Original Article

Highly Conserved Keratin-Associated Protein 7-1 Gene in Yak, Taurine and Zebu Cattle

(bovine / evolution / function / keratin-associated protein 7-1 / polymorphism)

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Abstract. Keratin-associated proteins (KRTAPs) play a critical role in cross-linking the keratin intermediate filaments to build a hair shaft. The genetic polymorphisms of the bovine *KRTAP7-1* gene were investigated for the first time in this study. The complete coding sequence of the *KRTAP7-1* gene in 108 domestic yak, taurine and zebu cattle from China and Indonesia were successfully amplified using polymerase chain reaction and then directly sequenced. Only two single-nucleotide polymorphisms (one non-synonymous at c.7C/G and another synonymous at c.21C/T) and three haplotypes (*BOVIN-KRTAP7-1*A, B* and *C*) were identified in the complete coding sequence of the bovine *KRTAP7-1* gene among all animals. There was no polymorphism across three Chinese indigenous yak breeds and one Indonesian zebu cattle population, all sharing the *BOVIN-KRTAP7-1*A* haplotype. The four taurine cattle populations also had *BOVIN-KRTAP7-1*A* as the most common haplotype with a frequency of 0.80. The frequency of novel haplotype *BOVIN-KRTAP7-1*B* was only 0.07 present in one heterozygous animal in each of the four taurine cattle populations, while *BOVIN-KRTAP7-1*C* was only found in a Simmental and a local Chinese Yellow cattle population with frequencies of 0.17 and 0.36, respectively. The monomorphic yak *KRTAP7-1* gene in particular, and highly conserved bovine, sheep and goat *KRTAP7-1* genes in general, demonstrated its unique intrinsic structural property (*e.g.*, > 21% high glycine content) and primary functional importance in supporting the mechanical strength and shape of hair.

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Abbreviations: CDS – coding sequence, HGT – high glycine-tyrosine, KIFs – keratin intermediate filaments, KRTAPs – keratin-associated proteins, PCR-SSCP – polymerase chain reaction–single-stranded conformational polymorphism, SNP – single-nucleotide polymorphism, UTR – 5'-untranslated region.

Introduction

Keratin-associated proteins (KAPs) are found in an amorphous matrix around keratin intermediate filaments (KIFs) in wool and hair. They play a crucial role in cross-linking the KIFs through extensive disulphide bonds to form a hair shaft. KAPs are characterized by their high/ultrahigh cysteine or high glycine-tyrosine (HGT) content. They are usually encoded by a large number of multi-gene families (termed as *KRTAPs*) with each gene generally composed of a single exon. Currently, more than 100 *KRTAP* genes with high sequence homology within each family have been identified from around 30 *KRTAP* families. These gene families are found to present in clusters in several genomic regions of the mammalian genome (McLaren et al., 1997; Shibuya et al., 2004; Rogers and Schweizer, 2005; Shimomura and Ito, 2005; Parry et al., 2006; Barthélemy et al., 2012; Gong et al., 2012a; Matsunaga et al., 2013). It was

suggested that many high/ultrahigh cysteine *KRTAP* genes have followed concerted evolution with frequent gene conversion events, while the HGT *KRTAP* genes have evolved more dynamically, with limited gene conversion events possibly due to positive selection (Wu et al., 2008). The high homology and polymorphism within individual *KRTAP* families have been considered as one of the challenges in application of modern 'omics' tools to the genetic improvement of sheep wool (Plowman et al., 2012).

Only one member, originally referred to as *HGT-C2* gene, was identified in the *KRTAP7* family from a Merino sheep. It has 258 nucleotides in the coding sequence (CDS) encoding 85 amino acids and is named *KRTAP7-1* gene (Kucek and Rogers, 1987; Gong et al., 2012b). This protein has a calculated molecular mass of 9.1 kDa based on the sequences of 27 non-redundant peptides with an accumulated score of 1387.7 (Rogers et al., 2002; Clerens et al., 2010). The first goat *KRTAP7-1* cDNA was isolated from the skin cDNA library of an Inner Mongolian Cashmere goat. It also encodes 85 amino acids and has a sequence identity of 96.8 % to its sheep counterpart (Zhang et al., 2006). Similarly to the specific expression pattern of the *KRTAP7-1* gene observed in human hairs (Rogers et al., 2002; Rogers and Schweizer, 2005), the goat *KRTAP7-1* gene was found to be expressed in the cortical layer and inner root sheath of the primary hair follicles, while its expression was significant in the cortical layer, inner root sheath and hair matrix of the secondary hair follicles of both Inner Mongolian and Liaoning Cashmere goats (Zhang et al., 2006; Jin et al., 2011; Wang et al., 2011). No polymorphism was identified in the nearly complete or complete CDS of the *KRTAP7-1* gene among five Chinese goat populations based on polymerase chain reaction–single-stranded conformational polymorphism (PCR-SSCP) analysis (Zhao et al., 2007; Yu, 2009).

