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Mitochondrial DNA Part A

DNA Mapping, Sequencing, and Analysis

ISSN: 2470-1394 (Print) 2470-1408 (Online) Journal homepage: http://www.tandfonline.com/loi/imdn21

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To cite this article: Jie Wang, Yu Shi, Mauricio A. Elzo, Shuzhang Dang, Xianbo Jia & Songjia Lai (2017): Genetic diversity of ATP8 and ATP6 genes is associated with high-altitude adaptation in yak, Mitochondrial DNA Part A

To link to this article: http://dx.doi.org/10.1080/24701394.2017.1285292

Published online: 17 Mar 2017.

MITOCHONDRIAL DNA PART A, 2017
http://dx.doi.org/10.1080/24701394.2017.1285292

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RESEARCH ARTICLE

Genetic diversity of *ATP8* and *ATP6* genes is associated with high-altitude adaptation in yak

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ABSTRACT

ATP synthase 8 (ATP8) and ATPase synthase 6 (ATP6) play an important role in mitochondrial ATPase assembly. Mutations in either of these units could affect the ATP processing and the respiration chain in mitochondria. To find out if there were differences in gene diversity between Tibetan yaks and domestic cattle, we sequenced the ATP8 and ATP6 genes in 66 Tibetan yaks and 81 domestic cattle. We identified 20 SNPs in the ATP8 gene and 60 SNPs in the ATP6 gene. Ten SNPs detected in ATP8 were probably positively associated with high-altitude adaptation, of which SNPs m.8164 G > A, m.8210 G > A, m.8231 C > T and m. 8249 C > T resulted in amino acid changes. Similarly, SNPs m.8308A > G, m.8370A > C, m.8514G > A of ATP6 also appeared to be associated with high-altitude adaptability. Specifically, m.8308 A > G, located in the overlap region, might bring in a conserved region found in cytochrome b561 which play an important role in iron regulation, thus it might help the Tibetan yaks with this mutation to utilize rare oxygen efficiently. Considering all mutations, three of eight haplotypes identified in gene ATP8 were present only in Tibetan yaks, and six (H3 to H8) out of 21 haplotypes (H1 to H21) in gene ATP6 were restricted to Tibetan yaks. Haplotypes present only in Tibetan yaks could be positively associated with high-altitude adaptation.

ARTICLE HISTORY
Received 17 December 2016
Accepted 18 January 2017

KEYWORDS
High-altitude; adaptation; ATP8 gene; ATP6 gene; Tibetan yak

Introduction

Mitochondria are known as the 'energy factory' of cells. They supply more than 95% of the energy in eukaryotic cells (Scott et al. 2011) by mainly oxidizing pyruvate and NADH. This process depends on the presence of oxygen and plays an important role in metabolism, illness, programed cell death and aging (Cao et al. 2006), thus it could be affected by hypoxia at high altitudes (Gnaiger 2003; Scott et al. 2009). Transmembrane ATPases are membrane-bound enzyme complexes/ion transporters that use ATP hydrolysis to drive the transport of protons across a membrane. There are different types of transmembrane ATPases, differing in function, structure and in the type of ions they transport (Cross & Muller, 2004). F-ATPases (F1F0-ATPases) are found in mitochondria, chloroplasts and bacterial plasma membranes where they are the prime producers of ATP, using the proton gradient generated by oxidative phosphorylation (mitochondria) or photosynthesis (chloroplasts). F1F0-ATPases are composed of two linked complexes, an F1 ATPase complex and an F0 ATPase complex. The F1 ATPase complex is a catalytic core and is composed of 5 subunits (alpha, beta, gamma, delta, and epsilon), whereas the F0 ATPase complex is a membrane-embedded proton channel, in which four of the F0 subunits (OSCP, F6, b, d) form a 'stalk' that connects the F1 and F0 moieties (Anderson et al. 1981).

Subunit 8 of the mitochondrial FiF0-ATP synthase (*ATP8*), an inner membrane polypeptide of the F0 component, is responsible for the correct assembly of ATP synthase holoenzyme (Tzagoloff et al. 2004). Studies demonstrated that mice with mutated *ATP8* had a higher ATP-TO-ADP ratio than wild-type mice (Eipel et al. 2011). In addition, two other studies (Yu et al. 2009a; Yu et al. 2009b) showed that *ATP8* variation resulted in oversized mitochondria and loss of cristae structure in kidney cells, in agreement with the results of Weiss et al. (Weiss et al. 2012).

Subunit 6 of the mitochondrial FiF0-ATP synthase (*ATP6*) is also an inner membrane polypeptide as *ATP8*, encoded by the mitochondrial genomes of all eukaryotic organisms examined to date (Dewey et al. 1985). The length of the *ATP6* gene of *Bos taurus* is 681 bp long and encoded for 223 amino acids. *ATP6* is a key component of the proton channel (Bao et al. 2008). Cross-links between *ATP6*, CI, and p subunits may indicate a connection between F0 and F1 (Enns & Criddle 1977; Todd & Douglas 1981). Previous studies have shown that a mutation in position 8993 of the *ATP6* gene could result in muscle weakness, ataxia, retinitis pigmentosa (NARP) (Holt et al. 1990; Puddu et al. 1993), and severe infantile lactate acidosis and encephalomyopathy (Houstek et al. 1995). Mutations of *ATP6* may also contribute to a reduction in the synthesis of ATPases (Hartzog & Cain 1993; Tatuch et al. 1994; Tatuch & Robinson 1993). Another mutation in nucleotide

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9101 of the *ATP6* gene found in a LHON family (Lamminen et al. 1995) was associated with lower efficiency of oxidative phosphorylation in lymphoblast mitochondria, resulting in a higher mitochondrial respiration rate.

