**RESEARCH ARTICLE**

# Phylogenetic Analysis of Tibetan Mastiffs Based on Mitochondrial **Hyper variable Region I**

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**ABSTRACT:** As precious germplasm resource and cultural heritage, the population of Tibetan Mastiffs is decreasing sharply. To be convenient for resource protection, rational development and utilization of Tibetan Mastiffs, there is a need for studies on genetic diversity and phylogenetic relationship with other canine species. We sequenced hypervariable region I of mitochondrial DNA of 110 individuals from Tibet region and Gansu province. A total of 12 polymorphic sites were identified which defined eight haplotypes. H4 and H8 were unique to Tibetan population with H8 being first identified. 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 did 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 a population decline. **Keywords:** Tibetan Mastiffs, Hypervariable Region, Genetic Diversity, MtDNA

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## INTRODUCTION

Tibetan Mastiffs are a species unique to China and native to Tibetan plateau, which are characterized with lion head type and tiger head type. Tibetan Mastiffs have the closest relationship with humans, e.g. keeping house, accompanying and herding. However, along with a nomadic lifestyle changes, excellent Tibetan mastiffs from the plateau have disappeared and genetic diversity of Tibetan mastiffs has dropped dramatically. In addition, due to weak awareness of protecting descent, original Tibetan Mastiffs have interbreed with local Shepherd Dog, which has resulted in a great decrease in the purebred. Meanwhile, due to non-Tibetan 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

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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. Furthermore, D-loop sequence consisted of conservatively intermediate sequence and two hypervariable regions (HVRI and HVRII). HVRI is preferred to study molecular evolution of animals in that functional region in HVRII was related to mtDNA replication and translation which caused more limited mutations than HVRI (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 investigated dogs were domesticated and originated from East Asia (Savolainen *et al.* 2002; Pang *et al.* 2009). Based on 582bp of mtDNA, Sourthern East Asia displayed the most genetic diversity, revealing the original center of wolf domestication (Ardalan *et al.* 2011). Analysis of 582bp 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 582bp of D-Loop, supporting that it was an ancient oriental breed derived from East Asia (Yan Li and Zhang 2012). However, study on evolutionary relationship and maternal origin of the Tibetan Mastiffs in different geographical regions has not been reported.

Therefore, in order 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*

In order to amplify HVRI, the primer pair (5'-CTCTTGCTCCACCATCAGC-3' and 5'-AAACTATATGTCCTGAAACC-3') was designed by Premier 5.0 software. PCR reactions were performed in a 40μL total volume containing 20μL 2×EsTaq MasterMix, 1μL each primer, 2μL genomic DNA, and 16μL RNase-Free Water. The thermal cycling program consisted of predenaturation at 95°C for 5 min, 30 cycles of 94°C for 30 s, 53°C for 30s, and

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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, the number of singleton variable sites, the number of parsimony informative sites and genetic distances between haplotypes were determined by MEGA5.05 software (Tamura *et al.* 2011). Published sequences from 25 domestic dogs, 4 gray wolves, 2 Tibetan wolves and 3 coyotes and 8 Tibetan Mastiff haplotypes in present study were used for comparison. Phylogenetic trees were constructed by Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods of MEGA 5.05 software, using the Kimura 2-parameter model with 1,000 bootstrapping replicates. In addition, median-joining network between 8 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 (Yan 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 (h), haplotype diversity, nucleotide diversity, average number of nucleotide differences (K) and genetic variation coefficient (Fst). In addition, Neutral test was performed and mismatch distribution was drawn.

## RESULTS

***Sequence variation of HVRI***

The sequence alignment among 110 mtDNA sequences revealed that 1 indel was observed, and 12 sites which accounted for 1.85% of the analyzed sites were polymorphic with 0 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, which 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, H3 ), consisting of ≥ 10 samples. The haplotypes (H4, H5, H7, H8) was less popular. Genetic distance among 8 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 population and Tibetan population possessed similar Hd. In terms of total population, although the number of Tibetan Mastiffs haplotypes from Gansu was lower than that from Tibet region, genetic diversity indices of Gansu population were sightly higher than Tibetan population (Table 1).