Four alleles were identified in a 413 base pair (bp) long fragment covering the complete CDS of the sheep *KRTAP7-1* gene by the *Msp*I Southern restriction fragment length polymorphism method (McLaren et al., 1997). However, no polymorphism was present in the same fragment of two flocks including pure Merino and Merino × Romney crosses based on the PCR-SSCP protocol (Itenge, 2007). Although three genotypes determined by two alleles were detected in a 388 bp long fragment spanning the complete CDS of 126 Tibetan sheep, one single-nucleotide polymorphism (SNP) changing T to C was found to be located in the 5'-untraslated region (UTR) of the Tibetan sheep *KRTAP7-1* gene (Wang et al., 2010). A PCR-SSCP screening of 309 Chinese Merino, 73 German Merino and 130 F1 crossbreds of the two Merino breeds also detected no polymorphism in their nearly complete CDS (data not shown). Nevertheless, only two alleles differing in one non-synonymous mutation (c.173G/A, p.Ser58Asn) were detected in the complete CDS among 250 Romney-cross sheep (Gong et al., 2012b). Another missense mutation (c.93C/A, p. Asp31Lys) was recently identified only in a Chinese Merino flock but not in other flocks of Romney, Hu sheep and Chinese Merino × Hu sheep crosses assayed using direct and cloning sequencing of their complete CDS (Liu, 2013).

The yak (*Bos grunniens*) is a long-haired bovine species distributed throughout the Himalayan region of southern Central Asia. It is the most important livestock species producing meat, milk, fibre, hide and dung fuel. Yak is also used by nomadic Tibetans to transport their possessions. All these functionalities support the livelihoods for the nomads, mostly the Tibetans, in the region (Wiener et al., 2003; Rhode et al., 2007; Qi et al., 2008, 2010). As the only bovine species which has been explored for its fibre, the yak produces three types of fibres: coarse outlier hair, mid-type hair and fine down fibre. The latter two types shed naturally in early summer. The coarse hairs are long, often medullated and larger than 52.5 μ in diameter. The mid-type hairs are relatively short, have a diameter between 25 μ and 52.5 μ, and some of them have latticed medulla. These hairs are collected traditionally for making rope, carpet and tent. The fine down fibres, which grow before the winter season and protect the yak against extreme colds due to its high degree of heat insulation, are shorter than 30 mm, un-medullated and finer than 25 μ in diameter. They have been collected and explored extensively by the textile industry since the 1970' (Wiener et al., 2003). Therefore, understanding the molecular biology of the yak fibre is similarly essential to the genetic improvement of efficiency and quality of sheep wool or goat cashmere among other natural fibres with their cortical cells made predominantly from keratins and KAPs. This study was the first attempt to search for possible functional polymorphism in the *KRTAP7-1* gene of yak as well as taurine and zebu cattle using the direct DNA sequencing approach.

Material and Methods

Samples

Sixty-one domestic yak (nine Gannan yak in Maqu county and 14 Tianzhu White yak in Tianzhu county of Gansu Province, China; 38 Jiali yak in Amdo, Dangxiong and Mangkang counties of central and south-eastern Tibetan Autonomous Region, China), 30 taurine cattle (nine Simmental sampled in Shandong Province; six Holstein-Friesian collected in Beijing; 15 local Chinese Yellow cattle sampled in Pingliang (8) and Tianzhu (7) counties of Gansu Province, China), and nine zebu cattle (Aceh Province, Indonesia) samples were included in this study. Another eight blood samples of F_1 yak-cattle hybrids collected in Qinghai Province, China were used to facilitate the haplotype reconstruction of heterozygous genotypes. The sample collection for this study only involved domesticated yak and cattle in state-owned land but none of their wild relatives under protection in any protected area; therefore, no specific per-

