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RESEARCH ARTICLE

Genetic diversities of *MT-ND1* and *MT-ND2* genes are associated with high-altitude adaptation in yak

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ABSTRACT

Tibetan yak (*Bos grunniens*) inhabiting the Qinghai-Tibet Plateau (QTP) where the average altitude is 4000 m, is specially adapted to live at these altitudes. Conversely, cattle (*B. taurus*) has been found to suffer from high-altitude hypertension or heart failure when exposed to these high altitudes. Two mitochondrial genes, *MT-ND1* and *MT-ND2*, encode two subunits of NADH dehydrogenase play an essential role in the electron transport chain of oxidative phosphorylation (OXPHOS). We sequenced these two mitochondrial genes in two bovine groups (70 Tibetan yaks and 70 Xuanhan cattle) and downloaded 300 sequences of *B. taurus* (cattle), 93 sequences of *B. grunniens* (domestic yak), and 2 sequences of *B. mutus* (wild yak) from NCBI to increase our understanding of the mechanisms of adaptability to hypoxia at high altitudes in yaks compared to cattle. *MT-ND1* SNP m.3907 C > T, present in all Tibetan yaks, was positively associated with high-altitude adaptation ($p < .0006$). Specially, mutation m.3638 A > G present in all cattle, resulting in the termination of transcription, was negatively associated with high-altitude adaptation ($p < .0006$). Additionally, *MT-ND2* SNPs m.4351 G > A and m.5218 C > T also showed positive associations with high-altitude adaptation ($p < .0004$). *MT-ND1* haplotypes H2, H3, H4, H6, and H7 showed positive associations but haplotype H20 had a negative association with high-altitude adaptation ($p < .0008$). Similarly, *MT-ND2* haplotypes Ha1 Ha8, Ha10, and Ha11 were positively associated whereas haplotype Ha2 was negatively associated with adaptability to high-altitudes ($p < .0008$). Thus, *MT-ND1* and *MT-ND2* can be considered as candidate genes associated with adaptation to high-altitude environments.

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High-altitude adaptation; *MT-ND1* gene; *MT-ND2* gene; Tibetan yak

Introduction

The role of genetics in determining an individual's susceptibility to high altitude is complicated. Mitochondria, double membrane-bound organelles found in all eukaryotic cells, are known for their function in energy supply, apoptosis (Green & Reed 1998), tumours (Magnon et al. 2005; Chae et al. 2012; Midzak et al. 2014), and relationships to other human diseases (Kelley et al. 2002; Wang et al. 2009). By consuming oxygen, mitochondria supply more than 95% of the energy in eukaryotic cells (Scott et al. 2011). The Tibetan yak, a Chinese indigenous bovid, inhabits the Qinghai-Tibet Plateau (QTP) on the west of China characterized by high altitudes (4000 m on average; (Guo et al. 2006), low temperatures, and low oxygen levels. Although the environment is harsh, more than 14 million domestic yaks inhabit the QTP and the adjacent Asian highlands, i.e. North India, Pakistan, Kyrgyzstan, Mongolia, and Russia (Schaller 1998; Wiener et al. 2003). Yaks supply milk, meat, dung for fuel, and serve as transport and protection for tents for local people (Wiener et al. 2003). Unlike yaks, cattle are susceptible to high-altitude hypertension and would experience heart failure when exposed to a high-altitudes (Weir et al. 1974), symptoms attributed to their genetic makeup (Newman et al. 2011). As mitochondria cannot properly function without an adequate supply and intracellular partial-pressure of oxygen, they would be affected by hypoxia at high altitudes (Gnaiger 2003; Scott et al. 2009). Xuanhan, a domestic cattle breed, inhabits Xuhuan, Sichuan province, China where the average altitude is 780 m, the highest area is 2349 m and the lowest area is 277 m. Xuanhan cattle is a dual-purpose breed used to produce meat and plough the fields.

Mitochondrial subunit *ND1* (mtND1) gene, encoding one of the seven subunits of respiratory complex 1, plays an important role in the first step of the electron transport chain of oxidative phosphorylation (OXPHOS) (Yusnita et al. 2010). The complete length of *MT-ND1* gene is 957 bp. Previous research indicated that electrons could be transferred from NADH molecules to ubiquinone (Yusnita et al. 2010). Variants of the *MT-ND1* gene were associated with cancers (Polyak et al. 1998; Parrella et al. 2001; Akouchekian et al. 2011), type 2 diabetes mellitus (T2DM)(Yu et al. 2004; Tang et al. 2006), and other human diseases. Pulmonary oedema (HAPE) resulting from the breakdown of the alveolar capillary membrane

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and the accumulation of a protein-rich fluid in the respiratory tract normally occurs when animals or humans are exposed to altitudes above 3000 m. Two SNPs, m.3397 A > G and m.3552 T > A, were correlated with HAPE susceptibility, and the HAPE group had a significantly higher occurrence of these mutations (Luo et al. 2012). There are other mtDNA-associated diseases including Leber's hereditary optic neuropathy (LHON), a mitochondrial and maternally inherited eye disorder (Newman 1993). A mutation in nucleotide 9101 of the *ATP6* gene was correlated with LHON and, in addition, this variant was associated with low efficiency of oxidative phosphorylation in lymphoblast mitochondria with a higher mitochondrial respiration rate (Lancet 1995).

