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Genetic Diversity and Molecular Phylogeny of Iranian Sheep Based on Cytochrome b Gene Sequences

Research Article

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ABSTRACT

Phylogenetic relationships and genetic variation between two Iranian sheep breeds were analyzed using cytochrome b (cyt-b) gene sequences. The genomic DNA was isolated by salting out method and amplified cytochrome b gene using polymerase chain reaction restriction (PCR) method with a pair of primer. A partial sequence of cyt-b gene of Iranian sheep is 780 bp and contained 13 variable sites and 11 haplotypes. Phylogenetic analysis of haplotype in the combination with the sheep from GenBank showed that Iranian sheep made a separated cluster. This study is provided useful information for understanding relationships between breeds from different parts of the world. This study may simplify the future researchers and breeders for better understanding the genetic structure and breed differentiation for designing future breeding strategies to the conservation of animal genetic resources.

KEY WORDS genetic diversity, Iranian sheep, mitochondrial cytochrome b gene, phylogenetic analysis.

INTRODUCTION

Sheep and goats are two important livestock species in Iranian rural areas. More than 57% of the available animal units in the country are sheep and goats. More than 27 breeds of sheep with a variety of sizes, shapes, types and color have been recognized in Iran (Mobini, 2013). All Iranian native sheep, except the Zel are fat-tailed breeds (Mobini, 2013). Ghezel and Shal are of the predominant sheep breeds in Iran and being very well adapted to harsh environmental conditions. They are fat tailed sheep used mainly for meat production (Atashi and Izadifar, 2012). Animal genetic resources are mainly facing two challenges. On one side, the demand for livestock products are increasing in developing countries as estimated by Food Agriculture Organization (FAO, 1993) and the demand for milk and meat from livestock have increased twice than usual. On the other hand, animal genetic resources are menaced because of the aimless development (Ruane *et al.* 2006). Strategies for genetic progress of domestic animals mainly involve the use of the genetic variation. Genetic diversity studies in livestock aim at evaluating genetic diversity within and between breeds, since the breed is the management unit for which factors such as inbreeding are controlled (Tolonea *et al.* 2012). Therefore, a molecular genetics study of the population diversity may improve the comprehension of the genetic resources (Tolonea *et al.* 2012). Mitochondrial DNA (mtDNA) is the genetic material that exists outside the nucleus in eukaryotic cells (Burgstaller *et al.* 2015). mtDNA is highly conserved and its relatively

slow mutation rates (compared to other DNA regions such as microsatellites) make it useful for studying the evolutionary, relationships, phylogeny of organisms. It has multiple copies, has a rapid evolutionary rate and follows maternal inheritance. The cytochrome b (cyt-b) gene is one of the important coding genes in mtDNA; it is about 1.2 kb in length (Sawaimul *et al*. 2014). Because of its maternal inheritance, its well-known gene structure and sequence, the occurrence of low recombination and other characteristics, the cyt-b gene has been widely used for phylogenetic evolution of several animal species (Patwardhan *et al*. 2014). The purpose of this study was to investigate the genetic diversity and phylogenetic evolution of two Iranian sheep (Ghezel and Shal) based on the analysis of the partial sequence of the cyt-b gene. This investigation will be helpful for the conservation, utilization, and exploitation of the genetic resources of the indigenous Iranian sheep.

MATERIALS AND METHODS

Population sampling

Blood samples from two Iranian sheep breeds (Ghezel and Shal) were considered for the study. Samples were collected from sheep that were judged to be true to type with the phenotypic characteristics of that breed. The animals selected had unrelated parents based on the information provided by the owners. A total of 50 individuals from different locations were sampled and the blood was stored at 4 ˚C up to 21 days. Genomic DNA was extracted from fresh blood according to standard procedures (Javanrouh *et al*. 2006) and was quantitated by spectrophotometry (Nanodrop ND1000).

PCR amplification and sequencing

At the first step, cyt-b of the mtDNA was amplified and sequenced. To amplify the cyt-b region of sheep mtDNA, a pair of primers was designed using the known sheep mtDNA sequence (GenBank Accession No NC_001941.1). The primers cyt-b-F 5′-CATTCTCCTCTGTAACCCACATCTG-3′ and cyt-b-R 5′-GTCCAATAATGATGTAGGGGTGTTC-3′ were used to amplify an 870 bp DNA fragment. PCR amplifications were conducted in a 30 µL volume containing 5 µL of 10x reaction buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 0.2 u*M* each primer, 1U Taq DNA polymerase (TaKaRa Biosystems) and approximately 150 ng genomic DNA. The PCR mixture underwent 4 min at 95 ˚C, 35 cycles 50 s at 94 ˚C, 1 min at 60 ˚C and 1 min at 72 ˚C and 5 min at 72 ˚C. PCR products were purified by using PCR Purification Kit (Watson BioTechnologies, Shanghai) and then sequenced using ABI PRISM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3130 Geneti Analyzer (Applied Biosystems, Foster City, USA).

