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Genetic Diversity of Urial Population in Northeast of Iran

Ali Javadmanesh[1], Shahrokh Ghovvati[2], Mahdi Soltani[3], Mohammadreza Nassiri[1*]

[1] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
[2] Department of Animal Science, Faculty of Agriculture, University of Guilan, Rasht, Iran
[3] Department of Animal Science, Khorasan Razavi Agricultural and Natural Resources Research and Education Center, AREEO, Mashhad, Iran

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Abstract
Habitat eradication and loss of animal species have created a new international hazard for wildlife conservation. National parks are considered as suitable places that can serve dual functions of biodiversity conservation and ecotourism. As recommended by the Food and Agriculture Organization (FAO) and ISAG, microsatellites have been used for animal biodiversity assessment. For this reason, Iranian urials population genetic diversity was studied by analyzing of 10 microsatellite markers in 75 skeletal muscle samples that were collected from Tandooreh National Park, Northeastern of Iran. Species of samples validated by sequencing of the control region from mtDNA. Allelic frequencies for each locus in the population and different measurements of within-breed genetic variations were computed by the POPGENE32 software. The number of alleles per locus counted from five to eight, with an average of 6.1. The polymorphism information content was calculated between 0.66-0.74 with the average of 0.7. Observed heterozygosity ranged from 0.223 (MaF214) to 0.776 (OarFCB128) with an average about 0.584 while the average expected heterozygosity for all studied loci was 0.785 ranging from 0.765 (BM8125) to 0.807 (MaF36). High levels of expected heterozygosity can be attributed to some factors such as low level of inbreeding, low selection pressure, and high allele number. However, findings of the present study of the high variability of the Iranian urials showed the presence of a possible 'hot spot' genetic diversity for wild urial population in the Northeast of Iran. In conclusion, values of genetic diversity revealed that the Iranian urial population harbor unique and appreciable reservoirs of diversity.

Key words: *Wild sheep, Urial, Genetic Diversity, Microsatellites, Iran.*

INTRODUCTION

The growing amount of habitat extermination and loss of animal species have aroused a new international perception for habitat and wildlife conservation (Bulte and Rondeau. 2007). Iran, as a large country in the Middle East with diverse ecosystems, is one of the great areas for the conservation of biodiversity (Farshi and Shariati. 2017). As a result of undisciplined hunting and habitat elimination, Iran has already lost two of its most worthy carnivores, the Caspian Tiger and the Persian Lion (NBSAP 2000). Some other

Corresponding Author: *nassiryr@um.ac.ir*

valuable species are threatened such as Persian crocodile, Persian Bactrian camel, Jebeer, etc (Afsharian et al. 2018; Azghandi et al. 2017; Javadmanesh et al. 2012), which indicate the immediate need for conservation management (NBSAP 2000).

However, one way of conserving biological diversity is the incorporation of environmental concerns in all regional and national development policies (Mace and Baillie 2007). For this goal, the national parks are unique areas of keeping biodiversity in natural ecosystems. Relatively broad natural areas holding specific characteristics and national significance from the geological, biogeographical, ecological, and scenic areas points of view are chosen as national parks with the purpose of keeping the natural and biological conditions, improvement of the populations of animals and vegetation sites. National parks are useful places for research and educational activities as well as ecotourism. In order to protect and maintain the biological diversity, ecological integrity, genetic reserves and scenic areas, residential and consumer utilizations are banned in these areas. (Ramírez and Santana 2019). Outside these areas, the biological diversity conservation is fragile and ecologically sustainable development policies have not been applied effectively (Jowkar et al. 2016).

Tandureh National Park, which is located at Northeast of Iran, has an area level of 37080 hectares. This park is the habitat of a vast variety of plants and wild animals.

Little is known about the actual numbers of Iranian urials. The International Union of the Conservation of Nature (IUCN) lists the *Ovis orientalis* vulnerable to extinction and their numbers are decreasing (Valdez 2008). It has been estimated that about 5000 – 6000 urials are living in Tandureh national park **(**https://en.wikipedia.org/wiki/Tandooreh_National_Park**)**. Urial's height is between 80 to 90 cm at the shoulder in males and weighs around 35 to 80 Kg. Females are smaller than males (Valdez 1976; Bon et al. 1993). The gestation period is between 150 – 180 days and single or twin lambs are born in mid-April to early May in Tandureh. Their life span is about 10 – 11 years. Their powerful hearing, sight, and sense of smell are all well-developed. They are extremely wary, based on quick detection of approaching danger and fight for their survival (Ptak et al. 2002).

