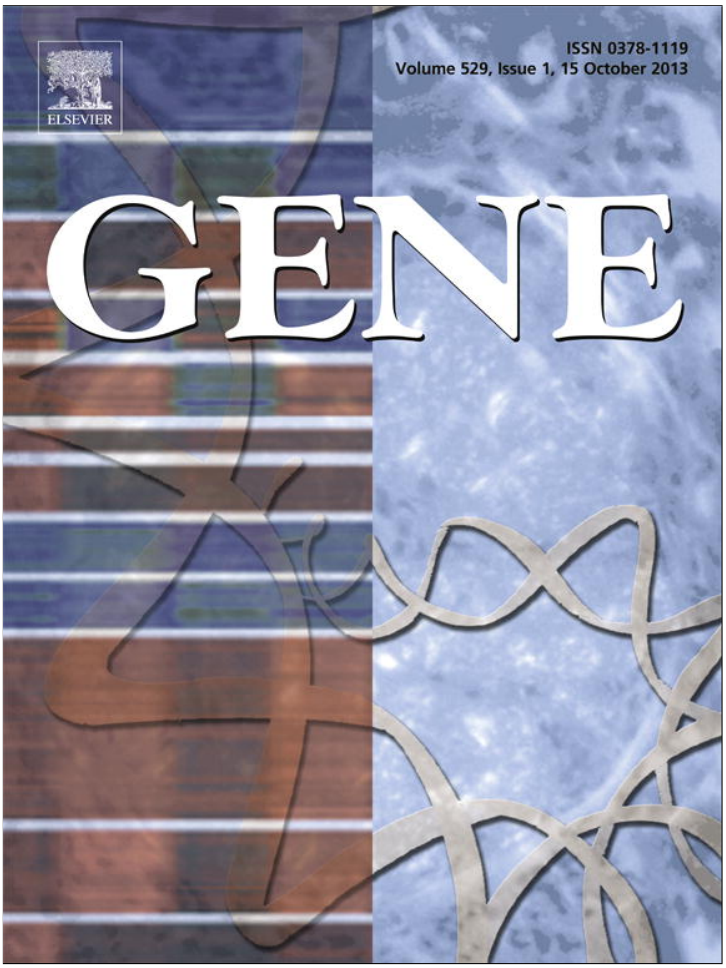

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Short Communication

Association between *MT-CO3* haplotypes and high-altitude adaptation in Tibetan chicken

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ABSTRACT

Genetic mutation in cytochrome c oxidase subunit III gene (*MT-CO3*) could influence the kinetics of cytochrome c oxidase (COX), which catalyzes oxygen transport capacity in oxidative phosphorylation. However, the potential relationship between *MT-CO3* variants and high-altitude adaptation remains poorly understood in Tibetan chicken. Here, we sequenced *MT-CO3* gene of 125 Tibetan chickens and 144 Chinese domestic chickens in areas at a low elevation (below 1000 m). Eight single nucleotide polymorphisms (SNPs) were detected; and five of them (m.10081A>G, m.10115G>A, m.10270G>A, m.10336A>G and m.10447C>T) shared by Tibetan chicken and lowland chicken with the significant difference in their respective allele frequencies. Nine haplotypes (H1–H9) were finally defined. Among them, haplotype H4 was positively associated with high-altitude adaptation whereas haplotypes H6, H7 and H8 had negative association with high-altitude adaptation. The Median-joining profile suggested that haplotype H5 had the ancestral position to the other haplotypes but had no significant relationship with high-altitude adaptation. However, there was only m.10081A>G mutation differed from haplotype H4 and H5. Results also suggested that chickens with A allele at m.10081A>G, had over 2.6 times than those with G allele in the probability of the ability to adapt hypoxia. It suggests that the synonymous mutation m.10081A>G may be a prerequisite for shaping high-altitude adaptation-specific haplotypes.

1. Introduction

Tibetan chicken (*Gallus gallus*), an aboriginal poultry breed in the Qinghai-Tibetan Plateau (QTP), has been completely adapting to the hypoxia environment at the altitude of over 4000 m. Compared with chicken breeds at lower altitudes, Tibetan chicken has unique physiological properties to overcome the environment of harsh extremes, including the increasing number of red blood cell, oxygen affinity in blood and hemoglobin concentration (Zhang et al., 2007), and the decreasing mean corpuscular volume. In addition, Tibetan chicken has 80% of the hatchability (Zhang et al., 2005) in hypoxia condition, which was higher than that of lowland chicken breeds (only 30%) (Zhang et al., 2006). Tibetan chicken is less sensitive to some mountain sickness, including pulmonary hypertension syndrome (PHS) and hypocapnia (Liu et al., 2003; Wideman et al., 2002). But the genetic factors configuring this high-altitude adaptation of Tibetan chicken remain unknown.

