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Evolutionary Divergence of Signal Transducer and Activator of Transcription 5A (*STAT5A*) Gene in Riverine Buffalo

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AN designed the study. MN carried out the genomic work. MFB collected samples. MN, MJ and RBK wrote the manuscript. AN and MJ analyzed the data. AN revised the manuscript.

Key words
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ABSTRACT

Signal transducer and activator of transcription 5A (STAT5A), also called mammary gland factor (MGF), is a key mediator of signal transduction within mammary gland and uterine epithelial cells. It is a member of placental lactogen (PL) and interferon-tau (IFN-τ) signal pathway. It is also the main mediator of growth hormone. When cells encounter growth hormones and cytokines, STAT5A is activated to regulate gene transcription. STA5A has an important role in fertilization, embryonic survival and milk production traits in farm animals. The genomic characterization of *STAT5A* gene has not been assessed before in Nili Ravi Buffalo. In present research work, the sequence of the bovine *STAT5A* gene was analyzed to identify single nucleotide polymorphisms and its effect on evolutionary divergence. Nine polymorphisms, six in intronic and three in exonic regions, were identified in *STAT5A* gene. One exonic polymorphism G→A, in exon 5, was significant that causes a non-synonymous amino acid change from Serine (S) to Asparagine (N). Further, 64 genetic variants were also identified in *STAT5A* gene and these variants were due to cattle and buffalo differences. Phylogenetic analysis and evolutionary divergence were also estimated. The sequence was submitted to GenBank (NCBI) with accession number MN712202. Our results represent a preliminary step towards the identification of polymorphisms in *STAT5A* gene of Nili Ravi Buffalo. Further studies are required for the association of genetic polymorphisms of *STAT5A* gene with fertility related traits.

INTRODUCTION

Signal transducers and activators of transcription (STAT) are basically transcription factors that mediate the actions of cytokines and many peptide hormones within the target cells (Darnell *et al*., 1994; Schindler and Darnell Jr, 1995). STATs proteins comprise of STAT1, 2, 3, 4, 5A, 5B and 6 that are present in different mammals. They play their role as signal transducers in the cytoplasm and as transcription activators within the nucleus of the cell (Kisseleva *et al*., 2002).

STAT5 gene was reported initially as a single form in sheep but later, two isoforms (STAT5A and STAT5B) have been identified in cattle, rat, human and mouse cells which are encoded by two different genes (Goldammer *et al*., 1997; Ripperger *et al*., 1995; Hou *et al*., 1995; Liu *et al*., 1995). *STAT5A* and *STAT5B* encoding genes are originated from a single primordial gene and share almost 96% similarity in sequence (Seyfert *et al*., 2000). There is a few amino acids difference is found between the two isoforms (Moriggl *et al*., 1996). In cattle, *STAT5A* gene is located on chromosome 19; it has 19959 base pair and 19 exons which encode a 794 amino acid protein (Seyfert *et al*., 2000). STAT locus also contains *STAT3* and *STAT5B* genes (Seyfert *et al*., 2000; Molenaar *et al*., 2000).

STAT5 is also called mammary gland factor (MGF) because it is a mediator of prolactin signaling and can activate the transcription of milk protein genes (Watson, 2001). It is also the main mediator of growth hormone (Argetsinger and Carter-Su, 1996). The STAT5A protein is a member of placental lactogen (PL) and interferon-tau (IFN-τ) signal pathway. It plays an important role in signal transduction within mammary gland and uterine epithelial cells (Khatib *et al*., 2008). The PL induces the formation of homodimers of STAT5A, resulting in the transcription of osteopontin and bovine uterine milk protein genes (Stewart *et al*., 2002; Spencer and Bazer, 2002, 2004). Nakasato *et al*. (2006) identified the role of *STAT5A* gene

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in fertilization and embryonic development processes. Before fertilization, STAT5A is expressed in oocytes and after fertilization it is detected in 2 cell, 4 cell, morula and blastula stages. The survival of the embryo depends upon the two mechanisms which are associated with STAT5A protein. In the first process, various sperm factors are liable for low fertilization and in the second mechanism, incompatibility was found between the male and female pronucleus which ultimately leads to the embryonic death before the blastocyst stage (Wakasugi, 2007).