The microsatellites have become a standard method for estimating genetic diversity (Nassiri et al. 2018; Peter et al. 2007). High levels of variation, merged with the simple analysis of the PCR, makes microsatellites as one of the most extensively used methods for genetic analysis in sheep (Sherrif and Aleymayehu 2018). Microsatellites have been suggested by the Food and Agriculture Organization (FAO) and International Society for Animal Genetics (ISAG) for the identification of animal biodiversity (Baumung et al. 2004; Cortes et al. 2022). Our study was based on the genotyping of ten microsatellite loci in 75 wild sheep samples from Tandureh National Park's Urials to estimate the genetic diversity.

MATERIAL AND METHODS

Study area

The studied population was distributed over 9250 ha of Tandureh National Park which is located at Northeast highlands of Iran (37°29'E to 37°33'E, 58°33'N to 58°54'N) in Khorasan Razavi province near to Turkmenistan border. From the topography point of view, the area is a mountainous region with an elevation of 900-2586 m. Harirroud, Kashafroud, and Daroungar are the three main rivers around this park. Climatically, Tandureh is a cold rainy area during winters and dry hot summers depend on Siberian and Mediterranean high-pressure circulations. The average annual percipitation is about 370 mm. An annual temperature difference is relatively high as July (the hottest month) has an average temperature of 34.15°C while temperature falls down to 2.7°C in January. From a geological point of view, Tandureh belongs to Koppedaq zone and consists of limestone, sandstone, marn, shale, and conglomerate. The human population density within the park is near zero.

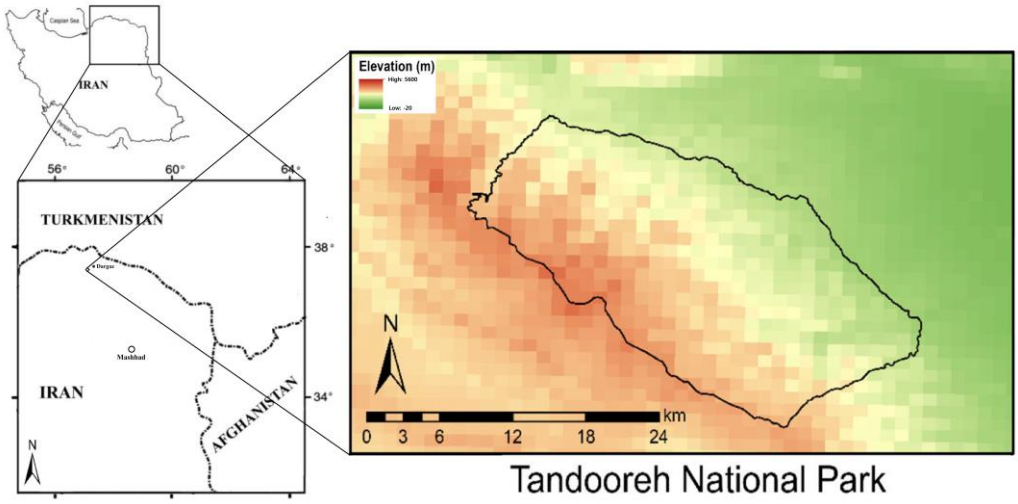

FIGURE 1. The study area.

Sample collection

Meat samples were collected from 75 individuals of legally harvested Iranian urials by hunters from three geographic regions of Tandureh National Park by the permission of Department of Environment, Khorasan-e-Razavi, Iran. Small pieces of skeletal muscle tissues were stored at -20°C as soon as possible.

DNA extraction

Wizard SV Genomic DNA Purification Kit (Promega, USA) was used for DNA extraction according to the manufacturer's instruction. The quantity and purity of extracted DNA samples were assessed using the NanoDrop ND1000 spectrophotometer (Thermofisher Scientific, USA) and electrophoresis in 0.8% agarose gels. Then DNA samples were diluted to 10 ng/µL concentration using TE buffer (10mM Tris-HCl, pH 8.0, 0.1mM EDTA).