The NADH dehydrogenase subunit 2 (ND2) enzyme is encoded by the *MT-ND2* gene and its size is 39 kDa (Schauer et al. 2015). As *MT-ND1*, *MT-ND2* is one of the 13 mitochondrial genes and it also takes part in the transfer of electrons and hydrogen to produce ATP through the progress of phosphorylation. It is presumed that *MT-ND2* plays an important role in proton translocation across the inner mitochondrial membrane, which contributes to pH regulation in cells (Schauer et al. 2015). An interesting research about polymorphism of *MT-ND2* among the longevity of Japanese centenarians and younger individuals showed an association between mutation A5178T and longevity (Tanaka et al. 1998). Another study found that the alloxan-resistant (ALR) mouse strain, a mutant group in nt4738 (mt-ND2^{a}) showed resistance to the development of spontaneous type-1 diabetes and a reduced ROS level compared with non-obese diabetic mice. Conversely, the cytosine-containing allele (mt-ND2^{c}) resulted in elevated mitochondrial ROS production (Gusdon et al. 2008). Additionally, polymorphism C5178A, causing an amino acid change from leucine to methionine, has been widely reported to be associated with various physiological phenomena including serum lipid levels (Kokaze et al. 2001), myocardial infarction (Mukae et al. 2003; Takagi et al. 2004) and onset of type 2 diabetes mellitus (Wang et al. 2001). Further, polymorphism C5178A was significantly associated with red blood cell (RBC) amount and haematocrit (HCT) value in Korean men, which indicated that there was a correlation between *MT-ND2* and blood iron metabolism (Kang et al. 2010). The tight link between iron metabolism and oxygen transport, suggests that a shortage of Fe can lead to a low-oxygen condition (hypoxia). Also, the lack of hepcidin, an essential regulator of iron metabolism, would be decreased by anaemia and hypoxia (Peyssonnaux et al. 2007). On the other hand, an increased iron availability would enhance oxygen uptake (Chepelev & Willmore 2011).

Thus, the objectives of this study were to increase our understanding of the mechanisms of hypoxia adaptability in Tibetan yaks and to confirm if there was a correlation between *MT-ND1* and *MT-ND2* genes and adaptation to high-altitude hypoxia by sequencing the complete length of the *MT-ND1* gene and partially sequence the *MT-ND2* gene in Tibetan yaks and Xuanhan cattle. We compared the nucleotide composition, gene diversity, haplotypes, and the function of predicted protein domains of these two genes in Tibetan yaks and Xuanhan cattle.

Materials and methods

Sampling and DNA extraction

Ear tissue samples were collected from 70 domesticated Tibetan yaks (*Bos grunniens*) and from 70 Xuanhan cattle (*B. taurus*), which were immediately put into 1.5 ml EP tubes filled with 1 ml of ethanol. Afterwards, samples were stored in a laboratory freezer at −20 °C. The domesticated Tibetan yaks were from Tibet, China (altitude ranging from 2000 to 5000 m) and the domesticated Xuanhan cattle come from Xuanhan, Sichuan province, China (average elevation of 780 m). 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). The PCR product with 1348 bp from the *MT-ND1* gene was amplified using the primers F: GGCTTAGTTAAGGTGGCA: and R: CAAACCCGATTCAGACAA, and then it was cut into 957 bp, which was the complete length of *MT-ND1*. A 980 bp sequence from the *MT-ND2* gene was amplified using the primers F: AAAACTCTTCGTGCTCCC and R: TTTGAAGGCTCTTG GTCT. All the primers were designed based on a complete mitochondrial genome sequence of *B. 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 °C to 60 °C (58 °C was designed for *MT-ND1*; 60 °C was designed for *MT-ND2*) 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 *B. mutus* (GenBank Accession Number KM233417) was used as the reference sequence for determining the variation sites of cattle *MT-ND1* and *MT-ND2* genes. In addition, 300 sequences of *B. taurus*, 93 sequences of *B. grunniens*, and 2 sequences of *B. mutus* were downloaded from NCBI and added to the pool of sequences utilized in the analysis. The yak and cattle sequences were edited and aligned with DNASTAR SeqMan software (DNASTAR Inc., Madison, WI). Sequence variations were identified using software MEGA 6.0 (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 *MT-ND1* and *MT-ND2* 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

Nicotinamide adenine dinucleotide (NAD) is a coenzyme found in all living cells, which exists in two forms: NADH is the oxidized form and the NAD^+ is the reduced form. NADH plays a key role in the transfer of H^+ and e^-, thus changes in its protein domain may influence its function and affect the respiratory chain. Thus, variants in the coding region of *MT-ND1* and *MT-ND2* genes may hinder the normal function of NADH dehydrogenase. Consequently, software SMART (http://smart.embl-heidelberg.de/) was used to identify protein domain structures and the location of mutations in genes *MT-ND1* and *MT-ND2*. The complete mitochondrial genome of *B. mutus* (GenBank Accession Number KM233417) was used as the reference sequence.

Statistical analysis

Tibetan yaks were regarded as the bovine group with high-altitude adaptation and Xuanhan cattle were considered to be the bovine group without high-altitude adaptation. Comparison of SNP frequencies for genes *MT-ND1* and *MT-ND2* in Tibetan yaks and Xuanhan cattle were conducted using Pearson's chi-square tests or Fisher' exact tests with Bonferroni's correction. Comparisons between *MT-ND1* and *MT-ND2* haplotype frequencies in Tibetan yaks and Xuanhan cattle were conducted using Fisher's exact tests with Bonferroni's correction, and odds ratios (OR) with 95% confidence intervals using the Mito Tool (http://www.mitotool.org/index.html).

Results

Base composition, sequence diversities in MT-ND1 and MT-ND2 genes

Base composition of the *MT-ND1* and *MT-ND2* genes calculated using MEGA 6.0 are presented in Table 1. For yak and cattle, base A possessed the highest percentage in both *MT-ND1* and *MT-ND2* (mean of 32.4% in *MT-ND1* and 36.3% in *MT-ND2*), and the base with the lowest percentage was G (mean of 12.2% for *MT-ND1* and 8.8% for *MT-ND2*). The A + T% represented over 50% of all bases for both genes in yaks and cattle. All percentages were computed with and without the 395 downloaded sequences; results in both cases were nearly identical. Additionally, there were no significant differences in base composition between yaks and cattle.

The complete sequence of *MT-ND1* (957 bp) and the partial sequence of *MT-ND2* (980 bp) were amplified (GenBank Accession Number KM233417; neither insertion nor deletions were detected). Number of haplotypes, haplotype diversity (Hd), average number of differences (K), and nucleotide diversity (π) of genes *MT-ND1* and *MT-ND2* are summarized in Table 2. Clearly, cattle had more segregating sites than that of yaks. In addition, cattle had more haplotypes that differed from wild yak than domestic yak. The two wild yaks were assigned to a separate haplotype for *MT-ND1*. Haplotype Ha1 from *MT-ND2* was present in most yaks including the two wild yaks.