Data on polymorphisms in the bovine *STAT5A* gene is limited. In the present research, a candidate gene approach was used to identify novel polymorphisms in *STAT5A* gene in Nili Ravi buffaloes and validate their allele and genotype frequencies that can be assessed as potential markers of fertility traits for future animal selection and breeding plans.

MATERIALS AND METHODS

Sample collection

Blood samples were collected from Nili Ravi buffalo breed of Pakistan in EDTA-anticoagulated vacutainer tubes from different livestock farms.

PCR amplification of STAT5A *gene*

Genomic DNA was extracted from whole blood by using standard organic DNA extraction method (Sambrook *et al*., 2001). Every DNA sample concentration was quantified by a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and the concentration of each sample was adjusted to 50ng/µl for PCR. The integrity of DNA was checked by electrophoresis on a 0.8% agarose gel. The specific primers were designed by using Primer3 Input (version 0.4.0) software for the amplification of exonic and intronic regions of *STAT5A* gene after retrieving the sequence from NCBI database (NC_037346.1). PCR Primer Stats (Stothard, 2000) and OligoCalc (Kibbe, 2007) were used for the optimization of primer sequences. In Silico PCR was used for the confirmation of specificity of primers (Kent *et al*., 2002). Touchdown PCR was performed on a 25µl reaction mixture containing: 2.5µl 10x PCR buffer (500 mM KCL, 100 mM Tris-HCl, pH 9.0), 2.5µl dNTP (2.5mM/µl each), 1µl of each primer (10 pmol/µL), 1.5µl $MgCl_2$ (2.5mM/µl), 0.3µl *Taq* DNA Polymerase (5U/µl), 1.0 µl of genomic DNA (50ng/ µl) and 15.2µl deionized H2O. The PCR mixture was placed on an Eppendorf T100™ Thermal Cycler and subjected to thermal conditions, consisting of an initial DNA denaturation (95°C/5 min), 35 cycles of amplifications with temperatures of denaturation (94°C/30 s), annealing (65°C to 55°C /30 s), extension 72°C/30 s), and final extension (72°C/10 min). The confirmation of PCR products was done by agarose gel electrophoresis along with 1 *kb Plus GeneRuler* DNA *ladder*.

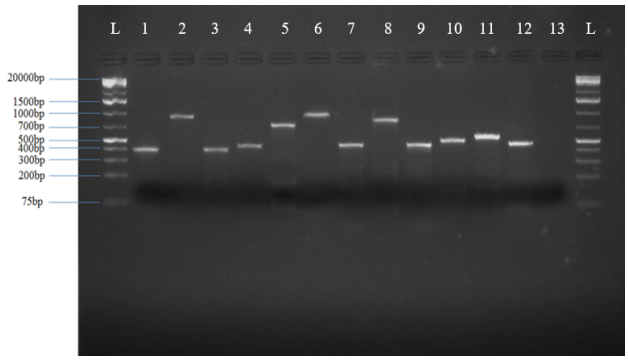

Fig. 1. PCR amplification products of one sample with all primers. Lane L, 1 kb Plus ladder; Lane 1, PCR product of primer 1 (381bp); Lane 2, PCR product of primer 2 (883bp); Lane 3, PCR product of primer 3 (390bp); Lane 4, PCR product of primer 4 (331bp); Lane 5, PCR product of primer 5 (731bp); Lane 6, PCR product of primer 6 (847bp); Lane 7, PCR product of primer 7 (446bp); Lane 8, PCR product of primer 8 (837bp); Lane 9, PCR product of primer 9 (456bp); Lane 10, PCR product of primer 10 (500bp); Lane 11, PCR product of primer 11 (589bp); Lane 12, PCR product of primer 12 (471bp); Lane 13, negative control (H_2O).