Species validation by mtDNA sequencing

To confirm the species of each sample, a 1075 bp from the control region of mitochondrial DNA was amplified, sequenced, and sequence homology was analyzed by the NCBI BLAST tool (Altschul et al. 1990) based on a method described by Hamadalahmad et al. (2020).

Microsatellite genotyping

Ten microsatellite markers were chosen for this study (Table 1) based on a recommendation by the International Society of Animal Genetics (ISAG) under FAO's MoDAD program for sheep diversity studies (http://dad.fao.org/).

Individual microsatellites were genotyped by polymerase chain reaction (PCR). All primer sets were amplified in separated reactions. Multiplexing information, primer sequences, size ranges, and PCR programs of the microsatellite markers are accessible from the FAO website (http://dad.fao.org/en/home.htm) and shown in Table 1. PCR amplification was accomplished in a total volume of 25 µl containing 2.5 µl of 10X reaction buffer, one unit of Platinum *Taq* DNA Polymerase (Invitrogen, USA), 0.2 mM each of dNTPs, 1.5 mM MgCl2, 10 pmol of each primer and 10 ng of extracted DNA. According to the MoDAD project, thermocycler condition was: primary denaturation at 94°C for 3 min, 30 cycles of 30 s at 94°C, 75 s at the specific annealing temperature (Table 1) and followed by a final extension for 5 min at 72°C. The PCR was performed with no extension step based on the MoDAD project data. Genotyping was performed by electrophoresis on an 8% denaturing polyacrylamide gel at 75 W (Bio-Rad, USA) and visualized by silver nitrate staining method (Bassam and Gresshoff 2007). Allele sizes were approximated using Invitrogen™ M12 and M50 bp DNA Ladders (Invitrogen, USA).

TABLE 1. Microsatellite markers and sequencing primers used in this study.

Locus	Accession no.	Chromosome	T_a	Primer
OarFCB128	L01532	2	64	F: CAGCTGAGCAACTAAGACATACATGCG R: ATTAAAGCATCTTCTCTTTATITCCTCGC
McM527	L34277	5	63	F: GTCCATTGCCTCAAATCAATTC R: AAACCACTEACTACTCCCCAA
OarFCB304	L01535	19	67	F: CCCTAGGAGCTTTCAATAAAGAATCGG R: CGCTGCTGTCAACTGGGTCAGGG
MAF36	M80519	22	65.5	F: TTGCGAAAGTTGGACACAATTGAGC R: CATATACCTGGGAGGAATGCATTACG
MAF65	M67437	15	64	F: AAAGGCCAGAGTATGCAATTAGGAG R: CCACTCCTCCTGAGAATATAACATG
MAF214	M88160	16	69.5	F: GGGTGATCAGGGAGGTTTTGGAGG R: AATGCAGGAGATCTGAGGCGGACG
OarHH47	L12557	18	63	F: TITATTGACAAACTCTCTTCCTAACTCCACC R: GTAGTTATI'TAMAAAATATCATACCTCTTAAGG
CSSM031	U03838	23	56.5	F: CCAAGTITAGTAC'TTGTAAGTAGA R: GACTCTCTAGCACTITATCTGTGT
BM8125	G18475	17	63	F: CTCTATCTGTGGAAAAGGTGGG R: GGGGGTTAGACTTCAACATACG
OarCP34	U15699	3	66	F: GCTGAACAATGTGATATGTTCAGG R: GGGACAATACTGTCTTAGATGCTGC
Control region	NC_001941	mt	58	F: AACTTGCTAAAACTCCCAAACATAC R: GTTGGAGTATGAATTTGAGTATTGAG

Statistical analysis

Allelic frequencies calculated once genotypes were determined. Allele frequencies for each loci were calculated by dividing the number for each allele by the total number of alleles (for every specific loci). Parameters of within breed genetic variations like expected and observed heterozygosity, polymorphic parameters (the number of actual alleles and the number of effective alleles) and Shannon index were calculated by POPGENE32 software (Yeh et al. 1999).

The intra-breed genetic variation was calculated based on observed heterozygosity (Ho) and the mean unbiased estimates of gene diversity (He) (Nei 1978). To assess the population's genetic structure, all F-statistics parameters (Weir and Cockerham 1984) were estimated using POPGENE32 computer program.

