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Research Article

Mitochondrial DNA single nucleotide polymorphism associated with weight estimated breeding values in Nelore cattle (*Bos indicus*)

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Abstract

We sampled 119 Nelore cattle (*Bos indicus*), 69 harboring *B. indicus* mtDNA plus 50 carrying *Bos taurus* mtDNA, to estimate the frequencies of putative mtDNA single nucleotide polymorphisms (SNPs) and investigate their association with Nelore weight and scrotal circumference estimated breeding values (EBVs). The PCR restriction fragment length polymorphism (PCR-RFLP) method was used to detect polymorphisms in the mitochondrial asparagine, cysteine, glycine, leucine and proline transporter RNA (tRNA) genes ($tRNA^{asn}$, $tRNA^{cys}$, $tRNA^{gly}$, $tRNA^{leu}$ and $tRNA^{pro}$). The 50 cattle carrying *B. taurus* mtDNA were monomorphic for all the *tRNA* gene SNPs analyzed, suggesting that they are specific to mtDNA from *B. indicus* cattle. No $tRNA^{cys}$ or $tRNA^{gly}$ polymorphisms were detected in any of the cattle but we did detect polymorphic SNPs in the $tRNA^{asn}$, $tRNA^{leu}$ and $tRNA^{pro}$ genes in the cattle harboring *B. indicus* mtDNA, with the same allele observed in the *B. taurus* sequence being present in the following percentage of cattle harboring *B. indicus* mtDNA: 72.46% for $tRNA^{asn}$, 95.23% for $tRNA^{leu}$ and 90.62% for $tRNA^{pro}$. Analyses of variance using the $tRNA^{asn}$ SNP as the independent variable and EBVs as the dependent variable showed that the G → T SNP was significantly associated ($p < 0.05$) with maternal EBVs for weight at 120 and 210 days ($p < 0.05$) and animal's EBVs for weight at 210, 365 and 455 days. There was no association of the $tRNA^{asn}$ SNP with the scrotal circumference EBVs. These results confirm that mtDNA can affect weight and that mtDNA polymorphisms can be a source of genetic variation for quantitative traits.

Key words: bovine, mitochondria, mtDNA, SNP, weight.

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Introduction

Mitochondria are eukaryotic cell organelles involved in various cellular functions, including cell proliferation, apoptosis and, mostly important, energy production (Birch-Machin, 2006) by oxidative phosphorylation (Taanman, 1999). These organelles are responsible for approximately 90% of the energy produced by the mammal cell (Boettcher *et al.*, 1996b). Mitochondria have their own genome, which is maternally inherited in mammals and is an important source of cytoplasmic genetic variation (Gibson *et al.*, 1997; Birky, 2001). In cattle, direct maternal effects have been discussed since Wagner (1972) but the contribution of cytoplasmic effects estimated for growth and milk production are not consistent between populations (Gibson *et al.*, 1997).

Boettcher *et al.* (1996b) demonstrated bias in the heritability, permanent environmental variance, and accuracy estimation in the animal model when the cytoplasmic effects were ignored. Moreover, the effect of cytoplasmic inheritance has been studied in respect to milk production (Bell *et al.*, 1985; Tess *et al.*, 1987; Schutz *et al.*, 1992; Boettcher *et al.*, 1996a) and beef cattle growth traits (Tess and Robison, 1990; Northcutt *et al.*, 1991; Tess and MacNeil, 1994; Quintanilla *et al.*, 1999).

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Recently, molecular biology has furthered our understanding of the function and inheritance of the mitochondrial genome and its importance on livestock development and production (Smith and Alcivar, 1993; Smith *et al.*, 2000). Since Anderson *et al.* (1982) published the complete mitochondrial DNA (mtDNA) sequence, nucleotide variants in coding and non-coding regions have been studied to associate molecular markers with the production of *Bos taurus* breeds (Ron *et al.*, 1993; Suzuki *et al.*, 1993; Mannen *et al.*, 1998; Mannen *et al.*, 2003). Pegoraro *et al.* (1996) identified sequence alterations in mitochondrial transfer RNA (tRNA) genes and in the origin of light strand (Ori L) replication of *Bos indicus* Nelore cattle as compared to *B. taurus* and described a single nucleotide polymorphism in the Nelore Ori L region and *asparagine tRNA* (*tRNA*^{asn}) gene. The polymorphisms were later confirmed by the complete genomic sequence of the *B. indicus* mitochondrial DNA (mtDNA) and deposited in the GenBank under accession number AY126697.