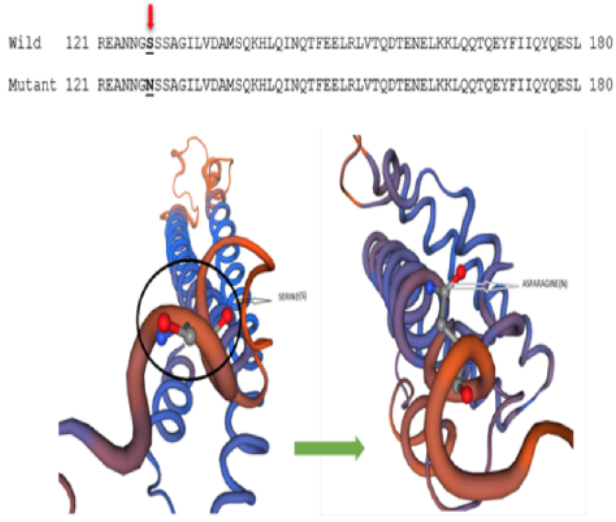

Fig. 2. Change in structure of *STAT5A* protein due to change in amino acid (Serine to Asparagine).

DNA sequencing and submission of sequence to NCBI

The amplified PCR products were purified with 70% ethyl alcohol and then sequenced on Genetic Analyzer (ABI 3130XL) by using BigDye Terminator (Applied Biosystems Inc). The sequence was submitted to GenBank (NCBI) with accession number MN712202.

Table I. Identified polymorphisms with allele and genotype frequencies in *STAT5A* gene.

Sr. no	SNP ID	Chromosome position*	Mutation	Change in codon	Region of mutation	Change in amino acid	Change type	Allele frequency		Genotype frequency			X^2 test *p*-value
								A	B	AA	AB	BB	
1	Stat-NR1	42396549	A>G	GCA>GCG	Exonic	Alanine > Alanine	Synonymous						
2	Stat-NR2	42396751	G>C	-	Intronic	-	-	0.69	0.31	0.66	0.06	0.28	0.002951
3	Stat-NR3	42401235	G>A	AGT>AAT	Exonic	Serine >Asparagine	Non-synonymous						
4	Stat-NR4	42407895	C>G	-	Intronic	-	-	0.61	0.39	0.60	0.02	0.38	0.000146
5	Stat-NR5	42408077	C>T	-	Intronic	-	-	0.62	0.38	0.56	0.12	0.32	0.001330
6	Stat-NR6	42411161	C>A	-	Intronic	-	-	0.56	0.44	0.52	0.08	0.40	0.002280
7	Stat-NR7	42414196	A>G	CCA>CCG	Exonic	Proline > Proline	Synonymous						
8	Stat-NR8	42415118	G>A	-	Intronic	-	-	0.67	0.33	0.62	0.10	0.28	0.006567
9	Stat-NR9	42415163	G>T	-	Intronic	-	-	0.67	0.33	0.58	0.18	0.24	0.004037

*Accession Number: NC_037346.1

Bioinformatics analysis

The analysis of results was done by NCBI Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/) after alignment of sequencing results with *STAT5A* gene reference sequence (NC_037346.1). For the alignment of multiple sequences, BioEdit software was used. Single nucleotide polymorphisms were recognized after every sequence observation. ExPacy bioinformatics tool was used for the translation of nucleotide sequence of mRNA into the amino acid sequence of *STAT5A* gene (Gasteiger *et al*., 2003).

Protein structure

SWISS-MODEL software was used for designing the three-dimensional structure of STAT5A protein (Waterhouse *et al*., 2018) and then after changed structure was compared with the normal structure of STAT5A protein.

Phylogenetic analysis

The nucleotide sequences of *STAT5A* gene from different species including some model organisms (*Bos taurus, Bubalus bubalis, Bos mutus, Ursus maritimus, Capra hircus, Homo sapiens, Mus musculus, etc.*) were selected for the construction of phylogenetic tree by using MEGA 6 software (Tamura *et al*., 2013).

Statistical analysis

POPGENE 1.31 was used for the calculation of allele and genotype frequencies of all identified polymorphisms (Yeh *et al*., 1997).

