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Short Communication

Genetic diversity of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) genes in cattle breeds

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Abstract

DNA from four cattle breeds was used to re-sequence all of the exons and 56% of the introns of the bovine tyrosine hydroxylase (TH) gene and 97% and 13% of the bovine dopamine β-hydroxylase (DBH) coding and non-coding sequences, respectively. Two novel single nucleotide polymorphisms (SNPs) and a microsatellite motif were found in the TH sequences. The DBH sequences contained 62 nucleotide changes, including eight non-synonymous SNPs (nsSNPs) that are of particular interest because they may alter protein function and therefore affect the phenotype. These DBH nsSNPs resulted in amino acid substitutions that were predicted to destabilize the protein structure. Six SNPs (one from TH and five from DBH non-synonymous SNPs) were genotyped in 140 animals; all of them were polymorphic and had a minor allele frequency of > 9%. There were significant differences in the intra- and inter-population haplotype distributions. The haplotype differences between Brahman cattle and the three *B. t. taurus* breeds (Charolais, Holstein and Lidia) were interesting from a behavioural point of view because of the differences in temperament between these breeds.

Key words: behavior, *B. t. indicus*, *B. t. taurus*, catecholamine, temperament.

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Basic cattle behavioural traits include social behaviour such as aggression and temperament (Buchenauer, 1999). These traits may have a direct economic impact and can be included in selection strategies (Mormède, 2005; Nkrumah *et al.*, 2007; Core *et al.*, 2009). Studies that have investigated an association between behaviour and production traits have found that nervous or aggressive animals have a decreased milk flow and yield (Rushen, cited by Hiendleder *et al.*, 2003). Temperament, which is defined as the animal's response to handling by humans (Burrow and Dillon, 1997), has been studied in different breeds, and has been shown to affect growth, health, performance and carcass merit (Fox *et al.*, 2004; Nkrumah *et al.*, 2004, 2007; Core *et al.*, 2009).

There are important temperament differences between *Bos taurus taurus* and *Bos taurus indicus* breeds and their crosses. Grandin (1980) found that the flight distances of Brahman cattle were longer than those of British breed cattle. Hearnshaw and Morris (1984) reported significant differences in the temperament of *B. t. taurus*- and *B. t. indicus*-derived breeds and among calves sired by *B. t. indicus* bulls; in contrast, there were no significant differences amongst calves sired by Hereford bulls.

Genes that encode different regulatory enzymes, transporters and receptors of the serotonin and dopamine signalling pathways may have a marked influence on genetic variation and phenotypic effects. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for dopamine biosynthesis (Marsden, 2006). Genetic polymorphisms in the TH gene have been associated with different neurological and psychiatric conditions in humans (Giegling *et al.*, 2009). Another target gene is dopamine β-hydroxylase (DBH), which is one of the most important enzymes for noradrenaline (NA) production. In humans, the genetic variation in this gene has been associated with maternal behaviour, feeding behaviour, impulsive behaviour, motivation, behaviour reinforcement and stimuli that produce stress (Hoebel 1985; Thomas and Palmiter 1997; Szczypka *et al.*, 1999; Adriani *et al.*, 2008; Geiger *et al.*, 2009; Luksys *et al.*, 2009).

The TH and DBH genes have also been studied in non-human mammals. Takeuchi *et al.* (2005) screened for polymorphisms in brain samples from ten unrelated Beagles and found two single nucleotide polymorphisms (SNPs) in DBH and four in TH. These SNPs were subse-

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quently studied in 193 samples from five dog breeds. With the exception of one SNP from each gene, there was significant variation in the allelic distributions among the breeds, which suggested (based on known functions of these genes) that these SNPs could be used as candidate markers to evaluate behavioural traits in dogs.

