RESEARCH ARTICLE

# Phylogenetic analysis of Tibetan mastiffs based on mitochondrial hypervariable region I

ZHANJUN REN*, HUILING CHEN, XUEJIAO YANG and CHENGDONG ZHANG

*College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, People's Republic of China*

## Abstract

Recently, the number of Tibetan mastiffs, which is a precious germplasm resource and cultural heritage, is decreasing sharply. Therefore, the genetic diversity of Tibetan mastiffs needs to be studied to clarify its phylogenetics relationships and lay the foundation for resource protection, rational development and utilization of Tibetan mastiffs. We sequenced hypervariable region I of mitochondrial DNA (mtDNA) of 110 individuals from Tibet region and Gansu province. A total of 12 polymorphic sites were identified which defined eight haplotypes of which H4 and H8 were unique to Tibetan population with H8 being identified first. The haplotype diversity (Hd: 0.808), nucleotide diversity (Pi: 0.603%), the average number of nucleotide difference (K: 3.917) of Tibetan mastiffs from Gansu were higher than those from Tibet region (Hd: 0.794; Pi: 0.589%; K: 3.831), which revealed higher genetic diversity in Gansu. In terms of total population, the genetic variation was low. The median-joining network and phylogenetic tree based on the mtDNA hypervariable region I showed that Tibetan mastiffs originated from grey wolves, as the other domestic dogs and had different history of maternal origin. The mismatch distribution analysis and neutrality tests indicated that Tibetan mastiffs were in genetic equilibrium or in a population decline.

[Ren Z., Chen H., Yang X. and Zhang C. 2017 Phylogenetic analysis of Tibetan mastiffs based on mitochondrial hypervariable region I. *J. Genet.* **96**, xx–xx]

## Introduction

Tibetan mastiffs are a species unique to China and native to Tibetan plateau. They are characterized by lion and tiger head types. Tibetan mastiffs have close relationship with humans in guarding house, accompanying and herding. However, along with the nomadic lifestyle changes, excellent Tibetan mastiffs have disappeared from the plateau and the genetic diversity has dropped drastically. In addition, due to weak awareness of protecting bloodline, original Tibetan mastiffs have interbreed with local Shepherd Dog, which has resulted in a great decrease in the purebred. Meanwhile, due to nonTibetan residents' enthusiasm for Tibetan mastiffs and the huge profits, the outflows of a large number of high quality Tibetan mastiffs have caused rapid loss of germplasm resources from the plateau. At present, how to maintain and increase the genetic diversity of Tibetan mastiffs is one of the important tasks in preservation of germplasm resources. Therefore, to provide reference values for resource protection, rational development and utilization of Tibetan mastiffs, there is a need for studies on genetic structure and phylogenetic relationship.

Mitochondrial DNA (mtDNA) is informative for indicating genetic diversity and phylogenetic analysis of animals because of its specific characteristics, such as small molecular weight, high variability and maternal transmission (Long *et al.* 2003; Achilli *et al.* 2012; Dadi *et al.* 2012; Imes *et al.* 2012; Melo-Ferreira *et al.* 2012). Because of length variation and little selective pressure, D-loop sequence accumulates much more mutations than the rest of the molecule and provides extensive polymorphism information, making it a useful tool for studying short-term evolutionary phenomena. Further, D-loop sequence consisted of conservatively intermediate sequence and two hypervariable regions (HVRI and HVRII). The functional region in HVRII is responsible for mtDNA replication and translation, thus limited mutations are present in HVRII. Compared with HVRII, higher mutation rate makes HVRI preferred to study molecular evolution of animals (Meyer *et al.* 1999; Picornell *et al.* 2005; Santos *et al.* 2008; Rout *et al.* 2012; Theyab *et al.* 2012). Recently, analysis of origin of dog by sequencing mtDNA has been reported. The previous studies indicated that dogs were

---

*For correspondence. E-mail: renzhanjun@nwsuaf.edu.cn.

