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Aggressive behavior in cattle is associated with a polymorphism in the *MAOA* gene promoter

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Summary

Molecular mechanisms underlying aggressive behavior are primitive and similar among the subphylum Vertebrata. In humans, a primary goal in the study of aggression is to determine the neurobehavioral molecular factors triggering violence. Although several species have been used to study agonistic responses, researchers are limited by the difficulty of artificially inducing aggression in animals not selected for it. Conversely, the Lidia cattle breed has been selected since the eighteenth century to display agonistic responses based on traits such as aggressiveness, ferocity and mobility, all of them showing significant heritability values. This intensive selection may have driven shifts in specific allele frequencies. In a previous analysis across the autosomes, we revealed long-term selection regions including genes involved in behavioral development. In the present study, we focus on mapping recent signatures of selection associated with aggressiveness at chromosome X, by comparing Lidia cattle samples with two non-specialized Spanish breeds showing tamed behavior. The most significant markers peaked around the monoamine oxidase A (*MAOA*) gene, and thus the associations of three functionally important regions located near the promoter of this gene were further investigated. A polymorphism consisting of a variable number of tandem repeats of the nucleotide 'C' (BTX:105,462,494) and displaying lower number of repetitions in the Lidia breed when compared with the tamed breeds was detected. *In silico* analyses predicted that the g.105,462,494delsinsC variant may code for the Sp1 binding motif, one of the major transcription factors controlling the core promoter and expression of the *MAOA* gene in humans.

Keywords behavior genetics, *Bos taurus*, Lidia cattle breed, polymorphisms, selection signatures

Introduction

Aggressiveness is a primitive yet highly conserved animal behavior, and as such, the molecular mechanisms underlying aggression are expected to be similar and common among the subphylum Vertebrata (Nelson & Chiavegatto 2001). A traditional prerequisite for domestication has been to breed for docility and discard aggressive responses (Belyaev *et al.* 1985). Consequently, livestock breeders and caretakers have sought behaviors based on friendly responses to humans in order to avoid aversive handling, which is a risk to their safety and may have negative effects on economically important traits (Belyaev *et al.* 1985; Fordyce *et al.* 1988), such as milk yield, milk protein and fat contents in dairy cattle (Breuer *et al.* 2000) or daily weight gain in beef cattle (Fordyce & Goddard 1984).

In humans, studies on the molecular basis underlying aggression are pivotal to determine the neurobehavioral factors triggering violence. Aggression forms are highly prevalent at both the intrapersonal (suicide) and the interpersonal (homicide) level, and show a clear genetic component with heritabilities near to 50% (Miles & Carey 1997; de Boer *et al.* 2003). According to the World Health Organization report (Krug *et al.* 2002), each year an estimated 1.6 million people die as a direct result of injuries resulting from violence, with aggressive acts being a serious and costly public health problem worldwide. A large number of preclinical studies in various animal species have shed significant light on the genetic background of human

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aggressiveness (de Boer *et al.* 2003); however, the molecular mechanisms involved in its initiation and progression are still unknown. Initially, murine species were the model of choice for studies on aggression. However, most strains of laboratory mice (*Mus musculus*) are bred to be docile and, consequently, they must be put into artificial situations to promote aggression (Nelson & Chiavegatto 2001). Other wt strains of various species have also been artificially developed to investigate the genetics of behavior, such as the successful experimental breeding program of silver foxes developed in 1956. This generated both a population that responds to humans in a friendly/tamed manner and a strain displaying aggressive responses to humans (Belyaev *et al.* 1985; Trut 1999, 2001). As for cattle, some traditional breeds that have retained primitive behavioral features, known as primitive breeds (Upadhyay *et al.* 2017), have been selected to develop agonistic–aggressive behavior, for example the Lidia bovine breed in Spain, along with the Italian Valdostana and the Swiss Herens. Lidia individuals have been classified and selected since the eighteenth century according to their aggression and fighting ability for use in bullfighting events (Eusebi *et al.* 2018a). Breeders use a set of traits registered on a categorical scale, including aggressiveness, ferocity and mobility as the main genetic parameters, all of them showing significant heritability values that range from 0.20 to 0.36 (Silva *et al.* 2006; Menendez-Buxadera *et al.* 2017).