In this work, we examined the genetic variation in the DBH and TH genes of cattle breeds that display different temperaments depending on their genetic background (*i.e.*, *B. t. taurus* and *B. t. indicus*). Eleven DNA samples from four cattle breeds (three Holstein, two Charolais, three Brahman and three Lidia) were used to assess genetic variation in the DBH and TH genes. Whole blood DNA was extracted from these cattle breeds and from additional bovine samples using commercial Wizard® Genomic DNA purification kits (Promega Corporation, Madison, WI, USA). The TH and DBH gene sequences obtained from GenBank (accession numbers NC_007330 and NC_007309, respectively) were used to design primers with Primer Select version 7.0.0 software (Lasergene, DNASTAR, Madison, WI USA). Table S1 shows that most of the primers were located at intron-exon borders. For TH, these primers facilitated the resequencing of 100% and 54% of the coding and non-coding sequences, respectively, whereas for DBH they facilitated the resequencing of 97% of the coding sequences (11 exons) and 13% of the non-coding sequences, which included a complete intron. Polymerase chain reaction (PCR) amplifications were done in 25 μL volumes on a Thermocycler DNA Engine TETRAD 2 Peltier thermal cycler (MJ Research, Inc.,Waltham, MA, USA). The reaction mixtures contained 5-100 ng of genomic DNA, 1.5 or 2.5 mM $MgCl_2$, 0.1 μM of each primer, 0.4 mM dNTPs and 2.5 U of Go*Taq* polymerase (Promega). A touchdown method was used and the amplification profile included an initial denaturation step of 95 °C for 10 min, five three-step cycles of 45 s at 95 °C, an annealing step for 45 s that started at 68 °C but decreased by 2 °C during each cycle and 45 s at 72 °C, and 25 three-step cycles of 45 s each at 95 °C, 65 °C and 72 °C. Bidirectional sequencing was done using the BigDye® Terminator procedure and an ABI PRISM 3100 Genetic Analyzer DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were assembled into contigs using Cap Win V6.0.6000 (Huang and Madan, 1999) and were aligned with ClustalX 2.0.8 (Larkin *et al.*, 2007).

The resulting sequence information was used as an initial polymorphism screening step. We compared the data obtained from 11 animals belonging to different breeds with sequences deposited in the NCBI database. In this analysis, the presence of SNPs was determined by visually inspecting the sequence chromatograms. The SNPs were defined according to their presence in the screening population using the three expected genotypes.

Tyrosine hydroxylase and dopamine β-hydroxylase are critical enzymes in the dopamine and norepinephrine pathways. The resequencing of 4.3 kb and 3.2 kb of the TH and DBH bovine genes, respectively, of 11 animals from four breeds revealed a microsatellite motif and 64 SNPs (22 located in the exons and 42 located in the introns of both genes).

For the amino acid conservation analysis, the published *B. t. taurus* (AF118638), *Equus caballus* (AB029430), *Homo sapiens* (BC017174), *Mus musculus* (S50200), *Rattus norvegicus* (L12407) and *Canis familiaris* (Q68CI2) DBH sequences were aligned using ClustalX 2.0.8. The possible effects of the amino acid substitutions introduced by the eight nsSNPs were predicted using the IPTREE-STAB server (Huang *et al.*, 2007). This software predicted the stability of the mutant proteins produced by the eight nsSNPs. The parameters used for the IPTREE-STAB server included a pH of 7 and a temperature of 25 °C.

Four restriction fragment length polymorphism (PCR-RFLP) and two amplification-created restriction site (PCR-ACRS) assays were designed to genotype one TH SNP and five of the eight DBH non-synonymous SNPs (nsSNPs) (Table 1). One hundred and forty animals from the four breeds (Lidia, n = 19; Charolais, n = 50; Brahman, n = 50; Holstein, n = 21) were genotyped using these assays.

The observed genotype frequencies were compared with the expected frequencies based on the Hardy-Weinberg equilibrium using Genepop version 4.0.10 (Rousset, 2008). The EM algorithm in the Arlequin version 3.11 software package was used to estimate the maximum likelihood haplotype frequencies (Excoffier *et al.*, 2005) within and between the four tested populations. A correspondence analysis done with the program JMP_v3.2.2 (SAS Institute Inc., Cary, NC, USA) was used to assess the influence of the haplotypes in the four breeds.