Meirelles *et al.* (1999) reported the existence of cattle carrying the *B. taurus* or *B. indicus* mitochondrial genome in Brazilian Nelore cattle. These animals, with same breed composition but harboring different mitochondrial genomes, constitute an interesting model to investigate the influence of mtDNA polymorphisms on quantitative traits. During the work described in the present paper we tested the hypothesis that cattle with different specific nucleotide variations differ in estimated breeding values (EBVs) for growth and reproductive traits. The aims of this study were to estimate the frequency of single nucleotide polymorphisms of the mitochondrial genes for asparagine tRNA (*tRNA*^{asn}), cysteine tRNA (*tRNA*^{cys}) (Pegoraro *et al.*, 1996), glycine tRNA (*tRNA*^{gly}), leucine tRNA (*tRNA*^{leu}) and proline tRNA (*tRNA*^{pro}) in *B. indicus* and *B. taurus* mtDNA from public GenBank sequences AY126697 and AY526085 in Brazilian Nelore cattle and to investigate the effects of these polymorphisms on weight and scrotal circumference estimated breeding values at different ages.

Material and Methods

Cattle sample

The sample (n = 119) consisted of 69 purebred adult Nelore cattle registered as pure origin imported (POI) in the Brazilian Association of Zebu Breeder's Herdbook and identified as carriers of *B. indicus* mtDNA plus a further 50 purebred *B. indicus* Nelore cattle which were carriers of *B. taurus* mtDNA and not registered as POI. A blood sample was collected from each animal and total DNA extracted according to Sambrook *et al.* (2001). The presence of *B. indicus* and *B. taurus* mtDNA was confirmed by the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method by the absence (*B. indicus*) or the presence (*B. taurus*) of *Hind*III restriction site at nucleotide 12178 on the NADH dehydrogenase subunit 5 gene (Meirelles *et al.*, 1999).

Detection of polymorphisms

The single nucleotide substitutions (SNP) in five transfer RNA (tRNA) mitochondrial genes were detected by PCR-RFLP using endonuclease enzymatic digestion of the amplicons. Primers were designed using the *B. indicus* mtDNA sequence as a reference (GenBank AY126697). The PCR was performed using 150 ng of total DNA, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, Invitrogen, Brazil), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer (Table 1) and 1.5 units of Taq DNA polymerase (Invitrogen, Brazil) in a final volume of 50 µL. Cycling was set for 5 min at 95 °C followed by 35 cycles of 40 s at 95 °C, 30 s at the specific temperature of each primer and 40 s at 72 °C, with a final 72 °C extension for 5 min. The PCR products (15 µL) were digested with 1 unit of *Bsr* I, *Mnl* I, *Spe* I, *Dra* I or *Bsm AI* restriction endonuclease specific for each tRNA gene sequence for one hour at the temperature recommended by the supplier (New England Biolabs, USA). The mitochondrial tRNA PCR amplicons, specific primer sequences, annealing temperatures, SNP positions and restriction enzymes used in this study are pre-

Table 1 - Gene names, primers, annealing temperatures (AT), polymerase chain reaction (PCR) amplicon (AP) sizes in base pairs (bp), single nucleotide polymorphism (SNP) base position and restriction enzymes (RE) used in the study. The SNP position indicates the difference between the cut *Bos taurus* sequence (GenBank AY526085) and the uncut *Bos indicus* sequence (GenBank: AY126697).

tRNA gene	Primer sequence (5'-3')	AT (°C)	AP (bp)	SNP position	RE
Asparagine (*tRNA*^{asn})	TCCTCACTAGACTGGTGG GGTTGAGAATAGTCAGCG	52	277	G5501 → T	*Bsr* I
Cysteine (*tRNA*^{cys})	AGCTAACTGGCTTCAATC ACCAAATAGTAGATAAAGG	50	257	C5612 → T	*Mnl* I
Glycine (*tRNA*^{gly})	ACGTCATCATTGGGTCCAC GAATGCGATGATGACGAGTAG	55	300	T9768 → C	*Spe* I
Leucine (*tRNA*^{leu})	AGAAGCCCGGTAATTGCTTTA ACCTACGACATTTGGACC	52	189	A3051 → G	*Dra* I
Proline (*tRNA*^{pro})	GACAGGTCTTTGTAGTACTC GTAGGTAATTCATTCTGTGG	55	302	T15751 → C	*Bsm* AI

sented in Table 1. Electrophoresis was carried out on 2% (w/v) agarose gel and DNA was stained with ethidium bromide and the fragments visualized under ultraviolet light. Mitochondrial transfer RNA SNP frequencies were obtained by direct counting of the PCR-RFLP results.