Recently, the availability of genomic tools such as SNP panels (Bovine HapMap Consortium 2009) enable the identification of selection sweeps in populations under intense selection for a particular trait (Pritchard *et al.* 2010). Also, various software programs are available, such as SelEstim (Vitalis *et al.* 2013) and BayeScan (Foll & Gaggiotti 2008), both widely used and implemented to detect signatures of long-term positive selection using algorithms based on allele frequency differences among populations. In a previous analysis of the same sample set we identified two genomic regions in chromosomes BTA3 and BTA8 showing signals of selection and including several genes involved in behavioral traits (Eusebi *et al.* 2018b). However, similarly to most studies on selective sweeps, these analyses focused only on autosomes because of the unique analytical challenges that the X chromosome presents (Nature Medicine 2017).

Taking into account that the X chromosome includes several polygenic regions associated with variations in behavior in different species (Brunner *et al.* 1993; Cases *et al.* 1995; Sabol *et al.* 1998; Craig & Halton 2009; Pavlov *et al.* 2012), we sought signals in the bovine chromosome X (BTX) driven by recent human selection for agonistic responses. We used SNP array data from the BTX of individuals belonging to the aggressive Lidia population and two tamed Spanish breeds, Asturiana de los Valles (RAV) and the Morenas Gallegas racial group (MG; including the Vianesa, Frieiresa and Limiá breeds), in which aggressiveness is not a desirable behavior and thus has been selected against. The selection signatures were analyzed through the population-extended haplotype homozygosity (XP-EHH) method (Sabeti *et al.* 2007). A genomic region under positive selection was identified close to the *MAOA* gene, widely studied for its association with aggressiveness in humans and other species (Sabol *et al.* 1998; Craig 2000; Popova *et al.* 2001; Karere *et al.* 2009). Thus, the association of three functionally important regions located near the promoter region of this gene with the different behavioral responses displayed by aggressive and tamed cattle breeds was further investigated.

Materials and methods

Samples

For the selection signatures scan, a total of 303 cattle blood samples were collected in Magic Buffer tubes (Biogen Diagnostica, Spain) and maintained at 15 °C until use, with the conservation buffer guaranteeing unlimited DNA integrity (Dunner & Cañón 2006). Genomic DNA was extracted using a standard phenol/chloroform method (Sambrook *et al.* 1989).

Lidia cattle samples were classified into three different classes of aggressiveness: (i) 100 individuals belonging to Spanish lineages that display extreme aggressive behavior (Spa+); (ii) 65 showing intermediate aggressiveness (Spa−); and (iii) 48 individuals from Mexico (Mex) demonstrating mild aggressive responses. The three Lidia groups were defined according to the fragmentation of the racial group into small and isolated populations named lineages or 'encastes' (Cañón *et al.* 2008), in which differences in the three main behavioral characteristics traditionally scored in the breed – aggressiveness, ferocity and mobility – can be detected (Silva *et al.* 2006; Cañón *et al.* 2008). Concordantly, the lineages with higher, mild and lower behavior scores also showed the highest genetic differentiation among the Lidia breed lineages (Eusebi *et al.* 2018b).

As a control group, for the selection signatures scan, the tamed RAV breed ($n = 60$) and the MG racial group ($n = 30$) were analyzed. For the MAOA sequencing, RAV, MG, and Lidia samples were analized, together with samples belonging to beef cattle breeds used worldwide, such as Limousin and Charolais, and also three Iberian breeds (Avileña, Retinta and Rubia Gallega) never selected for production traits and, may be considered as the most representative modern autochthonous Iberian cattle breeds (Table 1).