The coding sequence of DBH contained one SNP per 85 bp. This value was higher than the average for human genes (one SNP per 185 bp) and also higher than the average for some highly polymorphic genes, such as the bovine myostatin gene which contains one SNP per 100 bp (Dunner *et al.*, 2003). High polymorphism rates in genes with important biological roles may be related to the selection of *de novo* mutations because this mechanism increases the variability of the gene in question (Dunner *et al.*, 2003). As expected, most of the SNPs in the bovine DBH gene were located in introns (66%); however, a significant number, were also found in exons. The latter included 13 synonymous SNPs and eight nsSNPs, six of which resulted in non-conservative amino acid substitutions (Table 2). Analysis of these nsSNPs showed that the resulting amino acid changes could affect the stability of the DBH protein structure. Protein destabilization is a common mechanism by which amino acid substitutions cause human diseases (Teng *et al.*, 2010). The effect of these changes on gene function and phenotype needs to be assessed in specific ex-

Table 1 - Allelic frequencies of six coding SNPs from bovine TH and DBH genes.

Gene	SNP ID	Assay/(Enzyme)	Breed	A	C	G	T
TH	rs109268356	PCR-ACRS (*Sma I*)	Lidia		0.74		0.26
			Charolais		0.84		0.16
			Brahman		0.91		0.09
			Holstein		0.66		0.34
DBH	rs109353933	PCR-RFLP (*Acc I*)	Lidia	1.00		0.00	
			Charolais	0.85		0.15	
			Brahman	0.02		0.98	
			Holstein	0.72		0.28	
	rs110234325	PCR-RFLP (*Hinf I*)	Lidia			0.00	1.00
			Charolais			0.16	0.84
			Brahman			0.40	0.60
			Holstein			0.28	0.72
	rs110606764	PCR-RFLP (*Hha I*)	Lidia	1.00		0.00	
			Charolais	0.85		0.15	
			Brahman	0.06		0.94	
			Holstein	0.72		0.28	
	rs109388371	PCR-ACRS (*Pst I*)	Lidia	1.00	0.00		
			Charolais	0.85	0.15		
			Brahman	0.04	0.96		
			Holstein	0.72	0.28		
	rs109805094	PCR-RFLP (*Apa I*)	Lidia	1.00		0.00	
			Charolais	1.00		0.00	
			Brahman	0.91		0.09	
			Holstein	1.00		0.00	

periments designed to address this question. To assess amino acid conservation in the DBH nsSNPs we compared the DBH amino acid sequences across five mammalian species (*Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Equus caballus* and *Canis familiaris*). Two of the eight nsSNPs (rs109440428 and rs110234325) encoded amino acid residues that were conserved among these species. Notably, only one amino acid residue in the previously reported bovine DBH gene sequence was conserved among the five species examined.

The level of polymorphism in the bovine TH coding regions corresponded to one variant for every 733 bp. This low level of polymorphism could be related to the functional role of this enzyme as the limiting step in the entire dopamine production process. Currently, GenBank reports the presence of 25 polymorphisms in the bovine TH gene. However, none of the SNPs found here has been reported before, even in other non-human mammals. Takeuchi *et al.* (2005) reported that four variants were located in the coding region of canine TH; however, their locations were different from those of the SNPs present in the bovine gene.

In TH, the allelic frequencies of SNP rs109268356 revealed that the C allele predominated in the four genotyped bovine populations. Since this is a synonymous mutation it could be considered to be inconsequential for the primary protein structure and/or function; however, a growing number of studies have shown that synonymous mutations are also under evolutionary pressure and that they can be implicated in disease (Hunt *et al.*, 2009). Until recently, bovine behavioural traits had not been associated with a particular gene. However, a whole genome study by Hiendleder *et al.* (2003) identified a QTL on chromosome 29 that was linked to temperament. The authors proposed that TH could be one of several candidate genes that influence temperament in cattle. Our results provide additional support for the hypothesis that the TH gene is a strong candidate associated with bovine behaviour.

