between IMF and *DGAT1* in different muscles due to small sample sizes, an association analysis in our population with a significant number of individuals could extend the results of this preliminary study and also confirm the good genetic base of the BC, BN, and CA breeds for this marker. This study must consider that these breeds have never been selected for improved meat quality traits and have always been described as late-maturing breeds with fibrous meat and high amount of connective tissue (25).

Further studies will be necessary to develop marker assisted selection in these endangered breeds because the effects of these markers are also modulated by factors such as diet, management or environmental conditions. These breeds have small populations, and consequently, it is crucial to promote the parameters that add value to the product within the small number of animals of each breed and avoid potential negative effects resulting from selection strategies. Conservation must be the first objective in the management of these populations, to avoid increases in inbreeding and genetic drift.

Considering that no differences in frequency distributions have been detected between sexes (with the exception of the *CAST* marker in the BC breed), it will be necessary to specify the design of the matings and to promote the use of sires for artificial insemination. The CA breed will need more attention due to the small size of its sire census. First, males carrying the favorable alleles must be identified to promote their use as sires, with genetic distances considered to avoid inbreeding. This strategy would provide a maximum spreading of the desired alleles. This use of marker assisted selection may be an appealing strategy to promote consumer awareness about the positive qualities of the product to create and promote a quality label or similar tool linked to the breed and to the particular rearing system.

The accurate estimation of the effect of the three markers on the phenotype will be necessary to achieve this objective. The genotyping of sires would be a key step in pairing conservation with selection and genetic progress.

CONCLUSIONS

The three breeds genotyped in this study exhibited high frequencies of the *CAPN1, CAST,* and *DGAT1* alleles associated with meat quality in *B. taurus* breeds relative to the Continental breeds they are usually crossed with (Charolais and Limousine) to improve their productive characteristics and performance.

The maintenance of this frequency distribution may be crucial to conserve these breeds and to produce meat with differential quality. However, the association between the favorable alleles and meat quality must be confirmed in the meat of animals belonging to the BC, BN, and CA breeds.

The disequilibrium situation shown by the studied populations for the markers related to meat quality cannot be due to selection because the heterozygosities estimated by neutral molecular markers were of medium to high level. Therefore, the high homozygosity level must be a random effect or a coincidence of selecting animals from the same family in the sampling process.

The absence of differences in the frequency distributions between the sexes is advantageous in mating planning because it implies that there is no handicap to be overcome for the conservation program and it would allow the use of sires to promote the spread of improvements within a short period of time.

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