Estimated breeding values

The cattle selected for mtDNA genotyping were part of the Nelore cattle breeding program (NCBP) at the University of São Paulo (ANCP, http://www.ancp.org.br/sumarios) which estimated breeding values for approximately 900,000 cattle. The estimated breeding values used in this study for association analysis were kindly provided by the NCBP.

The traits analyzed were maternal effects for weight (MW) at 120 and 210 days (MW_{120} and MW_{210}), animal weight (AW) at 120, 210, 365, and 450 days (AW_{120}, AW_{210}, AW_{365}, and AW_{450}) and scrotal circumference (SC) at 365, 450, and 550 days (SC_{365}, SC_{450}, and SC_{550}).

Briefly, breeding values for each animal were estimated using an animal model applying the best linear unbiased predictor (Boldman *et al.*, 1995). The model used for genetic parameter estimations was $y = X\beta + Z1a + Z2m + Z3p + e$, where y is a N x 1 vector of records, β the vector of fixed effects (herd-year-season, sex, dam age class), X is the matrix associating β with y, a is the vector of estimated breeding values for direct genetic effects, $Z1$ is the matrix associating a with y, m is the vector of estimated breeding values for maternal genetic effects, $Z2$ is the matrix associating m with y, p is the vector of permanent environmental non-additive genetic effects contributed by dams to the records of their progeny, $Z3$ is the matrix associating p with y and e is the vector of residual effects (Gunski *et al.*, 2001).

Association analysis

Maternal and animal estimated breeding values were independently compared between animals with and without the *Bsr* I restriction site (G or T nucleotide) on the $tRNA^{asn}$ gene by analysis of variance (ANOVA). The linear model used was: $y_{ij} = \mu + N_i + e_{ij}$, where y_{ij} is the breeding value for each trait of the ij^{th} animal, N is the fixed effect of the i^{th} $tRNA^{asn}$ nucleotide (G or T) and e_{ij} is the random error effect associated with the ij^{th} observation. Means were compared using the Tukey Studentized Range Test in the SAS program (SAS Institute Inc., 2001). Differences with $\alpha < 0.05$ were considered statistically significant.

Nelore Heard-book genealogy data incorporated in the breeding value estimations allowed us to obtain the estimated breeding values for all parents and relatives from each genotyped animal. However, only those ascending the animals harboring *B. indicus* mtDNA were considered for further analysis. Because mitochondrial DNA is inherited maternally, females and their offspring from the same matrilineal lineage were assumed to have the same mitochondrial $tRNA^{asn}$ nucleotide at position 5501. This allowed us to group females from the same lineage with previously genotyped animals. The new data set was based on 345 observations for each characteristic analyzed and was used for ANOVA and the Tukey test.

Results and Discussion

All the cattle genotyped showed no sign of heteroplasmy for the five single nucleotide polymorphisms (SNPs) analyzed, suggesting maternal inheritance and homoplasmic distribution within tissues (Attardi, 1985; Smith *et al.*, 2000;Birky, 2001). The 50 cattle genotyped as carrying *B. taurus* mtDNA were monomorphic for the five mitochondrial tRNA gene SNPs analyzed in this study, suggesting that the SNPs evaluated are specific to mtDNA from *B. indicus* cattle.

In this Nelore sample (n = 119) no mitochondrial $tRNA^{cys}$ or $tRNA^{gly}$ mutations were found using PCR-RFLP. However, we did detect polymorphic SNPs in the $tRNA^{asn}$, $tRNA^{leu}$ and $tRNA^{pro}$ mitochondrial genes of the cattle harboring *B. indicus* mtDNA (n = 69) where the *B. taurus* sequence (Anderson *et al.*, 1982) was present in 72.46% of animals for $tRNA^{asn}$, 95.23% for $tRNA^{leu}$ and 90.62% for $tRNA^{pro}$, the remaining animals having a variant tRNA pattern similar to the *B. indicus* mtDNA GenBank sequence AY126697. Tracing the matrilineal genealogy, we found that each of the 19 cattle genotyped as having *B. indicus* $tRNA^{asn}$ were descendants of different females imported from India, suggesting that the nucleotide mutations in the $tRNA^{asn}$, $tRNA^{leu}$ and $tRNA^{pro}$ mitochondrial genes, characteristic of *B. indicus* cattle, occurred in India and were brought to Brazil with imported Indian cows.