Mitochondria afford more than 95% of eukaryotic cell energy by consuming oxygen and producing adenosine triphosphate, which influence the adaptation ability for high-altitude hypoxia condition (Scott et al., 2011; Wang et al., 2011). Cytochrome c oxidase (COX), one of the terminal ingredient of the mitochondrial respiratory chain, plays a vital role in oxidative phosphorylation by reducing the transformation of molecular oxygen into water molecules and storing energy as a proton-pumping across the inner cell membrane (Capaldi, 1990; Ferguson-Miller and Babcock, 1996). It contains 14 protein subunits in mammals and three of them (COX-1, COX-2 and COX-3 enzymes) are synthesized in the mitochondria (Balsa et al., 2012). COX-3 protein, a mitochondrial-coded subunit in COX protein complex that is encoded by the *MT-CO3* gene, plays an important role in the regulation of energy transduction in cytochrome oxidase, specially in the assembly and stability of subunits I and II (Brunori et al., 1987).

Evidence demonstrated that, there was minor difference in *MT-CO1* and *MT-CO2* gene between bar-headed geese and lowland geese, as well as mRNA and protein expression of COX-3 and COX-4, but a nonsynonymous substitution at the site in *MT-CO3* gene that is conserved across vertebrates resulted in a functional amino acid change (Trp-116 → Arg). This mutation was predicted to alter the

Abbreviations: Arg, arginine; ATP, adenosine triphosphate; COX, cytochrome c oxidase; dNTP, deoxyribonucleoside triphosphate; Gly, glycine; MT-CO1, mitochondrially encoded cytochrome c oxidase subunit I; mtDNA, mitochondrial DNA; mRNA, messenger RNA; NADH, reduced form of nicotinamide-adenine dinucleotide; *ND3*, NADH dehydrogenase subunit 3 gene; SNP, single nucleotide polymorphism; Trp, tryptophan; tRNA, transfer RNA; ′ (prime), denotes a truncated gene at the indicated side.

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interaction between COX-3 and COX-1, which contributed to adaptation in mitochondrial enzyme kinetics and O2 transport capacity and may finally contribute to the ability of bar-headed geese to fly at high altitude effect (Scott et al., 2011). Tibetan chicken is another bird that lives in high altitude and has adapted itself well to hypoxia. A valuable study of Bao et al. (2008a) compared the whole mtDNA genome of 18 Tibetan chickens and two lowland chicken populations (14 Silkies and 13 Shouguang chickens), which showed that a unique nonsynonymous mutation (m.10065T>C) of *MT-CO3* gene was present in Tibetan chickens but no missense mutation specific to Tibetan chicken has been detected in *MT-CO1* gene and *MT-CO2* gene. Therefore, we guess that *MT-CO3* gene may be a hot candidate gene for studying high-altitude adaptation of Tibetan chicken.

A missense mutation (m.10115G>A) in *MT-CO3* gene of Tibetan chicken resulted in an amino acid substitution (p.V62I) (Tong et al., 2006). But two following reports from Bao and his research group caught our attention. They found two other SNPs (m.10270G>A and m.10447C>T) that shared by Tibetan chicken and lowland chickens, and a unique missense mutation (m.10065T>C) of *MT-CO3* gene in Tibetan chickens that caused the replacement of amino acid methionine into threonine although with a low frequency (less than 1%) (Bao et al., 2008a). At the same year, only single nucleotide polymorphism (SNP) of m.10081A>G was shown in *MT-CO3* gene that was shared by expanded samples of 56 Tibetan chickens and 152 lowland chickens with different frequency in distribution ($P < 0.0001$) (Bao et al., 2008b). Although no information of precise locality for collecting Tibetan chicken samples was shown in these two studies of Bao and his colleagues, the above disputed results seems to be associated with the sampling size of Tibetan chicken. Therefore in the present study, we increased the numbers of Tibetan chickens and lowland chicken breeds, to investigate the specific SNPs in *MT-CO3* gene, and discuss their possible contribution for high-altitude adaptation of Tibetan chicken.

2. Materials and methods

2.1. Sampling and DNA extraction

In total, 125 purebred Tibetan chickens were collected from the local conservation farms in Daocheng, Xiangcheng, Batang Counties in Garzi Tibetan Autonomous Prefecture, Sichuan Province, China; and three Chinese native chicken breeds (Bairong Silkie, Xinghua and Yanxia) and one strain (E1 Dwarf strain) that were collected from coastal lowland (below 1000 m); and four Chinese chicken populations (Sichuan Mountain Black-bone, Jiuyuan, Chahua and Junlian strain) were sampled from inland lowland areas (below 1000 m) (the details are shown in Table 1). Only 1 mm blood of each individual was collected from the vein of animal underwing. No animal was slaughtered or injured unexpectedly in the process of catching birds. The protocol was approved by the Committee on the Care and Use of Laboratory Animals of the State-level Animal Experimental Teaching Demonstration Center of Sichuan Agricultural University (Approval ID: Decree No. 20 [2003]).