There were 120 variable sites for *MT-ND1* and 122 variable sites for *MT-ND2* genes, with 17 missense mutations for *MT-ND1* and 25 missense mutations for *MT-ND2* (Tables 3 and 4). Only two *MT-ND1* nonsynonymous mutations were present in domestic yaks. Conversely, there were 15 missense mutations in the cattle group, and 8 of them (m.3303 A > G, m.3330 T > A, m.3378 C > T, m.3601 T > C, m.3616 C > T, m.3638 A > G, m.3880 T > C and m.4007 A > G) were present in almost all animals in the Xuanhan cattle group. It should be mentioned that the mutation m.3638 A > G resulted in an early termination of transcription according to the termination codon. SNP m.3907 C > T, present in all the Tibetan yaks, was considered to be positively associated with high-altitude adaptation ($p < .0006$). A total of 25 missense mutations were detected in gene *MT-ND2*; three of them were present in the yak group and the other 22 were present only in the cattle group. Likewise, several mutations (m.4286 C > T, m.4650 A > T, m.4741 A > G, m.4827 G > A, m.4895 C > T, m.4946 C > T, m.4990 A > G, m.5167 G > A and m.5226 A > G) were found in most cattle. Yak SNPs m.4351 G > A and m.5226 A > G showed positive association with high-altitude adaptation ($p < .0004$).

Haplotype analysis of MT-ND1 and MT-ND2 genes

Twelve *ND1* haplotypes were analyzed in Tibetan yaks and Xuanhan cattle (Table 5). Five *MT-ND1* haplotypes were found

Table 1. Percentage of nucleotides for *MT-ND*1 and *MT-ND2* gene sequences in yaks and cattle populations.

Gene	Population	Number of samples	Downloaded sequences	T%	C%	A%	G%	T + A%
MT-ND1	Yak	70	93	26.3	29.1	32.4	12.2	58.7
	Cattle	70	300	27.6	27.9	31.8	12.6	59.4
MT-ND2	Yak	70	93	27.4	27.4	37.3	7.9	64.7
	Cattle	70	300	28.3	26.6	36.3	8.8	64.6

Table 2. Genetic diversity parameters for *MT-ND1* and *MT-ND2* gene sequences in yaks and cattle.

Gene	Population	Number of segregating sites	Number of haplotypes	Haplotype diversity (Hd)	Average number of differences (K)	Nucleotide diversity (π)
MT-ND1	Yak	20	15	0.592	1.193	0.00125
MT-ND2	Yak	16	13	0.734	2.206	0.00225
MT-ND1	Cattle	110	52	0.530	2.455	0.00257
MT-ND2	Cattle	113	60	0.452	1.652	0.00169

Table 3. Mutations in gene *MT-ND1* in yaks and cattle.

Sites	Variants	Amino acid	MN[a] Yak	MN[a] Cattle	TMN	*p* Value[b]	OR	95%CI
3187	G→C	Gly→Ala	0	1	1	1.00000	–	–
3271	T→C	Ile→Thr	0	5	5	1.00000	–	–
3303	A→G	Thr→Ala	0	358	358	2.553E-09**	–	–
3311	A→G	Ile→Met	0	1	1	1.00000	–	–
3330	T→A	Leu→Ile	0	370	370	1.700E-09**	–	–
3356	G→A	Met→Ile	0	2	2	1.00000	–	–
3378	C→T	His→Tyr	0	370	370	1.700E-09**	–	–
3405	G→A	Var→Ile	0	4	4	1.00000	–	–
3537	C→G	Leu→Var	0	1	1	1.00000	–	–
3561	C→G	Leu→Var	0	3	3	1.00000	–	–
3601	T→C	Ile→Thr	0	337	337	1.094E-09**	–	–
3616	C→T	Thr→Ile	1	370	370	2.798E-08**	0.04335	0.00605–0.31056
3638	A→G	Trp→ter	0	370	370	1.700E-09**	–	–
3876	T→C	Phe→Leu	0	6	6	1.00000	–	–
3880	T→C	Ile→Thr	0	364	364	1.582E-09**	–	–
3907	C→T	Ser→Phe	159	0	159	6.255E-269**	–	–
4007	A→G	Ile→Met	0	358	358	2.553E-09**	–	–

[a]MN: mutation number (only missense mutations were considered here); TMN: total mutation number; OR: odds ratio; 95%CI: 95% confidence interval; ter: the termination codon.
[b]Two-tailed Fisher's exact test with Bonferroni's correction: $^{*}p < .0029$ and $^{**}p < .0006$ (.05/17 = .0029, .01/17 = .0006).

Table 4. Mutations in gene *MT-ND2* in yaks and cattle.

Sites	Variants	Amino acid	MN[a] Yak	MN[a] Cattle	TMN	*p* Value	OR	95%CI[b]
4286	C→T	Thr→Ile	0	370	370	.00027**	–	–
4329	A→G	Ile→Met	0	15	15	1.00000	–	–
4348	A→G	Leu→Phe	0	1	1	1.00000	–	–
4351	G→A	Val→Ile	39	0	39	8.365E-70**	–	–
4357	A→G	Ile→Val	0	1	1	1.00000	–	–
4371	A→G	Ile→Met	0	1	1	1.00000	–	–
4538	A→G	Asn→Ser	0	2	2	1.00000	–	–
4650	A→T	Leu→Phe	0	370	370	.00027**	–	–
4700	T→C	Ile→Thr	0	1	1	1.00000	–	–
4741	A→G	Ile→Val	0	368	368	.00026**	–	–
4756	A→C	Ile→Leu	0	1	1	1.00000	–	–
4827	G→A	Met→Ile	0	370	370	.00027**	–	–
4895	C→T	Thr→Ile	0	370	370	.00027**	–	–
4946	C→T	Thr→Ile	0	368	368	.00026**	–	–
4962	C→G	His→Gln	0	1	1	1.00000	–	–
4976	C→T	Thr→Ile	0	4	4	1.00000	–	–
4990	A→G	Ile→Val	0	370	370	.00027**	–	–
5003	C→T	Ala→Val	1	0	1	.02269	–	–
5147	T→C	Ile→Thr	0	4	4	1.00000	–	–
5167	G→A	Ala→Thr	0	370	370	.00027**	–	–
5218	C→T	Pro→Ser	38	0	38	7.110E-68**	–	–
5221	C→A	Leu→Ile	0	1	1	1.00000	–	–
5225	T→C	Ile→Thr	0	2	2	1.00000	–	–
5226	A→G	Ile→Met	0	368	368	.00026**	–	–
5239	T→C	Phe→Leu	0	2	2	1.00000	–	–