**Keywords.** Tibetan mastiffs; hypervariable region; genetic diversity; mitochondrial DNA.

**Journal of Genetics**, DOI 10.1007/s12041-017-0753-3

domesticated and originated from east Asia (Savolainen *et al*. 2002; Pang *et al*. 2009). Based on 582 bp of mtDNA, Southeast Asia displayed the most genetic diversity, revealing the original centre of wolf domestication (Ardalan *et al.* 2011). Analysis of 582 bp of mtDNA control region in Australian dingoes showed dingoes stemmed from a dog population which came from east Asia (Savolainen *et al.* 2004). Maternal origin of Tibetan mastiffs has been also studied. For example, high genetic diversity were observed in Tibetan mastiff by sequencing 582 bp of D-Loop, supporting that it was an ancient oriental breed derived from east Asia (Li and Zhang 2012). However, study on evolutionary relationship and maternal origin of the Tibetan mastiffs in different geographical regions have not been reported.

Therefore, to reveal phylogenetic relationship of Tibetan mastiffs in different geographical regions, we collected 110 samples from Tibet and Gansu, and analysed genetic diversity and phylogenetic evolution of two populations by sequencing mitochondrial HVRI. Analysis of genetic diversity indices and phylogenetic relationship showed low genetic variation and different history of maternal origin, which laid a basis on the protection and rational utilization of Tibetan mastiffs.

## Materials and methods

### *Sample collection and DNA extraction*

Blood samples were collected from the following populations: 48 samples from Wuwei city, Gansu province, and 62 samples from Lhasa and surrounding areas in Tibet region. Total DNA was extracted by BloodGen Mini Kit, and the quality of DNA was examined by Nanodrop 2000 after electrophoresis in 1.0% agarose.

### *PCR amplification and sequencing*

To amplify HVRI, the primer pair (5′-CTCTTGCTCCACCA TCAGC-3′ and 5′-AAACTATATGTCCTGAAACC-3′) was designed by Premier 5.0 software. PCR reactions were performed in a 40 $\mu$L total volume containing 20 $\mu$L 2× EsTaq MasterMix, 1 $\mu$L each primer, 2 $\mu$L genomic DNA, and 16 $\mu$L RNase-Free Water. The thermal cycling programme consisted of predenaturation at 95°C for 5 min, 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 40 s, followed by a final extension at 72°C for 10 min. PCR amplification products were visualized by 1.5% agarose gels and then sequenced.

### *Phylogenetic analysis*

MtDNA sequences were viewed by Chromas, and edited by DNAStar 7.1, excluding inaccurate bases. The number of polymorphic sites, number of singleton variable sites, number of parsimony informative sites and genetic distances between haplotypes were determined by MEGA 5.05 software (Tamura *et al.* 2011). In the present study, published sequences from 25 domestic dogs, four gray wolves, two Tibetan wolves and three coyotes and eight Tibetan mastiff haplotypes were used for comparison. Phylogenetic trees were constructed by neighbour-joining (NJ) and maximum parsimony (MP) methods of MEGA 5.05 software using the Kimura 2-parameter model with 1000 bootstrapping replicates. In addition, median-joining network between eight haplotypes was constructed using Network 4.6.1.2 (Bandelt *et al.* 1999) as well as that between the haplotypes of Tibetan mastiffs from the present study and the previous result (Li and Zhang 2012).

The following indices were calculated by DnaSP 5.10 (Librado and Rozas 2009) to estimate the genetic diversity of the mtDNA data: number of haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi), average number of nucleotide differences ($K$) and genetic variation coefficient ($F_{\mathrm{st}}$). In addition, neutral test was performed and demographic expansions (mismatch distribution) was drawn.

## Results

### *Sequence variation of HVRI*

The sequence alignment among 110 mtDNA sequences revealed that one indel was observed, and 12 sites which accounted for 1.85% of the analysed sites were polymorphic, with zero singleton variable site and 12 parsimony informative sites. One transversion and 11 transitions were found, which showed transitions were significantly more than transversions (figure 1).

