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Short communication

IDENTIFICATION OF NOVEL SINGLE NUCLEOTIDE POLYMORPHISMS IN THE *PRKAG3* GENE OF PAKISTANI RIVER BUFFALO

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Summary

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Pakistani river buffalo is classified in five breeds out of which Kundi buffalo has been least documented. This study is designed to find single nucleotide polymorphisms (SNPs) in exonic and intronic regions of *PRKAG3* (AMP-activated, Gamma3 non-catalytic subunit) gene in the Kundi buffalo. The *PRKAG3* gene of 95 animals each from Kundi and Nili-Ravi were sequenced for identification of novel SNPs. Comparing with the Nili Ravi breed of buffalo *PRKAG3* gene, six SNP sites were identified in the Kundi buffalo. The novel SNPs found in this work can function as a genomic indicator for genetic-phenotypic relationship of *PRKAG3* gene with milk and meat production in buffalo. This is the first report of SNPs in *PRKAG3* gene of Kundi Buffalo.

Key words: AMPK, Pakistan, *PRKAG3*, river buffalo, single nucleotide polymorphism

Water buffaloes are of prime importance in the lives of farmers and thus in the economies of many countries worldwide. They are not only draught animals, but also a source of meat, horns, skin, and particularly milk, which may be processed into cream, butter, yoghurt, and many kinds of cheese (Michelizzi *et al.*, 2010). The number of water buffaloes in the world has increased rapidly over the past few decades and according to FAO statistics (FAO, 2011) there are about 195 million buffaloes in the world. The current world scenario has glimpsed an amplified loss in wildlife diversity due to ever increasing human intrusion into ecological

habitats. Although the trends of loss in livestock diversity are comparatively different, many breeds are near to losing their genetic identity due to the forced gene flow of superior traits from economically healthier populations. The commercially underestimated livestock breeds could provide great economic stimulus in the future in terms of the beneficial allele spectra they have gained because of adaptation to their environments. Therefore, the FAO Domestic Animal Diversity Information System (DAD-IS) and Domestic Animal Genetic Resources Information System (DAGRIS) have begun a worldwide campaign for the conservation of within- and between-breed genetic diversity in livestock for their sustainable future use befitting their economic and social value. The within- and between-breed genetic variability in livestock predicted by phenotypic attributes can nowadays be validated using molecular markers and their analysis with sophisticated statistical techniques (Toro *et al.*, 2009).

Pakistani river buffalo has been grouped in five distinct breeds out of which Kundi is the least genetically documented. Home tract of Kundi lies on both sides of river Indus (Shah, 1994). The Pakistani buffalo breeds are conventionally reared and bred and there is an imperative need to study and improve the indigenous buffalo breeds for maximum production-efficiency. The sustainable growth in meat production in livestock needs not only managing resources but also reliance on the development of animal genetics. Adenosine monophosphate-activated protein kinase (AMPK) is one of most significant cellular enzyme involved in energy metabolism in the body of maximum of the mammals including buffaloes (Babar *et al.*., 2008). AMPK influences the meat quality traits comprising meat colour, tenderness, drip loss and cooking loss (Milan *et al.*, 2000; Ciobanu *et al.*, 2001). AMPK complex consists of seven subunits, amongst which two subunits (α1 and α2) are a member of catalytic alpha subunit, two (β1 and β2) of regulatory beta subunit and three (γ1, γ2 and γ3) of regulatory gamma subunit (Carling, 2004; Crute *et al*., 1998). Protein kinase AMP-activated non-catalytic subunit gamma 3 (*PRKAG3*) gene encodes γ3-peptide which is one of the three γ-isoforms (γ 1, γ 2 and γ 3) of the γ-regulatory subunit of AMPK and with muscle-specific expression (Cheung *et al.*, 2000). The relationship between *PRKAG3* gene polymorphism and milk & meat quality traits have already been described in bovine and porcine (Kowalewska-Luczak & Kulig, 2011; Ryan *et al.*, 2012). Due to the geographical seclusion Pakistani buffalo breed; Kundi can be a smart model for identification of novel single nucleotide polymorphisms (SNPs) in *PRKGA3* gene and its association with meat and milk production and meat production traits. In the current study, we described through DNA sequencing, the locus specific novel polymorphisms in *PRKAG3* gene of riverine buffalo; Kundi. Furthermore, it will provide information on the genetic relatedness of the Kundi with reference to the Nili Ravi, which can be employed to support the natural populations in the coming years if necessary and infer strategies for the enrichment of the GenBank samples for buffalo in Pakistan.

Samples collection. Ninety-five unrelated (n=95) individuals of Kundi and Nili-Ravi breeds with typical phenotypic features were collected from the wild as well as from captive locations after rather extensive field search in their natural habitats. Ten mL blood was collected asepti-

cally from each animal from the jugular vein of Kundi breeds which was approved by the Institutional Board of Study of Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore with 0.5M ethylenediamine tetra-acetic acid (EDTA) as an anticoagulant. The blood samples were stored on ice immediately after collection. They were then brought to the laboratory and further stored temporarily at –20 ^{o}C prior to DNA extraction. Additionally, 95 blood samples of Nili-Ravi breed; another well characterised Pakistani buffalo were obtained for reference study.

DNA extraction and quantification. The stored samples were thawed (at room temperature using water bath) for the genomic DNA isolation using DNA extraction kit (BioBasic, Canada) as per manufacturer's guidelines and stored at –20 ^{o}C for further use. Quantification of the extracted DNA samples was carried out with the help of agarose gel electrophoresis (0.8 %) as well as NanoDrop (Thermo scientific, USA). Standard DNA/DNA ladder was added. All samples were brought to same level of concentration of 50 ng/µL.

