Partial sequencing of the *TOX* and *NCOA2* genes in buffaloes

de Camargo, G.M.F.[1@]; Cardoso, D.F.[1]; Baldi, F.[1]; Regitano, L.C.A.[2@] and Tonhati, H.[1]

[1]Universidade Estadual Paulista (Unesp). Faculdade de Ciências Agrárias e Veterinárias. Campus Jaboticabal. Departamento de Zootecnia. Jaboticabal-SP. Brasil.
[2]Empresa Brasileira de Pesquisa Agropecuária (Embrapa). Centro Pecuária Sudeste. São Carlos-SP. Brasil.

Additional keywords

Bubalus bubalis.
SNP.
miRNA.
Transcription factors.

SUMMARY

Buffaloes play an important role in food production and in the socioeconomic development of tropical regions. The characterization of genes permits the study of traits of this species and the development of animal production technology. This work partially studied the thymus high mobility group box protein *(TOX)* and the Nuclear Receptor Coactivator 2 *(NCOA2)* genes in female Murrah buffaloes using a PCR-sequencing technique. Six SNPs were identified in each gene. Two adjacent SNPs in *TOX* gene create/destroy a mature miRNA production site and are good candidates to be further studied. The homology of the regions of these genes with the cattle correspondents is very high (99 %).

Sequenciamento parcial dos genes *TOX* e *NCOA2* em bubalinos

Palavras chave adicionais

Bubalus bubalis.
SNP.
miRNA.
Fatores de transcrição.

RESUMO

Os bubalinos são animais com uma importância grande para a produção de alimentos, além da ação sócio-econômica na região tropical. A caracterização de genes possibilita o estudo de características próprias da espécie e o desenvolvimento de tecnologia para sua produção. Esse trabalho estudou parcialmente os genes thymus high mobility group box protein *(TOX)* e o Nuclear Receptor Coactivator 2 *(NCOA2)* em femêas bubalinas da raça Murrah pela técnica de PCR-sequenciamento. Seis SNPs foram identificados em cada gene. Dois SNPs adjacentes no gene *TOX* criaram/destruíram um sitio de produção de um miRNA e são bons candidatos para serem estudos no futuro. A homologia das regiões desses genes com as correspondentes em bovinos é muito alta (99 %).

Información

Cronología del artículo.
Recibido/Received: 29.4.2014
Aceptado/Accepted: 5.12.2014
On-line: 16.3.2015
Correspondencia a los autores/Contact e-mail:
gregoriocamargo@hotmail.com
luciana.regitano@embrapa.br

INTRODUCTION

Buffaloes (*Bubalis bubalis*) play an important role in meat and milk production worldwide, especially in Asia, where 97,1 % of the bubaline population is located (FAO, 2013). Interestingly, most individuals of this species are found in developing or less-developed countries. Therefore, improvement of buffalo production will actively improve the socioeconomic status of these countries. The higher is the profit generated by the livestock production, the higher is the benefit for the human population.

Although cattle and buffaloes are related species, the reproduction of these species differs in some aspects and species-specific technologies need to be developed for a proper husbandry (Gimenes *et al*., 2011; Carvalho *et al*., 2013). Reproductive traits of buffaloes show low heritability (Seno *et al*., 2010) or are measured late in life (Galeazzi *et al*., 2010a,b). These facts make these traits candidates to be studied with molecular markers.

In a study on cattle, Fortes *et al*. (2011) showed that some genes that act as hypothalamic transcription factors, which include *TOX* and *NCOA2*, seem to be important for the development of puberty, since these transcription factors are used in the transcription of several genes.