[a]MN: mutation number (only missense mutations were considered here); TMN: total mutation number; OR: odds ratio; 95%CI: 95% confidence interval.
[b]Two-tailed Fisher's exact test with Bonferroni's correction: $^{*}p < .002$ and $^{**}p < .0004$ (0.05/25 = .002, .01/25 = .0004).

Table 5. Haplotype distribution for gene *MT-ND1* in yaks and cattle.

Haplotype	Number of yaks	Number of cattle	*p* Value[a]	Odds ratio	95%CI
H1	2	0	.02697	–	–
H2	9	0	5.530E-08**	–	–
H3	30	0	2.368E-27**	–	–
H4	4	0	.00069*	–	–
H5	3	0	.00434	–	–
H6	6	0	.00002**	–	–
H7	98	0	3.758E-07**	–	–
H8	0	3	1.00000	–	–
H9	0	8	.36228	–	–
H10	0	6	.59487	–	–
H11	0	18	.05272	–	–
H12	0	4	1.00000	–	–
H13	0	3	1.00000	–	–
H14	0	3	1.00000	–	–
H15	0	4	1.00000	–	–
H16	0	5	.59757	–	–
H17	0	4	1.00000	–	–
H18	0	6	.59487	–	–
H19	0	3	1.00000	–	–
H20	0	246	7.336E-35**	–	–

[a]Two-tailed Fisher's exact test with Bonferroni's correction.
$^{*}p < .0025$. $^{**}p < .0005$ (0.05/20 = 0.0025, 0.01/20 = 0.0005).
Except the group of wild yak, the other haplotypes in which the number of cattle of yak under three were not considered.

only in Tibetan yaks (H1, H9, H10, H11, and H12) and seven *MT-ND1* haplotypes were found only in Xuanhan cattle (H2, H3, H4, H5, H6, H7, and H8). No *MT-ND1* haplotype was present in both yaks and cattle. Most Tibetan yaks contained *MT-ND1* haplotype H1 ($n = 50$) followed by haplotype H12 ($n = 17$), whereas the *MT-ND1* haplotype with the highest frequency in Xuanhan cattle was H2 ($n = 33$). All other haplotypes in Tibetan yaks and Xuanhan cattle were found in less than six animals. Conversely, seven haplotypes were analyzed for *MT-ND2* in Tibetan yaks and Xuanhan cattle (Table 6). Three haplotypes (Ha1, Ha2, and Ha4) were found only in Tibetan yaks, and haplotype Ha2 was present in the largest number of Tibetan yaks ($n = 34$). Four haplotypes were present only in Xuanhan cattle and most animals contained haplotype Ha7 ($n = 44$). No *MT-ND2* haplotype existed in yaks and cattle simultaneously. Amino acid changes resulting from these missense mutations are shown in Table 5 for *MT-ND1* and in Table 6 for *MT-ND2*.

Twenty haplotypes of *MT-ND1* and 12 haplotypes of *MT-ND2* were analyzed in yaks and cattle (Tables 5 and 6). More *MT-ND1* haplotypes were found in the cattle group than in the yak group (Table 5). Haplotype H20, present in over half of the animals in the cattle group, was negatively associated with high-altitude adaptability ($p < .0005$). Conversely, *MT-ND1* haplotypes H2, H3, H4, H6, and H7 in yak showed positive associations with high-altitude adaptation ($p < .0025$). Most Tibetan yaks possessed *MT-ND1* haplotype H7; however, wild yaks possessed a different *MT-ND1*

haplotype. A small number of Tibetan yaks harboured *MT-ND1* haplotype H3.

There were two main *MT-ND2* haplotypes in yak (Ha1 and Ha11; Table 6), and wild yaks had *MT-ND2* haplotype Ha1. Four *MT-ND2* haplotypes (Ha1, Ha8, Ha10, and Ha11), present only in yaks, had positive associations with high-altitude adaptation ($p < .0042$), whereas haplotype Ha2, present in most cattle, was negatively associated with ability to adapt to hypoxic environments ($p < .0008$). Surprisingly, haplotype Ha9, present in four Tibetan yaks, showed no association with high altitude adaptation.

Table 6. Haplotype distribution for gene *MT-ND2* in yaks and cattle.

Haplotype	Number of yaks	Number of cattle	*p* Value[a]	Odds ratio	95%CI
Ha1	58	0	6.698E-3**	–	–
Ha2	0	275	8.594E-930**	–	–
Ha3	0	11	.01901	–	–
Ha4	0	7	.10119	–	–
Ha5	0	6	.18556	–	–
Ha6	0	3	.55414	–	–
Ha7	0	3	.55414	–	–
Ha8	34	0	5.370E-18**	–	–
Ha9	4	0	.01234	–	–
Ha10	6	0	.0013*	–	–
Ha11	55	0	4. 547E-30**	–	–
Ha12	0	6	.18556	–	–

[a]Two-tailed Fisher's exact test with Bonferroni's correction.
*$p < .0042$.
**$p < .0008$ (0.05/12 = .0042, .01/12 = .0008).
Haplotypes in which the number of cattle of yak under three were not considered.