### *Genetic structure of population*

Eight haplotypes were identified among 110 samples (haplotype sequences were deposited under accession numbers KJ934223–KJ934230), and H2 and H6 were dominant haplotypes, accounted for 55.45%. The most popular H2 and H6 consisted of 31 and 30 samples from two populations, respectively, followed by two haplotypes (H1 and H3) consisting of ≥10 samples. The haplotypes H4, H5, H7 and H8 were less popular. Genetic distance among eight haplotypes ranged from 0.002 to 0.014, and the average genetic distance was 0.007 according to the model of Kimura-2-parameter. Gansu and Tibetan populations possessed similar Hd. In terms of total population, although the number of Tibetan mastiff haplotypes from Gansu was lower than that from Tibet region, genetic diversity indices of Gansu population were slightly higher than Tibetan population (table 1).

### *Phylogenetic analysis*

The distance between different haplogroups from Tibetan mastiffs, domestic dogs and wolves revealed significantly less genetic distance between Tibetan mastiffs and gray wolves than between Tibetan mastiffs and Tibetan wolves as well as coyotes (table 2).

The MJ network for HVRI haplotypes showed the distribution of haplotypes in two populations and evolutionary

| Haplotypes | Polymorphic sites | | | | | | | | | | | | Number of Gansu population | Number of Tibetan population |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H1 | T | T | G | T | A | C | A | G | C | A | T | A | 10 | 7 |
| H2 | . | . | A | C | G | T | . | A | . | . | . | . | 12 | 19 |
| H3 | . | . | A | . | . | . | . | . | T | . | C | . | 10 | 4 |
| H4 | . | . | A | . | . | . | T | . | T | . | . | . | 0 | 6 |
| H5 | . | . | A | . | . | . | . | A | T | . | . | . | 1 | 3 |
| H6 | C | C | A | C | G | T | . | A | . | . | . | . | 11 | 19 |
| H7 | . | . | A | C | G | T | . | A | . | G | . | . | 4 | 1 |
| H8 | . | . | A | . | . | . | . | . | T | . | C | G | 0 | 3 |

**Figure 1.** Sequence variations in HVRI detected in eight haplotypes of Tibetan mastiffs, '.' represents matched bases.

**Table 1.** Genetic diversity of Tibetan mastiffs based on HVRI.

| Population | Number of sequences | Haplotype | Hd | Pi/% | K |
|---|---|---|---|---|---|
| Gansu | 48 | 6 | 0.808 | 0.603 | 3.917 |
| Tibet | 62 | 8 | 0.794 | 0.589 | 3.831 |

**Table 2.** The distance between different haplogroups based on HVRI sequences of dogs and wolves.

| | TM | OD | GW | TW | C |
|---|---|---|---|---|---|
| TM | | 0.004 | 0.004 | 0.010 | 0.011 |
| OD | 0.018 | | 0.003 | 0.010 | 0.010 |
| GW | 0.017 | 0.018 | | 0.010 | 0.010 |
| TW | 0.064 | 0.068 | 0.068 | | 0.013 |
| C | 0.044 | 0.045 | 0.043 | 0.077 | |

Below the diagonal are genetic distances while the above are standard errors. TM, Tibetan mastiff; OD, other domestic dogs; GW, gray wolves; TW, Tibetan wolves; C, coyotes.

relationships between haplotypes (figure 2). H1–H3 and H5–H7 were shared by Gansu and Tibetan populations, whereas H4 and H8 were specific to Tibetan population. In the present study, we inferred populations from Gansu and Tibet which were connected by an undetected haplotype. H8 was novelly identified from H3 based on one mutation site (position 575). H6 was defined by sites 65 and 66, and H7 was characterized by site 210. All haplotypes, except H8 was identical to the published haplotypes. H8, A3, A51, A11, A18, A19, A117, A44, A29 and A95 covered the centre nodes, showing a star-like phylogenetic pattern, whereas the A55 subclade (A53, A45, A184, A185 and A151) were clustered alone (figure 3), which implied a different origin.