Tibetan yaks are distributed over a large area in the Qinghai-Tibet Plateau in the west of China at altitudes ranging from 2000 to 5000 m under environmental conditions of hypoxia and low temperatures. More than 14 million domestic yaks provide meat, milk, transportation, dung for fuel and hides for tents to local Tibetans and other nomadic pastoralists living in high-altitude areas (Wiener et al. 2003). Modern domestic yaks, derived from *Bos grunniens*, were first domesticated in Tibet (Guo et al. 2006). Although both yaks and cattle are bovine, they have quite different high-altitude and hypoxia adaptations. Cattle have been found to be susceptible to high-altitude hypertension and heart failure, two characteristics with an important genetic component. Because of the lack of adaptation to high altitudes, cattle in China are kept in low altitude areas (e.g. Sichuan basin, 300–700 m altitude).

In order to gain a better understanding the mechanisms of hypoxia adaptability, we sequenced the *ATP8* and *ATP6* genes in 66 Tibetan domesticated yaks and 81domesticated cattle and compared the DNA diversities of these two groups. We also determined if there was a correlation between the *ATP8* and *ATP6* genes in yaks and cattle with hypoxia adaptability at high altitudes.

Materials and methods

Sampling and DNA extraction

Ear tissue samples were collected from 66 domesticated Tibetan yaks and from 81 Holstein-Friesian cows from Sichuan, which were immediately put into 1.5 ml EP tubes filled with 1 ml ethanol. Afterwards, samples were stored in a laboratory freezer at −20 °C. All samples were successfully used in this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Sichuan Agricultural University, China.

DNA extraction, amplification and sequencing

The mtDNA of each animal was extracted from ear tissues using a DNA extraction kit (TIANGENamp Genomic DNA Kit, TIANGEN Biotechnology Company, Ltd, Beijing, China). A sequence of 1379 bp of PCR products was amplified using the primers F:TGAACCAAACAACCCTTAT: and R:GAGGCTTGAA TGGTAGAATG. These primers were designed based on a complete mitochondrial genome sequence of Bos Grunniens (GenBank Accession Number KM233416). The PCR procedure was as follows: pre-degeneration at 94 °C for 5 min; with 35 cycles of denaturation at 94 °C for 30 s, annealing at 58–60 °C for 30 s and elongation at 72 °C for 30 s, followed by a final elongation step at 72 °C for 10 min. Then, the PCR products were detected by 1.5% gel electrophoresis and preserved at 4 °C. Subsequently, the PCR products were purified using a PCR Clean Up Kit (Beyotime Biotechnology Company, Ltd. Shanghai, China). The purified PCR products were sequenced in both directions at the Chengdu Qingke Biotechnology Company, Ltd. (Chengdu, Sichuan, China).

Sequence data analysis

The complete mitochondrial genome sequence of *Bos taurus* (GenBank Accession Number V00654) was used as the reference sequence for determining the variation sites of cattle *ATP8* and *ATP6* genes. The yak and cattle sequences here were edited and aligned with DNASTAR SeqMan software (DNASTAR Inc., Madison, WI). Sequence variations were identified using software MEGA 5.5 (http://www.megasoftware.net). All sequences were exported as a FASTA file. Haplotype diversity (Hd), average number of differences (K), and nucleotide diversity (π) of the *ATP8* and *ATP6* genes were determined using software DnaSP V5 (http://www.ub.edu/dnasp/). A median-joining network analysis of haplotypes was performed using program Network 4.611 (http://www.fluxus-engineering.com/sharenet.htm).

Protein structure domain prediction

ATP8 and *ATP6* both encode an inner membrane polypeptide of the F0 component that takes part in the assembly of mitochondrial ATPase. The F0 ATPase complex is a membrane-embedded proton channel. Thus, mutations that occur in the DNA region that encode for the trasmembrane protein could affect proton transportation. Software SMART (http://smart.embl-heidelberg.de/) was used to identify protein domain structures and the location of mutations in genes *ATP8* and *ATP6*. The complete mitochondrial genome of *Bos taurus* (GenBank Accession Number V00654) was used as the reference sequence.

Statistical analysis

The Tibetan yaks were regarded as the bovine group with high-altitude adaptation and the Holstein-Friesian cows were considered to be the bovine group without high-altitude adaptation. Comparison of SNP frequencies for genes *ATP8* and *ATP6* in Tibetan yaks and Holstein-Friesian cows were conducted using the Pearson chi-square tests or Fisher' exact tests with Bonferroni correction. Comparisons between haplotype frequencies for genes *ATP8* and *ATP6* in Tibetan yaks and Holstein-Friesian cows were performed using Fisher's exact tests with the Bonferroni correction, and odds ratios (OR) with a 95% confidence interval using program Mito Tool (http://www.mitotool.org/index.html).