Because the mitochondrial $tRNA^{cys}$ and $tRNA^{gly}$ genes were not polymorphic for the SNPs tested in our sample and the mitochondrial $tRNA^{leu}$, and $tRNA^{pro}$ genes lacked sufficient variability, we used the SNP on the $tRNA^{asn}$ gene to compare the estimated breeding values of the cattle harboring *B. indicus* mtDNA in our sample.

As stated above, maternal and animal estimated breeding values were independently compared between the cattle in our sample which had, or lacked, the *Bsr* I restriction site (G or T nucleotide). The guanidine to thymine substitution was significantly associated ($p < 0.05$) with changes in maternal breeding value for weight at 120 and 210 days and the animal breeding value for weight at 210, 365 and 455 days (Table 2). Cattle with the mitochondrial $tRNA^{asn}$ guanidine SNP had higher estimated breeding values compared to those with the thymine SNP (Table 3), although there were no differences for the scrotal circumference estimated breeding values between cattle with different nucleotides at this position (Tables 4 and 5).

Gunski *et al.* (2001) studied mitochondrial genome effects to compare pre-weaning growth traits between were in Nelore cattle harboring *B. taurus* mtDNA and *B. indicus* mtDNA, but found no significant ($p > 0.05$) differences for maternal and animal estimated breeding values.

Table 2 - Analysis of variance between weight estimated breeding values (EBV) for the G5501 → T (G, n = 247; T, n = 71) mitochondrial *tRNA^{asn}* gene single nucleotide polymorphism (SNP). The table shows degrees of freedom (DF) and the mean square values for maternal EBV for weight (MW) at 120 and 210 days and animal EBV for weight (AW) at 120, 210, 365 and 450 days.

		Estimated breeding values (mean squares)					
Source	DF	MW_{120}	MW_{210}	AW_{120}	AW_{210}	AW_{365}	AW_{450}
Genotype	1	57.71	68.16	14.46	146.02	757.27	884.01
Error	343	6.11	10.50	15.96	37.65	102.06	132.39
Pr > F		0.00	0.01	0.34	0.04	0.00	0.01

Table 3 - Weight estimated breeding values (EBV) for the G5501 → T (G, n = 247; T, n = 71) mitochondrial *tRNA^{asn}* gene single nucleotide polymorphism (SNP). The table shows the means and standard errors of the mean (SE) for maternal EBV for weight (MW) at 120 and 240 days and animal EBV for weight (AW) at 120, 240, 365 and 450 days.

	Weight estimated breeding values (means ± SE, kg)					
tRNA^{asn}	MW_{120}	MW_{240}	AW_{120}	AW_{240}	AW_{365}	AW_{450}
G	1.59 ± 0.15^{a}	2.40 ± 0.19^{a}	-0.06 ± 0.25^{a}	-0.87 ± 0.38^{a}	2.86 ± 0.62^{a}	1.12 ± 0.68^{a}
T	0.58 ± 0.28^{b}	1.30 ± 0.37^{b}	-0.57 ± 0.38^{a}	-2.48 ± 0.65^{b}	-0.80 ± 1.07^{b}	-2.83 ± 1.42^{b}

^{a,b}values in columns with different superscript letters are significantly different by the Tukey test ($p < 0.05$).

Our results suggest an association between polymorphism in the mitochondrial *tRNA^{asn}* gene with growth traits in *B. indicus* Nelore cattle. The differences for maternal and direct weight estimated breeding values were not caused by a *B. taurus* nuclear genome effect since this sample were known to harbor the *B. indicus* mtDNA and were progeny of registered POI sires and cows.

There are reports associating cytoplasmic lineage and growth and milk traits effects in various cattle breeds. Tess *et al.* (1987) reported that cytoplasmic effects influenced pre-weaning and milk traits in Hereford cattle but later results did not confirm this effect (Tess and Robison, 1990), while Schutz *et al.*, (1993) found that maternal lineage was responsible for 4% to 15% of the phenotypic variation in milk traits of Holstein-Friesian cattle. Boettcher *et al.*, (1996b) reported that the inclusion of the maternal lineage effect in a simulated animal model gave better estimations of heritability and permanent environment variance and also accurate estimated breeding values. However, due to small number of different lineages in our data set we could not test if cytoplasmic lineage was associated with breeding value variation.