The phylogenetic analysis of eight haplotypes of Tibetan mastiffs and HVRI sequences of two Tibetan wolves, four grey wolves and 25 domestic dogs covering all phylogenetic clades was conducted by using coyotes (GenBank accession number NC-008093) as an outgroup. The NJ and ML analyses gave identical topological structures, thus only the ML tree is presented in figure 4. We found Tibetan wolves were clustered alone as one separate group as well as coyotes, whereas Tibetan mastiffs, domestic dogs and grey wolves were clustered into another group with grey wolf haplotypes dispersing over domestic dog haplotypes. This result indicated Tibetan mastiffs originated from grey wolves as the other domestic dogs. H1, H3–H5 and H8 were grouped together, whereas H2, H6 and H7 were clustered in specific subclades, which further confirmed the inference in figure 3.

### *Population history*

Analysis of history from two populations by using Tajima's *D* test and Fu test suggested Tajima's *D* value and Fu's Fs value of Gansu population were 2.12659 ($P < 0.05$) and 4.140 ($P < 0.05$), while that of Tibetan population were 1.42473 ($P > 0.1$) and 2.627 ($0.05 < P < 0.1$). In the present study, *D* value and Fs value were positive, indicating the loss of some haplotypes and a reduction of population size (Tajima 1989; Fu 1997). In addition, HVRI haplotypes in two populations showed a multimodal distribution (figure 5), which implied Tibetan mastiffs stayed in a balanced phase or declined, and did not undergo a population expansion (Rogers and Harpending 1992; Ray *et al.* 2003).

## Discussion

Previous studies showed high genetic diversity in Tibetan mastiff (Ren *et al.* 2009; Li and Zhang 2012). In the present study, Hd based on HVRI sequences of 110 samples from two populations was 0.806. Compared with the result that Hd of 154 dogs from 88 breeds was 0.929 (Sugiyama *et al.* 2013), Hd of 110 samples in the present study was lower. Further, a comparison of the result that 14 haplotypes were detected based on 582-bp HVRI sequences of 47 Tibetan individuals (Li and Zhang 2012), we found only eight haplotypes in 110 samples. In comparison with other mammals, the genetic diversity indices identified in total population were also low. Pi and average genetic distance (P) between haplotypes could uncover degree of mtDNA genetic variation, and Pi considered the frequency of mtDNA haplotypes in populations, thus could reveal genetic diversity. The Hd

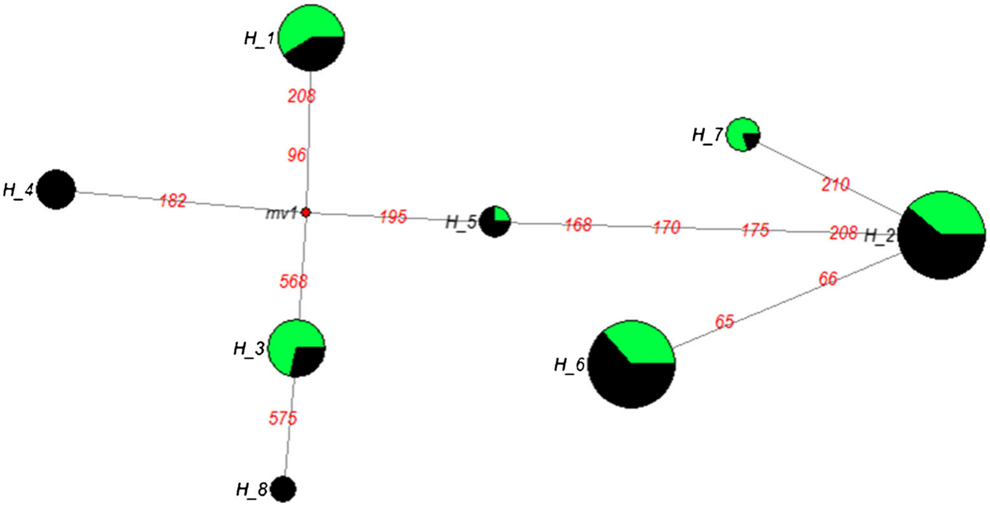

**Figure 2.** Median-joining haplotype network of populations from Gansu and Tibet based on HVRI sequence. Red dot are a postulated haplotype, and circle areas are proportional to haplotype frequencies, while green and black portions respectively represents the proportions of the same haplotype that occurs in Gansu and Tibetan populations.