***Phylogenetic analysis***

The distance between different haplogroups from Tibetan Mastiffs, domestic dogs and wolves revealed significant less genetic distance between Tibetan Mastiffs and gray wolves than that 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 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

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population. We infered populations from Gansu and Tibet were connected by an undetected haplotype in present study. 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 for H8, were identical to the published haplotypes. H8, A3, A51, A11, A18, A19, A117, A44, A29 and A95 covered the center 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 8 haplotypes of Tibetan Mastiffs and HVRI sequences of 2 Tibetan wolves, 4 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 analysis gave identical topological structures, thus only the ML tree was 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 did other domestic dogs. H1, H3-5 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 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).

## DISSCUSSION

Previous studies showed high genetic diversity in Tibetan Mastiff (Ren *et al.* 2009; Yan Li and Zhang 2012). In present study, haplotype diversity, nucleotide diversity and the average number of nucleotide difference of 110 samples were 0.806, 0.595% and 3.867, respectively. Compared with the result that haplotype diversity based on the 660bp HVRI haplotypes of 154 dogs from 88 breeds was 0.929 (Sugiyama *et al.* 2013), Hd of 110 samples in present study was lower. Furthermore, a comparison of the result that 14 haplotypes were detected based on 582bp HVRI sequences of 47 Tibetan individuals (Yan Li and Zhang 2012), we found only 8 haplotypes in 110 samples. In comparison with other mammals, genetic diversity indices identified in total population were also lower. Nucleotide diversity (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 haplotype diversity and nucleotide diversity based on a 479bp 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). Lower genetic diversity indices and less haplotypes implied lower genetic diversity of two

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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, KF857719). Consequently, H8 and H4 were specific to Tibetan population with H8 being 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 ($p > 0.1$) (Yan Li and Zhang 2012). The results of neutral test and mismatch distribution were explained by rapid loss of germplasm resources and decrease of the haplotype number from the plateau which were caused by the outflows of high quality Tibetan Mastiffs . The genetic erosion which was induced by human disturbance affected the reconstruction of evolutionary branch and genetic diversity of Tibetan Mastiffs. Although HVRI sequence was relatively short, the result could reflected 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 (Fst=-0.00055) and strong gene flow among even 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, the same as other domestic dogs did. Meanwhile, Tibetan mastiff haplotypes and grey wolf haplotypes were clustered into one group. These results investigated Tibetan mastiffs derived from grey wolves in accord with the previous studies (Q. Li *et al.* 2008; Y. Li *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 (Yan 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 ( clade A, B, C, D, E and D ) (Savolainen and Zhang et al. 2002; Pang and Kluetsch et al. 2009), in which dometic dog and wolf haplotypes of our study belonged to clade A and clade B with all Tibetan mastiff haplotypes forming clade A. Due to an origin of clade A in East Asia (Savolainen and Zhang et al. 2002), we further confirmed Tibetan mastiffs were an ancient oriental breed.

Recently, as precious germplasm resource and cultural heritage, the number of Tibetan Mastiffs 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

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potential for adapting to new ecological environment and increase of epidemic disease. Compared with commercial populations, the population generationally living in the place of origin constituted it's 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 is not enough to comprehensively uncover phylogenetic relationship of different groups, so larger sample sizes are required in the next study.

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**Table 1** Genetic diversity of Tibetan Mastiffs based on HVRI

| Population | Number of sequences | Haplotypes | 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. Tibetan Mastiff = TM, other domestic dogs = OD, gray wolves = GW, Tibetan wolves = TW, coyotes = C.

| 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 |

**Fig 1.** Sequence variations in HVRI detected in 8 haplotypes of Tibetan Mastiffs, "." represents matched bases.

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**Fig 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.