Population genetic relationships among the different cattle breeds included into the analysis can be observed from their projection on the two main axes obtained after performing a PCA using genotype data from the Illumina SNP Beadchip (Fig. S1). Figure S1 shows the dispersion of the tamed cattle breeds (a), although they are grouped together when the Lidia breed is included in the PCA (b).

Identification of selection signatures

All samples were genotyped with the 50K SNP BeadChip (Http://www.illumina.com). The SNPs with a MAF below 1% and call rates less than 90% were removed from the dataset using PLINK v.1.90 (Purcell *et al*. 2007). All missing data were also pruned from the merged data set leaving ~400 SNPs in BTX for downstream analyses. Haplotype reconstruction was performed with BEAGLE v.5.0 (Browning *et al*. 2018). The XP-EHH method (Sabeti *et al*. 2007) was used as implemented in the software SELSCAN v.1.2.0 (Szpiech & Hernandez 2014) to identify recent selective sweeps by comparing the three Lidia groups (Spa+, Spa− and Mex) with the tamed RAV and MG groups. This estimation is based on cross-population comparisons to identify alleles that have been swept near to fixation within a population (Pickrell *et al*. 2009). We used 750 kb spanning windows and the XP-EHH scores were standardized across the whole genome. The scores exceeding 5% of the standardized distribution were identified as potential locations for positive selection signatures in each Lidia group, using the criteria of at least two adjacent significant SNPs present in both pair-wise comparisons – Lidia and the two tamed groups (RAV and MG).

Gene annotation

Information on the candidate genes included in regions under selection was extracted from the NCBI Bovine Genome database (ftp://ftp.ncbi.nih.gov/genomes/Bos_taurus/mapview/seq_gene.md.gz), and as annotation of the bovine genome is still incomplete, BioMart from Ensembl Archive release 90 (www.ensembl.org/biomart) was used to determine the orthologous human gene ID for each candidate gene.

MAOA sequencing design

Three genomic regions within the promoter of the *MAOA* gene were amplified in the aggressive ($n = 31$) and tamed groups ($n = 41$). The three selected regions are located close to or within a putative regulatory region that is highly conserved and GC rich (Fig. 1), containing different simple tandem repeats.

Table 1 Breeds included in the aggressive and tamed groups sequenced for the *MAOA* gene

Group	Breed name	Sample size
Aggressive	Spa+	15
	Spa−	12
	Mex	4
	Total	31
Tamed	Asturiana de los Valles	11
	Morenas Gallega	7
	Retinta	4
	Rubia Gallega	4
	Avileña	3
	Limousine	5
	Charolaise	7
	Total	41

The GenBank NCBI genome ID 189361(Bos_taurus_UMD_3.1.1) was used for primer design. Table S1 provides the primer sequences, fragment sizes and annealing temperatures for the three amplified regions. Extracted DNA (10 ng) was added to the reaction mixture consisting of 0.5 mM $MgCl_2$, 0.4 mM Taq polymerase, 0.2 mM dNTPs and 0.5 pmol of each primer. The three amplicons were obtained after an initial 4 min cycle of 94 °C followed by 34 cycles of PCR (94 °C for 15 s, 57 °C for 50 s and 72 °C for 50 s) and a final 10 min incubation at 72 °C. The amplicons were sequenced using Big Dye TERMINATOR version 3.1 (Life Technologies, Madrid, Spain) in the ABI PRISM 3500 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence assembly and identification of genomic polymorphisms were performed using the BIOEDIT v7.0.5 software (Hall 2005). Simple repeat elements in the *MAOA* promoter sequences were predicted with the TANDEM REPEAT FINDER program (Benson 1999) and the putative functional characterizations of genetic polymorphisms identified in the promoter region of the *MAOA* gene was investigated using the web-based software SIGNAL SCAN (Prestridge 1991). Table 1 shows the animals sampled – only Lidia, RAV and MG cattle were initially genotyped with the 50K SNP BeadChip and the rest of the samples were used for validation of the g.105,462,494delsinsC variant.