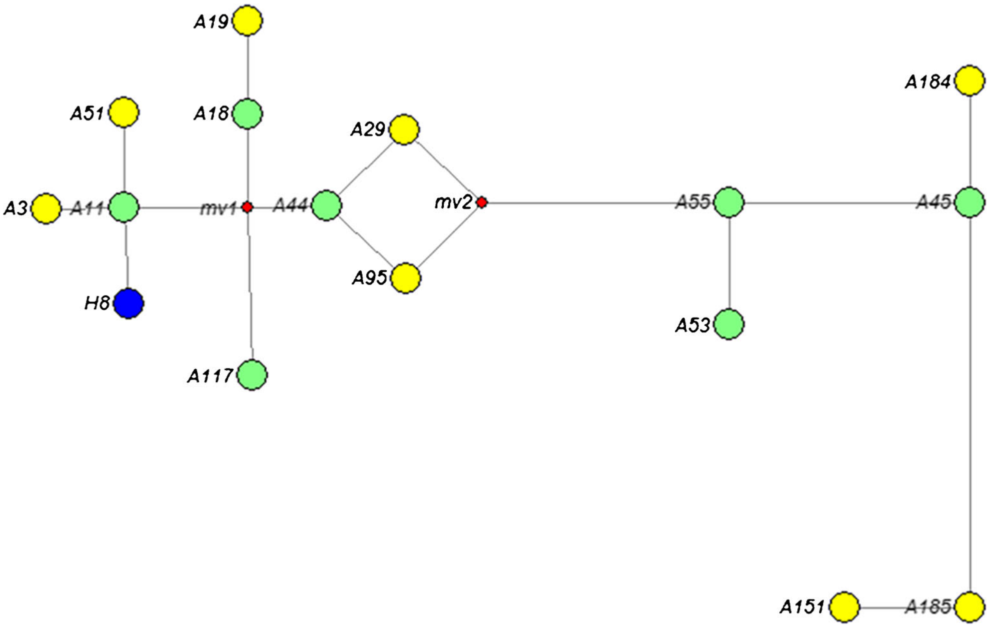

**Figure 3.** MJ haplotype network from the present study and the previous result. Red dots are postulated haplotypes, and green dots are haplotypes found from the present and previous result. Blue and yellow dots are detected from the present study and previous result, respectively.

and Pi based on a 479 bp fragment of D-loop of 132 Balkan donkeys from 10 regions were 0.982 and 1.7% (Pérez-Pardal *et al.* 2014). Hd and Pi based on D-loop sequences of 963 individuals from 16 Chinese indigenous breeds that distributed seven geographic regions were 0.961 and 3.165% (Zhao *et al.* 2013). However, nucleotide diversity based on HVRI sequences of 110 samples from two populations was 0.595%. Lower genetic diversity indices and less haplotypes implied lower genetic diversity of two populations in our study, which may be attributed to the smaller original founders or the closer genetic relationship among some individuals.

Eight haplotypes defined by HVRI sequences were compared with 20 reported haplotypes downloaded from NCBI (GenBank accession number AB007383, AB007385, AB007392, AB007396, AF531656, AF531695, AF531696, AF531702, AF531704, AF531706, EU223768, EU816468, EU816522, JN695048, JN695049, EU408300, EU740415, HM048871, JF342862 and KF857719). Consequently, H8 and H4 were specific to Tibetan population with H8 being