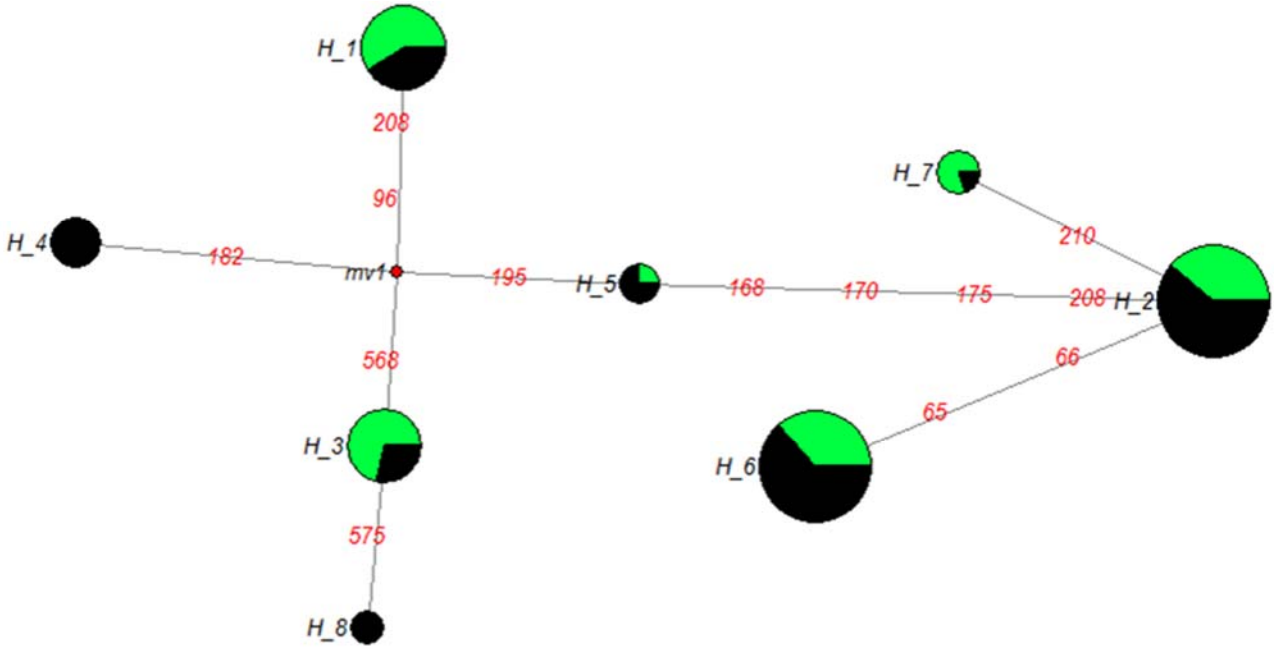


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**Fig 3**. Median-joining haplotype network from the present study and the previous result.

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.