Phylogenetic reconstruction

The quality of the 780 bp cyt-b gene sequence for individuals was firstly evaluated on the basis of sequecing peak value and then these sequences were manually edited using program Chromas version 2.23. Then sequences were arranged using the BioEdit program and were aligned using CLUSTALW (http://ebi.ac.uk/clustalw) software. These results were compared with other sequences obtained from GenBank. To investigate genetic relationship between mitochondrial sequences, phylogenetic tree unweighted pair group method with arithmetic mean (UPGMA) and neighbor joining (NJ) were constructed using the Tamura–Nei distance method (Tamura and Nei, 1993). The phylogenetic tree construction is incorporated in the MEGA version 6.1 (Tamura *et al*. 2013). DnaSP 5.0 (Librado and Rozas, 2009) was used to analyze the diversity parameters including haplotype diversity (HD), nucleotide diversity (π) and the average number of nucleotide differences.

RESULTS AND DISCUSSION

A total of 780 base pairs (bp) of the cyt-b region (from np 14410 to np 15190) were obtained for 50 samples. There were no insertions/deletions in 50 sequences of cyt-b region. The average percentage of nucleotides T, C, A and G were 26.3, 28.7, 29.71 and 13.65%, respectively. Percentage of nucleotide pairs A + T and C + G was 56% and 44%, respectively, suggesting that A + T nucleotides were higher in the cyt-b region of mtDNA Iranian sheep breeds. Because of the well-known gene structure and lack of recombination, the cyt-b gene has been generally used alone or in combination with other mtDNA encoding genes and hyper variable regions for phylogenetic studies between species (Chen *et al*. 2006). Generally, the AT content is always higher than the GC content in cyt-b (Sawaimul *et al*. 2014) which is consistent with our results. However, the result was different from that of Sawaimul *et al*. (2014), probably because of the differences for the sheep breeds and the length of the sequences that were studied. The cyt-b sequences were polymorphic. Fifty sequences rendered 11 divergent haplotypes with 13 variable sites defined. The largest haplotype group consisted of 5 individuals. The number of haplotypes detected in each breed ranged from 4 in Ghezel to 7 in Shal (Table 1). As an encoding gene of mtDNA, the incidence of mutation of the cyt-b gene is medium compared to mutation in the D-loop and other encoding genes (Chen *et al*. 2006). The nucleotide sequence of cyt-b genes revealed several nucleotides differences with Iranian sheep (Table 2).

These variable sites showed similarities among of the Iranian sheep breeds, but clearly were different with other sheep breeds.

Table 1 Haplotypes, parsimony informative sites, singleton and polymorphic sites for each breed

Breed	n	Haplotypes	PSI	Singleton sites	Polymorphic sites
Shal	25	7	11	1	13
Ghezel	25	4	10	0	12

PSI: parsimony informative sites.

Table 2 Analysis of genetic variations based on mtDNA cytochrome b gene refers to the other sheep breeds

Population	Site												
	67	81	86	151	154	253	271	454	471	493	565	571	748
Iran (Shal)	A	C	T	C	C	A	T	C	T	T	G	G	T
Iran (Ghezel)	-	T	-	C	T	-	-	-	-	C	-	A	-
AF010406 (Germany)	C	T	C	T	T	G	C	T	-	C	A	A	C
NC001941 (Germany)	T	T	C	T	T	G	C	T	-	C	A	A	C
AY858379 (Korea)	T	T	C	T	T	-	C	T	-	C	A	A	C
EF490451 (Austria)	T	T	C	T	T	G	C	T	C	C	A	A	C
HE577849 (Israel)	C	T	C	T	T	G	C	T	-	C	A	A	C
HM236179 (Australia)	C	T	C	-	-	-	C	-	-	-	A	-	-
JX235837 (Pakistan)	T	T	C	T	T	-	C	T	-	C	A	A	C
JX567831 (China)	T	T	C	T	T	G	C	T	-	C	A	A	C
KF938345 (China)	T	T	C	T	T	-	C	T	-	C	A	A	C
KF229236 (China)	T	T	C	T	T	-	C	T	-	C	A	A	C
KU899150 (China)	T	T	C	T	T	-	C	T	-	C	A	A	C
KF302446 (Italy)	T	T	C	T	T	-	C	T	-	C	-	A	C