Results

Base composition, sequence diversities in ATP8 and ATP6 genes

Base composition of *ATP8* and *ATP6* genes calculated using MEGA 5.5 are presented in Table 1. The base with the highest percentage in genes *ATP8* and *ATP6* in both Tibetan yaks and

Holstein-Friesian cattle was A (about 40% in *ATP8* and 33% in *ATP6*), and the base with the lowest percentage was G. Genes *ATP8* and *ATP6* differed in their proportion of A bases ($P = 0.0201$) and C bases ($P = 0.0227$; Data not shown; Pearson chi-square). The T + A% percentage was over 60% in both *ATP8* and *ATP6* genes. The complete lengths of genes *ATP8* and *ATP6* were amplified for a total of 842 bp (GenBank Accession Number V00654; neither insertion nor deletions were detected).

Twenty variable sites in *ATP8* and 60 variable sites in *ATP6* were obtained (Tables 2 and 3). Nine SNPs in gene *ATP8* were only present in Tibetan yaks, and 14 SNPs had allele frequencies that were significantly different between Tibetan yaks and Holstein-Friesian cows (Table 2). Similarly, 38 SNPs in gene *ATP6* existed only in Tibetan yaks, and the allele frequencies of 47 SNPs differed significantly between Tibetan yaks and Holstein-Friesian cows (Table 3). Ten variable sites in gene *ATP8* showed positive associations with high-altitude adaptation. Among which SNPs m.8164 G→A, m.8210 G→A, m.8231 C→T and m.8249 C→T resulted in amino acid changes. There were 21 nonsynonymous mutations in gene *ATP6* of which 7 were present in Tibetan yaks and 11 in Holstein-Friesian cows. The SNPs m.8308A > G ($P = 0.00005$), m.8370A > C ($P = 0.491 \times 10^{-8}$) and m.8514G > A ($P = 0.00005$) were significantly associated with high-altitude adaptation.

Table 1. Nucleotide composition of ATP8 and ATP6 gene sequences in Tibetan yaks and Holstein-Friesian cattle.

Genes	Population	Number of samples	T%	C%	A%	G%	T + A%
ATP8	Yak	66	29.3	23.0	41.8	6.0	71.1
	Cattle	80	28.2	24.0	39.7	8.1	67.9
ATP6	Yak	66	27.8	27.6	33.5	11.10	61.3
	Cattle	80	29	27	33	11	62

Haplotype analysis in ATP8 *and* ATP6 *genes*

Number of haplotypes, haplotype diversity (Hd), average number of differences (K) and nucleotide diversity (Pi) of genes *ATP8* and *ATP6* are summarized in Table 4. Holstein-Friesian cows had a larger number of *ATP8* haplotypes ($n = 9$) than Tibetan yaks ($n = 3$; Table 5). Most Tibetan yaks contained *ATP8* haplotype H4 ($n = 56$). Conversely, the most frequently found *ATP8* haplotype in Holstein-Friesian cows was Haplotype H8 ($n = 55$; Table 5).

There were 21 haplotypes for *ATP6* among Tibetan yaks and Holstein-Friesian cows. No haplotype existed in both groups as the same with that of *ATP8* gene. Tibetan yaks harboured 6 unique haplotypes (Haplotype H3, H4, H5, H6, H7 and H8) and Holstein-Friesian cows had 15 unique haplotypes (Table 6). Haplotype H5 was the most common haplotype in Tibetan yaks (present in 50 out of 66 yaks) and haplotype H17 was the most frequent in Holstein-Friesian cows (present in 45 out of 81 cows). The sequence variations are shown in Table 7 for gene *ATP8* and in Table 8 for gene *ATP6* for sample sizes larger than or equal to 3.

Median-joining network of haplotype

Median-joining network charts were constructed using the 12 haplotypes of gene *ATP8* shown in Table 5 (Figure 1) and the 21 haplotypes of gene *ATP6* shown in Table 6 (Figure 2). Haplotypes H3, H4 and H5 in *ATP8* were restricted to Tibetan yaks. These 3 haplotypes were close to Haplotype H1 present in Holstein-Friesian cows, and there were 8 mutations that could link haplotype H1 to H4. Haplotypes H3 and H5 were derived from Haplotype H4, according to Figure 1. Haplotype H8 appeared to be the ancestor of the other haplotypes in gene *ATP8*. Haplotypes H3–H8 in gene *ATP6* were present only in Tibetan yaks. Haplotype H5 was identified as the

Table 2. Mutations in gene ATP8 in Tibetan yaks (TY) and Holstein-Friesian (HF) cattle.

			MN[a]					
Sites	Variants	Amino acid	TY	HF	TMN[b]	*p*-Value[c]	OR[d]	95%CI[e]
8134	G→A	Pro	66	0	66	0.00027**	–	–
8164	G→A	Met→Ile	66	0	66	0.00027**	–	–
8168	T→C	Leu	0	14	14	8.73×10^{-14} **	–	–
8173	A→G	Ser	0	3	3	0.00183*	–	–
8188	T→C	Leu	60	21	81	0.00052*	0.35225	0.20465–0.60631
8194	C→T	Ile	66	15	81	0.07827	0.58343	0.32029–1.063
8204	C→T	Leu	66	0	66	0.00027**	–	–
8210	G→A	Val→Ile	60	21	81	0.00052*	0.35225	0.20465–0.60631
8230	T→C	Iao	66	0	66	0.00027**	–	–
8231	C→T	His→Tyr	66	0	66	0.00027**	–	–
8236	T→C	Asn	0	3	3	0.00183*	–	–
8245	G→A	Leu	66	0	66	0.00027**	–	–
8249	C→T	Pro→Ser	66	0	66	0.00027**	–	–
8259	T→C	Ile→Thr	4	0	4	1	–	–
8285	A→G	Thr→Ala	0	15	15	9.55×10^{-15} **	–	–
8304	A→G	Tyr→Cys	0	1	1	0.1232	–	–
8308	A→G	Leu	66	15	81	0.07827	0.58343	0.32029–1.063
8316	T→C	Leu→Ser	6	0	6	1	–	–
8318	T→C	Leu	0	2	2	0.01506	–	–
8326	G→A	Leu	66	1	67	0.00176*	10.028	1.378–72.988

