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Sequence analysis of 12S rRNA and 16S rRNA mitochondrial genes in Iranian Afshari sheep

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Abstract. This study was to determine phylogenetic relationships and genetic variation in Iranian Afshari sheep breed. For this reason, phylogenetic relationships and genetic variation were analyzed by using 12S rRNA and 16S rRNA gene sequences. The genomic DNA was isolated by salting out method and amplified 12S rRNA and 16S rRNA genes using PCR method. PCR amplification of 12S and 16S rRNA generated PCR amplicons at 859 and 1053 bp lengths, respectively. Sequence analysis was performed using Bio–Edit software. Phylogenetic tree was constructed using MEGA software. Phylogenetic analysis of haplotype in the combination with the sheep from Gen–Bank showed that Iranian Afshari sheep made a close to the Australian sheep cluster. There was found informative for establishing relationships between breeds from different parts of the world. This may facilitate the future researchers and breeders for better understanding the genetic interactions and breed differentiation for devising future breeding and conservation strategies to preserve the rich animal genetic reservoir of the country.
Keyword*:* 12S rRNA, 16S rRNA, Phylogenetic analysis, Iranian Afshari sheep.

Introduction

Sheep and goats form are the most important group of ruminants in Iran mainly in rural areas. More than 57 % of the available animal units in the country are sheep and goats. More than 27 breeds of sheep have been recognized in Iran which appears in a variety of size, shapes, types and color [TAVAKOLIAN, 2000]. All Iranian native sheep breeds are fat–tailed, except the Zel breed [SAADAT NOORI and SIAH MANSOOR, 1982. BONCIU *et al.,* 2018; BONEA *et al.,* 2018; GROZEA et al., 2017, STOLERU *et al.,* 2016].

The Afshari sheep is one of the heaviest and largest mutton breeds in Iran and is widely distributed in the mountainous areas in the west of the country. Today, a large percentage of the Afshari sheep population is raised in the Zanjan province. This breed has a large litter size, high fertility and appropriate growth characteristics compared with other Iranian sheep breeds [GHAFOURI–KESBI et al., 2009]. Livestock genetic resources are currently facing two challenges. On one side, the demand for livestock products is increasing in developing countries as estimated by Food Agriculture Organization (FAO), that the demand for milk and meat from livestock have increased twice than usual. On the other hand, livestock genetic resources are threatened because of the aimless development [RUANE et al., 2006, VARDANIAN *et al.,* 2018; STOLERU *et al.,* 2018].

Mitochondrial DNA (mtDNA) is the genetic material that exists outside the nucleus in eukaryotic cells [IRWIN et al., 1999, BONEA *et al.,* 2017, PENTEA et al., 2016, STOLERU *et al.,* 2012]. It has a simple molecular structure. It does not undergo recombination with nuclear DNA and has no identical sequence with nuclear DNA [IRWIN et al., 1999, BUTU *et al.,* 2014c. SAMFIRA *et al.,* 2015, BUTNARIU *et al.,* 2015b; BUTU *et al.,* 2014b].

It has multiple copies, has a rapid evolutionary rate, and follows maternal inheritance. The mitochondrial 12S rRNA gene could be a useful genetic marker, as it is more intra specifically conserved than mitochondrial protein–coding genes. A lot of studies have proven that mitochondrial

12S rRNA and 16S rRNA gene sequences of animals reveal the appropriate level of interspecific variation but the high level of intraspecific homogeneity [ROJAS et al., 2010; PUTNOKY *et al.*, 2013, BUTNARIU *et al.*, 2014; BUTNARIU and GIUCHICI, 2011].

Consequently, 12S rRNA and 16S rRNA genes are suitable for taxonomy and species identification, especially for the discrimination of even closely related species. Girish and collab. [GIRISH et al., 2007] used mitochondrial 12S rRNA gene sequences to identify between cattle and buffalo and between sheep and goat.