**Figure 4.** Phylogenetic tree of domestic dogs and wolves based on HVRI haplotype reconstructed by the ML method.

novelly identified and no unique haplotypes were detected in Gansu population. Various haplotypes in Tibetan population may result from complex geography and various climate types implying the migration path of Tibetan mastiffs. Coincident with the result of Tajima's *D* test and Fu test in our study, mismatch distribution of two populations implied Tibetan mastiffs stayed in a balanced phase, which was consistent with the report that Fu's Fs test for the TM showed no significant signal for population expansion ($P > 0.1$) (Li and Zhang 2012). The results of neutral test and mismatch distribution were explained by rapid loss of germplasm resources and decrease in haplotype number from the plateau which were caused by the outflows of high quality Tibetan mastiffs. The genetic erosion that was induced by human disturbance affected the reconstruction of evolutionary branch and genetic diversity of Tibetan

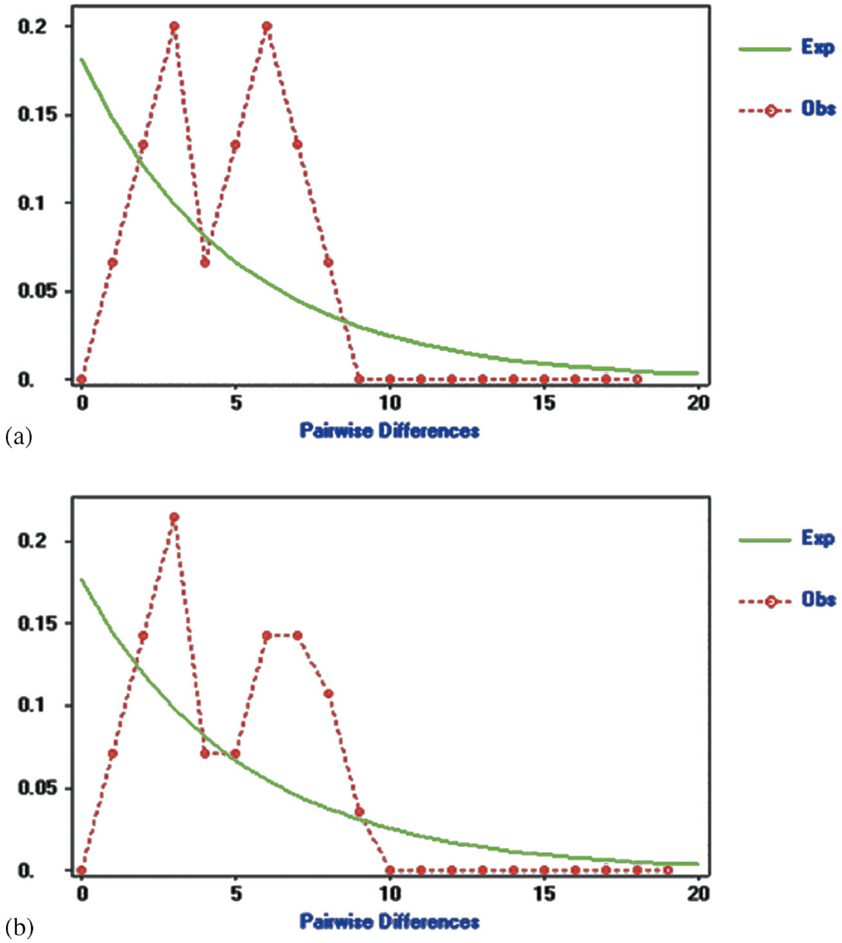

**Figure 5.** (a) Mismatch distribution of Gansu population; (b) Tibetan population.

mastiffs. Although, HVRI sequence was relatively short, the result could reflect evolutionary history of Tibetan mastiffs to some extent.

Tibetan mastiffs distributed in Tibetan plateau are divided into Tibetan, Qinghai and Hequ types. The appearance, temperament type and adaptability are significantly different among different areas and even within the same area, which is the embodiment of high genetic diversity of Tibetan mastiffs. Our analysis indicated low genetic differentiation ($F_{st} = -0.00055$) and strong gene flow among different populations which are geographically separated. This may be related to migration of nomads as well as commercial farming and business in recent years.