Results and discussion

We used the XP-EHH approach (Sabeti *et al*. 2007) to disentangle the differences in BTX that can be explained by the recent differential selection for aggressiveness in the Lidia population when compared with the tamed RAV and MG breeds. Chromosome X differs from autosomes in terms of gene divergence, patterns of gene expression and rates of gene movement between chromosomes (Sabol *et al*. 1998). In cattle, the X chromosome is ~148 Mb in size and accounts for 1.7% of the whole bovine genome.

Selection signatures analysis

A region under selection in BTX was retrieved for each Lidia group (Table 2), all of them partially overlapping from BTX 97,384,518 to 116,407,814 bp (Fig. 2), and considered as a joint-in region in downstream analyses. The joint-in region under selection is a polygenic area containing numerous genes, some of them previously identified as playing a key role in behavioral features, such as: *MAOA* (BTX: 105 380 191–105 445 070), encoding the enzyme monoamine oxidase A, which is the primary enzyme in the degradation route for synaptic serotonin and norepinephrine during neurodevelopment (Sabol *et al*. 1998); ubiquitin

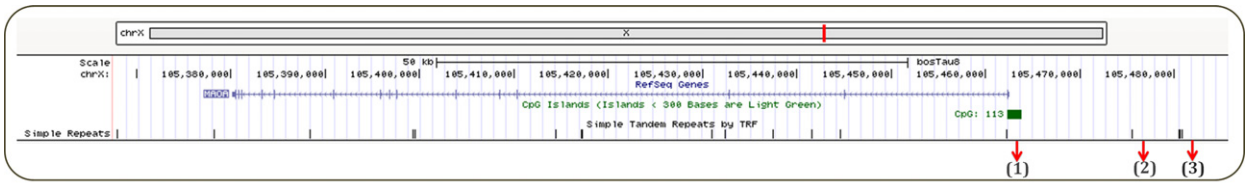


Figure 1 Overall genomic structure and strategy for sequencing the bovine *MAOA* gene (available at https://genome.ucsc.edu/cgi-bin/hgGatewa y). In brackets is given the position of each of the three target regions, previously reported for displaying simple tandem repeats: (1) CpG_Island; (2) GT_fragment; and (3) Far_fragment.

specific peptidase 9 X-linked (*USP9X*, BTX: 107 853 417–107 862 594), involved in X-linked intellectual disability (Homan *et al.* 2014); mediator complex subunit 14 (*MED14*, BTX: 108 241 528–108 303 280), a co-activator of a transcription factor SP1, whose overexpression increases *MAOA* promoter activity (Chen *et al.* 2006); ATPase H+ transporting accessory protein 2 (*ATP6AP2*, BTX: 108 396 795–108 424 453), playing an important role in cognitive function processes and brain development (Ramser *et al.* 2005); and tetraspanin 7 (*TSPAN7*, BTX: 110 055 861–110 188 380), associated with X-linked cognitive disability and neuropsychiatric diseases (Bassani *et al.* 2012). Thus, this region gathers pivotal genes associated with neuronal processes and pathways implicated in the modulation of offensive aggression (Craig & Halton 2009).

The remaining genes located within the joint-in region are presented in Table S2, most of them associated with different metabolic functions that may be related to reproductive traits. The mammalian X chromosome contains an atypical high proportion of two classes of genes, those implicated in mental performance and those associated mainly with reproduction-related traits (Graves & Delbridge 2001), as reflected in the associated joint-in region detected here (Table S2).

In contrast to recombination hotspots, which are associated with low LD (Hermisson & Pennings 2005), the large length of the selective sweep detected here (9.4 Mb) may be associated with strong LD across the target region. The Manhattan plot presented in Fig. 2 shows SNPs under selection within the Lidia groups along the X chromosome. A high overlap proportion is observed across groups as well as a clustering pattern of markers around specific regions, leaving silent gaps along the chromosome.

