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 (Buchenuar, 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-
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* (Q68C12) 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 from 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-
Experiments 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 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 |      |      |
|      | rs11060764 | 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 |      |      |
the motif was AGC6AC9 AG in Charolais, Hereford and Holstein animals, AGC8AC4AG in Lidia and Beefmaster animals, AGC12AC2AG3 in Brahman and Bradford animals and AGC13AC3AG3 in Nellore animals.

The presence of the microsatellite rs109798407 AGC6/8/12/13A(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 < 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

| 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 |
| TH  | 13   | C/T    | Pro (P)         | No change         | 401            |
| DBH | 1    | A/g    | Ile /Val*       | No change         | 45             |
|     | 1    | A/g    | Ile /Val        | No change         | 52             |
|     | 1    | G/A    | Leu             | No change         | 66             |
|     | 1    | T/C    | Phe             | No change         | 67             |
|     | 1    | G/A    | Glu             | No change         | 76             |
|     | 1    | T/C    | Leu             | No change         | 80             |
|     | 1    | G/A    | Val             | No change         | 82             |
|     | 1    | T/C    | Phe             | No change         | 92             |
|     | 3    | T/g    | Ser/Ala*        | Polar, neutral/ Non polar, neutral | 162 |
|     | 3    | C/T    | Asn             | No change         | 164 |
|     | 3    | A/g    | His/Arg         | Polar, neutral/ polar, positively charged | 169 |
|     | 3    | A/C    | Lys/Gln         | Polar, positively charged/ polar, neutral | 184 |
|     | 3    | C/T    | Pro             | No change         | 188 |
|     | 4    | C/T    | Asn             | No change         | 237 |
|     | 4    | G/A    | Ala             | No change         | 239 |
|     | 4    | C/T    | Ala             | No change         | 250 |
|     | 11   | T/C    | Pro             | No change         | 516 |
|     | 11   | C/T    | Ser             | No change         | 521 |
|     | 12   | A/g    | Ser/Gly         | Polar, neutral/ Non polar, neutral | 583 |
|     | 12   | A/g, G/A | Ser/Asp   | Polar, neutral/ Polar, negatively charged | 593 |

* Amino acid residues conserved among five species.
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 Brah-
man animals having the highest number (six haplotypes);
haplotype VII was the most frequent (55%) and was char-
acterized 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 (Ta-
ble 3).

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

| Haplotypes*   | 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             | GGGCA | 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          | GTCGA | 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. How-
ever, 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 phen-
types could be included in future selection strategies (Mor-
mède, 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.

Associate Editor: Alexandre Rodrigues Caetano
| Gene | Primer sequence | Amplicon size (bp) | Exon |
|------|----------------|-------------------|------|
| TH   | F-5'-TGGCCGTCTTCCTGTGGTCATC-3' R-5'-GGGAGGGCGAGTGCAGAGAA-3' | 321 | I |
|      | F-5'-GGGGCTGTGCTGTGGGTGAC-3' R-5'-GGTGAGGGGCCGGGGAGCACG-3' | 297 | II |
|      | F-5'-GGGGCTTTGGGGGCTGTGAGT-3' R-5'-GATGTGGTGCCGGGGAGGAA-3' | 296 | III |
|      | F-5'-TGGTGTCAGCAGCACCTCCATTTTA-3' R-5'-CCAGCGCGCGTGAGAGCAGTG-3' | 347 | IV |
|      | F-5'-CACCTGCCTTCTTCTTCT-3' R-5'-GCAGCGGGGGGGAGAGGTAA-3' | 197 | V |
|      | F-5'-GGGCCGCGCTGGGGGTCAT-3' R-5'-GGGGGAGCGAGGTCACAGC-3' | 331 | VI |
|      | F-5'-GTGAGGGGCCGGGGAGGAAG-3' R-5'-GGCAGCTACCGGGGCTGAG-3' | 560 | VII, VIII |
|      | F-5'-TGGTCCTGTCGTCGTCGTC-3' R-5'-GGGCGAGGCTGAGTGA-3' | 422 | X |
|      | F-5'-GGGGGCAGCCCCAGACG-3' R-5'-CGGGAGGGCCTGAGTCAC-3' | 408 | I |
|      | F-5'-AGGCCGCTGGGGGTGACTG-3' R-5'-GCGAGGGCCGCCCTCCT-3' | 421 | II |
|      | F-5'-GGCCGAGGGCCTCCCT-3' R-5'-GCTGCAGCTCCTGTTGT-3' | 453 | III |
|      | F-5'-GGCCGCGCCCTCCCTCCTCCATGAGTG-3' R-5'-GGGGGCCAGTTGGGGTGTT-3' | 489 | IV, V |
|      | F-5'-CCCAGGGCCCTGCGCTTGACT-3' R-5'-GCGAGGGCGAGTGACGAC-3' | 561 | VI, VII |
|      | F-5'-GCAGCGGGCGCCCGTCCCTCAG-3' R-5'-GCCGAGGGGCGCCATTG-3' | 490 | VIII, IX, X |
|      | F-5'-GGGGGCCAGGGATGCCAGCC-3' R-5'-GGCCAGGGGCTGAGTGGG-3' | 545 | XI, XII |
|      | F-5'-GGCCAGGAGGGCTCTTCGTA-3' R-5'-GAAGCTTATGGACAGAGATG-3' | 463 | XIII |
|      | F-5'-GGCCAGGAGGGCTCTTCGTA-3' R-5'-GGTGTTCAAGGAAAAGAGGTAT-3' | 509 | XIV |