[a]MN: mutation number.
[b]TMN: total mutation number.
[c]Two-tailed Fisher's exact test with Bonferroni correction: *$p < 0.0025$ and **$p < 0.0005$ (0.05/20 = 0.0025, 0.01/20 = 0.0005).
[d]OR: odds ratio.
[e]95% CI: 95% confidence interval.

Table 3. Mutations in gene ATP6 in Tibetan yaks (TY) and Holstein-Friesian (HF) cattle.

			MN[a]					
Sites	Variants	Amino acids	TY	HF	TMN[b]	*p*-Value[c]	OR[d]	95% CI[e]
8304	A→G	Leu	0	1	1	0.05887	–	–
8308	A→G	Thr→Ala	66	15	81	0.00005**	0.25614	0.14271–0.45974
8316	T→C	Phe	6	0	6	1	–	–
8318	T→C	Ile→Thr	0	2	2	0.00345	–	–
8326	G→A	Val→Ile	66	1	67	0.18235	4.211	0.58062–30.534
8332	T→C	Phe→Leu	66	0	66	0.03118	–	–
8343	T→C	Pro	60	0	60	0.04773	–	–
8352	C→T	Thr	66	0	66	0.03118	–	–
8358	C→T	Ile	0	3	3	0.0002*	–	–
8370	A→C	Pro	66	20	86	4.91×10^{-8} **	0.18553	0.10939–0.31465
8394	C→T	Asn	66	0	66	0.03118	–	–
8398	C→T	Leu	66	0	66	0.03118	–	–
8403	A→G	Val	66	0	66	0.03118	–	–
8405	G→A	Ser→Asn	66	0	66	0.03118	–	–
8406	C→T	Asp	60	0	66	0.04773		
8421	C→T	Thr	66	0	66	0.03118	–	–
8436	A→G	Ile→Met	66	0	66	0.03118	–	–
8439	T→C	Leu	66	0	66	0.03118	–	–
8466	T→C	Ser	0	15	15	1.87×10^{-19} **	–	–
8467	A→G	Ile→Val	4	0	4	1	–	–
8468	T→C	Ile→Thr	0	4	4	10^{--5} **	–	–
8475	T→C	Asn	66	1	67	0.18238	4.211	0.58062–30.534
8476	T→C	Thr→Ala	66	0	66	0.03118	–	–
8478	T→C	Ile→Met	66	0	66	0.03118	–	–
8494	A→G	Ala→Thr	0	15	15	1.87×10^{-19} **	–	–
8499	A→G	Leu	2	0	2	1		
8502	A→G	Ile→Val	0	1	1	0.05887	–	–
8503	T→C	Leu	0	15	15	1.87×10^{-19} **	–	–
8514	G→A	Leu	66	15	81	0.00005**	0.25614	0.14271–0.45974
8517	C→T	Ile	66	0	66	0.03118	–	–
8529	A→G	Gly	66	0	66	0.03118	–	–
8542	C→T	Leu	64	0	64	0.03103		
8550	A→G	Leu	66	0	66	0.03118	–	–
8571	A→G	Pro	0	15	15	1.87×10^{-19} **	–	–
8607	C→T	Ile	66	0	66	0.03118	–	–
8623	G→A	Ala→Thr	0	2	2	0.00345	–	–
8646	T→C	Asn	0	14	14	3.47×10^{-18} **	–	–
8656	G→A	Thr→Ala	66	0	66	0.03118	–	–
8691	C→A	Pro	66	0	66	0.03118	–	–
8710	C→T	Ile	0	3	3	0.0002**	–	–
8721	T→C	Ile	66	0	66	0.03118	–	–
8727	T→C	Thr	0	1	1	0.05887	–	–
8730	C→T	Ile	0	1	1	0.05887	–	–
8742	T→C	Ile	0	1	1	0.05887	–	–
8749	A→G	Ile→Val	0	15	15	1.87×10^{-19} **	–	–
8775	T→C	Ala	66	0	66	0.03118	–	–
8793	C→T	His	66	0	66	0.03118	–	–
8806	C→T	Leu	66	0	66	0.03118	–	–
8817	A→G	Gla	66	0	66	0.03118	–	–
8818	G→A	Ala→Thr	0	1	1	0.05887	–	–
8838	A→G	Ser	66	0	66	0.03118	–	–
8845	C→T	Thr→Ala	66	0	66	0.03118	–	–
8868	C→T	Phe	66	0	66	0.03118	–	–
8870	C→T	Thr→Ile	66	0	66	0.03118	–	–
8871	C→T	Thr→Ile	66	0	66	0.03118	–	–
8892	T→C	Ile	66	0	66	0.03118	–	–
8898	G→A	Glu	66	0	66	0.03118	–	–
8934	T→C	Thr	66	0	66	0.03118	–	–
8937	C→T	Leu	66	0	66	0.03118	–	–
8963	A→G	Asn→Ser	2	0	2	1	–	–

[a]MN: mutation number; TY: Tibetan Yak; HF: Holstein-Friesian cattle.
[b]TMN: total mutation number.
[c]Two-tailed Fisher's exact test with Bonferroni correction: $*p < 0.0025$ and $**p < 0.0005$ (0.05/60 = 0.0008, 0.01/60 = 0.0002).
[d]OR: odds ratio.
[e]95% CI: 95% confidence interval.