These silent regions may be partially due to constant selection, which gives rise to regions with reduced genetic diversity and low recombination, concentrating signals around gene-rich regions (Pritchard *et al.* 2010), coupled with the low recombination rate of chromosome X when compared with autosomes (Schaffner 2004). Similarly, Ma *et al.* (2014) attributed the absence of selection signals in some regions as a result of X-inactivation to sex-specific dosage compensation.

MAOA gene sequencing

MAOA has been identified as an important gene involved in pathological aggression, which includes a broad spectrum of psychiatric conditions such as dementia, manic depression, schizophrenia and addictive behaviors in humans, and offensive aggression in murine models (Sabol *et al.* 1998; Craig & Halton 2009). Thus, we selected this candidate gene to further explore the detected genomic signals given that: (i) this gene is located within the markers with higher XP-EHH scores in all Lidia groups, exceeding 95% of the standardized distribution (Fig. 2); and (ii) a functional variation within this widely studied gene has been associated with aggression and behavioral alteration in humans (Sabol *et al.* 1998). Several studies have associated a VNTR in the promoter region of the human *MAOA* gene (Lawson *et al.* 2003; Wendland *et al.* 2006; Alia-Klein *et al.* 2008), located 1137 bp upstream of the start codon, with aggression and mental illnesses, including depression, antisocial behavior and panic syndrome in humans. The different repetition numbers of the VNTR polymorphism are shown to affect the synthesis of the catabolic enzyme *MAOA* in the brain, where this gene is mainly expressed (Nelson & Chiavegatto 2001). Accordingly, several genetic polymorphisms were detected in intronic regions of the canine *MAOA* between different breed groups, the coding and promoter regions remaining conserved (Sacco *et al.* 2017),

Table 2 Genomic regions under selection in BTX per Lidia group

Group	No. of SNPs	BTAX position	Region length (Mb)
Spanish Lidia – extremely aggressive (Spa+)	4	107 043 386–116 407 814	9.36
Spanish Lidia – intermediately aggressive (Spa−)	2	97 384 518–107 052 079	9.67
Mexico – mildly aggressive (Mex)	6	107 040 858–113 052 079	6.30

Threshold is set at the 95% of the standardized distribution.