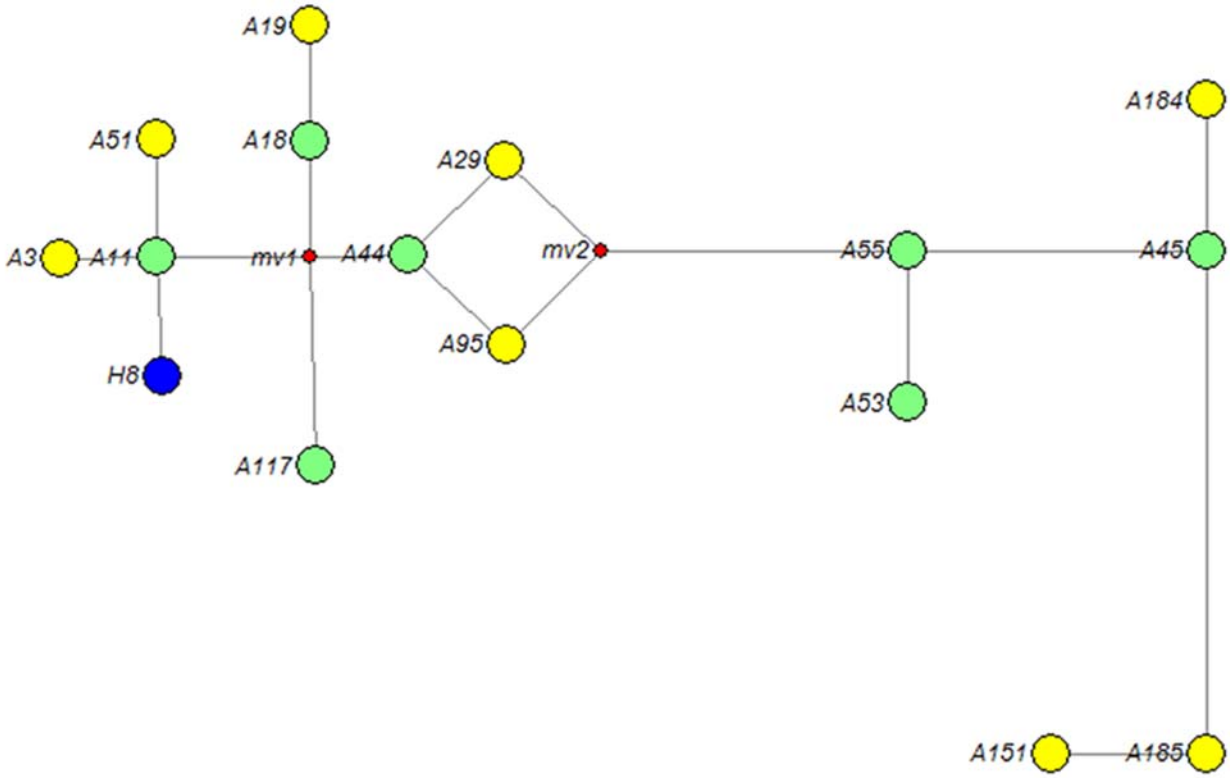


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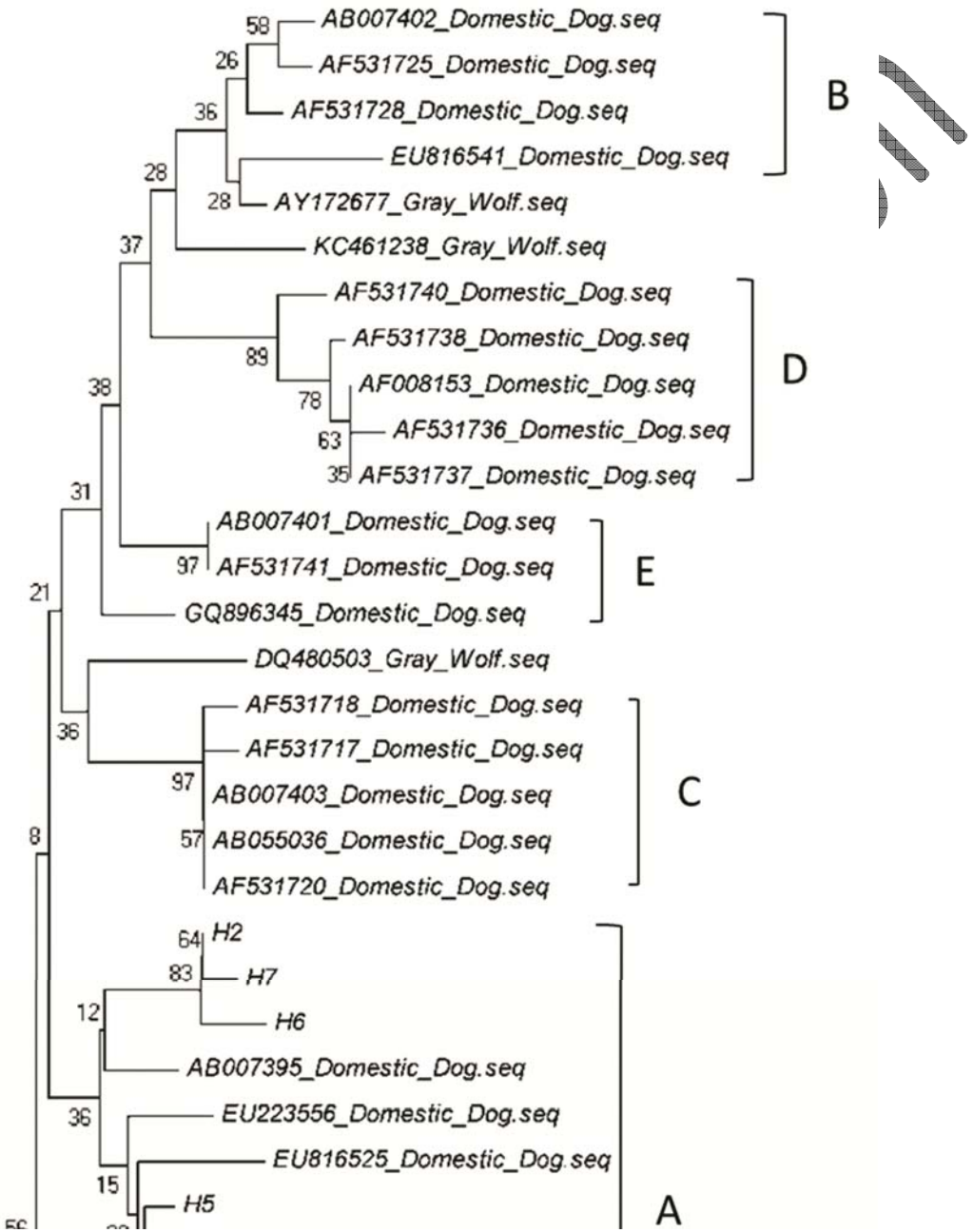
Fig 4. Phylogenetic tree of domestic dogs and wolves based on HVR haplotype reconstructed by the ML method.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H7
