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Genetic Identification of Three Pakistani Buffalo Breeds Through a Homozygosity Pattern in the PRKAG3 Gene

M. E. BABAR, M. IMRAN, M. NAWAZ, B. F. BENKEL, H. FARID, R. JABEEN, M. ABDULLAH, M. JAVED AND MOHSIN JAVED

Department of Livestock Production, University of Veterinary and Animal Sciences, Lahore, Pakistan (MEB, MI, MN, RJ, MA, MJ), Institute of Industrial Biotechnology, Government College University, Lahore (MJ) and Department of Plant and Animal Sciences, Nova Scotia Agricultural College, Truro, Nova Scotia, B2N 5E3, Canada (BFB, HF)

Abstract.- Adenosine monophosphate-activated protein kinase (AMPK) responds to both cellular and organismal level ATP decline in eukaryotes and plays a crucial role in the regulation of energy balance by stimulating energy-generating pathways and inhibiting energy-consuming pathways. AMPK is a heterotrimeric complex, consisting of a subunit α for its catalytic function and two other subunits β and γ for its regulatory properties. Although the characterization of each of genes encoding any of seven isoforms of α, β and γ subunits has been performed for different species including *Homo sapiens*, this task yet remains to be undertaken in buffalo (*Bubalus bubalis*), a species substantially contributing to many South Asian economies in terms of milk, beef, hides and employment. Owing to their economic importance, the genetic resource conservation of different buffalo breeds, especially Nili and Ravi breeds of Pakistan, is preferentially desirable to minimize any reduction in future economic returns from this species. Based on a polymorphism analysis of PRKAG3 (AMPKγ3) gene in three buffalo breeds (Nili, Ravi and Nili-Ravi), we present in this manuscript the identification of a homozygosity pattern ($T_{1070}T_{1382}A_{1489}$) which may serve as marker for the genetic resource conservation of Nili buffalo.

Key words: AMPK, polymorphism analysis, homozygosity pattern, buffalo, Pakistan

INTRODUCTION

AMPK family consists of seven isoforms encoded by distinct genes and is a heterotrimeric structural assembly of three subunits named as alpha (α), beta (β) and gamma (γ). Alpha subunit comprises of two isoforms (α1 and α2) and performs catalysis in functional AMPK assembly. The β and γ subunits have two (β1 and β2) and three (γ1, γ2 and γ3) isoforms, respectively, and play regulatory roles for physiological integration of AMPK (Crute *et al.*, 1998; Carling, 2004). It depends on tissue type whether AMPK contains a combination of α1, β1, and γ1 isoforms or some other combination from all twelve possible heterotrimers (α1β1γ1, α1β1γ2, α1β1γ3, α1β2γ1, α1β2γ2, α1β2γ3, α2β1γ1, α2β1γ2, α2β1γ3, α2β2γ1, α2β2γ2, and α2β2γ3) (Thornton *et al.*, 1998; Cheung *et al.*, 2000; Yu *et al.*, 2005; Birk and Wojtaszewski, 2006). The expression of γ3 is abundant and most often specific to skeletal muscles based on species under study (Cheung *et al.*, 2000; Mahlapuu *et al.*, 2004; Yu *et al.*, 2005; Costford *et al.*, 2007; Weyrich *et al.*, 2007).

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The function of AMPK complex is to respond to energy depletion caused by various environmental and nutritional stresses, which is achieved by modulating the fate of key regulators of biosynthetic pathways. For example, AMPK inhibits 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase and acetyl CoA carboxylase (the key regulatory enzymes for cholesterol and fatty acid synthesis) and activates the muscle glycogen phosphorylase and phosphofructokinase (the key regulatory enzymes for glycogen breakdown and glycolysis) to provide energy for the use in life-sustaining processes, such as circulation and breathing. Apart from these functions, AMPK also stimulates desire for taking energy in the form of eatables and is therefore considered as molecular switch which activates on energy depletion and corresponds to a variety of metabolic processes to keep the energy status in balance (Hardie and Carling, 1997; Hardie *et al.*, 1998; Kahn *et al.*, 2005; Hardie and Sakamoto, 2006; Xue and Kahn, 2006; Ramamurthy and Ronnett, 2006).