2.2. DNA extraction, amplification and sequencing

We extracted mtDNA by salt-extraction method (Miller et al., 1988); and the overall length of *MT-CO3* gene was amplified using the known primer pairs F9797: 5′-ACCAATAATACCATCAATCTCC-3′ and R10830: 5′-CGCTTAGTAGAAAGGATAGTGAG-3′ (Bao et al., 2008a), which were used to amplify ≈ 1034 bp length of mtDNA sequences that covered a fragment of ATP6 gene (≈136 bp), the whole length of *MT-CO3* gene region (784 bp), and a 68 bp tRNA-Gly sequence as well as a fragment of ND3 gene (≈46 bp). The position of primer pairs was determined according to the complete mitochondrial genome sequence of Cochin-Chinese Red Jungle Fowl (*Gallus gallus gallus*, GenBank accession number: NC_007236). PCR was carried out in a volume of 50 μL containing 100–150 ng DNA template, 5 μL 10× buffer, 25 mM $MgCl_2$, 2.5 mM of dNTP mixture, 2 mM of each primer, 1.25 U *Taq* Polymerase (Takara, Dalian, China) and was amplified by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C, preceded by a 5 min incipient denaturation at 94 °C and followed by a 7 min final extension at 72 °C. PCR products were detected by agarose gel (1.5%) electrophoresis and purified by TIANgel Midi Purification Kit (product ID: DP209-02). And the final purified PCR products were directly sequenced in both directions with Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Calif, USA) on the ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, USA) according to the manufacturer's instruction.

2.3. Sequence date analysis

The unripe sequences were edited and aligned by DNAstar Software (DNAstar Inc. Madison, WI, USA). Sequence variations, containing nucleotide composition and variable sites, were identified using MEGA 5 (Tamura et al., 2011). The complete mitochondrial genome sequence of Cochin-Chinese Red Jungle Fowl (*G. g. gallus*, GenBank accession number: NC_007236) was used as reference sequence for determining the variable sites of chicken *MT-CO3* sequences. We

Table 1
Sample information used in this study.

Population	Number of samples	Place or Habitat (elevation)	Group by elevation	Haplotype distribution (number of birds)
Tibetan chicken[a]	125	Conservation farms, Garzi Tibetan Autonomous Prefecture, Sichuan province (≈3100 m)	Highland chicken breed, with high-altitude adaptation (TC group)	H1 (2), H2 (42), H3 (6), H4 (49), H5 (5), H6 (2), H7 (12), H8 (5), H9(2)
Bairong Silkie[a]	28	Fujian province (800–900 m)	Lowland chicken breed, without high-altitude adaptation (LC group)	H5 (1), H6 (15), H7 (2), H8 (10)
E1 Dwarf strain[b]	27	Institute of Animal Science Guangdong Academy of Agricultural Science, Guangzhou City, Guangdong (≈110 m)		H4 (9), H7 (11), H8 (7)
Junlian strain[a]	23	Junlian County, Yibin City, Sichuan (719–931 m)		H4 (9), H7 (1), H8 (12), H9 (1)
Jiuyuan[a]	24	Conservation farm, Wangyuan City, Sichuan Province (726–786 m)		H4 (3), H5 (2), H6 (1), H7 (5), H8 (13)
Sichuan mountainous black-boned[a]	15	Sichuan Agriculture University Poultry Breeding Farm, Sichuan Province (≈600 m)		H4 (6), H5 (1), H7 (1), H8 (7)
Chahua[a]	14	Xishuangbanna City, Dai Autonomous Prefecture, Yunnan Province (700–900 m)		H4 (1), H6 (2), H7 (3), H8 (6), H9 (2)
Xinghua[b]	9	Fengkai County, Guangdong province, China (≈900 m)		H6 (2), H7 (6), H8 (1)
Xiayan[b]	4	Rong County, Guangxi Zhuang Autonomous Prefecture, Guangxi Province (≈998 m)		H4 (1), H5 (1), H7 (2)

[a] Samples were obtained and identified by Sichuan Agriculture University.
[b] Samples were supplied by Institute of Animal Science Guangdong Academy of Agricultural Science.

exported all sequences as an aligned FASTA file, and haplotype was defined using DnaSP V5 (Librado and Rozas, 2009). A median-joining network analysis was performed by using program Network 4.611 (http://www.fluxus-engineering.com/sharenet.htm).

2.4. Statistical analysis

We regarded Tibetan chickens as a group (with high-altitude adaptation, TC) and all lowland chickens as a whole group (without high-altitude adaptation, LC). Statistical difference in the haplotype frequencies of *MT-CO3* gene in TC and LC chickens were analyzed using Fisher's exact test, odds ratio (OR) and 95% confidence intervals (95% CIs) were also calculated. *P*-value < 0.05 was regarded as statistical significance. MitoTool (http://www.mitotool.org/), an online bioinformatics platform, was used for analyzing haplotype distribution frequency between TC group and LC group (Fan and Yao, 2011).

2.5. Protein secondary structure prediction

TMpred program was used for analysis of hydrophobicity and secondary structure changes of coding sequence caused by the variants of *MT-CO3* gene (http://www.ch.embnet.org/software/TMPRED_form.html). The complete mitochondrial genome of Cochin-Chinese Red Jungle Fowl (*G. g. gallus*) was used as the reference sequence (GenBank accession number: NC_007236).