H6
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C
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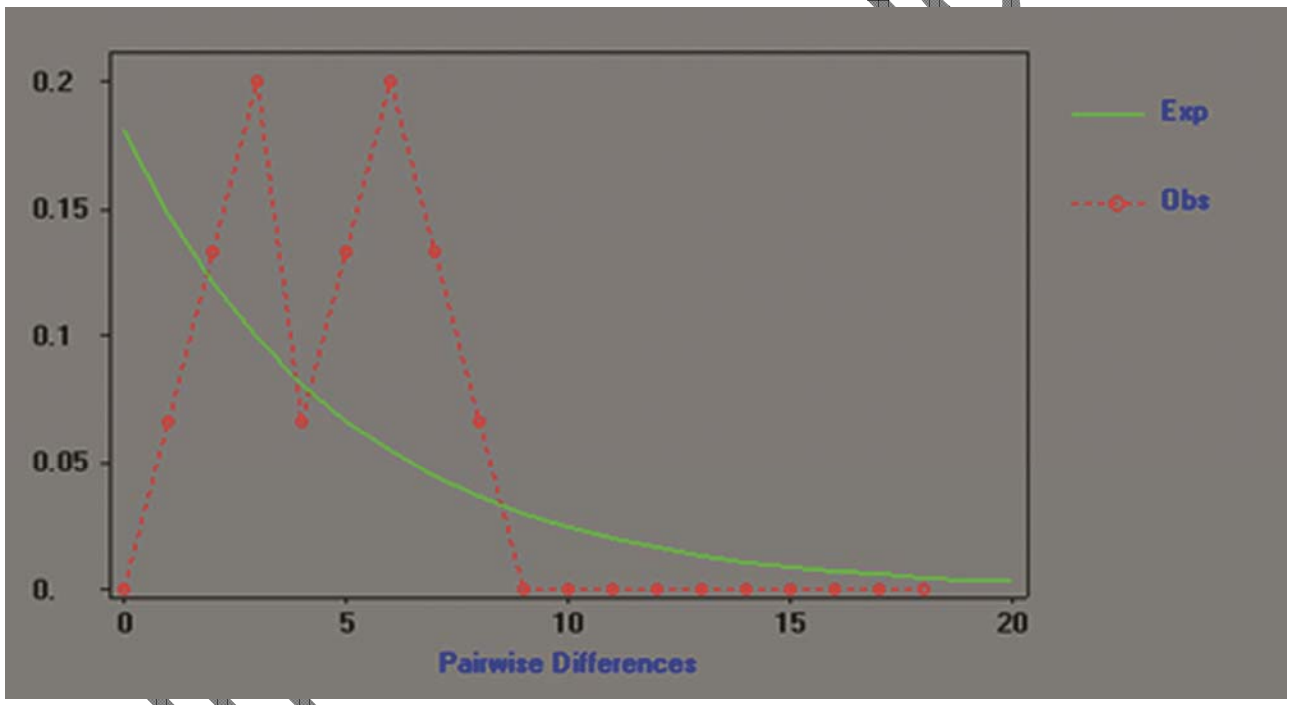


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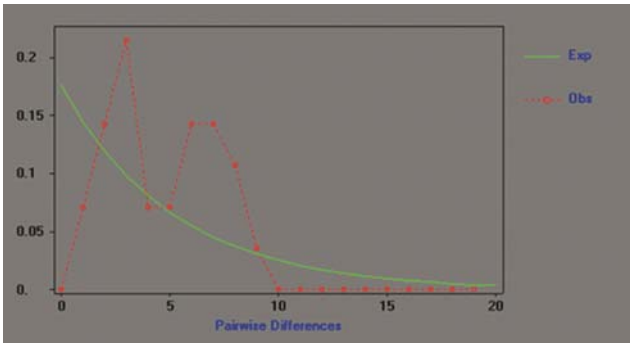


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