The novel microsatellite identified in TH was located approximately 84 bp from the start of exon 5 and was characterised by a cytosine mononucleotide core motif. This motif exhibited an adenine interruption where the cytosine residues showed length differences of 6 to 13 bp and were repeated 2 to 9 times in the 5' to 3' direction. We analysed this motif at the structural level and sequenced this region not only in Lidia, Charolais, Brahman and Holstein animals but also in Beefmaster, Bradford and Nellore animals. The subsequent sequence analyses revealed interbreed variability but no individual intrabreed variation. The structure of

Table 2 - Novel SNPs located in coding regions of bovine TH and DBH genes.

Gene	Exon	Allele	Amino acid (AA)	Change of AA class	AA position	Reference sequence (GenBank)
TH	13	C/T	Pro (P)	No change	401	rs109268356
DBH	1	A/g	Ile /Val*	No change	45	rs109440428
	1	A/g	Ile /Val	No change	52	rs109353933
	1	G/A	Leu	No change	66	rs109276478
	1	T/C	Phe	No change	67	rs110113254
	1	G/A	Glu	No change	76	rs109241989
	1	T/C	Leu	No change	80	rs109516590
	1	G/A	Val	No change	82	rs110078689
	1	T/C	Phe	No change	92	rs110945422
	3	T/g	Ser/Ala*	Polar, neutral/ Non polar, neutral	162	rs110234325
	3	C/T	Asn	No change	164	rs110301179
	3	A/g	His/Arg	Polar, neutral/ polar, positively charged	169	rs110606764
	3	A/C	Lys/Gln	Polar, positively charged/ polar, neutral	184	rs109388371
	3	C/T	Pro	No change	188	rs110843365
	4	C/T	Asn	No change	237	rs109649412
	4	G/A	Ala	No change	239	rs110811803
	4	C/T	Ala	No change	250	rs110135964
	11	T/C	Pro	No change	516	rs110950937
	11	C/T	Ser	No change	521	rs109572660
	12	A/g	Ser/Gly	Polar, neutral/ Non polar, neutral	583	rs109805094
	12	A/g, G/A	Ser /Asp	Polar, neutral/ Polar, negatively charged	593	rs110864292

*Amino acid residues conserved among five species.

the motif was AGC_6AC_9 AG in Charolais, Hereford and Holstein animals, AGC_8AC_4AG in Lidia and Beefmaster animals, $AGC_{12}AC_2AG_3$ in Brahman and Bradford animals and $AGC_{13}AC_3AG_3$ in Nellore animals.

The presence of the microsatellite rs109798407 $AGC_{6/8/12/13}A(C)_{2/3/4/9}$ AG motif in the bovine TH gene has not been reported before. The presence of this microsatellite is very interesting because there are several examples of polymorphic microsatellites within some genes, such as bovine growth hormone (bGH), calpastatin (CAST) and insulin-like growth factor 1 (IGF1). Genetic diversity analyses of these genes have shown an association between microsatellites and both productive traits and the allelic distribution and segregation between different genetic groups, including *B. t. taurus*, *B. t. indicus* and their crosses (Levéziel *et al.*, 1994; Nonneman *et al.*, 1999; Hale *et al.*, 2000; Curi *et al.*, 2005). As described here, the bovine TH microsatellite shows interbreed variation and contains at least four allelic forms with changes in their structure and length. Notably, the analysis of individuals from the eight breeds studied revealed no intrabreed differences. Although in mammals most of the repetitive sequences are located within introns the C-monomers are present at a frequency of 11.4%. In humans, the TH gene contains a four-nucleotide motif ($TCAT_n$) located in the first intron; this motif acts as a regulatory element in gene transcription (Meloni *et al.*, 1998). Further studies are needed to establish whether the microsatellite has any functional effect in bovine TH transcription and to determine whether there is any correlation between variation in microsatellite structure and productive traits in cattle.