3. Results

3.1. Sequence variations in MT-CO3 gene

The whole length of 269 *MT-CO3* sequences were truncated into 784 bp (GenBank accession numbers: KC847707–KC847975; no insertion/deletions were detected). A total of 8 single-nucleotide polymorphisms (SNPs) were found in the *MT-CO3* gene from 9 chicken breeds (Table 2). Table 3 contained the observed allele frequencies in each polymorphic site between Tibetan chicken and 8 lowland chicken breeds. There were six synonymous mutations (m.10081A>G, m.10270G>A, m.10336A>G, m.10369G>A, m.10447C>T and m.10669C>T) and 2 nonsynonymous substitutions (m.10115G>A and m.10370G>A). Three SNPs in *MT-CO3* gene (m.10115G>A, m.10336A>G and m.10370G>A) were unique to Tibetan chicken with the allele frequency of 35.2%, 4.8% and 1.6%, respectively. The other 5 SNPs (m.10081A>G, m.10270G>A, m.10369G>A, m.10447C>T and m.10669C>T) were shared between Tibetan chickens and Lowland chickens. Using Pearson chi-square test, except the SNP of m.10369G>A ($P = 0.76973$), the other four SNPs whose allele frequencies were significantly different between Tibetan chicken and any lowland chicken breed (the detailed *P* values are shown in Table 3).

Table 3
Distribution of SNPs in *MT-CO3* gene in Tibetan chickens and lowland chickens.

SNP sites	Allele distribution			*P*-value in Pearson chi-square test
	Allele	TC (%)[a]	LC (%)[b]	
10081	A	26 (20.8%)	115 (79.9%)	3.894×10^{-22}
	G	99 (79.2%)	29 (20.1%)	
10115	G	81 (64.8%)	144 (100%)	6.922×10^{-15}
	A	44 (35.2%)	0 (0.0%)	
10270	G	111 (88.8%)	93 (64.6%)	3.699×10^{-6}
	A	14 (11.2%)	51 (35.4%)	
10336	A	119 (95.2%)	144 (100%)	0.00784
	G	6 (4.8%)	0 (0)	
10369	G	123 (85.4%)	141 (97.9%)	0.76973
	A	2 (14.6%)	3 (2.1%)	
10370	G	123 (98.4%)	144 (100%)	0.12762
	A	2 (1.6%)	0 (0.0%)	
10447	C	118 (94.4%)	57 (39.6%)	5.922×10^{-21}
	T	7 (5.6%)	87 (60.4%)	
10669	C	113 (90.4%)	113 (78.5%)	0.00776
	T	12 (9.6%)	31 (21.5%)	

Relative content in parentheses meant the number of birds in corresponding allele in the specific polymorphic site.
[a] TC was the abbreviations for Tibetan chicken.
[b] LC was the abbreviations for lowland chicken.

3.2. Haplotype analysis in MT-CO3 gene

Nine haplotypes (H1 to H9) were found in 269 chickens, including 219 Chinese native chickens and two strains (27 E1 Dwarf and 23 Junlian chickens). Haplotype H1 was found in 2 Tibetan chickens. Haplotype H2 consisted of 42 Tibetan chickens and haplotype H3 was only found in 6 Tibetan chickens. Haplotype H4, the largest shared haplotype, consisted of 2 strains of 9 E1 Dwarf and 9 Junlian chickens, and 60 Chinese native chickens, covering 5 native chicken breeds (including 49 Tibetan chickens). Haplotype H5 consisted of 5 Tibetan chickens, 1 Bairong Silkie chicken, 2 Jiuyuan chickens, 1 Sichuan Mountain Black-bone chicken and 1 Xiayan chicken, covering 5 Chinese native breeds. Haplotype H6 consisted of 22 Chinese native chickens, including 2 Tibetan chickens, 15 Bairong Silkie chickens, 1 Jiuyuan chicken, 2 Chahua chickens and 2 Xinghua chickens. Haplotype H7 was found in 12 Tibetan chickens, 2 Bairong Silkie chickens, 5 Jiuyuan chickens, 1 Sichuan mountainous black-boned chicken, 11 E1 Dwarf chickens and 1 Junlian chicken. Haplotype H8 consisted of

Table 2
Sequence variations in the *MT-CO3* gene of chickens and the corresponding haplotype distribution.