Chen and collab. analyzed 179 mitochondrial 12S rRNA gene sequences of ten farm animal species to determine the intra species and species–specific variations and could be applied to species identity test [CHEN et al., 2012], commercial fraud, and wildlife crime.

The purpose of this study was to investigate the genetic diversity and phylogenetic evolution of Iranian Afshari sheep based on the analysis of the partial sequence of the 12S rRNA and 16S rRNA genes. This will be helpful for the conservation, utilization, and exploitation of the genetic resources of the indigenous Iranian sheep.

Material and methods

Population sampling. Blood samples were collected from sheep that were judged to be true to type with the phenotypic characteristics of that breed. The individuals selected had unrelated parents and grandparents based on the information provided by the owners and also cross checked with their neighbors. A total of 30 individuals from different locations were sampled and the blood was stored at 4 °C. Genomic DNA was extracted from fresh blood according to standard procedures [JAVANROUH et al., 2006; DIMITRIU *et al.*, 2016; GEORGIEVA *et al.*, 2018; BUTNARIU and CAUNII, 2013] and was quantitated by spectrophotometry (Nanodrop ND1000).

PCR amplification and sequencing. The first 12S rRNA and 16S rRNA of the mtDNA were amplified and sequenced. The mitochondrial genome fragments were amplified using the following primers: The primers 12S–F 5′–GTTTGGTCCCAGCCTTCC–3′ and 12S–R–TTTACTTGAGGAGGGTGA–3'; 16s–F: 5'–AAGGAGGATTTAGCAGTA –3' and 16s–R: 5'– TGAATCTTTCCTGGGTGC–3' were used to amplify an 859– and 1053 bp DNA fragment. PCR amplifications were conducted in a 30 µL volume containing 5 µL of 10 x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM 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 1min at 72 °C, and 5 min at 72 °C. PCR products were purified by using Watson PCR Purification Kit (Watson Biotechnologies, Shanghai).

PCR products were sequenced using ABI PRISM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3130 Genetic Analyzer (Applied Bio–systems, Foster City, USA).

Phylogenetic reconstruction. The quality of the 859 bp and 1053 bp 12S rRNA and 16S rRNA gene sequences for individuals were firstly evaluated on the basis of sequencing 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 that they obtained from Gen–Bank. To investigate genetic relationship between mitochondrial sequences, UPGMA phylogeny [SAITOU and NEI, 1987, CAUNII *et al.*, 2015; IANCULOV *et al.*, 2004] was 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]. Diversity parameters including haplotype diversity (HD) and nucleotide diversity (π) were estimated using DNASP (Sequence Polymorphism Software) 4.1 [ROZAS et al., 2003. SAMFIRA *et al.*, 2014; BUTNARIU; 2012; BUTU *et al.*, 2014a, STOLERU *et al.*, 2016].

Results and discussion

A total of 859 base pairs (bp) of the 12S rRNA region (from np 73 to np 932) and 1053bp of the 16S rRNA region (from

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np 841 to np 1894) were obtained for 30 samples.

There were no insertions/deletions in 30 sequences of these regions. The average percentage of nucleotides A, C, T and G were 38.2, 22.3, 21.68 and 17.73 %, respectively for 12S rRNA and A, C, T and G were 41.01, 19.99, 25.08 and 15.01%, respectively (figure 1, 2).