The only synonymous TH SNP and five of the eight DBH nsSNPs were genotyped in the animals of four breeds, which allowed their validation as SNPs based on their allelic frequencies (Table 2). Similarly, there were important differences in the genetic diversity parameters of the DBH nsSNPs among the populations tested. Although the Brahman breed had the highest number of polymorphic *loci* (five), the genetic variation was higher in Holstein and Charolais animals (32.6% and 20.9%, respectively). This increased genetic variation most likely reflected differences in the heterozygosity values, which were higher in the latter two breeds than in Brahman animals.

Table 3 shows the haplotype distributions calculated based on the five DBH nsSNP genotypes. There were significant differences ($p \leq 0.0001$) in the haplotype distributions among the *B. t. taurus* breeds and between the *B. t. taurus* and *B. t. indicus* breeds. The correspondence analysis based on these frequencies showed the clear separation of only *B. t. indicus* from breeds with a *B. t. taurus* background (Figure 1). Some shared haplotypes were identified among the four breeds. The breeds with a *B. t. taurus*

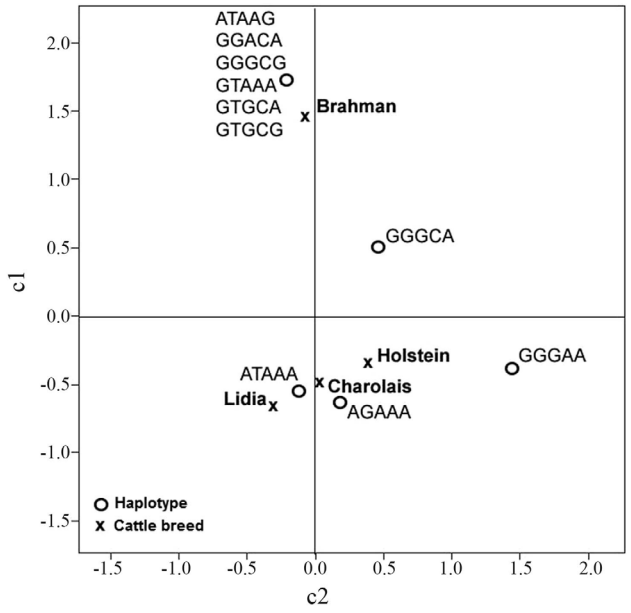

Figure 1 - Correspondence analysis showing the DBH haplotypes diversity observed in four cattle breeds with different genetic background.

background shared haplotype I, which was also the haplotype with the highest frequency among the breeds. Haplotype I was characterized by the absence of nsSNPs in the sequence (Table 3). Haplotype II was the second most frequent haplotype and was present in Charolais, Brahman and Holstein animals. Some exclusive haplotypes were also observed in each breed, with Brahman animals having the highest number (six haplotypes); haplotype VII was the most frequent (55%) and was characterized by the presence of three nsSNPs. Notably, we only observed haplotype VI in this breed; haplotype VI was characterised by the presence of five nsSNPs (Table 3).

Table 3 - Haplotype frequencies for DBHb in four cattle breeds.

Haplotypes*		Cattle breed			
		Lidia	Charolais	Brahman	Holstein
I	ATAAA	1.00	84.00	0.00	71.74
II	GGGCA	0.00	15.00	32.50	27.17
III	AGAAA	0.00	1.00	0.00	0.00
IV	ATAAG	0.00	0.00	2.50	0.00
V	GGACA	0.00	0.00	2.50	0.00
VI	GGGCG	0.00	0.00	5.00	0.00
VII	GTAAA	0.00	0.00	1.25	0.00
VIII	GTGCA	0.00	0.00	55.00	0.00
IX	GTGCG	0.00	0.00	1.25	0.00
X	GGGAA	0.00	0.00	0.00	1.09
Total haplotypes/breed		1	3	7	3

*The order of SNPs in the haplotypes was rs109353933, rs110234325, rs110606764, rs109388371 and rs109805094.