Median-joining network of haplotype

Median-joining network charts for *MT-ND1* and *MT-ND2* were constructed with information from the 20 *MT-ND1* haplotypes (Figure 1) and the 12 *MT-ND2* haplotypes (Figure 2). Figure 1 shows that *MT-ND1* haplotype H1 occupied the ancestral position relative to other haplotypes. Most yaks possessed *MT-ND1* haplotype H7 (Figure 1). There were only three variable sites (m.3575 G > A, m.3907 C > T and m.3728 C > T; only m.3907 C > T was a nonsynonymous mutation) that differed between *MT-ND1* haplotypes H7 and H1. The other five *MT-ND1* yak haplotypes (H2 to H6) also had a few mutations that differed from haplotype H1. Notably, there were more than 80 SNPs that differed between *MT-ND1* haplotypes H1 and H8 (data not shown). Cattle had almost twice the number of *MT-ND1* haplotypes compared to yaks, and the majority of cattle harboured haplotype H20. No *MT-ND1* haplotype was shared by yaks and cattle.

There were fewer haplotypes for *MT-ND2* (Figure 2) than for *MT-ND1* (Figure 1). Unlike *MT-ND1* haplotypes, most domestic yaks and the wild yaks possessed *MT-ND2* haplotype Ha1. In addition, Ha11, the nearest *MT-ND2* haplotype

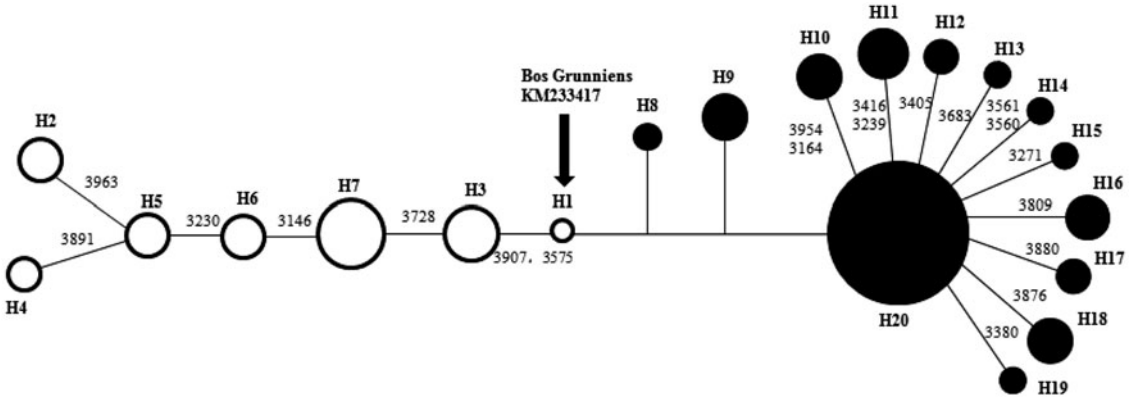

Figure 1. Median-joining network of *MT-ND1* haplotypes. Yaks and cattle are represented with white and black circles, respectively. Circle areas represent haplotype frequencies. The arrow indicates that the reference sequence belongs to haplotype H1.

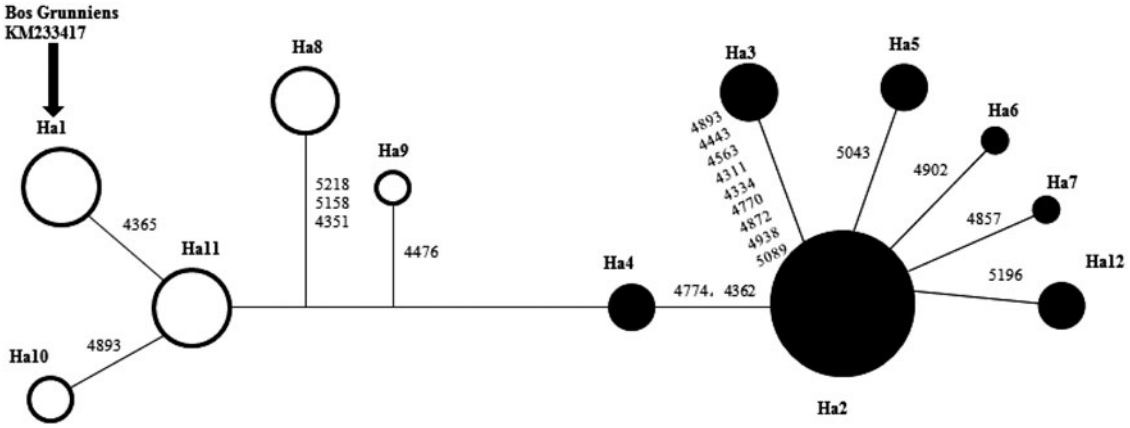

Figure 2. Median-joining network of *MT-ND2* haplotypes. Yaks and cattle are represented with white and black circles, respectively. Circle areas represent haplotype frequencies. The arrow indicates that the reference sequence belongs to haplotype Ha1.

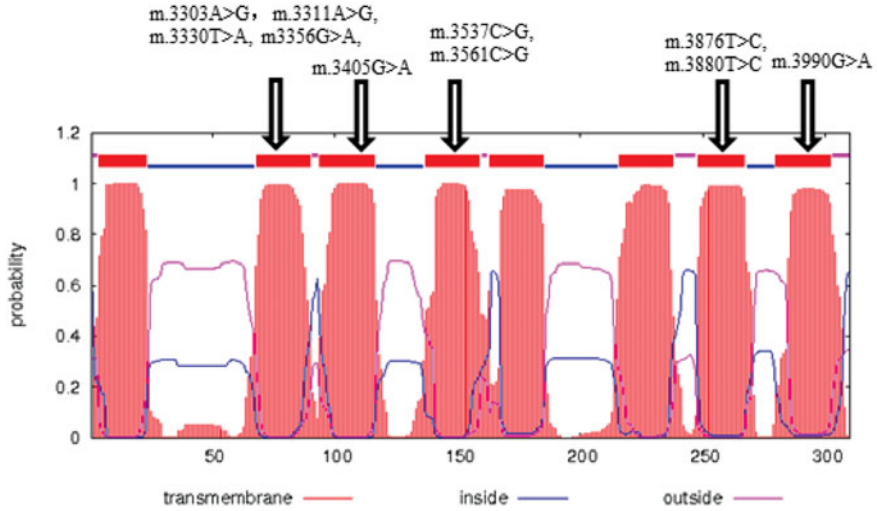

Figure 3. Diagram of the transmembrane structure of the *MT-ND1* protein predicted using the SMART program. The arrows indicate that the nonsynonymous mutations were present in each of the transmembrane regions.

