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Association analysis of polymorphism in thyroglobulin gene promoter with milk production traits in riverine buffalo (*Bubalus bubalis*)

P.K. Dubey [1], S. Goyal, S.K. Mishra, A.K. Yadav, P. Kathiravan [2], R. Arora, R. Malik [3], R.S. Kataria *

National Bureau of Animal Genetic Resources, Karnal 132001, Haryana, India

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ABSTRACT

Polymorphism within the promoter region of bovine thyroglobulin has been reported to be associated with milk and meat quality. In this study, we investigated the genetic variation within thyroglobulin promoter region of swamp and riverine buffaloes using PCR–SSCP technique and sequencing, and also analyzing association of polymorphism with the milk production traits. The study revealed four conformational patterns, A, B, C, and D among 323 buffaloes of two riverine breeds and different swamp populations. The frequency of SSCP variant 'A' was found to be invariably high among all buffalo populations. Variant 'C' was found to be absent in pure swamp population and present with higher frequency among riverine dairy breeds Mehsana and Nili Ravi. Frequency of D variant was observed to be highest in buffalo population, representing riverine and hybrid types. Sequencing of three representative PCR products of each of the SSCP variants, revealed three polymorphic sites responsible, 33C > T, 176G > T and 221C > T, in the buffalo *TG* promoter region. Further, association studies of SSCP variants with various milk production and milk quality traits indicated significant effect on fat percentage in buffaloes belonging to Mehsana and Nili Ravi dairy breeds. The preliminary results also showed the substantial variations in the distribution of SSCP variants' frequencies across swamp and riverine buffaloes, two distinct populations being reared for meat and milk production, respectively.

1. Introduction

Buffalo is an important livestock species contributing to meat, milk and draught power in Southeast Asia. Fat percentage is important parameter to assess the quality of milk as well as meat of both cattle and buffalo. A QTL region associated with fat deposition in skeletal muscles, has been identified in the centromeric region of bovine chromosome 14 (BTA14), among multiple cattle populations (Moore et al., 2003; Casas et al., 2005). Genes lying in this region include diacylglycerol-O-acyltransferase (DGAT1), thyroglobulin (*TG*) and adipose fatty acid binding protein (FABP4), found to be associated with fat percentage in both beef and dairy cattle (Barendse, 1999; Michal et al., 2006). Thyroid hormones play an important role in regulating the metabolism and can affect adipocyte growth, differentiation, and homeostasis of fat depots. Among these genes, *TG* is a glycoprotein hormone, synthesized in thyroid follicular cells and carrier for both triiodothyronine (T3) and thyroxine (T4), stored in the thyroid gland. Genetic variation in *TG* has been associated with back fat thickness and marbling in beef cattle as well as milk traits in dairy cattle (Moore et al., 2003; Barendse et al., 2004; Rincker et al., 2006; VanEenennaam et al., 2007). The genetic variations responsible are located in the 5′promoter region and 3′UTR of the *TG* gene, which have been widely used in marker assisted selection (MAS) programs to improve the predictability of marbling level and eating quality in beef (Barendse, 1999). An allele of the *TG* gene has also been identified to be having a significant association with marbling score in cattle (Gan et al., 2008). In Japanese Black cattle, marbling has been associated with both T3 and T4 (Mears et al., 2001). The polymorphism C/T (PsuI-RFLP) in 5′ upstream region was associated with higher marbling scores in cattle that had the homozygous cytosine (C) allele as favourable one (Wood et al., 2006).

India has two different populations of water buffaloes — swamp and riverine types, differing in their chromosome numbers as well as in habitat and utility. Phenotypically also two buffalo populations differ, with swamp buffaloes mainly found in Northeast region of India, being short and stout are utilized for draught and meat purpose, whereas riverine buffaloes not so muscular in body structure, are reared primarily for milk. Earlier reports have also shown significant variations in the

* Corresponding author at: Division of Animal Biotechnology, National Bureau of Animal Genetic Resources, P.O. Box 129, GT Road By-Pass, Karnal 132001, Haryana, India.
E-mail address: katariaranji@yahoo.co.in (R.S. Kataria).
[1] Present address: Immune regulation, WPI-IFREC, Osaka University, Osaka, Japan.
[2] Present address: Animal Production and Health Section, International Atomic Energy Agency, Vienna, Austria.
[3] Present address: Central Institute for Research on Buffaloes, Sub-campus, Nabha, Punjab, India.

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allelic frequencies of polymorphic loci in genes governing immune response and other traits across swamp and riverine buffaloes (Dubey et al., 2013), indicating two populations with variable genetic structure.

Majority of Indian buffalo population is riverine type, contributing more than 50% to total milk production. Buffalo species is well known for high fat percentage in its milk, quality of meat low in cholesterol and candidate gene polymorphism has been exploited to find the association with these production traits (Tanpure et al., 2012). Due to the important role of thyroglobulin in fat metabolism and association of polymorphism with meat and milk quality reported in cattle and because of the fact that no such analysis has been carried out in closely reported buffalo species the present study was undertaken to identify polymorphism within promoter region of thyroglobulin gene across riverine and swamp buffaloes and analyze its association with milk production and fat percentage traits in buffalo.