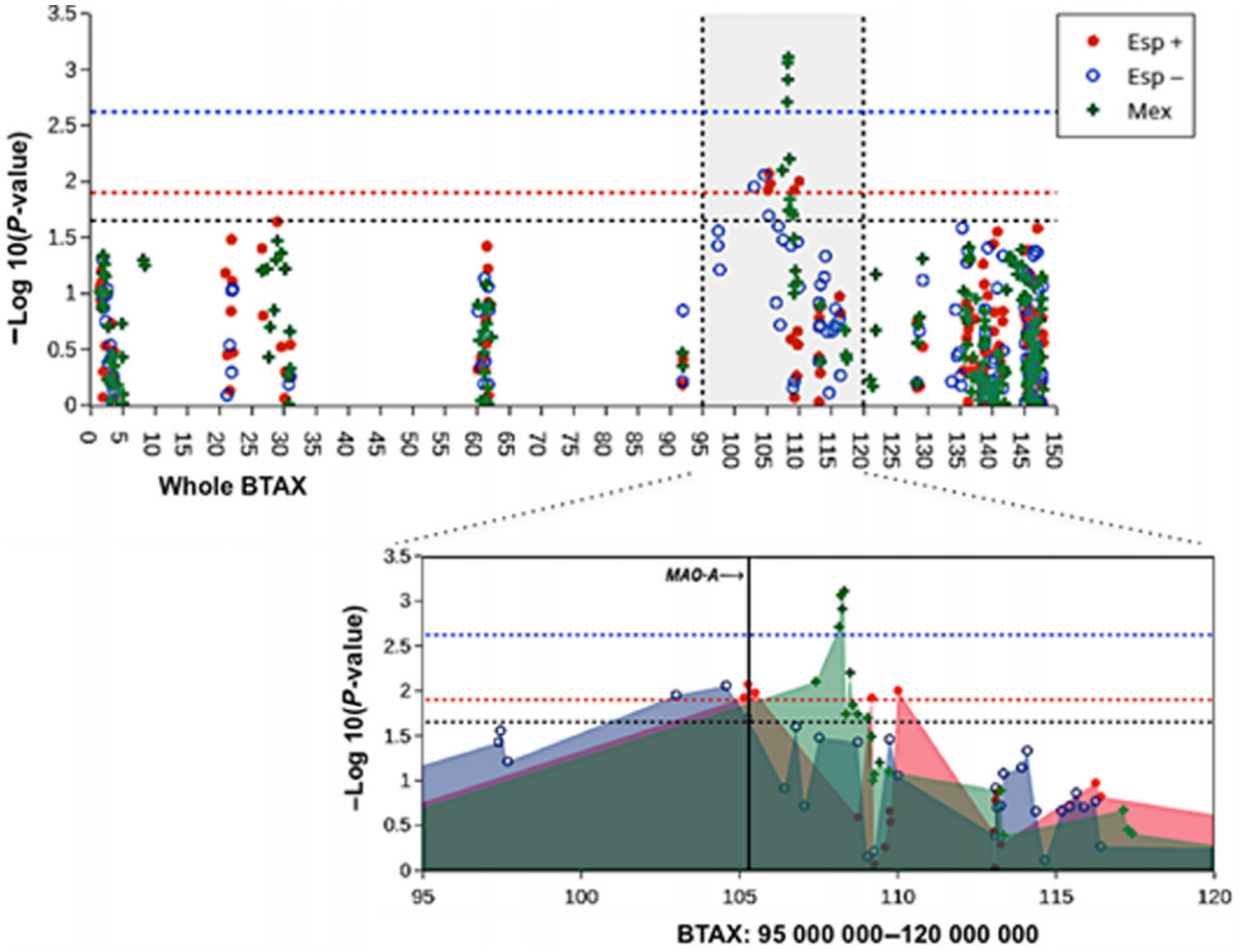
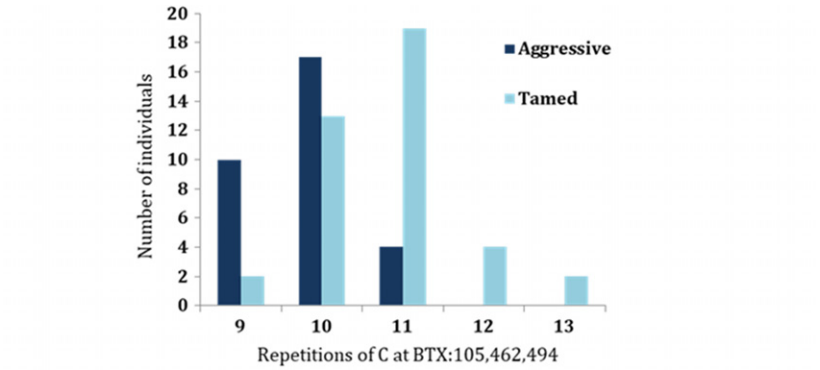


Figure 2 Plot of the SNPs under selection in the different Lidia groups – aggressive (Spa+), intermediate (Spa−) and mild (Mex) – located in BTX: 95 000 000–120 000 000. The black, red and blue dotted lines stand for the XP-EHH scores exceeding the 90, 95 and 99% upper quartiles of the standardized distribution respectively.

although these polymorphisms still lack validation studies on their association with aggressive behavior in the dog.