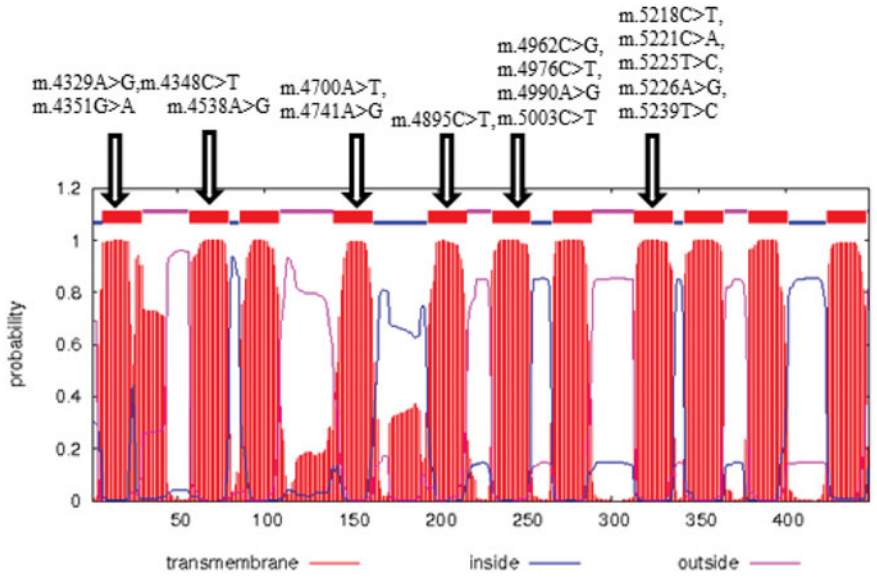

Figure 4. Diagram of the transmembrane structure of the *MT-ND2* protein predicted using the SMART program. The arrows indicate that the nonsynonymous mutations were present in each of the transmembrane regions.

to Ha1, had only one different synonymous mutation (m.4365 C > T) from Ha1, and it was present in the second biggest group of domesticated yaks (Figure 2). Contrarily, more than 50 variants existed between the yak *MT-ND2* haplotype Ha11 and cattle haplotype Ha4 (data not shown). Most cattle contained *MT-ND2* haplotype Ha2 and *MT-ND2* haplotype Ha3 was the second most numerous groups. No haplotype existed in both two groups. Thus, cattle had more *MTN-ND1* and *MT-ND2* haplotypes than yaks, and a distinct separation among haplotypes existed between yaks and cattle.

Prediction of the secondary structure changes in *MT-ND1* and *MT-ND2* genes. We found 10 missense mutations in gene *MT-ND1* located in five of the eight predicted transmembrane regions, with an enrichment of variants in the second transmembrane region (Figure 3). Conversely, the *MT-ND2* gene had 11 predicted transmembrane regions (Figure 4) and 16 SNPs located in regions 1, 2, 4, 5,6, and 8 regions; 9 SNPs were in regions 6 and 8 indicating a higher mutation rate in these two areas.

Association between haplotype distribution and high-altitude adaptation

It was obvious that there were distinct differences for *MT-ND1* and *MT-ND2* between Tibetan yaks and Xuanhan domesticated cattle. According to the Table 5, it suggested a positive correlation between the haplotype H1 and H12 and high-altitude adaptation while a negative relation between haplotype H2 and high-altitude adaptation (H1, $p = 3.755E\text{-}17^{**}$; H12, $p = .00008^{**}$; H2, $P = 1.257E\text{-}16^{**}$). For gene *MT-ND2*, two haplotypes Ha1 and Ha2 showed positive association with high-altitude adaptation. And at the same time, there were two haplotypes Ha3 and Ha7 negatively associated with high-altitude adaptation (Ha3, $p = .00005^{**}$; Ha7, $P = 1.338E\text{-}16^{**}$).

There were distinct differences between *MT-ND1* and *MT-ND2* in cattle and yaks. On one hand, wild yaks were in a separate *MT-ND1* haplotype, and only one missense mutation differed between *MT-ND1* haplotype H1 and haplotypes H3 and H7 (present in over half of all domestic yaks). *MT-ND1* haplotypes H3 and H7 showed positive associations with high-altitude adaptation (Table 5). In addition, haplotypes H2, H4 and H6 also had positive associations with high-altitude adaptability. On the other hand, wild yaks possessed *MT-ND2* haplotype Ha1, a haplotype present in most domestic yaks that showed a positive association with high-altitude adaptation. In addition, Ha11, the nearest *MT-ND2* haplotype to Ha1, was positively associated with high-altitude adaptation and *MT-ND2* haplotypes Ha8 and Ha10 were also positively associated with ability to high-altitude adaptation (Table 6).

Discussion

Tibetan yak is one of the most important domestic animals for high-altitude environments. It provides not only food but also transportation and fuel for native people and nomadic pastoralists in the Qinghai-Tibetan Plateau. Conversely, cattle suffer from severe pulmonary hypertension when exposed to the same habitat as yaks (Hecht et al. 1962; Weir et al. 1974; Will et al. 1975). Yaks' adaptability to high altitudes is likely due to adaptive evolutionary changes in anatomical and physiological traits including large lungs and hearts, lack of hypoxic pulmonary vasoconstriction, increased foraging ability, strong environment sense, and high energy metabolism. Genomic comparisons between yaks and cattle identified an enrichment of positively selected and rapidly evolving genes related to hypoxia in yaks as well as enrichment of protein domains involved in sensing extracellular environment and hypoxic stress (Qiu et al. 2012).