Haplotype	Position of variable sites in *MT-CO3* gene[a]								Chicken group[b]		Number of birds
	10081	10115	10270	10336	10369	10370	10447	10669	TC	LC	
Reference sequence	A	G	G	A	G	G	C	C			
H1	G	A	.	.	.	A	.	.	2 (1.6%)	–	2
H2	G	A	.	.	.	.	.	.	42 (33.6%)	–	42
H3	G	.	.	G	.	.	.	.	6 (4.8%)	–	6
H4	G	.	.	.	.	.	.	.	49 (39.2%)	29 (20.1%)	78
H5	.	.	.	.	.	.	.	.	5 (4.0%)	5 (3.5%)	10
H6	.	.	A				.	.	2 (1.6%)	20 (13.9%)	22
H7	.	.	A				.	T	12 (9.6%)	31 (21.5%)	43
H8							T	.	5 (4.0%)	56 (38.9%)	61
H9					A	.	T	.	2 (1.6%)	3 (2.1%)	5
AA Subst.		*Val62Ile*				*Ala147Thr*					

Dot (.) denoted the same single nucleotide to the reference sequence; Dashes (–) represented the absence of certain haplotype in the population; Nonsynonymous substitutes were marked in square frames; amino acid substitutions (AA Subt.) were listed below the nucleotide information and marked in italic.
[a] Position of variable sites in *MT-CO3* gene of chickens was obtain using the sequence of Cochin-Chinese Red Jungle Fowl mtDNA genome as the reference sequence (*G. g. gallus*, GenBank accession number: NC_007236).
[b] TC and LC were the abbreviation names for highland chicken (Tibetan chicken) and lowland chicken, respectively.

5 Tibetan chickens, 10 Bairong Silkie chickens, 13 Jiuyuan chickens, 7 Sichuan Mountain Black-bone chickens, 6 Chahua chickens, 1 Xinghua, 7 E1 Dwarf chickens and 12 Junlian chickens; and haplotype H9 consisted of 2 Tibetan chickens, 1 Junlian chickens and 2 Chahua chickens.

3.3. Median-joining network of haplotypes

The median-joining network chart (Fig. 1) was constructed using the 9 haplotypes. Haplotypes H1, H2 and H3 were restricted to Tibetan chickens, which were derived from haplotype H4. Haplotypes H5, H6, H7, H8 and H9 were scattered in Tibetan chickens and lowland chicken populations where haplotype H5 had the ancestral position to the other haplotypes.

3.4. Association between haplotype distribution and high-altitude adaptation

According to the altitude of sampling sites, 6 Chinese native chicken breeds (except for Tibetan chicken) and 2 strains belonged to lowland chickens. Haplotypes H4 to H9 were shared by 75 Tibetan chickens, 28 Bairong Silkie chickens, 24 Jiuyuan chicken, 15 Sichuan mountainous black-boned chicken, 14 Chahua chickens, 9 Xinghua chickens, 4 Xiayan chickens, 27 E1 Dwarf chickens and 23 Junlian chickens (Table 1). There was obvious difference in haplotype distribution between Tibetan chickens and lowland chickens (Table 2). Haplotypes H1 to H3 were unique in 50 Tibetan chickens, which occupied 40% of all Tibetan Chicken samples (50/125 = 40.00%) whereas 144 Lowland chickens were scattered in haplotypes H4 to H9 that shared with 75 Tibetan chickens. After examining the level of significance by Bonferroni correction, we found that 4 haplotypes significantly associated with high-altitude adaptation at the 0.05 level (Table 4). Specially, haplotype H4 was positively associated with high-altitude adaptation (adjusted *P*-value, 0.00046; OR, 2.626, 95% CI, 1.524–4.525), whereas H6, H7 and H8 showed a negative correlation with high-altitude adaptation (H6: adjusted *P*-value, 0.0016; OR, 0.102; 95% CI, 0.023–0.448; H7: adjusted *P*-value, 0.01172; OR, 0.394; 95% CI, 0.193–0.806; H8: adjusted *P*-value, 8.87×10^{-13}; OR, 0.067; 95% CI, 0.026–0.173).

3.5. Prediction of the secondary structure changes in MT-CO3 protein

We found that two Tibetan chicken specific-non-synonymous *MT-CO3* variants m.10115G>A and m.10370G>A were located along the outer surfaces of the inner mitochondrial membrane, but the reported SNP m.10065T>C (Bao et al., 2008a) was located in the transmembrane helical structure (Fig. 2). As shown in Fig. 3, the V62I changed caused by variant m.10115G>A and the amino acid change A147T caused by m.10370G>A did not change the hydrophobicity of the COX-3 protein. But the M45T change caused by a known mutation m.10065T>C that was exclusive to Tibetan chicken (Bao et al., 2008a) decreased the hydrophobicity of the COX-3 protein.

4. Discussion

An amino acid replacement at key sites usually alters protein function (Golding and Dean, 1998). A recent finding suggested that a nonsynonymous substitution in *MT-CO3* gene resulted in an amino acid change (Trp-116 → Arg) and may contribute to the ability of bar-headed geese to fly at high altitude effect (Scott et al., 2011). The Tibetan chicken, a Chinese native chicken breed, adapts itself well to the low pressure of high altitude in the plateau environment. But no greatness hitherto in the research of genetic mechanisms on hypoxia adaptation of Tibetan chicken has yet been made.