Hong *et al*. (1996) identified the *NCOA2* gene in mice, this gene interacts with hormone binding domains of glucocorticoids such as estrogen and androgen. According to Hong *et al*. (1996), *NCOA2* is the main component of the mechanism of RNA polymerase II transcription, acting as a transcriptional co-activator and forming a bridge between transcription and hormone-binding sites, i.e., this protein is required by transcription factors for the initiation of transcription. In another study, Hong *et al*. (1997) demonstrated the

presence of the *NCOA2* protein in the brain where it interacts with five steroid receptors and induces the hormone-dependent activation of mineralocorticoid, glucocorticoid and steroid receptors.

The thymus high mobility group box protein *(TOX)* was first isolated by Wilkinson *et al*. (2002) from thymus tissue of mice. This protein is part of the family of high mobility group proteins that bind to and induce sharp bends in DNA (Bianchi and Beltrame, 1998), thus regulating gene expression by altering the chromatin structure and modulating the formation of regulatory complexes of multiple proteins (Wilkinson *et al*., 1998). Wilkinson *et al*. (1998) showed that *TOX* participates in the development of thymus cells and causes changes in gene expression. Laz *et al*. (2007) described *TOX* gene, in mice, and concluded that transcription factor is expressed in liver and is regulated by growth. Wilkinson *et al*. (1998) demonstrated the presence of *TOX* in human brain and Fortes *et al*. (2011) detected this protein in the hypothalamus of cattle.

The characterization of genes is the initial step in the development of molecular markers. Therefore, the objective of the present study was to sequence partially the *TOX* and *NCOA2* genes in buffaloes and to evaluate possible genetic variations in these genes in order to obtain information to develop genetic markers.

MATERIAL AND METHODS

Twenty unrelated female Murrah buffaloes (*Bubalus bubalis*) were used for this study. The animals were sourced from commercial farm located in Dourado, São Paulo, Brazil. Animals were raised on pasture with feed supplementation during the dry period from April to September. The farm participates in the milk-recording program of the Animal Science Department, São Paulo State University (Unesp), Jaboticabal. São Paulo, Brazil.

Deoxyribonucleic acid (DNA) was extracted from hair follicles by the phenol-chloroform-isoamyl alcohol method (Sambrook and Fritsch 1989). The primers used for amplification, the amplicon size, and the region amplified are shown in **table I.**

The reaction mixture contained 1.5 µL DNA (105 ng), 1,5 µL of each primer (15 pM), 7,5 µL GoTaq Colorless Master Mix, and 4 µL nuclease-free water in a final volume of 15 µL. Amplification was performed in a Master Cycler Gradient 5331 thermal cycler (Eppendorf®, Germany, 2005) under the following conditions: denaturation at 95 ºC for 5 min, followed by 35 cycles of denaturation at 95 ºC for 1 min, specific annealing temperatures for each primer pair (according to de Camargo *et al*., 2014) for 1 min, and extension at 72 ºC for 1 min. A final extension step at 72 ºC for 5 min was included.

The PCR products were sequenced using both primers (forward and reverse) by the dideoxynucleotide chain termination reaction. Sequencing was performed in an automated ABI 3730 XL sequencer (Applied Biosystems) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

For identification of the polymorphisms, the sequences obtained were analyzed with the CodonCode Aligner program available at http://www.codoncode.com/aligner/download.htm. The Alibaba 2.1 TF Binding Prediction software available at http://www.generegulation.com/pub/programs/alibaba2/index.html was used for the identification of possible transcription factor-binding sites. The http://www.mirbase.org/search.shtmlsite was used to identify possible microR-

Table I. Primers used for amplification, amplified region, amplicon size, and annealing temperature of the primers (Os iniciadores utilizados para a amplificação, a região amplificada, em amplicons, e temperatura de emparelhamento dos iniciadores).