Percentage of nucleotide pairs A+T was 60 % and the C+G was 40 %, suggesting that A+ T nucleotides were higher in the cytB region of mtDNA Iranian sheep breeds. Because of the well–known gene structure and lack of recombination, the 12S rRNA and 16S rRNA genes have 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 speaking, the AT content is always higher than the GC content in 12S rRNA and 16S rRNA genes [CHEN et al., 2006, BUTNARIU and SAMFIRA, 2012; IANCULOV *et al.*, 2005, STOLERU *et al.*, 2018]. Our study was consistent with that, showing proportions of 60:40. As an encoding gene of mtDNA, the occurrence of mutation in the 12S rRNA and 16S rRNA genes are low compared to mutation in the D–loop and other encoding genes [CHEN et al., 2006, PETRACHE *et al.*, 2014, BUTNARIU *et al.*, 2014, BARBAT 2013, BUTU *et al.*, 2015]. There were no variable site and haplotypes of 16S rRNA sequences identified from the multiple alignment results between the thirty tested Afshari sheep sequences. Haplotypes diversity (H_d) and nucleotide diversity (π) values were low in Afshari sheep population (Hd=0.37, π= 0.0005).

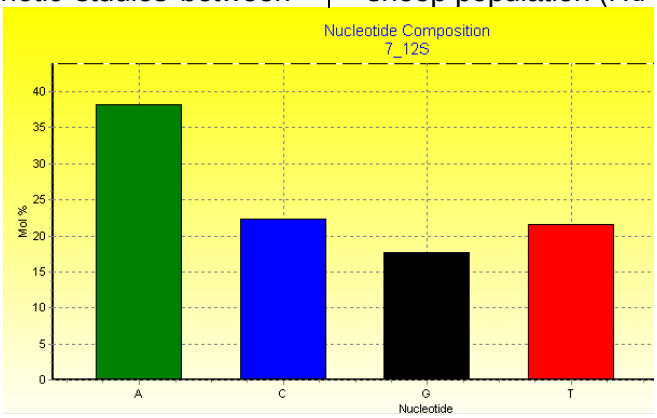

Figure1. Average base composition of the 12S rRNA region sequences in Afshari sheep

Nucleotide diversity and haplotype diversity of mtDNA 12S rRNA region are the important indices for assessing population polymorphism and genetic differentiation.

It was far lower than that of the D–loop region [JAVANROUH et al., 2016] indicating that the 12S rRNA gene is relatively conserved.

Most base substitutions did not change the coding of the amino acid. In addition, Molaee and collab. [MOLAEE et al., 2009, BAGIU *et al., 2012*; BUTNARIU and CORADINI, 2012] who studied in six Iranian indigenous sheep populations by investigating their nuclear DNA using microsatellite markers, and the result showed that the mean polymorphism information content (PIC) of the six breeds were low.

Phylogenetic analysis. The phylogenetic tree of Afshari sequences were constructed using UPGMA method with reported sheep sequences from Italy (KF302446), Korea (AY858379), China (KP998473, KR868678, KP981378, KF938359, KF938325), Austria (EF490451), Israel (HE577849, HE577850), Australia (HM236189, HM236183, HM236179), France (AY670663, AF091699) and Germany (AF010406) (Figure 2).

Phylogeny tree of 12S rRNA and 16S rRNA genes nucleotide showed that Iranian sheep clustered near the Australia sheep breed. This result is supported by the bootstrap value of 100 %.

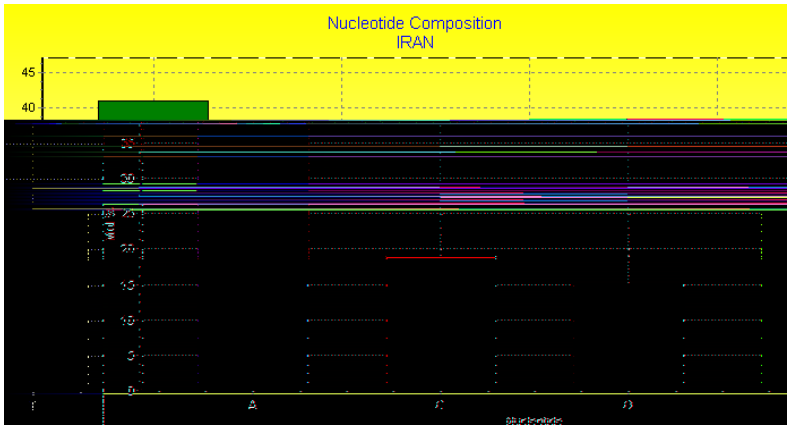

Figure 2. Average base composition of the 16S rRNA region sequences in Afshari sheep