In cattle, little is known about the genetics underpinning aggressive behavior. Recently, an analysis using a panel of SNPs in autosomic chromosomes in the Charolais breed revealed some candidate polymorphisms associated with temperament-related traits through their effect on dopamine- and serotonin-related genes, such as proopiomelanocortin (*POMC*), neuropeptide Y (*NPY*) and the solute carrier family 18, member 2 (*SLC18A2*) (Garza-Brenner *et al*. 2017). Regarding the *MAOA* gene in cattle, several polymorphisms located in different exons were reported in another study comparing two commercial breeds that display different handling behaviors (Lühken *et al*. 2010). The functional impact of the proteins affected by these polymorphisms was evaluated *in silico* in the same work, but no significant association was detected between these and the related scores of cattle behavior (Lühken *et al*. 2010). However, research on the possible effect of variants in the promoter region of the *MAOA* gene using specific cattle breeds selected for aggressiveness (i.e. the Lidia breed) has not been conducted so far.

We focused on three sequences in the promoter region of the *MAOA* gene (Fig. 1), which are key in the regulation of gene expression (Sabol *et al*. 1998). From the three sequences analyzed, the (2) GT_fragment and (3) Far_fragment did not render any variation, but a different pattern of cytosine (C) repetitions was observed between the Lidia and the tamed groups ($P < 0.0001$) in the fragment (1), which corresponds to a CpG_Island, named g.105,462,494delsinsC. Most Lidia individuals displayed a low number of repetitions (9 and 10 C; $P < 0.001$; Fig. 3). In contrast, the tamed group showed a noticeable variation in the distribution of 10 and 11 C repetitions ($P < 0.001$). The number of 9C repetitions observed in the tamed group belongs exclusively to a couple of individuals of the Limousine breed (Fig. 3). The presence of this low number of Cs in Limousines may be expected since, despite being considered tamed, this beef breed is known by breeders and farmers for its difficulty



C.105,462,494 allele repetitions	Lidia group			Tamed group						
	Spa +	Spa -	Mex	RAV	MG	Retinta	Rubia Gallega	Avileña	Charolaise	Limousine
9	5	3	2							2
10	7	9	1	3				2	6	2
11	3		1	7	6		4	1	1	
12				1		3				1
13					1					

Figure 3 Distribution of the variation in the number of g.105,462,494delsinsC repetitions across the two groups of cattle populations.

in handling and is reported as being behaviorally agitated (Grandin 1993).

A further analysis to predict candidate transcription factor binding sites *in silico* (Pickrell *et al.* 2009) reveals that the g.105,462,494delsinsC variant may code for the functionally important Sp1 transcription factor binding site, and can also generate a new site for the CACCC-box binding factor. In addition, the joint-in genomic region also pinpointed *MED14*, another co-activator of Sp1. Sp1 binding motifs constitute the major transcription factors controlling the core promoter and, hence, *MAOA* expression in humans (Zhu *et al.* 1994; Chen 2004). Our understanding of the regulatory elements of transcription in mammalian genomes is, however, far from comprehensive and no general rules have been proposed to account for the functional consequences of regulatory mutations (Sinnett *et al.* 2006). However, the variation in the number of Cs at g.105,462,494delsinsC when comparing aggressive and tamed cattle and its potential functional importance for the transcription of Sp1 and *MAOA* gene expression may indicate a possible influence on cattle behavior.

In summary, using SNP array data from BTX, a genomic region under recent positive selection has been identified in the Lidia cattle breed containing genes associated with aggressive behavior. Within this candidate region, the most significant markers were located near to the *MAOA* gene, where further analyses have revealed a genetic polymorphism in the form and means of a cytosine expansion (g.105,462,494delsinsC). This polymorphism is located in a CpG island of the gene promoter and displays a lower number of repetitions in the Lidia breed when compared with tamed Spanish local breeds. The exact functional implication in agonistic behavior of this expansion polymorphism is still to be determined.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The dataset of 303 bovines genotyped using the Illumina 50K BeadChip is available in the Figshare public repository (https://figshare.com/s/a63a54b3943a9bfed6b6). For the *MAOA* gene variants the nucleotide sequences accession codes go from MN367149 to MN367220.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Information on the amplified *MAOA* promoter regions.

Table S2 Additional genes located within the joint-in region under selection.

Figure S1 PCA using data from the SNP chip showing the dispersion of the tamed (a) and aggressive (b) cattle breeds.