Table 4. Genetic diversity parameters of ATP8 and ATP6 gene sequences in Tibetan yaks and Holstein-Friesian cattle.

Gene	Population	Number of polymorphisms	Number of haplotypes	Haplotype diversity (Hd)	Average number of differences (K)	Nucleotide diversity (Pi)
ATP8	Yak	2	3	0.272	0.28	0.0014
ATP6	Cattle	7	6	0.418	0.80	0.0012
ATP8	Yak	11	9	0.516	2.10	0.0104
ATP6	Cattle	22	15	0.667	3.37	0.0050

ancestor of the other five haplotypes (H3, H4, H6, H7, H8). Conversely, the ancestral haplotype in Holstein-Friesian cows was H17. Moreover no one haplotype was possessed by two groups simultaneously for both *ATP8* and *ATP6* genes.

Table 5. Haplotype distribution for gene ATP8.

Haplotype	Number of yaks	Number of cattle	*p*-Value[a]	Odds Ratio	95% CI
H1	0	14	0.00027**	–	–
H2	0	1	1	–	–
H3	6	0	0.00719	–	–
H4	56	0	1.174×10^{-30}**	–	–
H5	4	0	0.03860	–	–
H6	0	1	1	–	–
H7	0	3	0.25273	–	–
H8	0	55	1.449×10^{-20}**	–	–
H9	0	3	0.25273	–	–
H10	0	1	1	–	–
H11	0	1	1	–	–
H12	0	2	0.50182	–	–

[a]Two-tailed Fisher's exact test with Bonferroni correction: $^{**}p < 0.0008$ (0.05/12 = 0.0042, 0.01/12 = 0.0008).

Table 6. Haplotype distribution for gene ATP6.

Haplotype	Number of yaks	Number of cattle	*p*-Value[a]	Odds ratio	95% CI
H1	0	14	0.00027**	–	–
H2	0	1	1	–	–
H3	6	0	0.00719	–	–
H4	2	0	0.19989	–	–
H5	50	0	1.449×10^{-25}**	–	–
H6	4	0	0.03860	–	–
H7	2	0	0.19989	–	–
H8	2	0	0.19989	–	–
H9	0	1	1	–	–
H10	0	1	1	–	–
H11	0	2	0.50182	–	–
H12	0	3	0.25273	–	–
H13	0	1	1	–	–
Ha14	0	3	0.25273	–	–
H15	0	1	1	–	–
H16	0	1	1	–	–
H17	0	45	1.230×10^{-15}**	–	–
H18	0	4	0.12770	–	–
H19	0	1	1	–	–
H20	0	1	1	–	–
H21	0	2	0.50182	–	–

[a]Two-tailed Fisher's exact test with Bonferroni correction: $^{**}p < 0.0005$ (0.05/21 = 0.00238, 0.01/21 = 0.00048).

Association between haplotype distribution and high-altitude adaptation

There was a clear difference in haplotype distributions for genes *ATP8* (Table 5) and *ATP6* (Table 6) between Tibetan yaks and domesticated cattle. We found one haplotype positively associated with high-altitude adaptation in gene *ATP8* (H4; $P < 1.174 \times 10^{-30}$), and two haplotypes negatively associated with high-altitude adaptation (H1, $P < 0.00027$, and H8, $P < 1.449 \times 10^{-20}$; Table 5). Similarly, gene *ATP6* had one haplotype positively associated with high-altitude adaptation (H5, $P < 1.449 \times 10^{-25}$), and two haplotypes negatively associated with high-altitude adaptation (H1, $P < 0.00027$, and H17, $P < 1.230 \times 10^{-15}$; Table 6).

Prediction of the secondary structure changes in ATP8 *and* ATP6 *genes*

We found one nonsynonymous mutation (m.8164G > A) in the transmembrane region of *ATP8* gene (Figure 3), and 12 nonsynonymous mutations in the transmembrane region of *ATP6* (Figure 4). Among which the SNP m.8308A > G, located in the transmembrane region of *ATP6*, changed the threonine to alanine which brought in a conserved region containing six transmembrane helices in cytochrome b651 and homologous proteins including some ferric reductases.

Discussion

Adaptive divergence at a molecular level may be expressed by an increased rate of nonsynonymous changes within genes involved in adaptation (Bakewell et al. 2007). The 20 SNPs in gene *ATP8* and 60 SNPs in gene *ATP6* of Tibetan yaks and Holstein-Friesian cows contained 8 and 20 missense mutations, respectively. More than 50% of the nonsynonymous mutations in these two genes occurred in Tibetan yaks, which may be an indication that there was some selection pressure on Tibetan yaks for adaptation to high-altitudes. The SNP m.8308A > G in *ATP6* gene, a nonsynonymous mutation, resulted in an amino acid change from threonine to alanine which might bring in a conserved region, containing six transmembrane helices, found in cytochrome b651 and homologous proteins including some ferric reductases

Table 7. Sequence variation for gene ATP8, haplotype distribution, and amino acid changes in Holstein-Friesian cattle.