RESULTS AND DISCUSSION

Species validation

Sequencing and BLAST results showed that all samples belonged to the Transcaspian urial (*Ovis vignei*); Data not shown (Wilson, 2005). This urial sheep currently inhabitant in Turkmenistan, Uzbekistan, northern Iran, western Kazakhstan, Afghanistan, north Pakistan (Rezaei et al. 2010). The geographic distribution of wild sheep showed that naturally, Iran was the only country that inhabited two species of wild sheep with overlapping habitats. In addition, hybrids of these species, *O. vignei* and *O. orientalis* were reported (Nadler et al. 1973).

It has been shown that the control region of the mitochondria in sheep contributed the highest quantity of variation among other fragments of mtDNA (Meadows et al. 2011). Therefore, it can be concluded that this segment might be the best candidate for species identification. Although, there are other fragments have been used for diversity estimation or species identification, e.g. COXI and CytB in wild sheep as well as other species (Azghandi et al. 2017; Javadmanesh et al. 2017).

TABLE 2. True and effective number of alleles.

Locus	Size Range (bp)	*n_a	**n_e	***PIC	Shannon index (H')
OarFCB128	100-128	6	4.9901	0.72	1.6733
McM527	160-184	6	4.8254	0.74	1.6796
OarFCB304	154-191	5	4.4193	0.67	1.5370
MaF36	84-116	8	5.0314	0.71	1.7231
MaF65	160-184	6	4.4897	0.71	1.6297
MaF214	175-267	6	4.1961	0.67	1.5744
OarHH47	126-146	5	4.1977	0.66	1.5121
CSSM031	136-171	7	4.8033	0.74	1.7056
BM8125	108-120	6	4.1614	0.66	1.5490
OarCP34	105-133	6	4.5072	0.68	1.5779
Mean		6.1	4.5622	0.70	1.6162
SD		0.8756	0.3311	0.03	0.0758

*n_a = Observed number of alleles
**n_e = Effective number of alleles
***PIC= Polymorphism information content

Microsatellite loci

All ten microsatellites were amplified in the designed PCR reactions. All studied microsatellite loci were polymorphic and size ranges of amplified alleles were in agreement with the FAO report (2004). Electrophoresis images of all loci are presented in the supplementary material.

The polymorphism information content (PIC) was calculated between 0.66-0.74 with the average of 0.7. The number of alleles per locus counted from five (OarFCB304, OarHH47) to eight (MaF36) for all loci, with an average of 6.1 (Table 2). These results indicated a considerable level of polymorphism among studied loci since all of calculated PICs are above 0.5 (Botstein et al. 1980). We observed a high positive correlation between PIC and both effective allele number and average heterozygosity, 0.84 and 0.81, respectively. It could be inferred that all loci were informative and selected ten loci could be used as an effective panel for estimating genetic diversity.

Allele number is a measure of genetic diversity that has a direct impact on breed development within a species (Buchanan et al. 1994). A total of 61 alleles were detected by the ten microsatellite markers. Also, the highest mean effective number of alleles (n_e) was 5.0314 for MaF36 locus (Table 2). The Chi-square and likelihood ratio tests implemented to evaluate the population for Hardy– Weinberg equilibrium (HWE). Two of independent tests for Hardy-Weinberg equilibrium were rejected at $p < 0.05$. Excess of homozygotes in MaF214 caused its deviation from HWE ($P < 0.05$). On the other hand, a slight excess of homozygotes in OarFCB304 did not affect HW proportions and this locus was still in HWE ($P < 0.05$). In other studied loci, there was not any excess of homozygotes; therefore, no significant departure ($P < 0.05$) from HWE proportions was revealed in these loci. In general, observed HWE deviations were not persistent across all studied loci.

Genetic variation

Observed heterozygosity (H_o) varied from 0.223 (MaF214) to 0.776 (OarFCB128) with an average of about 0.584 while the average expected heterozygosity (H_e, gene diversity) for all loci was 0.785 with variation between 0.765 (BM8125) and 0.807 (MaF36). MaF36 showed the highest level of intrapopulation variation in terms of expected heterozygosity, while BM8125 displayed a lower variability than the other loci. The average means for different genetic diversity parameters, implied that all the studied loci have a high level of genetic variability. All loci were in the relatively similar level of within-breed diversity in terms of H_e ($P < 0.05$).