RESULTS

STAT5A gene was selected for the first time for the identification of polymorphisms in Nili Ravi Buffaloes as it has an important role in transmitting signals for fertilization and early embryonic development. Some genes are proposed as potential candidates which have an association with dairy fertility traits and *STAT5A* gene seems to be promising among them. Polymorphisms occurring within such genes may influence the fertilization rate and be an effective DNA marker of a subregion of the dairy cattle genome.

Genomic DNA of all samples were amplified and the amplified products were confirmed on 1.2% agarose gel. The results showed that amplification fragment sizes have a good specificity and were consistent with the target ones. PCR products of one sample with all primers were shown in Figure 1.

Sequence analysis revealed nine single nucleotide polymorphisms in exonic and intronic region of *STAT5A* gene of Nili Ravi buffaloes. Six polymorphisms were found in introns and three polymorphisms named Stat-NR1, Stat-NR3, Stat-NR7 were found in exon 2, 5 and 18, respectively (Table I). Stat-NR3 polymorphism (G>A) causes the amino acid change Serine (S) to Asparagine (N) due to the change in codon from AGT>AAT (Fig. 2). Sequence analysis also revealed 64 variants in exonic and intronic regions of *STAT5A* gene and these variants are due to cattle and buffalo differences (Table II).

Phylogenetic tree represents that *STAT5A* gene has extended sequence homogeneity in Nili Ravi buffalo,

Table II. Identified novel variants in *STAT5A* gene sequence of Nili Ravi buffalo.

Sr. No.	Variant ID	Chromosome position*	Change in nucleotide	Exonic/ intronic	Sr. No.	Variant ID	Chromosome position*	Change in nucleotide	Exonic/ intronic
1	Stat-1	42395561	G>A	Exonic	33.	Stat-33	42407777	T>G	Intronic
2	Stat-2	42395562	C>T	Exonic	34.	Stat-34	42407786	C>G	Intronic
3	Stat-3	42395569	A>C	Exonic	35.	Stat-35	42407874	A>G	Intronic
4	Stat-4	42395617	C>T	Intronic	36.	Stat-36	42407896	A>G	Intronic
5	Stat-5	42396609	G>C	Intronic	37.	Stat-37	42407927	A>G	Intronic
6	Stat-6	42396619	A>C	Intronic	38.	Stat-38	42407945	C>A	Intronic
7	Stat-7	42396772	G>C	Intronic	39.	Stat-39	42407967	T>C	Intronic
8	Stat-8	42396824	G>C	Intronic	40.	Stat-40	42408113	G>A	Intronic
9	Stat-9	42397014	T>A	Exonic	41.	Stat-41	42408114	T>G	Intronic
10	Stat-10	42397074	G>A	Intronic	42.	Stat-42	42408142	C>A	Exonic
11	Stat-11	42398189	C>T	Exonic	43.	Stat-43	42408220	T>C	Exonic
12	Stat-12	42398347	C>T	Intronic	44.	Stat-44	42408322	T>C	Intronic
13	Stat-13	42401415	G>A	Intronic	45.	Stat-45	42409893	C>G	Intronic
14	Stat-14	42401486	G>A	Intronic	46.	Stat-46	42410939	C>T	Intronic
15	Stat-15	42401523	T>C	Intronic	47.	Stat-47	42411013	T>G	Intronic
16	Stat-16	42401527	G>A	Intronic	48.	Stat-48	42411048	C>T	Intronic
17	Stat-17	42406851	C>G	Intronic	49.	Stat-49	42411049	T>C	Intronic
18	Stat-18	42406873	C>G	Intronic	50.	Stat-50	42411058	C>A	Intronic
19	Stat-19	42406881	T>C	Intronic	51.	Stat-51	42411166	A>C	Intronic
20	Stat-20	42406909	T>C	Intronic	52.	Stat-52	42412461	C>A	Intronic
21	Stat-21	42407140	G>C	Intronic	53.	Stat-53	42412738	G>T	Intronic
22	Stat-22	42407151	G>A	Intronic	54.	Stat-54	42412817	A>G	Intronic
23	Stat-23	42407153	G>A	Intronic	55.	Stat-55	42412994	C>T	Exonic
24	Stat-24	42407167	C>G	Intronic	56.	Stat-56	42413857	C>T	Intronic
25	Stat-25	42407168	A>C	Intronic	57.	Stat-57	42413883	C>A	Intronic
26	Stat-26	42407169	G>T	Intronic	58.	Stat-58	42413901	C>T	Intronic
27	Stat-27	42407194	C>T	Intronic	59.	Stat-59	42413920	A>C	Intronic
28	Stat-28	42407235	G>A	Intronic	60.	Stat-60	42413923	T>C	Intronic
29	Stat-29	42407263	T>G	Intronic	61.	Stat-61	42413943	G>A	Intronic
30	Stat-30	42407599	T>C	Exonic	62.	Stat-62	42414068	C>T	Intronic
31	Stat-31	42407719	A>C	Exonic	63.	Stat-63	42414139	C>T	Exonic
32	Stat-32	42407754	G>A	Intronic	64.	Stat-64	42414154	T>C	Exonic