Pakistan supports about 28.4 million buffaloes representing five breeds (Nili, Ravi, Nili-

Ravi, Kundi and Aza-Kheli), among which Nili-Ravi and Kundi constitute majority and are considered best performing breeds for meat and milk production (GOP, 2006-2007). It is generally thought that Nili and Ravi breeds have been merged to single Nili-Ravi since 1960s, due to intensive crossbreeding. However, typical specimens of these two distinct breeds are still present in their original home tract that extends between the belt of Sutluj and Ravi rivers of Punjab province (Khan *et al.,* 2005).

The present study was designed to differentiate three Pakistani buffalo breeds (Nili, Ravi and Nili-Ravi) on the basis of SNP detection in sequences of a part of AMPKγ3-isoform-encoding bovine PRKAG3 gene. PRKAG3 gene was selected due to its conservation in eukaryotic species and an 885 bp segment of this gene was amplified and sequenced to investigate genetic diversity for this locus of buffalo genome.

MATERIALS AND METHODS

Livestock Production Research Institute (LPRI) in Bahadarnagar, Okara and in Rakh Dera Chahl, Lahore were visited to select unrelated animals belonging to 2 domestic buffalo breeds (Ravi and Nili-Ravi) for sampling. Unrelated animals from another Nili breed were sampled from Arifwala, Hasilpur, Bahawalnagar and their surroundings, a breeding tract for this breed. Most of animals of Ravi breed were sampled from its breeding tract, *i.e*. Tandhlianwala, Pakpattan and their surroundings. Blood samples from the jugular vein were collected in EDTA vaccutainers, transferred immediately to icebox and later stored at -20°C to protect the blood from decomposition. Genomic DNA extraction from peripheral blood was performed as described previously by Grimberg *et al.* (1989). The concentration of extracted DNA was adjusted to fifty nanograms per microlitre in TE buffer (pH 8.0) using spectrophotometery and agarose gel electrophoresis.

An 885 bp DNA fragment including the bulk of exon 1, the whole exon 2 and an intron 1 of AMP-activated protein kinase gamma subunit (PRKAG3) (GeneBank accession number AY692035) was amplified using a forward primer U1A (5□GAGCAAGGAGACAGCACTTCA3□) (918-938) and a reverse primer U2B (5□ACCTGTAGCA TGGTGTCGAAGA3□) (1781-1802) in polymerase chain reactions. In addition to these primers, forward UP SEQ1 (5'GACCTCAGCATCC AGGCT3') (1295-1312) and reverse UP SEQ2 (5□GACAGTAACTCCATCTTCCA3') (1362-1381) primers were used in PCR sequencing reactions. PRKAG3 fragments were amplified in 25 µl reaction mixtures containing 50 ng genomic DNA, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM dNTPs, 10 pM each primer, 1.5 units Ampli Taq Gold (Applied Biosystems, Foster City, CA). The acceptable yield of desired DNA fragment was obtained at temperature conditions consisting of an initial step of denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 1 min. A step of final extension was programmed at 72°C for 10 min. Amplificons were analyzed and quantified by resolving PCR products on 1.5% agarose gel mixed with DNA-intercalator ethidium bromide and illuminating gel under ultraviolet radiations.

PCR products from amplification PCR reactions were purified by Exo SAP-IT (Amersham) according to the manufacturer's instructions and used in sequencing PCR reactions for bidirectional sequencing. After purification, the sequencing PCR products were resolved on an ABI 3130 capillary DNA analyzer (Applied Biosystems, Inc., Foster City, CA). Sequence analyses were performed using the Chromas Lite (2.01) software and two-sequence-alignment program available at NCBI (www.ncbi.nlm.nih.gov.library.vu.edu.au/BLAST/).