Transversions occurred only at one position 67 (A/C) and in all the other positions, transitions occurred (G/A, 3 and T/C, 9). Haplotypes diversity values were moderate in two populations. Values ranged from 0.791 ± 0.021 in Shal to 0.623 ± 0.014 in Ghezel. As can be seen from Table 3, synchronous with haplotype diversity enhancement, the nucleotide diversity of mtDNA and polymorphism of the population were increased. The nucleotide diversity (π) ranged from 0.013 ± 0.011 (Ghezel) to 0.014 ± 0.002 (Shal). The average number of nucleotide differences (k) was quite relevant and the highest was for the Shal breed (6.641) (Table 3). Nucleotide diversity and haplotype diversity of mtDNA cyt-b region are the important indices for assessing population polymorphism and genetic differentiation.

Table 3 Values of haplotypes diversity (HD), nucleotide diversity (π) and average number of nucleotide differences (k) for each breed

Breed	(HD±SD)	(π±SD)	k
Shal	0.791±0.021	0.014±0.002	6.641
Ghezel	0.623±0.014	0.013±0.011	6.157

SD: standard deviation.

It was far lower than that of the D-loop region (Javanrouh *et al.* 2016) indicating that the cyt-b gene is relatively conserved and that most base substitutions did not change the coding of the amino acid. The extent of gene differentiation of these sheep breeds was in accordance with that obtained from microsatellites (Molaee *et al.* 2009). Molaee *et al.* (2009), studied six Iranian indigenous sheep populations by investigating their nuclear DNA using microsatellite markers and the result showed that the mean polymorphism information content of the six breeds were moderate.

The cyt-b gene has been used to study other aspects such as intra or interspecific relationships and gene flow as well (Alves *et al.* 2003). It is generally recognized that the domestic animals experience a bottleneck effect after domestication (Xin *et al.* 2006). But in this study, none of the sheep population expansion events irrespective of the size of the population. Out of the 11 haplotypes observed in this study, only 4 haplotypes are common to these breeds, suggesting that a moderate level of genetic diversity was present within each of these breeds. This unique pattern of haplotype distribution may also be attributed to reproductive isolation due to harsh geographical structure of the country and unique husbandry practices (migratory farming system) associated with to this specific region. We identified 1 singleton sites and 10 parsimony informative sites. A singleton site contains at least two types of nucleotides (or amino acids) with, at most, one occurring multiple times. DNAsp identifies a site as a singleton site if at least three sequences contain unambiguous nucleotides or amino acids.

A site is parsimony-informative if it contains at least two types of nucleotides (or amino acids) and at least two of them occur with a minimum frequency of two (http://www.megasoftware.net).

Phylogenetic relationship on Iranian sheep breeds

The phylogenetic trees of Shal and Ghezel sequences were constructed using UPGMA method with reported sheep sequences from Italy (KF302446), China (KP229236, KF938345, KU899150, JX567831), Korea (AY858379), Austria (EF490451), Australia (HM236179), Pakistan (JX235837) and Germany (NC001941 and AF010406), as

in groups and with goat (AB004070.1) and cattle (AB074964.1) sequences as out groups (Figure 1).

Phylogeny tree of cyt-b gene nucleotide showed that Iranian sheep made a separated cluster.