*Accession Number, NC_037346.1

Table III. Distance estimation of evolution among the species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. NR Buffalo_Pak		0.001	0.001	0.018	0.003	0.001	0.018	0.033	0.017	0.057	0.073	0.003	0.034	0.020	0.054
2. Bos taurus (Cattle) NC_037346.1	0.003		0.002	0.018	0.003	0.001	0.018	0.033	0.017	0.057	0.073	0.003	0.034	0.020	0.054
3. Bubalus bubalis (Italian Mediterranean Buffalo) NC_037547.1	0.020	0.023		0.018	0.003	0.001	0.017	0.032	0.017	0.056	0.073	0.003	0.034	0.020	0.054
4. Ursus maritimus (Polar Bear) NW_007907187.1	0.365	0.365	0.363		0.019	0.020	0.002	0.032	0.014	0.055	0.095	0.019	0.033	0.010	0.050
5. Capra hircus (Goat) NC_030826.1	0.052	0.052	0.050	0.370		0.003	0.018	0.033	0.018	0.059	0.074	0.001	0.034	0.022	0.057
6. Bos mutus (Wild Yak) NW_005394079.1	0.012	0.009	0.025	0.399	0.054		0.019	0.034	0.018	0.057	0.073	0.003	0.035	0.021	0.055
7. Ailuropoda melanoleuca (Giant Panda) NW_003217842.1	0.358	0.357	0.355	0.044	0.361	0.389		0.031	0.013	0.053	0.094	0.018	0.032	0.009	0.049
8. Homo sapiens (Human) NC_000017.11	0.679	0.679	0.678	0.678	0.688	0.698	0.664		0.032	0.062	0.135	0.032	0.001	0.031	0.066
9. Equus caballus (Horse) NC_009154.3	0.360	0.360	0.354	0.279	0.365	0.390	0.275	0.685		0.055	0.093	0.017	0.034	0.016	0.053
10. Mus musculus (House Mouse) NC_000077.6	1.041	1.042	1.030	1.005	1.060	1.047	0.998	1.123	1.033		0.147	0.059	0.059	0.057	0.012
11. NC_007114.7 Danio rerio (zebrafish)	1.237	1.237	1.242	1.452	1.247	1.236	1.448	1.815	1.441	1.914		0.074	0.137	0.099	0.139
12. Ovis aries (sheep) NC_040262.1	0.054	0.054	0.052	0.369	0.019	0.057	0.360	0.683	0.364	1.054	1.247		0.034	0.021	0.057
13. Pan troglodytes (chimpanzee) NC_036896.1	0.708	0.708	0.706	0.705	0.710	0.728	0.690	0.011	0.707	1.093	1.830	0.713		0.031	0.064
14. Canis lupus familiaris (Dog) NC_006591.3	0.411	0.410	0.410	0.184	0.436	0.414	0.182	0.652	0.296	1.070	1.494	0.428	0.652		0.056
15. Rattus norvegicus (Norway rat) NC_005109.4	1.015	1.015	1.012	0.952	1.037	1.022	0.950	1.169	1.009	0.216	1.865	1.033	1.147	1.043	

Bos taurs, Bubalus bubalis, Bos mutus, Capra hircus and Ovis aries (sheep) and it is highly divergent from *Danio rerio* (zebrafish) (Fig. 3). Further, distance estimation of evolution has manifested that cattle is the most similar with Nili Ravi buffalo among all other species (Table III).