Mitochondria play a key role in evolution and speciation. Mitochondrial energy metabolism support ATP for almost all the internal activities dependent on oxygen. Correlations between mitochondrial genes and high-altitude adaptability had been found in many studies (Torroni et al. 1994; Sun et al. 2013a; Zhao et al. 2016). Yet differences between mitochondrial genes *MT-ND1* and *MT-ND2* between Tibetan yak and lowland cattle have not been reported. We speculate that the polymorphisms of the two genes have some influence on the ability of adapting hypoxia when exposed to high-altitude environments. Polymorphisms in mitochondrial DNA could result in dysfunctional organelles and changes in the physiological status of animals (Penta et al. 2001). Animals inhabiting at different altitudes may have different haplotypes associated with altitude adaptability (Sun et al. 2013a; Zhao et al. 2016).

We found 80 polymorphic sites in gene *MT-ND1* and 67 in gene *MT-ND2*, of which we selectively analyzed only those corresponding to nonsynonymous mutations. No significant differences in base composition were detected between *MT-ND1* and *MT-ND2*. For *MT-ND1* gene, 12 missense mutations were detected in which six SNPs were present only in Tibetan yaks and two SNPs were present only in Xuanhan cattle. There was an interesting observation that all the Tibetan yaks had the mutation m.3637 G > A in *MT-ND1* gene, which changed to the ending codon might result the advanced termination of transcription. This mutation was located at the transmembrane region of NADH dehydrogenase subunit 1 and when the mutation happened, almost 140 acid amines were lost and the NADH dehydrogenase subunit 1 may have lost three transmembrane domains according to the protein prediction using software SMART.

To find SNP differences in genes *MT-ND1* and *MT-ND2* from Tibetan yaks and lowland cattle, we analyzed both the sequenced mitochondrial genes from the 70 Tibetan yaks and 70 Xuanhan lowland cattle in this research, and the 300 sequences of *B. taurus*, 93 sequences of *B. grunniens* and 2 sequences of *B. mutus* downloaded from NCBI. We found a total of 120 polymorphic sites in gene *MT-ND1* and 122 in gene *MT-ND2*, of which we selectively analyzed only those corresponding to nonsynonymous mutations. No significant differences in base composition were detected between *MT-ND1* and *MT-ND2*. 17 missense mutations were detected in gene *MT-ND1*; two SNPs were present only in yaks and the 15 others were present only in cattle. Eight SNPs (m.3303 A > G, m.3330 T > A, m.3378 C > T, m.3601 T > C, m.3616 C > T, m.3638 A > G, m.3880 T > C and m.4007 A > G) present in almost all the cattle showed negative associations with high-altitude adaptability. Interestingly, 358 cattle had mutation m.3638 A > G that produced a stop codon resulting in termination of transcription, thus four predicted transmembrane regions would be lost. As wild yaks contained a haplotype closely similar to haplotype H7 present in most Tibetan yaks, differences in *MT-ND1* polymorphisms between domestic yaks and cattle could be speculated to be the result of natural selection and evolution. *MT-ND1* and *MT-ND2* encode two subunits of NADH dehydrogenase which is the first enzyme complex of the mitochondrial respiratory chain (complex I) and can oxidize NADH to liberate electrons to help protons across the inner membrane to create a proton gradient (Mimaki et al. 2012). A failure in the assembly of the transmembrane protein will likely have a negative impact on the oxidative chain. Thus, mutation m.3638 A > G could have hindered the assembly of the NADH dehydrogenase resulting in inefficient proton transfer in lowland cattle.

However, all Tibetan yaks with this mutation in this study were supposed to well-adapted to the challenge of extreme high-altitude conditions. Therefore, this aspect need additional research to determine whether this mutation has some influence on adaptability to high-altitude or its effect could have been nullified via other pathways. In addition, there were five variants (m.3302 G > A, m.3329 A > T, m.3377 T > C, m.3906 A > G and m.3989 A > G) that may have been positively associated with high-altitude adaptation because they were present only in Tibetan yaks. Except for the mutation m.3377 T > C, the other four variants were located in the transmembrane which may have some influence on the transference of electrons.

Among the variants of gene *MT-ND2*, SNPs m.4328 A > G, m.4350 G > A, and m.5217 C > T were only present in Xuanhan cattle whereas all other SNPs were shared by all the Tibetan yaks and 15 Xuanhan cattle. No SNPs were present only in Tibetan yak.

Twenty five nonsynonymous mutations were detected in gene *MT-ND2*. Three mutations (m.4351 G > A, m.5003 C > T, and m.5218 C > T) were present in Tibetan yaks, and two of them (m.4351 G > A and m.5218 C > T) were also present in the sequences of other yaks downloaded from NCBI. All three SNPs showed positive associations with high-altitude adaptation. Nine variants of *MT-ND2*, present in most lowland cattle, were negatively associated with high-altitude adaptation, and four of them (m.4741 A > G, m.4895 C > T, m.4990 A > G, and m.5226 A > G) were located in transmembrane regions. We consider that all the missense mutations found in predicted transmembrane regions of lowland cattle here may potentially be the cause of their low adaptability to high-altitudes.

The haplotype H1 and H12 showed significant association with high-altitude adaptation ($p < .0008$). Haplotype H2, which harboured more than half of Xuanhan cattle, was negatively associated with adaptability to hypoxia. While *MT-ND2* haplotypes Ha1 and Ha2 were positively associated with adaptability to hypoxia, *MT-ND2* haplotypes Ha3 and Ha7 showed a negative association with this trait ($p < .0014$). Furthermore, among the missense mutations, two SNPs, m.4350 G > A and m.5217 C > T, that differed between haplotypes Ha1 and Ha2 and haplotype Ha3 suggested that these two SNPs may play a decisive role in determining the ability to survive in high-altitude environments. Moreover, these two SNPs were both located in the transmembrane regions, which suggested their important role in the electrons transfer. In detail, Tibetan yaks with alleles G and C SNPs separately for these missense mutations showed better adaptability to high-altitude than cattle with alleles A and T.