In terms of genetic distance, Tibetan mastiffs were closer to grey wolves, same as other domestic dogs did. Meanwhile, Tibetan mastiff haplotypes and grey wolf haplotypes were clustered into one group. These results indicated Tibetan mastiffs were derived from grey wolves in accord with the previous studies (Li Q. *et al*. 2008; Li Y. *et al*. 2011). The prior result discovered that the A55 subclade had a different origin from A3, A51, A11, A18, A19, A117, A44, A29 and A95, indicating an independent arrival to the Qing–Tibet plateau (Li and Zhang 2012). Because H8 were grouped together with A3, A51, A11, A18, A19, A117, A44, A29 and A95, we infered the origin of samples belonging to H8 also differed from the A55 subclade. Sequences of dog population worldwide were assigned into six clades (A, B, C, D, E and F) (Savolainen *et al.* 2002; Pang *et al.* 2009), in which dometic dog and wolf haplotypes of our study belonged to clades A and B, with all Tibetan mastiff haplotypes forming clade A. Due to an origin of clade A in east Asia (Savolainen *et al.* 2002), we further confirmed Tibetan mastiffs were an ancient oriental breed.

Recently, the number of Tibetan mastiffs, which is a precious germplasm resource and cultural heritage, is declining. At present, popular commercial populations on the mainland were multiplied from a few Tibetan mastiffs so that genetic diversity of these populations were deficient and many resistance genes were lost, which contributed to the deficiency of genetic potential for adapting to new ecological environment and increase of epidemic disease. Compared with commercial populations, the population living for many generation in the place of origin constituted its gene pool, which was the inexhaustible motive force and source of development of the breed. Therefore, how to prevent the loss of genetic diversity of Tibetan mastiffs was extremely urgent. This study showed that genetic variation between two populations was lower and Tibetan mastiffs had different history of maternal origin, which laid the foundation for the place of origin, the origin time and migration paths of different groups. Meanwhile, it provided reference values for avoiding the loss of genetic diversity, and then the conservation and rational utilization of germplasm resource. However, the present study not being large enough to comprehensively uncover phylogenetic relationship of different groups, thus larger sample sizes are required for future study.

### Acknowledgement

We thank Dr Chuzhao Lei for his kind help in manuscript improvement.

## References

Achilli A., Olivieri A., Soares P., Lancioni H., Hooshiar Kashani B., Perego U. A. *et al.* 2012 Mitochondrial genomes from modern horses reveal the major haplogroups that underwent domestication. *Proc. Natl. Acad. Sci. USA* **109**, 2449–2454.

Ardalan A., Kluetsch C. F., Zhang A. B., Erdogan M. and Uhlén M. 2011 Houshmand M.others Comprehensive study of mtDNA among Southwest Asian dogs contradicts independent domestication of wolf, but implies dog–wolf hybridization. *Ecol. Evol.* **1**, 373–385.

Bandelt H. -J., Forster P. and Röhl A. 1999 Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* **16**, 37–48.

Dadi H., Lee S. H., Jung K. S., Choi J. W., Ko M. S. and Han Y. 2012 Effect of population reduction on mtDNA diversity and demographic history of korean cattle populations. *Asian-Australas J. Anim. Sci.* **25**, 1223–1228.

Fu Y. -X. 1997 Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**, 915–925.

Imes D. L., Wictum E. J., Allard M. W. and Sacks B. N. 2012 Identification of single nucleotide polymorphisms within the mtDNA genome of the domestic dog to discriminate individuals with common HVI haplotypes. *Forensic Sci. Int. Genet.* **6**, 630–639.

Li Q., Liu Z., Li Y., Zhao X., Dong L., Pan Z. *et al.* 2008 Origin and phylogenetic analysis of Tibetan Mastiff based on the mitochondrial DNA sequence. *J. Genet. Gen.* **35**, 335–340.

Li Y. and Zhang Y. 2012 High genetic diversity of tibetan mastiffs revealed by mtDNA sequences. *Chine. Sci. Bull.* **57**, 1483–1487.

Li Y., Li Q., Zhao X., Xie Z. and Xu Y. 2011 Complete sequence of the Tibetan Mastiff mitochondrial genome and its phylogenetic relationship with other Canids (Canis, Canidae). *Animal* **5**, 18–25.