	Position of variation sites in gene ATP8																		Group[b]		
Hap[a]	8134	8164	8168	8173	8188	8194	8204	8210	8230	8231	8236	8245	8249	8259	8285	8308	8316	8326	TY	HF	*N*[c]
Ref	G	G	T	A	T	C	C	G	T	C	T	G	C	T	A	A	T	G			
H1	.	.	C	.	C	T	.	A	.	.	.	.	.	.	G	G	.	.	0	14	14
H3	A	A	.	.	C	T	T	A	C	T	.	A	T	.	.	G	C	A	6	0	6
H4	A	A	.	.	C	T	T	A	C	T	.	A	T	.	.	G	.	A	56	0	56
H5	A	A	.	.	C	T	T	A	C	T	.	A	T	C	.	G	.	A	4	0	4
H7	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	3	3
H8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	55	55
H9	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	0	3	3
AA		1						2		3			4	5	6			7			

Dot(.) denotes the same single nucleotide to the reference sequence. Haplotypes which the sample size under 3 were not considered.
[a]Hap = Haplotype; Ref = Reference sequence; H1 to H9 = Haplotypes 1 to 9; AA = Amino Acid change: 1 = Met→Ile; 2 = Val→Ile; 3 = His→Tyr; 4 = Pro→Ser; 5 = Ile→Thr; 6 = Thr→Ala; 7 = Leu→Ser.
[b]TY = Tibetan yaks; HF = Holstein-Friesian cattle.
[c]N = Number of yaks or cattle.

Table 8. Sequence variation for gene ATP6, haplotype distribution, and amino acid changes in Holstein-Friesian cattle.

Position of variation sites in gene ATP6

Hap[a]	8308	8326	8332	8343	8352	8358	8370	8394	8398	8403	8405	8406	8421	8436	8439	8466	8467	8468	8475	8476	8478	8494	8503
	A	G	T	T	C	C	A	C	C	A	G	C	C	A	T	T	A	T	T	T	T	A	T
H1	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	C
H3	G	A	C	.	T	.	C	T	T	G	A	.	T	G	C	.	.	.	C	C	C	.	.
H5	G	A	C	C	T	.	C	T	T	G	A	T	T	G	C	.	.	.	C	C	C	.	.
H6	G	A	C	C	T	.	C	T	T	G	A	T	T	G	C	.	G	.	C	C	C	.	.
H12	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
AA	1	2	3								4			5			6	7		8	9	10	

Hap[a]	8514	8517	8529	8542	8550	8571	8607	8646	8656	8691	8710	8721	8749	8775	8793	8806	8817	8838	8845	8868	8870	8871	8892	8898	8934	8937	Group[b]		N[c]
	G	C	A	C	A	A	C	T	G	C	C	T	A	T	C	C	A	C	A	C	C	C	T	G	T	C	TY	HF	
H1	A	.	.	.	.	G	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	0	14	14
H3	A	T	G	T	G	.	T	.	A	A	.	C	.	C	T	T	G	T	G	T	T	T	C	A	C	T	6	0	6
H5	A	T	G	T	G	.	T	.	A	A	.	C	.	C	T	T	G	T	G	T	T	T	C	A	C	T	50	0	50
H6	A	T	G	T	G	.	T	.	A	A	.	C	.	C	T	T	G	T	G	T	T	T	C	A	C	T	4	0	4
H12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	3	3
H14	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	3	3
H17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	45	45
H18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	4	4
AA									11				12								13								

Dot(.) denotes the same single nucleotide to the reference sequence. Haplotypes which the sample size under 3 were not considered.
[a]Hap = Haplotype; AA = Amino Acid change: 1 = Thr→Ala; 2 = Val→Ile; 3 = Phe→Ile; 4 = Ser→Asp; 5 = Ile→Met; 6 = Ile→Val; 7 = Ile→Thr; 8 = Ser→Pro; 9 = Ser→Pro; 10 = Thr→Ala; 11 = Ala→Thr; 12 = Ile→Val; 13 = Thr→Ile.
[b]TY = Tibetan yaks; HF = Holstein-Friesian cattle.
[c]N = Number of yaks or cattle.