So far, the association between mitochondrial respiratory function and the adaptation to hypoxia in Tibetan chicken has been demonstrated (Bao et al., 2007). MT-CO3 protein, a mitochondrial-coded subunit in COX, plays an important role in the regulation of energy

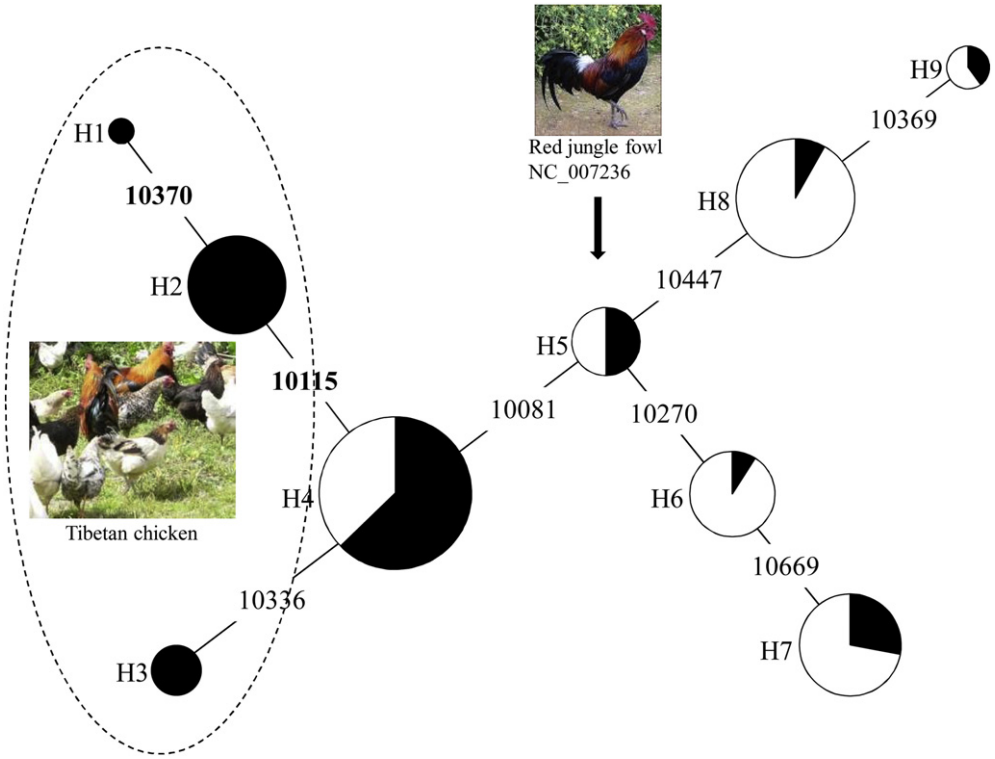

Fig. 1. Median-joining network of *MT-CO3* haplotypes. Tibetan chicken and lowland chicken were represented with black and white, respectively. The circle area represented haplotype frequency. The links are labeled by nucleotide positions to designed substitutes. Nonsynonymous mutations are also marked in bold type. Area in dashed circle indicated the haplotypes unique to Tibetan chicken.

Table 4
Effect of *MT-CO3* haplotypes on high-altitude adaptation.

Haplotype	Number (TC)[a]	Number (LC)[b]	*P*-value	Adjusted *P*-value[c]	OR	95% CIs
H2	42	0	7.710×10^{-17}	6.168×10^{-16}	–	–
H3	6	0	0.00894	0.07152	–	–
H4	49	29	0.00046	0.00046	2.626	1.524–4.525
H5	5	5	1.000	1.000	1.178	0.333–4.168
H6	2	20	0.0002	0.0016	0.102	0.023–0.448
H7	12	31	0.01172	0.01172	0.394	0.193–0.806
H8	5	56	8.87×10^{-13}	8.87×10^{-13}	0.067	0.026–0.173
H9	2	3	1.000	8.000	0.777	0.128–4.726
Total	123	144				

OR: odds ratio, CIs: confidence intervals.
Two tailed Fisher's exact statistical testing was carried out on the basis of the original data in Table 2.
Haplotype which the sample size under 5 was not considered here.
Adjustment of *P*-value was calculated by Bonferroni correction in the corresponding group that contained no more than 5 chickens.
[a] Number (TC) meant the number of Tibetan chicken (with high-altitude adaptation).
[b] Number (LC) meant the number of lowland chickens (without high-altitude adaptation).
[c] Adjustment of *P*-value was calculated by Bonferroni correction in the corresponding group that contained no more than 5 chickens.