Librado P. and Rozas J. 2009 DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451–1452.

Long J. R., Qiu X. P., Zeng F. T., Tang L. M. and Zhang Y. P. 2003 Origin of rabbit (Oryctolagus cuniculus) in China: evidence from mitochondrial DNA control region sequence analysis. *Animal Genet.* **34**, 82–87.

Melo-Ferreira J., Boursot P., Carneiro M., Esteves P. J., Farelo L. and Alves P. C. 2012 Recurrent introgression of mitochondrial DNA among hares (Lepus spp.) revealed by species-tree inference and coalescent simulations. *Syst. Biol.* **61**, 367–381.

Meyer S., Weiss G. and von Haeseler A. 1999 Pattern of nucleotide substitution and rate heterogeneity in the hypervariable regions I and II of human mtDNA. *Genetics* **152**, 1103–1110.

Pang J. F., Kluetsch C., Zou X. J., Zhang A. B., Luo L. Y., Angleby H. *et al.* 2009 mtDNA data indicate a single origin for dogs south of Yangtze River, less than 16,300 years ago, from numerous wolves. *Mol. Biol. Evol.* **26**, 2849–2864.

Pérez-Pardal L., Grizelj J., Traore A., Cubric-Curik V., Arsenos G., Dovenski T. *et al.* 2014 Lack of mitochondrial DNA structure in Balkan donkey is consistent with a quick spread of the species after domestication. *Animal Genet.* **45**, 144–147.

Picornell A., Gomez-Barbeito L., Tomas C., Castro J. A. and Ramon M. M. 2005 Mitochondrial DNA HVRI variation in Balearic populations. *Am. J. Phys. Anthropol.* **128**, 119–130.

Ray N., Currat M. and Excoffier L. 2003 Intra-deme molecular diversity in spatially expanding populations. *Mol. Biol. Evol.* **20**, 76–86.

Ren D., Yang Q., Ye J., Xu L., Zhao H. and Wu X. 2009 Strong heterozygote deficit in tibetan mastiff of China based on microsatellite loci. *Animal* **3**, 1213–1215.

Rogers A. R. and Harpending H. 1992 Population growth makes waves in the distribution of pairwise genetic differences. *Mol. Biol. Evol.* **9**, 552–569.

Rout P., Thangraj K., Mandal A. and Roy R. 2012 Genetic variation and population structure in jamunapari goats using microsatellites, mitochondrial DNA, and milk protein genes. *Scientific World J.* **2012**.

Santos C., Sierra B., Alvarez L., Ramos A., Fernandez E., Nogues R. *et al.* 2008 Frequency and pattern of heteroplasmy in the control region of human mitochondrial DNA. *J. Mol. Evol.* **67**, 191–200.

Savolainen P., Zhang Y. -p., Luo J., Lundeberg J. and Leitner T. 2002 Genetic evidence for an East Asian origin of domestic dogs. *Science* **298**, 1610–1613.

Savolainen P., Leitner T., Wilton A. N., Matisoo-Smith E. and Lundeberg J. 2004 A detailed picture of the origin of the Australian dingo, obtained from the study of mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **101**, 12387–12390.

Sugiyama S., Chong Y. H., Shito M., Kasuga M., Kawakami T., Udagawa C. *et al.* 2013 Analysis of mitochondrial DNA HVR1 haplotype of pure-bred domestic dogs in Japan. *Leg. Med.* **15**, 303–309.

Tajima F. 1989 Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S. 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Theyab J. B., Al-Bustan S. and Crawford M. H. 2012 The genetic structure of the Kuwaiti population: mtDNA inter-and intra-population variation. *Hum. Biol.* **84**, 379–403.

Zhao E., Yu Q., Zhang N., Kong D. and Zhao Y. 2013 Mitochondrial DNA diversity and the origin of Chinese indigenous sheep. *Trop. Animal Health Prod.* **45**, 1715–1722.

Received 25 January 2016, in revised form 15 April 2016; accepted 13 July 2016

Corresponding editor: N. G. Prasad