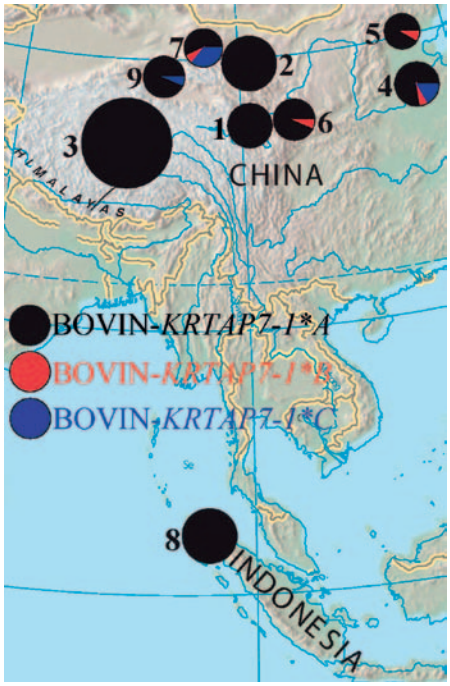

Fig. 1. Sampling locations and distribution of the three haplotypes of the bovine *KRTAP7-1* gene. **1**. Gannan, Gansu, China; **2** and **7**. Tianzhu, Gansu, China; **3**. Amdo, Dangxiong and Mangkang, Tibet, China; **4**. Shandong, China; **5**. Beijing, China; **6**. Pingliang, Gansu, China; **8**. Aceh, Indonesia; **9**. Qinghai, China. The size of each pie is proportional to the respective number of samples of each population (see Material section for details).

mission was required. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (CAAS). The geographic localities of the sampling sites are shown in Fig. 1.

Primer design, DNA extraction, PCR amplification and DNA sequencing

An incomplete CDS (GenBank accession no. HQ902901.1) of the yak *KRTAP7-1* gene was recently isolated from the genomic DNA of an Indian yak (Singh et al., 2012). We decided to search for the homologous sheep *KRTAP7-1* gene in taurine cattle *Bos taurus* (NC_007299.5 and NW_003103794.1 for Hereford chromosome 1) (Elsik et al., 2009; Zimin et al., 2009), zebu cattle *Bos indicus* (AGFL01000280.1 for Nelore chromosome 1) (Canavez et al., 2012) and yak *Bos grunniens* (NW_005395809.1 for yakQH1 unplaced genomic scaffold) (Qiu et al., 2012) genomic resources, and an identical CDS of the bovine *KRTAP7-1* gene coding for 87 amino acids was identified in these three species. A pair of primers (forward: 5'-CTT GGG TAA CTT GCT CCT CA-3'; reverse: 5'-CTG TTC TGC ACA CAG AGA A-3') covering the last 54 bp of 5'-UTR and the first 58 bp of 3'-UTR was designed based on these three bovine reference genomic sequences (Elsik et al., 2009; Zimin et al., 2009; Canavez et al., 2012; Qiu et al., 2012).

Genomic DNAs were extracted from blood or meat samples using the routine phenol/chloroform protocol (Sambrock and Green, 2012). The 50 µl PCR reaction contained 100 to 150 ng of genomic DNA, 8 pmol of each primer, 25 µl 2× *Taq* PCR Mix (Tiangen Biotech (Beijing) Co., Ltd., China), and 22 µl ddH_2O. A thermal-cycling profile included an initial denaturation for 5 min at 94 °C, followed by 35 cycles of 30 s each at 94 °C, 55.4 °C and 72 °C, and completed by a final extension for 10 min at 72 °C.

The PCR products were purified using a TIANgel Midi Purification kit (Tiangen Biotech) and then directly sequenced using the two PCR primers and a BigDye® Terminator v3.1 Cycle Sequencing Kit in an ABI 3730 Sequence Analyzer (Applied Biosystems, Foster City, CA). Because only two SNPs were found in the CDS among all samples, PCR products of two representatives of heterozygous taurine cattle samples carrying the two SNPs were chosen for re-sequencing the fragment using a cloning procedure involving a pBM19-T vector (Biomed Co., Ltd., Beijing, China). Three clones for the first sample and five clones for the second sample were selected for the re-sequencing using the PCR forward primer.