Primer sequences	Primer number	Amplicon size (bp)	Amplified region/gene	Annealing temperature (ºC)
5'-ACAAACGGATGTGAGGGAAG-3' 5'-GGCGGAAACAAAAGCAGAG-3'	1	271	Promoter, exon 1 (TOX)	59.8
5'-CAGGGTCAGGAAAGATGAC-3' 5'-TCAACAAATGCCAACTCTG-3'	2	466	Exon 2 (TOX)	55.4
5'-ATCTGAAGGGGTCCTGTGTG-3' 5'-CCCAACACAAATCAGGAAGC-3'	3	409	Exon 3 (TOX)	59.9
5'-AGGAGAAGGGTGGAAATGTG-3' 5'-TGTAACTGGACAAGCAGGTGA-3'	4	556	Exon 4 (TOX)	57
5'-AAATTAGGCTGGAAGAGGATGA-3' 5'-TACAGTCCGCAGGGTCATAA-3'	5	600	Exon 5 (TOX)	57.3
5'-CAAACCAACTGCCTCCACTC-3' 5'-CCAAGGGATGTTGTTCTGG-3'	6	542	Exon 6 (TOX)	54.9
5'-CCATTCCTCCTGAAACTGGA-3' 5'-ACACGATCAGCATATCTAAAATACAA-3'	7	501	Exon 1 (NCOA2)	59
5'-GTTGGGCAGATCATCCTTGT-3' 5'-CCATCTTTAGGGGATTGCTG-3'	8	603	Exon 2 (NCOA2)	59
5'-TTCTTGTGTCACTCTGTCCTTGA-3' 5'-CCTTCTTGGTGGTCCATTTT-3'	9	252	Exon 3 (NCOA2)	59
5'-TGCGGAGTACATCCATCTCA-3' 5'-CCCCAGTTACTGTTATCCCTGA-3'	10	639	Exon 4 (NCOA2)	58.4

NA (miRNA). The creation of possible miRNA sites for the suborder Ruminantia was investigated.

The sequences were edited according to the GenBank recommendations and deposited in its website.

RESULTS AND DISCUSSION

All primer pairs amplified fragments of the predicted size in DNA extracted from buffaloes, except for primer 1 of the *TOX* gene which did not amplify any fragment. Since the primers were designed based on the bovine sequence, this lack of amplification may be due to differences in the DNA sequences between these two species. The sequences generated were deposited in GenBank under the accession numbers KF418275 (*NCOA2* gene) and KF418277 (*TOX* gene). The regions of the *TOX* and *NCOA2* genes amplified in buffaloes showed 99 % homology with the respective sequences of these genes in the cattle genome (NC_007312.5), demonstrating that these genes are highly conserved in the two species.

Six single-nucleotide polymorphisms (SNPs) were identified in the *TOX* gene of buffaloes. Two SNPs were located in exon 5 (g.1812G/T and g.1884C/T) and four in intron 5 (g.2267G/A, g.2292A/G, g.2367A/G, and g.2368C/G). The polymorphisms in exon 5 do not cause amino acid substitutions and are silent mutations in the codon of an arginine (g.1812G/T) and of a serine (g.1884C/T). We also investigated whether the SNPs created/destroyed mature miRNA production sites in ruminants. The adjacent SNPs g.2637A/G and g.2638C/G created a production site of bta-miR-2285m with an A/C combination. The bta-miR-2285 family has over 40 members (Lawless *et al*., 2013) with production sites spanning the entire bovine genome (Guduric-Fuchs *et al*., 2012). Micro-RNAs are non-coding RNAs that regulate the expression of other genes in the organism. Therefore, these SNPs are candidates for subsequent association studies.

In addition, six interspecific SNPs (comparing the sequence of cattle and buffaloes) were identified in the coding regions of the *TOX* gene. Three of these SNPs were located in exon 3 (positions 692, 722 and 836) and three in exon 4 (positions 1176, 1245 and 1254). These positions are indicated in the GenBank sequence KF418277. However, all are silent mutations and encode the same amino acids in both species.