Seven haplotypes in yaks and 13 haplotypes in lowland cattle were identified for gene *MT-ND1*. *MT-ND1* haplotypes H2, H3, H4, H6, and H7, present only in yaks, were positively associated with high-altitude adaptation. Thus, variants in these haplotypes likely represent real genetic differences between yaks and cattle. Tibetan yaks were largely divided into those containing haplotype H3 and those harbouring haplotype H7. We speculated that differences between these two haplotypes could contribute to different ability levels of adaptation to high-altitude environments. There were only 12 haplotypes in *MT-ND2*, four present only in Tibetan yaks (Ha1, Ha8, Ha9, and Ha11) and 7 present only in lowland cattle (Ha2 to Ha7, and Ha12). Ha1, the *MT-ND2* haplotype with the highest frequency in yaks, occupied the ancestral position relative to other haplotypes, and it was close to haplotype Ha11. Results showed a positive association between *MT-ND2* haplotypes Ha1, Ha8, Ha10, Ha11, and high-altitude adaptation ($p < .0008$), whereas *MT-ND2* haplotype Ha2, present in most cattle, was negatively associated with high-altitude adaptation ($p < .0008$).

The median-joining network analysis showed fewer variants for genes *MT-ND1* and *MT-ND2* in yaks than in lowland cattle, where these genes had multiple haplotypes radiating from one central haplotype indicating that cattle had a substantially higher haplotype diversity than yaks. External environmental conditions may have played a major role in determining *MT-ND1* and *MT-ND2* haplotype diversity in yaks and lowland cattle.

The median-joining network analysis indicated that haplotype H2 had the ancestral position to other haplotypes in *MT-ND1* gene (Figure 1). Haplotypes H5, H7, and H8, which had no significant association with high- altitude adaptation, had only one different nucleotide from haplotype H2. For gene *MT-ND2* (Figure 2), seven haplotypes were divided into groups of cattle and yaks. Haplotype Ha7 occupied the ancestral position and contained 44 out of 70 cattle, whereas haplotype Ha3 was the second biggest group including 15 cattle and was comparatively close to the yak group. Three haplotypes Ha1, Ha2, and Ha4 were present only in Tibetan yaks, of which haplotype Ha2 harboured more than a half of the Tibetan yaks. Haplotypes Ha2 and Ha3 differed by four mutations (m.4859 C > T, m.4350 G > A, m.5162 T > C and m.5217 C > T), and m.4350 G > A and m.5217 C > T were nonsynonymous mutations that were located in transmembrane regions. This suggested that that these two mutations may change the structure or even the function of NADH dehydrogenase subunit 2 in the oxidative progress and enhance the adaptability to hypoxia of Tibetan yaks.

Previous studies confirmed the existence of a positive correlation between mitochondrial respiratory function and adaptation to high-altitude. Mitochondrion protein ND1 of HepG2 cells decreased 2.6 times when cultured for six circles (1% oxygen for 24 h and normal 21% oxygen for 24 h) and the ND1 protein of cells cultured at 1% oxygen for 48 h declined 42.9 times relative to HepG2 cells cultured in normal 21% oxygen (Wang et al. 2003). mtDNA encodes 13 core subunits of oxidative phosphorylation (OXPHOS), 2rRNA, and 22tRNAs (Kang et al. 2013), and OXPHOS supplies about 90% of the energy of human body demands, thus mtDNA polymorphisms probably affect the OXPHOS function and consequently they exert a major influence on oxygen utilization and hypoxia adaptation (Wallace et al. 2010).

Complex I, one of the "entry enzymes" of OXPHOS in the mitochondria, catalyzes the transfer of electrons from NADH to coenzyme Q. In complex I, *ND1*, combining with *ND4L* and *ND6*, may serve as a proton pump and have combining sites for coenzyme Q (Baradaran et al. 2013). Two nonsynonymous mutations (G3745A and T4216C) in gene *MT-ND1*, present only in Sherpa-specific lineages, may have an effect on complex I and are candidate variants for mtDNA adaptation to high-altitude environments (Kang et al. 2013). In addition, variants G3745A and T4216C were also considered to be associated with Leber's hereditary optic neuropathy (LHON) (Johns & Berman 1991; Brown et al. 1992) and *MT-ND1* variant T3394C was suggested to be a candidate for adaptation in Tibetan and Indian highlanders (Ji et al. 2012). In addition, a missense mutation in the *MT-ND2* gene showed significant association with production of reactive oxygen species in mitochondria but no significant association with the activity of complex I (Wang et al. 2013). As indicated previously, *MT-ND2* gene plays a key role in iron homeostasis, and increased iron availability will enhance oxygen uptake. Thus, *MT-ND2* is supposed to have a potential effect on oxidative utilization, which is tightly linked with high-altitude adaptation. In short, this study identified *MT-ND1*, and *MT-ND1* as candidate genes associated with adaptation to high-altitude environments. Furthermore, this is the first study that reports a potential

correlation of polymorphisms in the *MT-ND1* and *MT-ND2* genes and adaptation to high-altitude conditions in Tibetan yaks and cattle.

We sequenced the *MT-ND1* and *MT-ND2* genes of 70 Tibetan yaks and 70 Xuanhan cattle, and in order to obtain real differences between yaks and lowland cattle, we downloaded hundreds of cattle and yak sequences from NCBI to complement our sequenced data. We explored the mechanisms of high-altitude adaptation by detecting polymorphisms in genes *MT-ND1* and *MT-ND2* and through differences in haplotype distributions between yaks and cattle. We found that *MT-ND1* haplotypes H2, H3, H4, H6, and H7 and *MT-ND2* haplotypes Ha1, Ha8, Ha10, and Ha11 were positively associated with high-altitude adaptation. Many of the missense mutations in genes *MT-ND1* and *MT-ND2* only occurred in cattle indicating a negative association between these variants and high-altitude adaptation. In particular, mutation m.3638 A > G, present in almost all cattle in this study, yielded a stop codon that likely resulted in the termination of transcription, thus hindering the assembly of NADH dehydrogenase, decreasing the efficiency of oxygen utilization, and limiting cattle's adaptability to high-altitude environmental conditions.

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Disclosure statement

All authors declare to have no conflicts of interest.

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