



Polymorphism of insulin like growth factor binding protein 3 (IGFBP3) partial gene in mithun

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The insulin like growth factor binding proteins (IGFBPs) comprise of a family of peptides that play important role in mammalian growth and development. Out of the 6 IGFBPs the IGFBP3 is produced by kupffer cells of liver and functions as the major carrying protein for insulin like growth factors (IGF1 and IGF2) in the circulation and modulates IGF activity (LeRoith *et al.* 1995). It binds with IGFs to form a binary complex because of which the half-life is increased up to 6–12 h in comparison to 20 min in unbound form (Lee and Rechler 1995). IGFBP3 is also important in maintaining reservoirs of IGFs in circulation (Francis *et al.* 1990) and enhancing the mitogenic activity of IGFs *in vitro* (Zapf 1995). IGFBP3 is 8.9 kb long gene, made up of 5 exons (Martin and Baxter 1992) and mapped to chromosome 4 in cattle (Maciulla *et al.* 1997), 12 in human beings (Cubbage *et al.* 1990), 18 in pigs (Archibald *et al.* 1995) respectively. Molecular weight of IGFBP3 depends on glycosylation degree, thus non-glycosylated form has 29.48 kDa in comparison to 54 kDa in glycosylated form (Russell and Vanwyk 1995). No reports of IGFBP3 gene are available on mithun (*Bos frontalis*) - an important beef animal of North-Eastern Region of India, which plays a significant role in the economic, social and cultural life of tribal people. Owing to high meat value of this animal, the growth is the most desirable parameter for improvement. The present study is an attempt to characterize and ascertain polymorphism in the exon 2–3 of IGFBP3 gene of mithun to explore a DNA marker which would facilitate early selection of this animal.

Unrelated mithuns (90) maintained at National Research Centre, Jharnapani and Porba, Nagaland were used for the present investigation. Healthy animals of 4 strains, viz. Arunachalee, Nagamee, Manipuri, Mizoram, were selected for the study. Venous blood sample (15 ml) was collected

from each animal in sterile 15 ml polypropylene centrifuge tube containing 0.5 ml of 2.7% EDTA as anticoagulant. The blood samples were transported to the laboratory in an icebox and stored at –20°C in deep freezer till the isolation of genomic DNA (Sambrook and Russel 2001) and the precipitated DNA was dissolved in 200 µl of TE buffer. The quality of DNA was checked by performing agarose gel electrophoresis. The purity and concentration were evaluated by spectrophotometry. The samples that showed an OD ratio (OD₂₆₀/OD₂₈₀) in the range of 1.7–1.9 were assessed to be of good quality. Concentrations of DNA were calculated by the formula and were found to be in the range of 16–20 µg/ml of blood. A previously reported (Maciulla *et al.* 1997) primer set for cattle (5' CCA AGC GTG AGA CAG AAT AC 3' as forward primer and 5' AGG AGG GAT AGG AGC AAG TT 3' as reverse primer), which encompasses part of exon 2, intron 2, exon 3 and part of intron 3 of IGFBP-3 gene and amplified 651 bp fragment in cattle (AF305712) was used. The working primer solution was further prepared by 10-fold dilution of stock solution to have a final concentration of 30 p mole/µl. The PCR amplification were carried out in a final volume of 25 µl containing 1µl genomic DNA (80–100 ng), 2.5 µl 10 X PCR buffer (1.5 mM), 2.5 µl dNTP mix (0.2 mM), 1.5 µl MgCl₂ (1.5 mM), 1 µl forward primer (30 pmol/µl), 1 µl reverse primer (30 pmol/µl), 0.2 µl Taq DNA polymerase (5U/µl). Samples were amplified for 35 cycles with initial denaturation at 94°C for 5 min, 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min followed by final extension at 72°C for 5 min. After the final cycle the PCR products were stored at 4°C prior to resolving on agarose gel. IGFBP3 gene fragment was digested with *Hinf* I and the recognition site of the enzyme is ...G↓ANTC... (where N is any nucleotide). The RE digestion was carried out in 15 µl reaction mixture carrying 7 µl autoclaved triple distilled water, 2 µl 10X assay buffer for RE, 1 µl RE enzyme (10U/µl) and 5 µl PCR product. The incubation temperature and time were optimized to be 37°C and overnight for complete digestion. The amplicon of

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IGFBP3 gene was eluted, sequenced and submitted to Gen Bank. The obtained sequence was then aligned with other available sequences of different species (www.ncbi.nlm.nih.gov) using 'MEGALIGN' programme of 'DNASTAR' software and the phylogenetic and molecular evolutionary analyses were conducted.