RESULTS

The PRKAG3 gene sequences of three buffalo breeds, showed four polymorphisms C/T (1070), A/G (1344), C/T (1382) and G/A (1489), of which three were in the intronic region and a G/A (1489) polymorphism was in the coding region. This polymorphism (GCg→GCa) occurred in the codon-degenerating position of codon number 82 of the gene studied; and hence encoded the same amino acid (Ala→Ala). All of breeds were polymorphic

for all of nucleotide positions concerned, except Ravi breed that was monomorphic for nucleotide number 1344. Most of polymorphic animals showed three of the four polymorphisms, distributed with almost similar frequencies (Table I). A few animals were polymorphic for only one position 1344.

Table I.- Frequency distribution of SNPs in PRKAG3 gene of three buffalo breeds.

Breed	Polymorphic nucleotide position* (respective frequency)			
	1070	**1344**	**1382**	**1489****
Nili	C (0.66) C/T (0.17) T (0.17)	A (0.83) A/G (0.17)	C (0.66) C/T (0.17) T (0.17)	G (0.66) G/A (0.17) A (0.17)
Ravi	C (0.9) C/T (0.1)	Monomorphic	C (0.9) C/T (0.1)	G (0.9) G/A (0.1)
Nili-Ravi	C (0.55) C/T (0.45)	A (0.89) A/G (0.11)	C (0.55) C/T (0.45)	G (0.55) G/A (0.45)

*Nucleotide positions are comparable with PRKAG3 gene sequence provided in GeneBank accession number AY692035.
**Position 1489 indicates third nucleotide of 82nd codon (GCg→GCa encoding Ala→Ala) of PRKAG3 gene; the remaining positions exist in the intronic region.

Some animals (17%) of Nili breed showed homozygous changes from C→T (1070), C→T (1382) and G→A (1489). This polymorphic homozygosity separated a mutant lineage of Nili. These polymorphisms were also present in other breeds but only in heterozygous state. The morphological characterization of these breeds revealed excessive mixing of Nili and Ravi to develop crossbred Nili-Ravi. However, the animals from homozygous lineage of Nili represented their reliable and consistent morphology in terms of body weight and size, body color and head depressions etc.

DISCUSSION

The increase in loss of harmony of many species with their environment is an alarming situation to be considered for strategic planning of biodiversity conservation. About 35% mammalian breeds and 63% avian breeds are at the risk of extinction, with a loss of one breed per week (Gibson and Bishop, 2005). This accelerated pace of species extinction has ensued world-wide governmental and scientific campaigns to protect and preserve present genetic resources and to prevent the creation of ecological barriers that restrict species diversification. The genetic richness of species contributes to their adaptability and economic performance (Gibson and Bishop, 2005; Cardinale *et al.,* 2007). The present study explored a homozygosity pattern of three SNPs as marker across a part of PRKAG3 gene of Nili breed of Pakistani buffalo. The use of this SNP-based marker with consideration of characteristic morphological features of buffalo breeds can help minimize the risk of breed identity loss (the hap- hazardous mergence of different breeds into distinct crossbreds) in Pakistan.

Based on results summarized in Table I, it is clear that Ravi and Nili-Ravi breeds also represent polymorphisms for the three sites ($T_{1070}T_{1382}A_{1489}$) involved in formulating homozygosity pattern for Nili breed, but only in heterozygous state. The consideration of heterozygous state for these sites along with polymorphic position 1344 supports the idea of excessive migratory distribution of Nili buffalo, decreasing its number to minimum animals in its home tracts, to home tracts of Ravi buffalo. The idea of excessive migration of Nili has also been proposed by Shah (1991) on the basis of historical perspectives. The presence of all four polymorphic sites in Nili and Nili-Ravi indicate the proposed migratory trend in Nili and its intensive crossbreeding with Ravi.