Bootstrap value is a benchmark to determine the level of accuracy of phylogeny tree. The bootstrap analysis is a method to test how well the set of model data and bootstrap was supported by the software testing, the branches could be trusted [DHARMAYANTI, 2011]. Mitochondrial DNA (mt DNA) has become a very powerful tool in species identification and forensic sciences because of the high number of copies in each cell and the lack of recombination with paternal mtDNA.

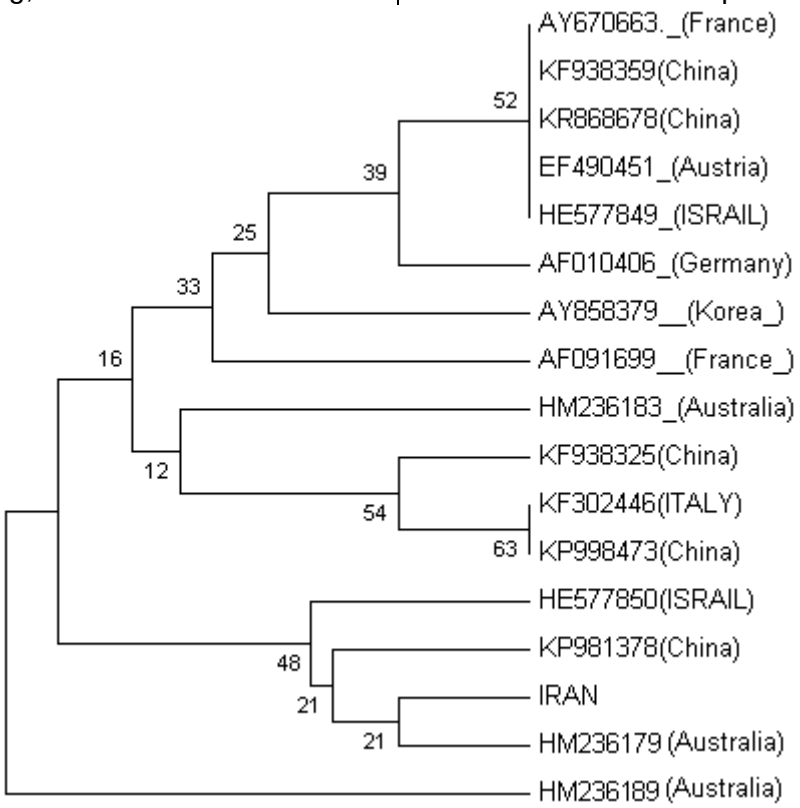

Figure 3. UPGMA phylogenetic tree constructed for Iranian sheep mtDNA sequences based on the combined sequences of the 12S and 16S rRNA genes

The high copy number results in increased sensitivity of species identification, a number of studies have adopted the universal primers introduced by Kocher and collab. [KOCHER et al., 1989, BUTNARIU *et al.*, 2012, STOLERU *et al.*, 2012] targeting the cyt b locus [BELLIS et al., 2003].

However, it is evident that regions residing in the 12S rRNA locus in the mitochondrial genome among mammals are more strictly conserved than the region in the cyt b locus.

These regions are located in both the loop and stem portions in the

secondary structure [SPRINGER and DOUZERY, 1996]. It should be noted that no such highly conserved region has been found in the cyt b region, which is commonly used as a means for species identification [BELLIS et al., 2003; WAN and FANG, 2003].

Conclusions

This study was found informative for establishing relationships between breeds from different parts of the world. In conclusion, this study may facilitate the future researchers and breeders for better understanding the genetic interactions and breed differentiation for devising future breeding and conservation strategies to preserve the rich animal genetic reservoir of the country.

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