transduction in cytochrome oxidase, specially in the assembly and stability of subunits I and II (Brunori et al., 1987). Bao et al. (2008a) identified three SNPs m.10081A>G, m.10270G>A and m.10447C>T of *MT-CO3* gene shared by 18 Tibetan chickens and 27 lowland chickens (14 Silkies and 13 Shouguang chickens), however, only one SNP (m.10081A>G) in *MT-CO3* gene was shared by an expanded samples of 56 Tibetan chickens and 152 lowland birds, and the allele frequency of coding variant 10081A was significantly higher in 47 Tibetan chickens than that in four lowland chicken breeds ($P < 0.0001$) (Bao et al., 2008b). Accordingly, in the present study, we increased the sampling number of Tibetan chickens ($n = 125$) and successfully identified 3 SNPs in *MT-CO3* gene of 52 Tibetan chickens, indicating that *MT-CO3* gene may be a mutation hotspot relevant to high-altitude adaptation of Tibetan chicken. At SNP loci 10081, Tibetan chicken harbored a high frequency of G allele (79.2%, 99/125) whereas a high frequency of A allele was present in lowland chickens (80.6%, 116/144), which was contrast to the previous report (Bao et al., 2008b) whereas the frequency of A allele in highland chickens is 83.9%. As is recorded (Wu and Li, 2012), Tibetan chickens are distributed into four geographical areas including Tibet Autonomous Region, Garze prefecture in Sichuan Province, Zhongdian County in Yunnan Province and Gansu province, at an average elevation from 2200 m to 4100 m. No information about the collection sites was present in the two studies of Bao's research group, and 125 Tibetan chickens used in this study were collected from Daocheng, Xiangcheng, Batang Counties in Garze prefecture, Sichuan Province, at the elevation of over 3100 m. Besides, we also expanded the collection of lowland chicken breeds in different altitude regions from the east coast to inland China, including two breeds on coastal land (27 E1 Dwarf strain and 28 Bairong Silkies) and six breeds in inland areas (23 Junlian strain, 24 Jiuyuan, 15 Sichuan mountainous black-boned, 14 Chahua, 9 Xinghua and 4 Xiayan chickens). Thus, we guess that different areas for collecting chickens may be one of important factors that ultimately contributed to our survey results of SNPs distribution in *MT-CO3* gene, unlike the above previous studies.

The known SNP (m.10065T>C) that was exclusive to Tibetan chicken (Bao et al., 2008a) was not detected in 125 Tibetan chicken samples, suggesting it may be a missense mutation in the MT-CO3 gene restricted to the reported local chicken population. Using the Tmpred program, this variant changed the amino acid M45I on the second transmembrane helical structure and decreased the hydrophobicity of the COX-3 protein (Figs. 2 and 3). Although the SNP of m.10115G>A was

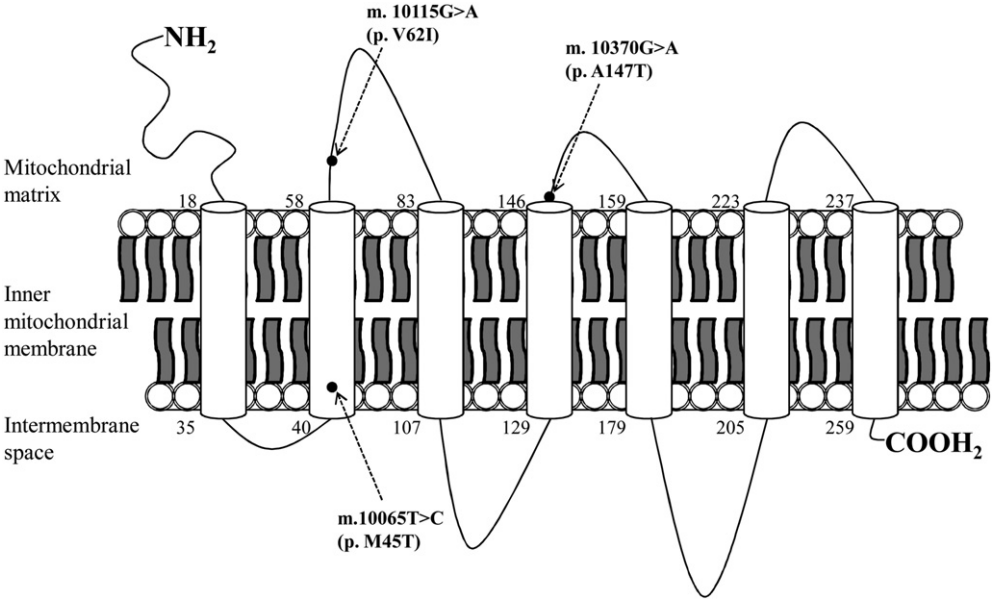

Fig. 2. Diagram of transmembrane structure of COX-3 protein predicted using the Tmpred program. The transmembrane structure of coding sequence by two newfound nonsynonymous mutations (m.10115G>A and m.10370G>A) and a known SNP (m.10065T>C) were predicted. The respective amino acid substitute are shown in the parentheses. Inserted cylinders represent transmembrane helices.