(SMART database). Cytochrome b561, a transmembrane ascorbate-dependent oxidoreductase (Nakanishi et al. 2009; Njus et al. 1983; Su & Asard 2006), is reducible by ascorbate (Wakefield et al. 1986). Cytochrome b561 (Cyt b_{561}) functions as an electron transporter that shuttles electrons across membranes from ascorbate to an acceptor molecule (Wakefield et al. 1986; Njus et al. 1987; Horemans et al. 1994). Additionally, Cytochrome b561 might play an important role in the iron regulation (Lane et al. 2015). It is known that the increased iron availability will enhance the oxygen uptake (Chepelev & Willmore 2011). Hence, it was likely that the Tibetan yaks with this mutation might had a better iron metabolism which would enhance the use of rare air efficiently. Moreover, the frequency of the mutant G base was significantly higher in Tibetan yaks (60 out of 66) than in Holstein-Friesian cows (14 out of 81). The higher frequency of m.8308A > G in Tibetan yaks suggests that it may be associated with high-altitude adaptability. Additionally, 5 SNPs (including m.8308A > G) were detected in the overlap region between *ATP8* and *ATP6* and all the variable sites were missense mutations located in the transmembrane region. These mutations resulted in a higher threshold for proteins of the *ATP6* gene (data not shown) according to the prediction of protein region using SMART (http://smart.embl-heidelberg.de/). In a human study (Boominathan et al. 2016), a patient's cytoplasmic hybrid cell line with a single point mutation in the overlap region of the *ATP8* and *ATP6* genes resulted in a premature truncation of *ATP8* gene and also a reduction of *ATP6* gene levels, which both contributed to the failure of assembly of Complex V (cytochrome c reductase core protein V), the impaired oxidative phosphorylation and cell viability. Herewith it had influence on the ATP levels and oxygen consumption. In another study in humans (Ware et al. 2009), SNP m.8528T > C (m.8291 T > C in this study), led to an amino acid change from tryptophan to arginine in the *ATP8* gene, and at the same time the start methionine for *ATP6* was converted to threonine. Both mutations impaired the translation of the *ATP6* gene. Mutations in the *ATP6* gene had been proven to result in substantially decrease of ATP synthesis (Nijtmans et al. 2001; Jesina et al. 2004; Houstek et al. 2006). During exposure to the hypoxia, the oxidative phosphorylation (OXPHOS) for ATP generation will be decreased in plus with insufficient ATP availability might result in cell death (Santore et al. 2002). In our study, the mutations located in the overlap region, such as m.8308A > G and m.8326G > A, were mainly represented in the group of Tibetan yaks. In addition, the m.8326G > A was contained by haplotypes H4 in *ATP8* gene and H5 in *ATP6* gene which both had positive association with high-altitude adaptation. So we thought there might be a more complex regulation mechanism about the ATP availability.

Interestingly, no haplotype was shared between Tibetan yaks and Holstein-Friesian cows although they shared some segregating sites. Tibetan yaks had fewer haplotypes than Holstein-Friesian cows which indicated that the diversity level for *ATP8* and *ATP6* may be lower in Tibetan yak than in Holstein-Friesian. The most frequent haplotypes for *ATP8* were H4 for Tibetan yaks (93%) and H8 for Holstein-Friesian cows (67.9%). Conversely, the haplotype with the highest

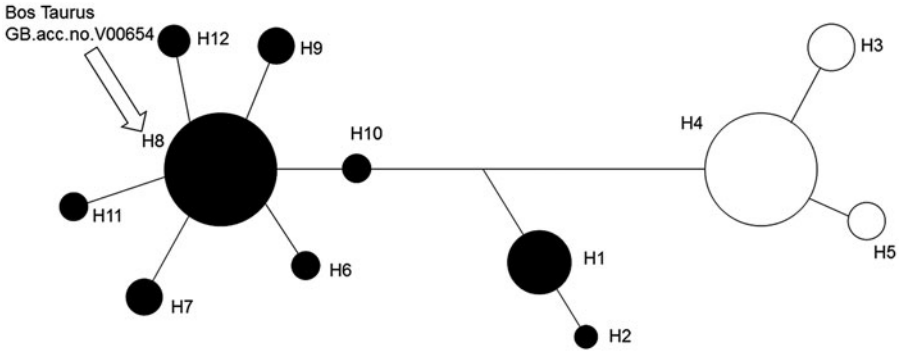

Figure 1. Median-joining network of ATP8 haplotypes. Tibetan yaks and Holstein-Friesian cattle were represented with white and black circles, respectively. Circle areas represent haplotype frequencies. The arrow indicates that the reference sequence belongs to haplotype H8.

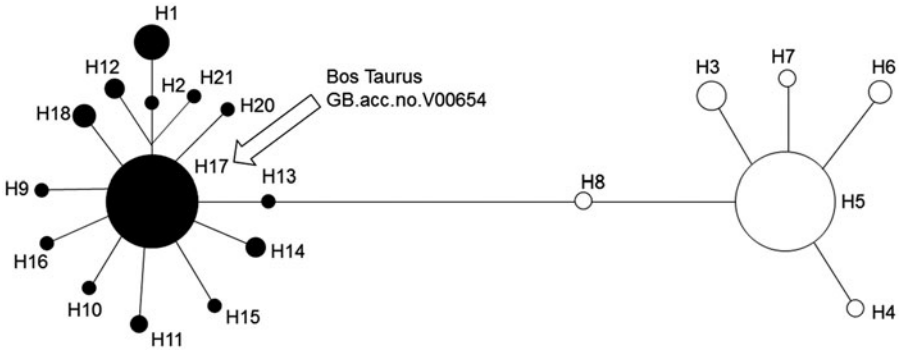

Figure 2. Median-joining network of ATP6 haplotypes. Tibetan yaks and Holstein-Friesian cattle were represented with white and black circles, respectively. Circle areas represent haplotype frequencies. The arrow indicates that the reference sequence belongs to haplotype H17.