In the present study, a 652 bp region of IGFBP3 gene in mithun was amplified which was 1 basepair more than cattle with the same set of primers. It comprised exon 2 (20 bp), intron 2 (474 bp), exon 3 (120 bp) and intron 3 (38 bp). The amplified fragment upon digestion with *Hinf*I (RE site G↓ANTC) gave 3 bands of 314, 206 and 132 bp (AA genotype). All the bands were distinctly visible (Fig. 1). This suggested that amplified fragment of IGFBP3 gene contained 2 RE site for *Hinf*I and this was confirmed to be at 206th and 338th position. No polymorphism was found with respect to this restriction enzyme in all the mithun screened hence the gene and genotype frequencies were 1.00. Panigrahi *et al.* (2009) also observed monomorphism in same fragment of IGFBP3 using *Nla*III restriction enzyme in mithun. On the other hand, Maciulla *et al.* (1997) found polymorphism at the *Hae*III site on 331st nucleotide of the same fragment in Holstein, Hereford and Angus breed of cattle. Alike mithun, lack of polymorphism was also observed in Murrah buffaloes (Padma 2000, Mishra 2005) using *Hae*III, *Msp*I and *Taq*I enzymes, indicine cattle (Chowdhary 2004) using *Nla*III, goats (Mishra 2005) and sheep (Kumar *et al.* 2002) using *Hae*III. Contrastingly, polymorphism with 2 alleles and 3 genotypes was observed in taurine and crossbred cattle (Chowdhary 2004, Chowdhary *et al.* 2007) using *Hae*III and *Nla*III REs.

The alignment report indicated that there was an insertion 'T' at 617th position in the third intron of mithun when compared to most of the species hence, the length of the fragment became 652 bp as compared to 651 bp in cattle. Most of the insertions, deletions and substitutions observed

in the second intronic region except the G to A at 614th position of exon 3, causing the wobble base to change and hence no effect on amino acid sequence i.e. glutamine (Gln) suggesting a conserved nature of the protein.

When mithun sequence was compared to that of small ruminant's, viz. goat and sheep, the exonic region showed seven substitutions, viz. A521G, G527A, C534G, T553C, G554A, G568C and C586T. Thus, it was observed that the IGFBP3 gene fragment of mithun was more homologous to the large ruminants as compared to small. Alignment report also indicated that 6 single nucleotide differences existed in mithun sequences when compared to cattle, which were identified to be T34C, C164G, T175C, A354G, T599C and G614A and homology between them was 98.2%. Despite the single nucleotide variations, the amino acid sequences showed 100% homology indicating that variations in these nucleotides did not affect the amino acid coding with respect to the amplified fragment.

In mithun 100% homology in amino acid sequences was also noticed when compared to buffalo, horse and pig. The sequence of A allele, which was the first report on IGFBP3 gene in mithun, was submitted to GenBank (Accession No. EF686017).

Further, if any polymorphism in this genes is identified in future, association of the polymorphs with growth traits like birth weight, weekly and monthly body weights, weight gain and carcass traits etc. could be used as a genetic marker for selection programme. More number of individuals belonging to different types of mithun should be screened with more number of restriction enzymes to find out the allelic variants in other exonic and intronic regions of these genes. Attempts should also be made at studying the polymorphism of other growth associated genes.

SUMMARY

A fragment of 652 bp corresponding to exon-2-3 of IGFBP3 gene was amplified in 90 mithun (*Bos frontalis*). The PCR- RFLP analysis showed the absence of polymorphism in this fragment with respect to *Hinf*I restriction enzyme and showed only 2 restriction sites, which produced 3 fragments of 314, 206 and 132 bp (AA genotype). Six single nucleotide differences viz. T34C, C164G, T175C, A354G, T599C and G614A existed in mithun sequences when compared to cattle. The nucleotide and amino acid sequence homology between them was 98.2 and 100% respectively. Mithun had minimum deviation from cattle with respect to nucleotide as well as amino acid sequences. The sequence of the amplicon, which was the first report on mithun IGFBP3, was submitted to GenBank (Accession number EF686017).

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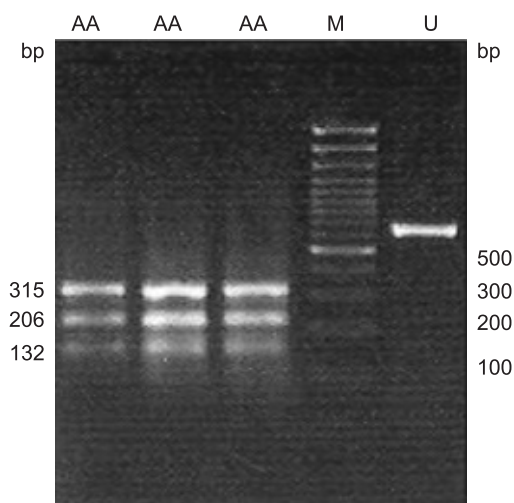


Fig. 1. IGFBP3-652bp amplicon Digestion with *Hinf*I/
M: 100bp marker; U: Undigested

rendered by Dr Chandan Rajkhowa, Director and Dr Arindam Dhali, Scientist, National Research Centre on Mithun, Jharnapani, Nagaland 797 106.

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