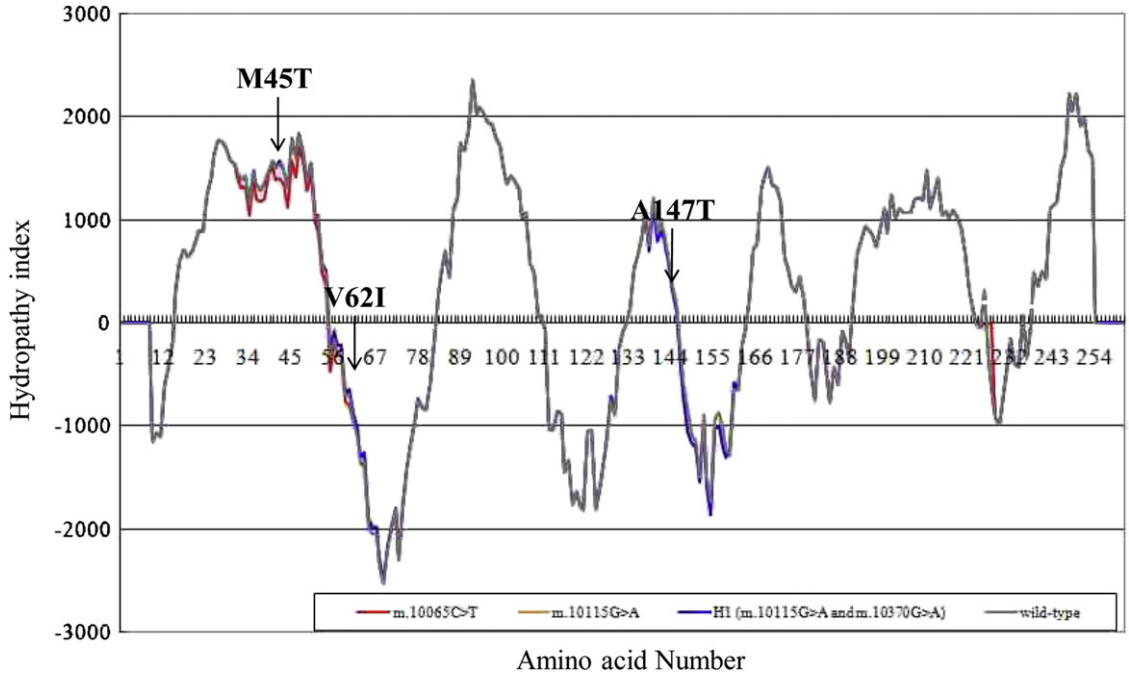

Fig. 3. Hydropathy plot for the COX-3 protein predicted using the Tmpred program. The *x*-axis represented the amino acid residues in the sequence, and the *y*-axis indicates the hydropathy index. Negative values indicated hydrophilic characteristics, and positive values indicated hydrophobic characteristics. We compared the hydrophobicity of haplotypes with different amino acid substitutes and that of Cochin-Chinese Red Jungle Fowl (*G. g. gallus*; GenBank accession number: NC_007236). The irregular curve in red meant the hydrophobic profile of coding sequence with mutation m.10065T>C; the irregular curve in yellow was the hydrophobic profile of coding sequence with mutation m.10115G>A; the irregular curve in blue was the hydrophobic profile of coding sequence with mutations m.10115G>A and m.10370G>A; the irregular curve in gray was the hydrophobic profile of coding sequence in the Red Jungle Fowl (*G. g. gallus*, wild type).

identified only in a Tibetan chicken mtDNA genome as an uncommon missense mutation (Tong et al., 2006), more than one third Tibetan chickens (44/125, 35.2%) harbored this mutation in the present study. It was suggested as a unique nucleotide variant in Tibetan chicken and may relate to adaptation to hypoxia environment.

Using Fisher's exact test, it showed the positive association between haplotype H4 and high-altitude adaptation (adjusted *P*-value, 0.00046; OR, 2.626; 95% CI, 1.524–4.525), suggesting that the probability of the ability to adapt hypoxia for chickens with G allele at G10081A polymorphic site of *MT-CO3* gene was over 2.6 times than that for chickens with A allele. But haplotypes H6, H7 and H8 indicated the negative associations with high-altitude adaptation, suggesting that the ability to adapt hypoxia for chickens with any of the three mutations (m.10447C>T, m.10270G>A and m.10669C>T) might be suppressed.

Five Tibetan chickens and 5 lowland chicken samples were distributed in haplotype H5 with the reference sequence of Cochin-Chinese Red Jungle Fowl (*G. g. gallus*; NC_007236). Based on the Median-joining profile of haplotypes distribution of *MT-CO3* gene, Haplotype H5 had the ancestral position to the other haplotypes but had no significant relationship with high-altitude adaptation (adjusted *P* value = 1.000; OR = 1.178; 95% CI, 0.333–4.168). However when adenine was replaced by guanine at the polymorphic site 10081, it resulted in the occurrence of haplotype H4, in which the individuals were provided the possibility of high-altitude adaptation. Then three haplotypes H1, H2, H3 unique to Tibetan chickens were produced through 2 nonsynonymous substitutions (m.10115G>A and m.10370G>A) and 1 synonymous mutation (m.10336A>G) (Fig. 1.). It suggested that the synonymous mutation m.10081A>G might be a prerequisite for shaping high-altitude adaptation-specific haplotypes. Although the synonymous SNP did not change coding sequence, it may affect the timing of contranslational folding, thereby altering the substrate specificity of MDR1 protein (Kimchi-Sarfaty et al., 2007). Therefore, we suggested that the "silent" polymorphism (m.10081A>G) in the *MT-CO3* gene may affect the specific functions of COX enzyme relating to high-altitude adaptation of Tibetan chicken.

Conflict of interest statement

All authors have no declared conflict of interest.

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