Six SNPs were identified in the *NCOA2* gene of buffaloes. Two SNPs are located in intron 1 (g.454A/G and g.733T/C), one in intron 2 (g.1109G/A), two in exon 3 (g.1366T/C and g.1390T/C), and one in intron 3 (g.1631A/T). The polymorphisms in exon 3 did not cause amino acid substitutions and were silent mutations in the codon of a threonine (g.1366T/C) and of an aspartic acid (g.1390T/C).

Investigation of interspecific SNPs in the *NCOA2* gene of buffaloes and cattle identified three changes in the promotor region (positions 13, 19 and 24) and three changes in exon regions, one in exon 1 (position 251) and two in exon 4 (position 1914 and 1929). These positions are indicated in the GenBank sequence KF418275. The change in the promotor region at position 13 created a binding site for the transcription factor Sp1 and the change at position 24 created two binding sites for the transcription factors USF and NF-1. If these transcription factors were used, this interspecific mutation could indicate possible differences in the expression of this gene between these species. The interspecific SNPs located in exons did not cause any amino acid substitution.

Some polymorphisms in these regions of *NCOA2* gene were associated with reproductive traits in zebu cattle (de Camargo *et al.*, 2014). The association shows that these genes influence reproduction in ruminants and may be useful in the future to genetic evaluation.

The present results show that the *TOX* and *NOCA2* genes are highly conserved in cattle and buffaloes. However, differences were observed in buffaloes that permit the development of molecular markers for these regions and future association studies with productive traits of interest. Differences were also observed between cattle and buffaloes, suggesting differential expression of these genes in the two species. Studies have shown low expression of the *NCOA2* gene in subfertile mice (Konno *et al*., 2010). In addition, *NCOA2* gene is known to be involved in the energy metabolism of cattle (Pryce *et al*., 2012), providing further important evidence for its study as a candidate gene.

Further studies characterizing additional regions of the *TOX* and *NCOA2* genes and evaluating the association of the SNPs found with reproductive performance would be useful to develop molecular markers for this species.

CONCLUSION

The regions studied in the *TOX* and *NCOA2* genes are highly conserved between cattle and buffaloes. However, some genetic variations were observed among buffaloes and they could be used for the development of molecular markers in association studies. Differences were also detected between cattle and buffaloes and they may indicate different expression mechanisms of the same genes between the species.

ACKNOWLEDGEMENTS

The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp) for the financial support and for the grant of the first author.

BIBLIOGRAPHY

Carvalho, N.A.T.; Soares, J.G.; Porto Filho, R.M.; Gimenes, L.U.; Souza, D.C.; Nichi, M.; Sales, J.S. and Baruselli, P.S. 2013. Equine chorionic gonadotropin improves the efficacy of a timed artificial insemination protocol in buffalo during the nonbreeding season. *Theriogenology,* 79: 423-428.

De Camargo, G.M.F.; Costa, R.B.; de Albuquerque, L.G.; Regitano, L.C.; Baldi, F. and Tonhati, H. 2014. Polymorphisms in TOX and NCOA2 genes and their associations with reproductive traits in cattle. *Reprod Fertil Dev*. http://dx.doi.org/10.1071/RD13360 (12-2-2014).

FAOSTAT (Food and Agriculture Organization of the United Nations-Statistics Division). http://faostat3.fao.org/browse/Q/QA/E. (24-1-2014)

Fortes, M.R.S.; Reverter, A.; Nagaraj, S.H.; Zhang, Y.; Jonsson, N.N.; Barris, W.; Lehnert, S.; Boe-Hansen, G.B. and Hawken, R. J. 2011. A single nucleotide polymorphism-derived regulatory gene network underlying puberty in 2 tropical breeds of beef cattle. *J Anim Sci,* 89: 1669-1683.

Galeazzi, P.M., Mercadante, M.E.Z.; Silva, J.A.IIV.; de Albuquerque, L.G.; de Camargo, G.M.F. and Tonhati, H. 2010a. Analysis of culling probability in dairy buffalo using survival models. *Animal*, 4: 1325-1329.