High levels of expected heterozygosity can be attributed to some factors such a low level of inbreeding, relatively low selection pressure, and high allele number. Since wild sheep breed naturally and no voluntary control made on their reproduction process, consequently, no artificial selection pressure or inbreeding can be imagined. Similar results indicating a high level of diversity was reported on wild sheep population from Northeastern of Iran, however, this study was conducted by the sequencing of the complete length of the control region of mtDNA (Hiendleder et al. 2012).

According to the 95% confidence interval of within-population inbreeding estimates (F_{IS}) were positive in all loci and significantly different from zero ($P < 0.05$). Although these samples did not allow for testing of Mendelian inheritance of the microsatellite alleles, the results revealed that the deficiency of heterozygotes at MaF214 and OarFCB304 microsatellites could be explained by the existence of non-amplifying null alleles. Positive F_{IS} estimates for two studied microsatellites could be assumed as an indication for inbreeding.

Although it is challenging to consider the exact basis of this departure, however, the existence of low-frequency null alleles segregating at these loci may be acknowledged as a possibility (Peter et al. 2007). This deviation could also be connected to reasonably high positive F_{IS} values (Mukesh et al. 2004; Mukesh et al. 2006) observed in the studied population ($P < 0.05$, Table 3). The low number of heterozygotes and high number of homozygotes might be attributed to several aspects such as population heterogeneity, sample relatedness, or null alleles (Peter et al. 2005). However, the main reason for significant F_{IS} values in this population might be the relationship of few individuals under range conditions.

TABLE 3 The observed (H_o), expected (H_e), average heterozygosity and Wright's fixation index (F_{IS}) for different loci.

Locus	H_o	H_e	Average Het.	F_{IS}
OarFCB128	0.7763	0.8049	0.7996	0.0291
McM527	0.6579	0.7980	0.7928	0.1701
OarFCB304	0.4605	0.7788	0.7737	0.4048
MaF36	0.5658	0.8066	0.8012	0.2939
MaF65	0.6184	0.7824	0.7773	0.2044
MaF214	0.2237	0.7667	0.7617	0.7063
OarHH47	0.6316	0.7668	0.7618	0.1709
CSSM031	0.6711	0.7971	0.7918	0.1525
BM8125	0.6053	0.7647	0.7597	0.2033
OarCP34	0.6316	0.7833	0.7881	0.1883
Mean	0.5842	0.7849	0.7798	0.2524
SD	0.1497	0.0160	0.0159	0.1865

Heterozygote deficiency analysis showed that one of the studied loci displayed significant deviations from HWE ($P < 0.05$). It may be due to the presence of low-frequency null alleles segregating at these loci (Peter et al. 2005). This lack of deviation could also be connected to a high negative F_{IS} value (Mukesh et al. 2004) observed in this study ($P < 0.05$).

From the demographic structure of this population, it is obvious that rams breed with some of the ewes in the herd, as male and females grazed together and no supervised mating can be imagined. On the other hand, in industrial husbandry systems, generally, few males breed with all the females in the herd. Using individuals with relationship for reproduction may cause high heterozygote deficiency observed in those systems. Generally, a small sample size in this study might be a cause of individual relatedness in the sample and it might be the reason for the heterozygote deficiency in some loci reported in this study.
Major efforts caused by unsuitable management practices to recover individuals and ensure intentional breeding, mating can cause an unwanted damage to the biodiversity (Goyache et al. 2003). Because of

limitations of sampling such as collecting samples from one region, and the small size of samples, the hidden genetic structure could not be ruled out.

The present results also displayed the high allelic variation in Iranian urial as represented by mean expected heterozygosity (0.765 to 0.807). The average Shannon index across all loci was calculated 1.62 ± 0.08 (Table 3). Direct comparison of diversity measures from other studies is difficult mainly due to quantity of analyzed individuals and differences in marker sets. However, findings of this study of high diversity of the Iranian urials found in this study is in accordance with other sheep biodiversity studies based on microsatellite markers. Domestic sheep breeds originating from the Near East, and neighboring regions such as the Southeast Europe and Caucasian regions, show elevated levels of genetic diversity because they have retained some diversity from the ancestral wild species, mouflon and urial (*Ovis orientalis*) (Tapio et al. 2006; Peter et al. 2007).

We presented a new report on the genetic diversity of the Iranian urial by analyzing microsatellite loci recommended by ISAG.

In conclusion, estimates of genetic diversity indicated that the Iranian urial population located at the Northeastern of the country; contain a unique and estimable reservoir of biodiversity.

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