Haplotype reconstruction and statistical analysis

All raw sequencing data was manually edited using the software Chromas version 1.45 (http://www.technelysium.com.au/chromas.html) and the cleaned sequences were aligned using the ClustalW method implemented in the MEGA program version 5.2.2 (Tamura et al., 2011). The haplotypes of heterozygous genotypes carrying one SNP were directly separated, while those with the two SNPs were deduced based on the sequences of relevant homozygous genotypes. Such haplotypes were validated using the sequences of F_1 yak-cattle hybrids and eight cloned PCR products. Sequences of identified haplotypes were deposited in the GenBank database with accession numbers of KJ551547-KJ551549. These bovine sequences together with relevant sheep and goat data (see Fig. 2) were then exported into the DnaSP program version 5.10.01 (Librado and Rozas, 2009) for analysing their polymorphisms.

Results

A 376 bp long PCR product of the bovine *KRTAP7-1* gene was generated and sequenced in all 108 samples. Following the predicted mRNA sequences for taurine cattle (XM_002702395.1) (Elsik et al., 2009; Zimin et al.,2009) and yak (XM_005908579.1) (Qiu et al., 2012), a 264 bp long complete CDS was identified and used for this analysis. Compared with the available goat and sheep *KRTAP7-1* CDS haplotypes either published (Zhang et al,. 2006; Gong et al., 2012b; Dong et al., 2013; Liu, 2013) or directly deposited into the GenBank

Haplotype	GenBank accession no.	7128991111111111111122 5182322222245667713 012345689384954
BOVIN-*KRTAP7-1*A*	KJ551547.1	CCCCACGAATTATACTTTAGC
BOVIN-*KRTAP7-1*B*	KJ551548.1	G.T..................
BOVIN-*KRTAP7-1*C*	KJ551547.1	..T..................
SHEEP-*KRTAP7-1*A*	JN091630.1	.T....T------..GCAGTT
SHEEP-*KRTAP7-1*B*	JN091631.1	.T....T------..GCA.TT
SHEEP-*KRTAP7-1*C*	JN707700.1	.T...AT------..GCAGTT
SHEEP-*KRTAP7-1*D*	FJ712657.1	.T....T------CTGCAGTT
SHEEP-*KRTAP7-1*E*	FJ712658.1	.T..C.T------..GCAGTT
GOAT-*KRTAP7-1*A*	AY510121.1	.T.T..T------..GCA.TT

Fig. 2. Polymorphic nucleotides within the coding sequences of bovine, sheep and goat *KRTAP7-1* gene haplotypes (in 5' to 3' directions). Dots indicate identical nucleotides to the *BOVIN-KRTAP7-1*A* haplotype while dashes show deletions. Numbers in vertical lines in the top-right corner are the positions of polymorphic nucleotides.

database (FJ712657.1 and FJ712658.1 from the Malpura sheep in India), the bovine *KRTAP7-1* CDS had additional six consecutive nucleotides (see Fig. 2), resulting in a bovine KAP7-1 protein with 87 amino acids.

There was no polymorphism in the CDS among all 61 samples of three Chinese yak breeds and also in nine Indonesian zebu cattle, both sharing a haplotype identical to the three bovine reference genomic sequences (Elsik et al., 2009; Zimin et al., 2009; Canavez et al., 2012; Qiu et al., 2012). It was therefore named *BOVIN-KRTAP7-1*A* (KJ551547.1) based on the updated nomenclature for KAPs (Gong et al., 2012a). Among 30 taurine cattle, only two SNPs, one non-synonymous (c.7C/G, p.Arg3Gly) and another synonymous (c.21C/T) (see Fig. 2), were found among the four populations. Altogether three haplotypes were validated, of which *BOVIN-KRTAP7-1*A* was the most common one with a frequency of 0.80 (ranging between 0.57 in Tianzhu Yellow cattle and 0.94 in Pingliang Yellow cattle). The novel haplotype *BOVIN-KRTAP7-1*B* (KJ551548.1) carrying the two SNPs was present only in heterozygous state in one sample each of the four taurine cattle populations, while *BOVIN-KRTAP7-1*C* (KJ551549.1) defined by the synonymous SNP was found in Simmental and Tianzhu Yellow cattle populations with frequencies of 0.17 and 0.36, respectively. The F_1 yak-cattle hybrids carried *BOVIN-KRTAP7-1*A* and *BOVIN-KRTAP7-1*C* haplotypes with frequencies of 0.94 and 0.06, respectively (see Fig. 1). The hybridization between yak and either taurine or zebu cattle has been a traditional breeding practice to generate F_1 hybrids with an improved milk and/meat yield (Wiener et al., 2003; Qi et al., 2010) Although the highly shared *BOVIN-KRTAP7-1*A* haplotype between yak and cattle confounded identification of the parental species of these F_1 animals, the presence of the *BOVIN-KRTAP7-1*C* haplotype in the F_1 population, despite low frequency, suggested a likely involvement of the local Chinese Yellow cattle, which had a sympatric distribution with domestic yak, *e.g.*, in Tianzhu, as one of their parents.