Galeazzi, P.M.; Mercadante, M.E.Z.; Silva, J.A.IIV.; Aspilcueta-Borquis, R.R.; de Camargo, G.M.F. and Tonhati, H. 2010b. Genetic parameters for stayability in Murrah buffaloes. *J Dairy Res*, 77: 252-256.

Gimenes, L.U.; Carvalho, N.A.T.; Sa Filho, M.F.; Vannucci, F.S.; Torres-Júnior, J.R.S.; Ayres, H.; Ferreira, R.M.; Trinca, L.A.; Sartorelli, E.S.; Barros, C.M.; Beltran, M.P.; Nogueira, G.P.; Mapletoft, R.J. and Baruselli, P.S. 2011. Ultrasonographic and endocrine aspects of follicle deviation, and acquisition of ovulatory capacity in buffalo *(Bubalus bubalis)* heifers. *Anim Reprod Sci*, 123: 175-179.

Guduric-Fuchs, J.; O'Connor, A.; Cullen, A.; Harwood, L.; Medina, R.J.; O'Neill, C.L.; Stitt, A.W.; Curtis, T.M. and Simpson, D.A. 2012. Deep sequencing reveals predominant expression of miR-21 amongst the small non-coding RNAs in retinal microvascular endothelial cells. *J Cell Biochem*, 113: 2098-2111.

Hong, H.; Kohli, K.; Garabedian, M.J. and Stallcup, M.R. 1997. GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol Cell Biol,* 17: 2735-2744.

Hong, H.; Kohli, K.; Trivedi, A.; Johnson, D.L. and Stallcuo, M.R. 1996. GRIP1, a novel mouse protein that serves as a transcriptional activator in yeast for the hormone binding domains of steroid receptors. *PNAS,* 93: 4948-4952.

Konno, T.; Graham, A.R.; Rempel, L.A.; Ho-Chen, J.K.; Alam, S.M.K.; Bu, P.; K Rumi, M.A. and Soares, M.J. 2010. Subfertility linked to combined luteal insufficiency and uterine progesterone resistance. *Endocrinology,* 151: 4537-4550.

Lawless, N.; Foroushani, A.B.K.; McCabe, M.S.; O'Farrelly, C. and Lynn, D.J. 2013. Next generation sequencing reveals the expression of a unique miRNA profile in response to a gram-positive bacterial infection. *PLOS ONE*, 8: e57543.

Laz, E.V.; Holloway, M.G.; Chen, C.S. and Waxman, D.J. 2007. Characterization of three growth hormone-responsive transcription factors preferentially expressed in adult female liver. *Endocrinology,* 148: 3327-3337.

Pryce, J.E.; Arias, J.; Bowman, P.J.; Davis, S.R.; Macdonald, K.A.; Waghorn, G.C.; Wales, W.J.; Williams, Y.J.; Spelman, R.J. and Hayes, B.J. 2012. Accuracy of genomic predictions of residual feed intake and 250-day body weight in growing heifers using 625,000 single nucleotide polymorphism markers. *J Dairy Sci*, 95: 2108-2119.

Sambrook, J. and Fristch, E.F. 1989. Molecular cloning. A laboratory manual, 2nd ed. Cold Spring Laboratory. New York.

Seno, L.O.; Cardoso, V.L. ; El Faro, L. ; Sesana, R.C.; Aspilcueta-Borquis, R.R.; de Camargo, G.M.F. and Tonhati, H. 2010. Genetic parameters for milk yield, age at first calving and interval between first and second calving in milk Murrah buffaloes. *Livest Res Rural Develop,* 22: 2. http://www.lrrd.org/lrrd22/2/ (12-12-2013)

Wilkinson, B.; Chen, J.Y.; Han, P.; Rufner, K.M.; Goularte, O.D. and Kaye, J. 2002. *TOX*: an HMG box protein implicated in the regulation of thymocyte selection. *Nat Immunol,* 3: 272-280.