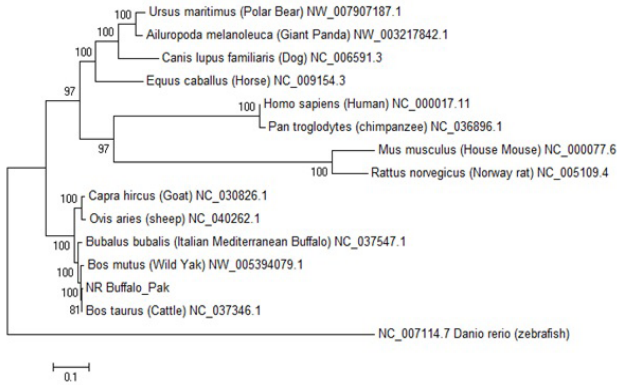

Fig. 3. Phylogenetic tree of *STAT5A* gene.

DISCUSSION

STAT5A gene was widely studied for the identification of polymorphisms in different breeds of bovine for embryonic survival, milk production and fertility related traits. Khatib *et al*. (2010) identified polymorphisms in *STAT5A* and *FGF2* genes in Holstein bulls and reported the association of polymorphisms with estimated relative conception rate and low bull fertility. The interesting thing is that these genetic variants were previously reported with high milk composition. And this association was reported not only in vitro but also in vivo experiments. Crepaldi *et al*. (2009) identified 30 single nucleotide polymorphisms in seven genes including *STAT5A*. Two polymorphisms were identified in exon 8 of *STAT5A* gene which have associated with bull fertility. Surprisingly reported polymorphisms were not identified in Nili Ravi buffalo.

STAT5A gene has an important role in lactogenesis and development of mammary gland (Liu *et al*., 1997). He *et al*. (2007) identified the single nucleotide polymorphism A9501G in *STAT5A* gene which had strong effects on milk yield, fat and protein percentage in Chinese Holstein cattle. Brym *et al*. (2004) also reported the mutation (A9501G) in Jersey cows which had significant association with milk production traits in the first and second lactations, whereas, the mutation (A9501G) was not significantly associated (P>0.05) with milk yield, protein yield, protein percentage, fat yield and fat percentage in Black-and-White cows. Selvaggi *et al*. (2009) identified a genetic polymorphism C6853T within the exon 7 of *STAT5A* gene in Italian Brown cattle. Cows with CC genotype produced more milk with higher protein content as compared to CT cows. In our findings, no polymorphism was found in exon 7.

Khatib *et al*. (2008) reported the mutation C>G at the position of 12195 in exon 8 of bovine *STAT5A* gene which had strong association with a significant decrease in protein and fat contents and with less significant decrease in somatic cell source (SCS), while, another genetic polymorphism A14217G in intron 9 had no significant association with milk yield, protein yield, protein percentage, fat yield, fat percentage and SCS. In Nili Ravi buffalo, no genetic mutation was found in exon 8 however, exon 2 and 18 were reported with one SNP each that was responsible for synonymous amino acid change (Table I).

Bao *et al*. (2010) reported two single nucleotide polymorphisms (A9501G and C12735T) in STAT5A gene of Chinese Holstein cattle which had strong association with milk production traits. The SNP A9501G was also previously reported by Brym *et al*. (2004). Significant association was identified in cows having GG genotype with higher milk yield as compared to cows having AA genotype.

CONCLUSION

The divergence of exonic and intronic single nucleotide polymorphisms revealed that *STAT5A* gene has high level of genetic variability. *STAT5A* gene has an important role in fertility related traits in bovine. The advanced research on *STAT5A* gene in Buffalo will be helpful for the determination of factors that are responsible for a mutation in *STAT5A* gene and for the selection of animals with good quality traits.

Statement of conflict of interest

The authors have declared no conflict of interest.

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