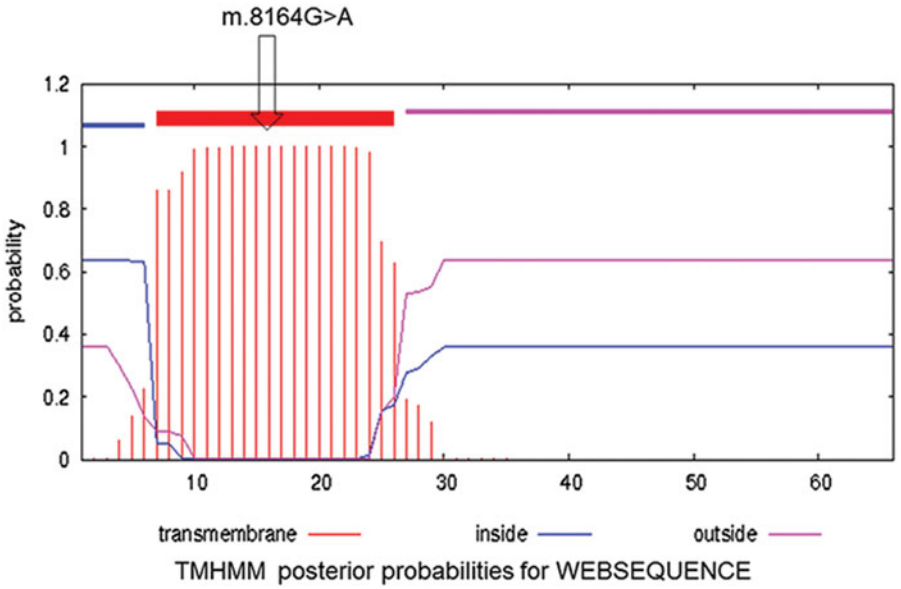

Figure 3. Diagram of transmembrane structure of the ATP8 protein predicted using the SMART program. The arrow indicates that the nonsynonymous mutation was distributed in the transmembrane region.

frequency for *ATP6* was H5 in Tibetan yaks (75.8%) and H17 in Holstein-Friesian cows (55.6%). It seems likely that haplotypes poorly represented in a group of animals may have been under high negative selection pressure. The zero representation of haplotypes H1 and H8 of *ATP8* and H1 and H17 of *ATP6* in Tibetan yaks indicated that these haplotypes were likely negatively associated the high-altitude adaptation and eliminated from the population. Conversely, the high occurrence of haplotypes H5 in *ATP8* and H17 in *ATP6* in Tibetan yaks may have been under positive selection pressure for their positive association with high-altitude adaptation.

Conclusions

The SNP m.8308A > G, contributing to the conversion of threonine, was predicted to have influence in the oxygen

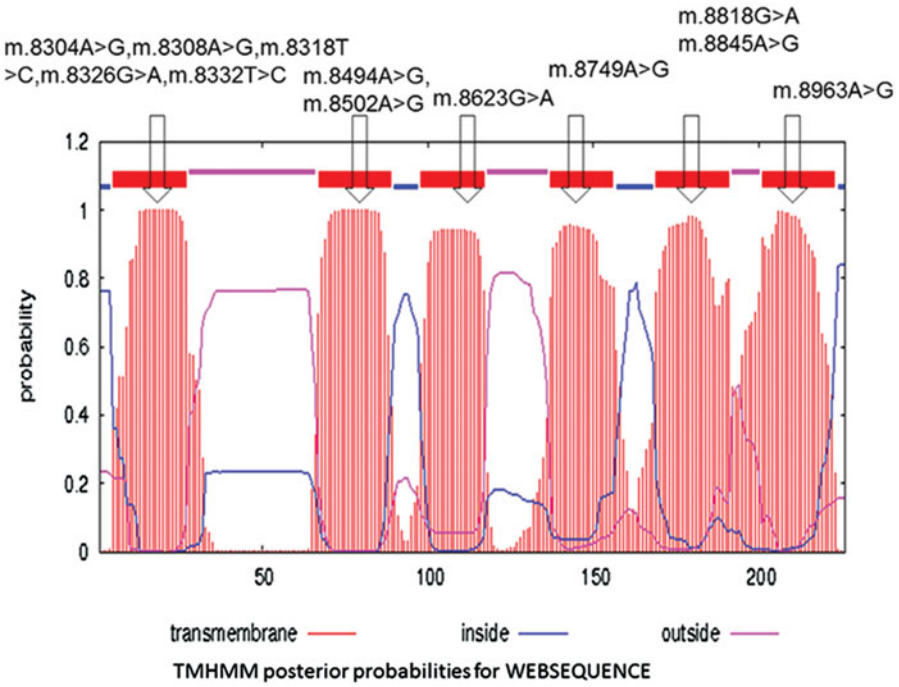

Figure 4. Diagram of transmembrane structure of the ATP6 protein predicted using the SMART program. The arrows indicate that the nonsynonymous mutations were distributed in each of the transmembrane regions.

uptake which may help Tibetan yaks to adapt to high-altitude hypoxic environments. Haplotypes H4 in *ATP8* and H5 in *ATP6* present only in Tibetan yaks were suggested to be positively associated with high-altitude adaptation.

Acknowledgments

This study was financially supported by 12th Five-Year Breeding Research projects in Sichuan (Grant No. 2011NZ0099-4), China Agricultural Research System (Grant No. CARS-44-A-2), the double-support project of Sichuan Agricultural University.

Disclosure statement

All authors have no declared conflict of interest.

Funding

This study was financially supported by 13th Five-Year Breeding Research projects in Sichuan (Grant No. 2016NYZ0046), China Agricultural Research System (Grant No. CARS-44-A-2), the double-support project of Sichuan Agricultural University.

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