Table 4 - Analysis of variance between scrotal circumference (SC, in centimeters) estimated breeding values for the G5501 → T (G, n = 247; T, n = 71) mitochondrial *tRNA^{asn}* single nucleotide polymorphism (SNP). The table shows degrees of freedom (DF) and the mean square SC estimated breeding values at 365, 455 and 550 days.

		Scrotal circumference estimated breeding values (mean squares)		
Source	DF	SC_{365}	SC_{455}	SC_{550}
Genotype	1	0.00	0.05	0.06
Error	343	0.22	0.56	0.51
P > F		0.97	0.75	0.72

Quantitative traits have also been associated with mtDNA polymorphisms. D-loop variation has been associated to carcass traits such as *longissimus* muscle area and beef marbling score (Mannen *et al.*, 1998), milk production as measured by yield, fat content and estimated milk energy (Schutz *et al.*, 1994), and calving rates (Sutarno *et al.*, 2002). Furthermore, polymorphic sites on rRNA genes have also been associated with milk traits (Boettcher *et al.*, 1996a).

The effect of variation in the mitochondrial genome on milk and beef traits is thought to be a consequence of changes in metabolic rate or the energy available for milk production and muscle development, the differences reported by us in this study tend to support this hypothesis.

We investigated scrotal circumference (SC) because this has been correlated with reproductive traits in Nelore cattle (Martins-Filho and Lôbo, 1991) and the heritability of scrotal circumference is considered moderate to high at 0.36 to 0.47 (Garnero *et al.*, 2001; Pereira *et al.*, 2002; Silveira *et al.*, 2004). However, no studies have suggested that the mitochondrial genome may influence fertility traits

Table 5 - Scrotal circumference (SC, in centimeters) estimated breeding values for the G5501 → T (G, n = 247; T, n = 71) mitochondrial *tRNA^{asn}* single nucleotide polymorphism (SNP). The table shows the means and standard errors of the mean (SE) for SC estimated breeding values at 365, 450 and 550 days.

	Scrotal circumference estimated breeding values (means ± standard errors, cm)		
tRNA^{asn}	SC_{365}	SC_{450}	SC_{550}
G	-0.17 ± 0.03^{a}	-0.28 ± 0.04^{a}	-0.31 ± 0.04^{a}
T	-0.17 ± 0.05^{a}	-0.31 ± 0.08^{a}	-0.34 ± 0.07^{a}

^{a}values in columns with the same superscript letter do not differ statistically by the Tukey test ($p > 0.05$).

and the polymorphism tested by us was not associated with scrotal circumference estimated breeding values.

In humans, there are approximately 108 disorders that are associated with SNPs of mitochondrial tRNA genes. Three of these disorders involve the *tRNA*^{asn} gene (Brandon *et al.*, 2005), indicating that SNPs of mitochondrial tRNA genes have an important role in mitochondria. Mitochondrial *tRNA*^{asn} gene SNP (G5501 → T) could affect maternal and direct weight estimated breeding values due to a direct effect on the functional efficiency of the *tRNA*^{asn} gene. Otherwise, the *tRNA*^{asn} gene SNP genotyped in our study may be associated to other functional polymorphisms or a group of polymorphisms (haplotype). While our results are not conclusive concerning the effect of the *tRNA*^{asn} gene SNP on Nelore estimated breeding values, they provide important evidence that nucleotide variation within the mitochondrial genome should be considered as genetic markers for the assisted selection of quantitative traits.

The *B. taurus* and *B. indicus* mitochondrial genomes have been completely sequenced but the existing polymorphisms between these two breeds need to be studied to further characterize the existing genetic variability in these breeds and elucidate the effects of such polymorphisms on production traits. The association of weight estimated breeding values with a *tRNA*^{asn} gene SNP confirms that mitochondrial DNA is a source of genetic variation that influences growth during the bovine pre-weaning and post-weaning periods.

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Internet Resource

Nelore cattle breeding program (NCBP) at the University of São Paulo, under the auspices of the Brazilian National Association of Breeders and Researchers of Nelore Cattle Associação Nacional de Criadores e Pesquisadores – ANCP) http://www.ancp.org.br/sumarios.

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