Primers and PCR amplification. Amplification used *PRKAG3* specific primers given in Table 1 and available at GenBank, National Centre for Biotechnology Information (NCBI) using Primer 3 software and Insilico PCR web facility (Rozan & Skaletsky, 2000). PCR was performed according to the protocol of primers set. DNA polymerase, polymerase chain reaction (PCR) buffer, dNTPs, $MgCl_2$, genomic DNA and nuclease-free water were used for the amplification using thermocycler (IcyclerBioRad, USA). PCR was performed in reaction volume of 25 µL using cycling conditions: initial denaturation at 95 ^{o}C for 4 min followed by 35 cycles of 94 ^{o}C for 1 min; 54 ^{o}C for 1 min; 72 ^{o}C for 1 min with final extension at 72 ^{o}C for 7 min.

Sequencing. PCR amplifications were seen by running 6 µL of PCR product mixed with 2 µL of loading dye on 1.5% agarose gel at a constant voltage of 100 V for 50 min in 1× TAE buffer. The resulting bands were visualised under UV light using gel documentation system (BioRad, USA). The amplified PCR products were purified using DP203-TIANquick Mini Purification Kit (China) as per provided instructions.The quality of DNA was examined on 2% agarose gel. Purified PCR products were then sent to Singapore for Sanger's sequencing.

Table 1. List of primers used to amplify different regions of the *PRKAG3* gene

Primer name	Primer sequence (5'–3')	Product size	Primer location
PRKAG3F1	GAGCAAGGAGACAGCACTTCA	891	Ex3, Int3, Ex4
PRKAG3R1	ACCTGTAGCATGGTGTCGAAGA		
PRKAG3.UPSEQ1	GACCTCAGCATCCAGGCT		
PRKAG3.UPSEQ2	GACAGTAACTCCATCTTCCA		
PRKAG3F2	CCAAGCAGCGCACCCTGCT	1313	Ex10, Int10,
PRKAG3R2	CTTACAATCACATCAAAGCGG		Ex11
PRKAG3.DNSEQ1	ATGGTAGAGAACACTGTGTA		
PRKAG3.DNSEQ2	TTCCACAGATCTGCCTGCT		
PRKAG3.DNSEQ3	ATATAGCCTGAATCTGCGTG		
PRKAG3.DNSEQ4	GAGGTGAAATGACATGATTC		

lic representation of inter-breed polymor-

Table 2. Genotypes and allelic frequencies of the *PRKAG3* gene

Genotypes				Allele Frequencies		
	Name	Allele	Position	Nili-Ravi (n=95)	Kundi (n=95)	Both breeds (n=200)
Ex3	NK1A	GG	967	0.67	1.0	0.835
	NK1B	AA		0.33	0	0.165
	NK2A	GG	1030	1.0	0.55	0.775
	NK28	GC		0	0.45	0.225
Int3	NK3A	CC	1070	0.53	1.0	0.765
	NK3B	CT		0.37	0	0.185
	NK3C	TT		0.10	0	0.05
	NK4A	CC	1382	0.47	1.0	0.735
	NK4B	CT		0.38	0	0.19
	NK4C	TT		0.15	0	0.075
Ex4	NK5A	GG	1489	0.46	0.32	0.39
	NK5B	GA		0.35	0	0.175
	NK5C	AA		0.19	0.68	0.435
				0.58	0.67	0.625
				0.42	0.33	0.375
Int10	NK6A	TT	4875	0.67	1.0	0.835
	NK6B	AA		0.33	0	0.165

Bioinformatics analysis. The sequences were aligned by using NCBI BLAST tool. CodoncCode Aligner software was used for sequence editing, alignment and detection of variable sites. Multiple sequence alignments were executed with Clustal W.

In this study six SNPs were detected in the *PRKAG3* gene of Pakistani Buffalo; Kundi and Nili-Ravi (Table 2). Two SNPs at position 1070 and 1489 were also included from this study in *PRKAG3* gene of Nili-Ravi buffalo. The polymorphisms were observed at exon 3, intron 3, exon 4 and intron 10 of *PRKAG3* gene of the Kundi buffalo. The variants NK1A and NK1B were found in Pakistani buffalo at 0.835 and 0.165 with heteromorphic pattern in the Nili-Ravi breed and monomorphic pattern in the Kundi breed (Table 2). NK1A to NK6B variants provides an allephism of the Kundi buffalo. NK2A (0.55), NK2B (0.45), NK5A (0.32), NK5C (0.68), NK6A (0.67) and NK6B (0.33) variants provides polymorphic pattern of *PRKG3* gene within the Kundi breed.

The present study provides the first source of information on genetic variation in Pakistani buffaloes assessed by using *PRKAG3* gene bovine chromosomes. Moderate levels of both allelic and genetic diversity were observed for the studied breeds. The SNPs in *PRKAG3* gene has been linked with meat quality traits in pig and milk production traits in cattle (Kowalewska-Luczak & Kulig, 2011; Ryan *et al.*, 2012). As some other genes were also associated with production traits in river buffalo (Dayal *et al.*, 2005; Pauciullo *et al.*, 2012) therefore it is desired to confirm these polymorphisms at extensive studies in the similar or additional

buffalo populations. These SNPs might be associated with production traits in river buffalo and the new SNPs establish in the Kundi buffalo can be used as genetic indicator of breed development programs is the practical application of the SNPs outcome. This is the first report of gene characterisation and SNPs identification in *PRKGA3* gene of the Kundi as well as Nili Ravi buffaloes. Altogether, the analyses presented in this study provide preliminary data on genetic diversity and population structure of Pakistani buffalo and might be helpful for similar studies in other livestock breeds of Pakistan.

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