The transmission of concerned polymorphisms (Table I) within and among breeds may be explained considering several possibilities of allele combinations (Fig. 1) which in turn may explain the phenomena of random breeding, outbreeding and inbreeding. The presence of three polymorphic sites in homozygous and heterozygous states in Nili demonstrates both inbreeding and random breeding in parallel. In Ravi and Nili-Ravi, the allele combination is of either outbreeding or random breeding nature. The question of whether the polymorphisms originate independently within breeds or they transmitted from one breed to other is justifiable considering the following assumptions: (1) The probability of originating all four polymorphisms in a single animal or generation is negligible. (2) The transmission of alleles with 2

polymorphisms and only one polymorphism maximum and is a result of either random breeding (excluding trisomic alleles' existence) is at

Existence of possible PRKAG3 alleles in studied buffalo breeds by introduction of 1st polymorphism

↓

CACG TACG CGCG CATG CACA [Probability of occurrence of 1st polymorphism either in ↓ or ↑ direction is 0.25]

CACG TACG CGCG CATG CACA AACG GACG CTCG CCCG CAGG
CAAG CACT CACC

First forward polymorphic event ↓↑ Allele formulation by all possible single reverse polymorphic events
[Genotypes formulation 1 (gf1)]

CACG TACG CGCG CATG CACA C/TACG CA/GCG CAC/TG CACG/A C/TA/GCG C/TAC/TG C/TACG/A
CA/GC/TG CA/GCG/A CAC/TG/A

↓

Existence of possible PRKAG3 alleles in studied buffalo breeds by introduction of 2nd polymorphism

↓

CACG TACG CGCG CATG CACA TGCG TATG TACA CGTG CGCA CATA
[Probability of occurrence of 2 spontaneous polymorphisms either in ↓ or ↑ direction is 0.063]

CACG TACG CGCG CATG CACA TGCG TATG TACA CGTG CGCA
CATA AACG GACG CTCG CCCG CAGG CAAG CACT CACC AGCG
GGCG ACCG ATCG TCCG TTCG GTCG GCGG TAGG TAAG AATG
GATG AAGG AAAG GAGG GAAG GACA AACA TATA TAAC GACC
GACT AACT AACA CCTG CTTG CGAG CGGG CCAG CCGG CTAG
CTGG CCCA CTCA CGCC GGCT CCCT CTCC CTCT CAGA CAAA
CATC CATT CAAC CAAT CAGC CAGT

Second forward polymorphic event ↓↑ Allele formulation by possible 2 spontaneous reverse polymorphic events
[Genotypes formulation 2 (gf2)]

With inclusion of genotypes formulated in (gf1) TGCG TATG TACA CGTG CGCA CATA TA/GCG TAC/TG TACG/A
C/TA/GC/TG C/TA/GCG/A C/TAC/TG/A C/TGCG CA/GC/TG/A CGCG/A CGC/TG C/TATG CA/GTG CATG/A
C/TACA CA/GCA CAC/TA TA/GC/TG TA/GCG/A C/TGC/TG C/TGCG/A C/TA/GC/TG/A TAC/TG/A C/TA/GTG
C/TATG/A C/TA/GCA C/TAC/TA CGC/TG/A CA/GTG/A CA/GC/TA

↓

Existence of possible PRKAG3 alleles in studied buffalo breeds by introduction of 3rd polymorphism

↓

CACG TACG CGCG CATG CACA TGCG TATG TACA CGTG CGCA CATA TGTG TGCA TATA CGTA
[Probability of occurrence of 3 spontaneous polymorphisms either in ↓ or ↑ direction is 0.016]

Allele formulation by 3 spontaneous reverse polymorphic events is not shown due to low probability value

Third forward polymorphic event ↓↑ Allele formulation by possible 3 spontaneous reverse polymorphic events
[Genotypes formulation 3 (gf3)]