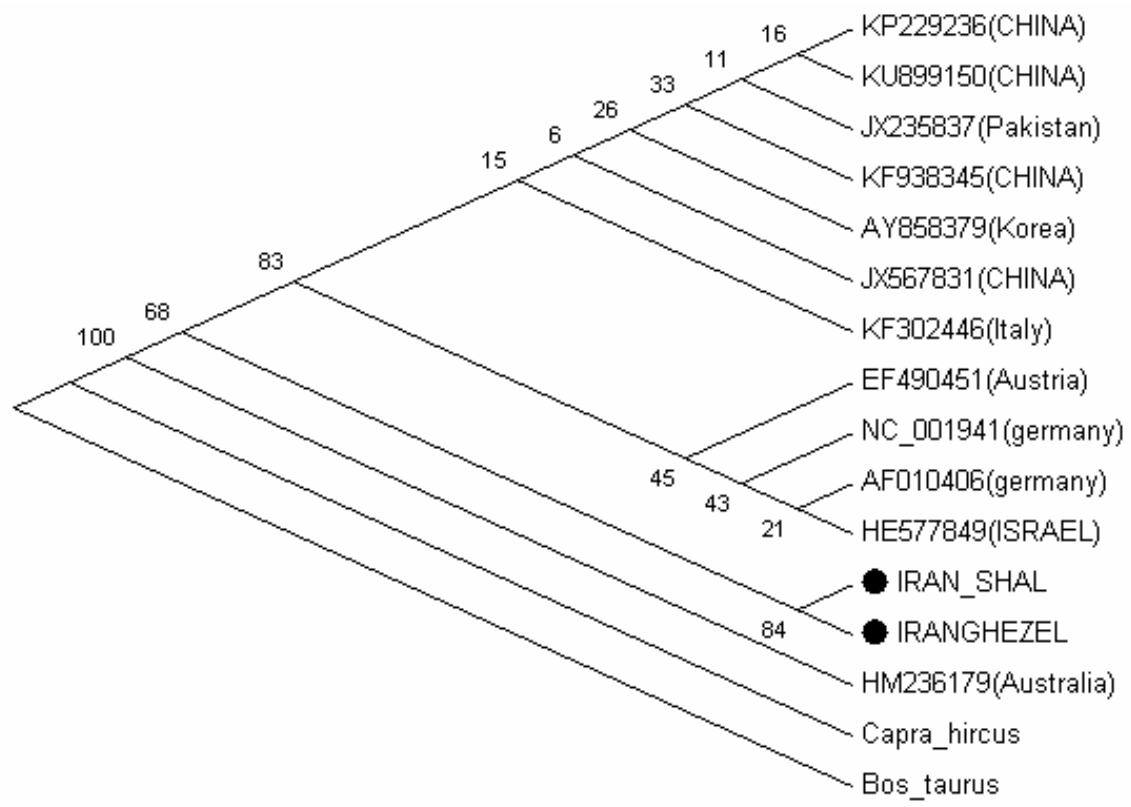

Figure 1 UPGMA phylogenetic tree constructed for Iranian sheep mtDNA sequences with the 12 reference sequences

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	1	2	3	4	5	6	7	8	9	10	11
1. IRAN											
2. GERMANY	0.009										
3. KOREA	0.007	0.003									
4. CATTLE	0.185	0.183	0.182								
5. GOAT	0.122	0.120	0.118	0.153							
6. AUSTRIA	0.010	0.001	0.004	0.182	0.121						
7. ISRAIL	0.009	0.000	0.003	0.183	0.120	0.001					
8. AUSTRALIA	0.011	0.013	0.011	0.176	0.118	0.014	0.013				
9. PAKISTAN	0.007	0.003	0.001	0.180	0.117	0.004	0.003	0.010			
10. CHINA	0.007	0.002	0.002	0.180	0.117	0.003	0.002	0.011	0.000		
11. ITALYA	0.008	0.004	0.003	0.182	0.118	0.005	0.004	0.011	0.001	0.002	

Figure 2 Estimates of mean distance over sequence pairs between groups

This result is supported by the bootstrap value of 100%. Bootstrap value is a criterion to determine the level of accuracy of phylogeny tree. The estimated genetic distances between populations also indicated that the Iranian and Australia sheep populations are far away (0.011) and Iranian sheep are closely related to Pakistani, Korea and China (0.007) sheep populations (Figure 2). Clustering the different sheep breeds within one branch of phylogeny is because of the low sequence substitutions in cyt-b gene (Sultana *et al*. 2003). Clustering also occurred in other comparator groups because of nucleotide substitutions in cyt-b gene. The present information could be used to strengthen the monitoring, characterization and conservation of animal genetic resources towards the sustainable rearing of the autochthonous sheep breeds. However, further studies involve the existing knowledge from microsatellite marker will help to unravel the history of domestication of Iranian sheep.

CONCLUSION

In the present study, we investigated the diversity and the organization of cyt-b region in Iranian sheep breeds. The cyt-b region of mtDNA using sequencing techniques was suitable tool for analyzing genetic variability, phylogenetic relationship and time of divergence between the Ghezel and Shal sheep breeds. The evolutionary divergence into distinct entities of Iranian sheep breeds based on cytochrome b sequence appear to closely follow their geographical distribution in Iran and this could have implications for management, improvement and conservation strategies in Iranian sheep.

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