There were significant differences in the haplotype frequencies within and among the breeds studied. These differences were more evident among the three *B. t. taurus* breeds when compared with the Brahman breed, which was considered to be representative of the *B. t. indicus* genetic background. The implications of these differences could be discussed from a behavioural perspective since there are important differences in temperaments both within and among cattle breeds. Previous work has shown that when Brahman crosses are restrained in a squeeze chute they show higher cortisol levels than English breed crosses (Grandin, 1997). For the moment, we are unable to associate any genotype with differences in temperament. However, the genetic diversity observed among the breeds examined in this study provides a basis for investigations into such associations. Since the overall aim of such studies is to identify DNA markers for cattle temperament, this trait may have direct economic value, and temperament phenotypes could be included in future selection strategies (Mormède, 2005; Nkrumah *et al.*, 2007; Core *et al.*, 2009).

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Supplementary Material

The following online material is available for this article:

Table S1 - Primer sequences used to analyse bovine TH and DBH genes.

This material is available as part of the online article from http://www.scielo.br/gmb.

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Table S1 - Primer sequences used to analyse bovine TH and DBH genes.

Gene	Primer sequence	Amplicon size (bp)	Exon
DBH	F-5'-TGGCCGTCTTCCTGGTCATC-3' R-5'-GGGAGGGCGAGTGCAGAGAA-3'	321	I
	F-5'-GGGGCTGTGCTGTGGGTGAC-3' R-5'-GGTGAGGGGCCCGGGATGCT-3'	297	II
	F-5'-CCGGGCTTGGGGCTGTGAGT-3' R-5'-GATGTGGTGCCGGGGGAAGC-3'	296	III
	F-5'-GGTGCAGCCCTCCCATTTTA-3' R-5'-CCAGCGGCGTAGAGGCAGTG-3'	347	IV
	F-5'-CACCTGCCCATCTGTCTTCT-3' R-5'-GCAGGCGGGGGCAGGTAA-3'	197	V
	F-5'-GAGGGCGGCTGGGGGTCAT-3' R-5'-GGGGGAGCGAGGTCACAGC-3'	331	VI
	F-5'-CTGACTGGGGAGGAGGAAG-3' R-5'-GGGCACTCACCGGCTGGAC-3'	560	VII, VIII
	F-5'-TCTGGCCTCGTGTGATGA-3' R-5'-GTGGGTGGGGGCCTGAT-3'	422	X
	F-5'-GATGGCACAAGAGATGAAGC-3' R-5'-GCACGGCGGCACACCTG-3'	349	XI
	F-5'-TTTGGTTTGGGAGAAGG-3' R-5'-TGCACCCGTCACACTCAA-3'	702	XII
TH	F-5'CGGACCTCGCCGGCACCAT-3' R-5'GAGCACAGCCTCCCTTCAGC-3'	408	I
	F-5'CGGGGCAGCCCCAGACG-3' R-5'CGGGAGGGGCCCCTCTCCT-3'	421	II
	F-5'AGGCCGCCTGGGGGTGACTG-3' R-5'GCCGAGGGCCGCCTCACCT-3'	453	III
	F-5CAGCGGCCCCCTCCCTGTGT-3' R-5'GGCCCGAGGATGGGGTGTGG-3'	489	IV, V
	F-5CCCCGAAGGGGCCTGGACT-3' R-5'GGCACCGCACCCTTCAGGAA-3'	561	VI, VII
	F-5CGCCCCCGCCCCAGCAGA-3' R-5'GCGAAGGGTGCCCATTGGTG-3'	490	VIII, IX, X
	F-5CGGGCCCCCAGCGGATCC-3' R-5'TCCCGGGTGGCCTGAGTCCA-3'	545	XI, XII
	F-5AGTCGCCTCGGGTCCTGAGA-3' R-5'GGCCAGGAAGGACCCATCTT-3'	463	XIII
	F-5CAAGCTTATGGACAGAGATG-3' R-5'GGCCAGGGCCTGCATCTCGT-3'	509	XIV