Discussion

Similarly to very limited polymorphisms identified in the sheep and goat *KRTAP7-1* CDS (Itenge, 2007; Zhao et al., 2007; Wang et al., 2010; Liu, 2013), the bovine *KRTAP7-1* CDS was also very conserved across yak, taurine and zebu cattle in general. In fact, a comparatively high genetic diversity within domestic yak populations has been reported based on the studies involving 'neutral' genetic markers, *e.g.*, mitochondrial DNA sequences (Guo et al., 2006;, Lai et al., 2007; Qi et al., 2008; Wang et al., 2010; Wiener et al., 2011) and nuclear autosomal microsatellite loci (Qi et al., 2005; Zhang et al., 2008). The monomorphic nature of the yak *KRTAP7-1* CDS detected in this study was particularly interesting. A similar pattern was also witnessed in other functional genes in the yak, *e.g.*, *Bogr-DRA* (An et al., 2012) and exon 2 of the tyrosinase gene (Zhang et al., 2012), to name but a few. The very specific expression patterns of the *KRTAP7-1* gene observed in human hairs and goat hair follicles (Rogers et al., 2002; Zhang et al., 2006; Jin et al., 2011; Wang et al., 2011) clearly supported its critical role in building a hair shaft.

The glycine content was the highest (> 21 %) among all amino acid residues of the predicted bovine, sheep and goat KRTAP7-1 proteins. The bidirectional binding property of HGT KAPs was proposed to contribute to the mechanical strength and shape of hair (Matsunaga et al., 2013). The HGT KAPs are very small molecules, and their amino acid composition rather than their sequences were thought to perform their function (Rogers and Schweizer, 2005; Parry et al., 2006). The general amino acid composition was recently suggested to be more important than amino acid order for the function of a protein (Shckorbatov and Berezhnoy, 2008). It was

found that the glycine residue rarely appears in active sites of proteins, but it is one of the most conserved amino acids. The proportion of glycine in a protein or peptide is negatively correlated with the rate of amino acid substitution, and this proportion alone explains about 38 % of the total variation in amino acid substitution rates among different protein families (Graur, 1985). The rate of protein evolution is also determined by the strength of functional requirements or selective constraints (Tourasse and Li, 2000).

High-altitude adaption showed various ways including evolutionary selection. Tibetans revealed candidates for high-altitude adaptations in the *EGLN1* and *EPAS1* genes, associated with lower haemoglobin concentration (Peng et al., 2011; Xu et al., 2010; Xiang et al., 2013) and convergent (sometimes called parallel) evolution (Wang et al., 2015) for organisms with different ancestors adopting similar adaptations to environmental stresses. *SOCS4* showed a signature of convergent evolution in yak (*Bos grunniens*) and Tibetan antelope (*Pantholops hodgsonii*), which are endemic to the Qinghai-Tibetan Plateau; both have numerous traits that are adaptive to high altitudes. In this case, the ruminant *KRTAP7-1* genes are anticipated to have a very high functional density with most of their amino acids being specific function sites that tend to 'freeze' their primary structure in the evolution (Zuckerkandl, 1976). It is known that the well-developed fleece coat helps the yak withstand the extremely cold climate on the world's highest and largest plateau – the Qinghai-Tibetan Plateau (Wiener et al., 2003); therefore, the lack of polymorphism in the *KRTAP7-1* gene of the yak in particular and the very limited functional diversity in this gene among the sheep, goat and bovine species in general demonstrated its unique intrinsic structural property and primary functional importance in supporting the mechanical strength and shape of hair.

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