2. Materials and methods

2.1. Animals, blood sample collection and DNA isolation

Blood samples were collected from unrelated buffaloes belonging to different breeds/populations from their native breeding tracts of different agro-climatic regions of the country. For SSCP analysis, the breeds/populations included were riverine type dairy breeds — Mehsana (136) and Nili Ravi (82) along with swamp type of Northeast Indian states including, Assam-riverine or hybrid (48), Assam – pure swamp (41) and Mizoram – pure swamp (16), bordering China and Myanmar (Table 1). Since buffaloes in India are mostly dairy type, riverine breeds of Mehsana (N = 144) and Nili Ravi (N = 22), for which milk production and fat records existed, were included for association analysis studies. Genomic DNA was extracted from the whole blood using standard SDS-Proteinase-K digestion and phenol/chloroform extraction procedure (Sambrook and Russel, 2001). Quality and quantity of DNA was assessed by measuring OD_{260} and OD_{280} using a Nanodrop, ND-1000 UV–vis Spectrophotometer (Thermo Scientific) as well as by running on ethidium bromide stained 0.8% agarose gel. The study was conducted following the ethical and regulatory guidelines in place at the institute.

2.2. Single strand conformation polymorphism analysis and sequencing

A set of oligonucleotide primers (forward: 5′-AGAGGGGAAAGGGGATGACT-3′ and reverse: 5′-GGGGGTGTGGCTTCATAATTC-3′) was designed from the cattle thyroglobulin gene promoter sequence using PrimerSelect program of Lasergene software (DNASTAR Inc., Madison, WI, USA), to amplified 300 bp region, comprising part of promoter region, −423 bp upstream to exon 1 start site (Dubey et al., 2014). Polymerase chain reaction was performed in a final reaction volume of 25 μL, containing ~100 ng of genomic DNA, 5 pmole of each primer, 200 μM of each dNTPs, 2.5 μL of 10× buffer with 15 mM $MgCl_2$ and one unit of *Taq* DNA polymerase (Bangalore Genei, India). Amplification was performed with an initial denaturation at 95 °C for 2.5 min followed by 35 cycles of 94 °C for 30 sec, annealing temperature at 58 °C for 30 sec and extension at 72 °C for 1 min, with a final extension of 5 min at 72 °C.

The PCR products were visualized on 2% ethidium bromide stained agarose gel. Amplified PCR products were subjected to single-strand conformational polymorphism (SSCP) analysis after optimization of non-denaturing PAGE conditions. The electrophoresis was carried out in 8% PAGE gel by using Protean II vertical electrophoresis unit (Bio-Rad, USA) using 1× TBE buffer. Gels were silver stained (Sambrook and Russel, 2001), dried and scored manually for SSCP variants. The SSCP variants were further sequenced for the identification of single nucleotide polymorphisms within promoter region of thyroglobulin gene using the PCR products from three representative samples of each variant, representing Mehsana, Nili Ravi riverine and swamp buffalo populations. Purified PCR products were sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems, USA) on ABI 3100 Genetic Analyzer and the raw sequence data was edited manually using Chromas Ver. 1.45 (http://www.technelysium.com. au/chromas.html). Each of the identified SSCP variants was sequenced from both ends using both forward and reverse primers and submitted to NCBI, GenBank (Table 2). Multiple sequence alignment of the buffalo sequences along with cattle sequence was carried out using MegAlign program of Lasergene software (DNASTAR, Inc., Madison, WI, USA) and different transcription binding factors were analyzed and compared using GPMiner and p-Match software (http://gpminer.mbc.nctu.edu.tw/, http://www.gene-regulation.com/pub/programs.html), as described previously for buffalo (Dubey et al., 2014).

2.3. Statistical analysis

For association analysis of SSCP variants with dairy performance traits, data related to different animals belonging to the Nili Ravi and Mehsana breeds of riverine buffalo, were collected and classified according to different seasons, years and herds etc. Effects of different non-genetic factors like seasons (rainy, mid-June to September; winter, October to February; summer, March to mid-June), year (2001–2005), parity and influence of different management practices in the farm and field conditions were included in the analysis. The effects of non-genetic factors were estimated by least squares analysis of variance for non-orthogonal data using a complete fixed model as described by Harvey (1987). The effect of different SSCP variants on various production traits milk yield, milk fat percentage and total fat yield at late stage of lactation, i.e. 305 days, was analyzed by least squares method using a mixed model with random effect of sire and fixed effect of SSCP variant or genotype as given below.

$$Y_{ijk} = \mu + Si + Gj + e_{ijk}, \text{ where}$$

Y_{ijk} is the dependent trait under study (milk yield, fat percentage and fat yield at different stages of lactation of kth individual with jth genotype and ith sire), μ is the overall population mean, $S_i$is the random effect of the ith sire, Gj is the fixed effect of the jth genotype, and e_{ijk} is the random error, assumed to be normally independently distributed with

Table 1
Frequency of SSCP variants in thyroglobulin promoter in different riverine and swamp buffalo breeds/populations.

Breed/population	Total no.	Variants (no. of observations)				Frequency			
		A	B	C	D	A	B	C	D
Mehsana	136	94	19	17	6	0.69	0.14	0.13	0.04
Nili Ravi	82	68	3	10	1	0.83	0.04	0.12	0.01
Total	218	162	22	27	7	0.75	0.10	0.12	0.03
Assamese (riverine/hybrid)	48	34	5	3	6	0.71	0.10	0.06	0.13
Swamp	57	39	5	0	13	0.69	0.08	0.00	0.23
Total	105	73	10	3	19	0.69	0.10	0.03	0.18
Overall	323	235	32	30	26	0.72	0.10	0.10	0.08

Table 2
Nucleotide changes observed at three loci in different SSCP variants of bubaline thyroglobulin promoter and their GenBank accession numbers.

SSCP variants	Nucleotide position			Accession numbers
	33	176	221	
Variant A	C	G	C	KC607819
Variant B	C/T	G	C	KC607820
Variant C	C	G/T	C/T	KC355364
Variant D	C/T	G/T	C/T	KC355365