With inclusion of genotypes formulated in (gf1) and (gf2) TGTG TGCA TATA CGTA TGC/TG TGCG/A
TA/GC/TG/A C/TGC/TG/A TA/GTG TATG/A C/TA/GTG/A TA/GCA TAC/TA C/TA/GC/TA C/TGTG CGTG/A
C/TGCA CGC/TA C/TATA CA/GTA TGC/TG/A TA/GTG/A C/TGTG/A TA/GC/TA C/TGC/TA C/TA/GTA

↓

Existence of possible PRKAG3 alleles in studied buffalo breeds by introduction of 4th polymorphism

↓

CACG TACG CGCG CATG CACA TGCG TATG TACA CGTG CGCA CATA TGTG TGCA TATA CGTA TGTA
[Probability of occurrence of 4 spontaneous polymorphisms either in ↓ or ↑ direction is 0.004]

Allele formulation by 4 spontaneous reverse polymorphic events is not shown due to low probability value

Fourth forward polymorphic event ↓↑ Allele formulation by possible 4 spontaneous reverse polymorphic events
[Genotypes formulation 3 (gf4)]

With inclusion of genotypes formulated in (gf1), (gf2) and (gf3) TGTA TGTG/A TGC/TA TA/GTA C/TGTA

Fig. 1 Allele-in-allele-out diagram for 4 polymorphic events considering all kinds of sexual matings (Inbreeding, out-breeding and random breeding) within and among Nili, Ravi and Nili-Ravi buffalo breeds.

or crossbreeding. (3) The polymorphism G_{1344} is inherited in conjunction with other 3 polymorphisms ($T_{1070}T_{1382}A_{1489}$) from Nili to Ravi developing new breed Nili-Ravi. (4) The independent origin of these polymorphisms in Nili-Ravi is negligible. (5) The homozygosity ($T_{1070}T_{1382}A_{1489}$) in Nili is an indication for G_{1344} origin at later stages, hence inbreeding of G_{1344} for its emergence in homozygous state is not found in any animal. (6) There is a possibility of having only two alleles $T_{1070}A_{1344}T_{1382}A_{1489}$ and $T_{1070}G_{1344}T_{1382}$ A_{1489} in the present population of Nili buffalo along with ancestral allele $C_{1070}A_{1344}C_{1382}$ G_{1489}. (7) A new polymorphism takes millions of years to occur within a gene. (8) The probability of developing homozygosity $T_{1070}T_{1382}A_{1489}$ over only few generations of Nili-Ravi is negligible. (9) The probability of wrong identification of animals of Nili-Ravi as of Ravi is considerable because the Government Livestock Farms from where sampling of Ravi and Nili-Ravi was performed, rear buffaloes from studied breeds classifying them as a single breed Nili-Ravi and the differentiation of Ravi from Nili-Ravi is somewhat difficult. Therefore, the sampling of animals from Ravi breed was problematic. (10) The animals of Nili were traced from their home tracts from farmers who maintained Nili generation after generation, so the probability of ambiguous sampling of Nili is negligible. (11) The transfer of T_{1070}, T_{1382} and A_{1489} polymorphisms from Ravi to Nili is negligible. (12) The studied polymorphisms are most probably linked due to small distance among them.

Considering the buffalo genome size equivalent to that of cattle (approx. 2.5 bbp) and a maximum mutation rate of 10^6 in the presence of compatible genome replication and repair machinery of host, there is a probability of occurrence of 2431 mutations within whole genome at one time. Thus, probability of occurrence of single mutation within a segment of 876 bp remains only 0.000876, which doubles (0.000000876), triples (0.000000000876) and so on, for second, third and more spontaneous mutations, respectively. By including, if there are also chances of reverse mutations to occur, the probability of occurrence of 1^{st} reverse mutation at studied polymorphic sites within studied portion of DNA is 0.000876*0.25 (Fig. 1), of 2^{nd} mutation is 0.000000876*0.063 and so on. The probability of occurring 2 polymorphisms spontaneously either in forward or reverse direction is negligible, therefore, favors assumption 1 and 7.

The alleles and genotypes given in Table I are the only alleles and genotypes found in studied buffalo breeds. No other combination (Fig. 1) was displayed by any of buffalo. If the polymorphisms occurred in different animals/animal lineages producing different alleles with one, two, or more polymorphisms which combined to give rise to heterozygous genotypes (Table I), then there must be allele which could produce homozygous genotype $T_{1070}A_{1344}T_{1382}A_{1489}$ with three polymorphisms. Alleles with 1 or 2 polymorphism can not change to $T_{1070}A_{1344}T_{1382}A_{1489}$ allele by recombination, as distance between them is only in hundreds. This clarifies that $T_{1070}A_{1344}T_{1382}A_{1489}$ was the only allele that was established in a buffalo lineage and then transferred to different areas through random matings of animals from $T_{1070}A_{1344}$ $T_{1382}A_{1489}$ lineage with those from lineages with ancestral allele $C_{1070}A_{1344}C_{1382}G_{1489}$. As no possible allele (Fig. 1) except alleles (Table I) was found in studied buffalo breeds, it is concluded that $T_{1070}A_{1344}T_{1382}A_{1489}$ allele was established independently in single linage despite multiple lineages. So the assumption 2 is disapproved and assumption 12 is approved by what we discussed in this paragraph.

The development of C/TA/GC/TG/A is possible by the combination of any 2 of 4 alleles $T_{1070}A_{1344}T_{1382}A_{1489}$, $C_{1070}G_{1344}C_{1382}G_{1489}$, $T_{1070}G_{1344}$ $T_{1382}A_{1489}$ and $C_{1070}A_{1344}C_{1382}G_{1489}$, but the absence of CA/GCG genotype from studied buffalo breeds indicates that G_{1344} occurred in allele T_{1070} $A_{1344}T_{1382}A_{1489}$. Moreover, this polymorphism occurred at the end as its frequency is at minimum among detected alleles (Table I). This qualifies assumptions 3, 5 and 6.

History provides an evidence of migration of Nili to home tracts of Ravi (Shah, 1991) for the development of crossbred Nili-Ravi. If the genetic

flow is considered from Ravi to Nili, then $T_{1070}A_{1344}T_{1382}A_{1489}$ allele frequency in Ravi does not corresponds to that in Nili-Ravi (Table I). Furthermore, Ravi is morphologically more close to Nili-Ravi than that of Nili, which is persistent with genetic flow from Nili to Ravi and environmental suppression of expression of Nili's morphology at equal level to that of Ravi. So, the sampled animals of Ravi with $T_{1070}A_{1344}T_{1382}A_{1489}$ may belong to Nili-Ravi and low frequency of $T_{1070}A_{1344}T_{1382}A_{1489}$ allele in Ravi supports this assumption. Therefore, the genetic flow from Nili to Ravi favors assumptions 3, 6, 9, 10 and 11.

If the detected polymorphisms occurred independently in crossbred Nili-Ravi, then the chance of receiving $T_{1070}A_{1344}T_{1382}A_{1489}$ allele by both Nili and Ravi in an environment of random and unplanned breeding is equal that is not met in the present study. This approves assumptions 4 and 8.

In the light of above discussion, it is concluded that $T_{1070}A_{1344}T_{1382}A_{1489}$ and $T_{1070}G_{1344}T_{1382}A_{1489}$ alleles originated in Nili buffalo breed and transferred to Ravi by intensive crossbreeding with this breed. Results indicate the lack of inbreeding within Nili-Ravi. The $T_{1070}A_{1344}T_{1382}A_{1489}$ and $T_{1070}G_{1344}T_{1382}A_{1489}$ alleles may be considered as markers along with morphological parameters for the identification of studied buffalo breeds. It is suggested that future studies should recruit these buffalo breeds after identification with these markers. These markers may also be used for strategic planning of ex-situ or in-situ conservation of studied buffalo breeds.

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