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Phylogenetic analysis of Persian Gazella, *Gazella subgutturosa* (Artiodactyla: Bovidae) based on cytochrome *b* in central Iran

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A B S T R A C T

Persian gazelle, *Gazella subgutturosa*, exists throughout arid and semiarid regions of Iran and has a key role in these frail ecosystems. Habitat degradation and population decline has placed it on the list of vulnerable species in 2008. The phylogenetic relationships of three Persian gazelle populations in the central part of Iran (i.e. Ghamishlou National Park and Wildlife Refuge, Mouteh Wildlife Refuge in Isfahan province and Kalmand-Bahadoran Protected Area in Yazd province) were investigated using parts and short fragments of mitochondrial cytochrome *b* gene (425 base pairs). A maximum likelihood phylogenetic tree separated the populations of Yazd and Isfahan provinces, but populations within the Isfahan province shared the same clade. All populations were classified as Persian gazelle. The studied populations are facing threats because of road construction, industrial development and urbanization. Accordingly urgent conservation plans are needed to preserve their genetic diversity and prevent them from falling into extinction.

Key words: Mammals; Desert ungulate; Cytochrome *b*; Conservation units

INTRODUCTION

The Persian gazelle, *Gazella subgutturosa* (Gueldenstaedt, 1780), which used to exist in very large numbers, lives in arid and semi-arid habitats from the Arabian peninsula through Mongolia [1-3]. Central Iran has been known to be one of the main habitats for the Persian gazelle. In the mid-1970s, Persian gazelles added up to many thousands, but now only about 20% of the former population remains [4], and the rest are nowadays threatened by extinction in Iran [1, 2, 4, 5]. The main reason can be intensified poaching, which has

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become far more efficient since the introduction of firearm based hunting from motorized vehicles [6-8]. Furthermore, habitat loss or deterioration as well as competition with domestic livestock have had major impacts on many migratory gazelles [9, 10]. These threats have already led to the IUCN (2008) classification of the Persian gazelle (*Gazella subgutturosa*) as 'vulnerable species'. Although morphology, taxonomic and habitat preference of the Persian gazelle have been studied already [1, 11, 12], using molecular markers to obtain information on its genetic diversity has been scarce [2].

Genetic diversity is one of the most important attributes of any population. Assessing genetic diversity is central to population genetics, and has extremely important applications in conservation biology [13]. Conservation genetics has major implications for the conservation of biodiversity by clarifying taxonomic relationships [14, 15]. On the other hand, an urgent need for gazelle conservation has long been felt [9, 16].

A main objective of the present study was to analyze the genetic diversity of Persian gazelle populations in central parts of Iran in order to understand their phylogenetic relationships. To achieve this, sequence variations of a mitochondrial marker (cytochrome *b*) of samples obtained from Persian gazelles were analyzed. It is concluded that although the populations are separated in central Iran, gen flow can be observed in close areas.

MATERIALS AND METHODS

Taxon sampling: Samples from Persian gazelles were collected from 3 different localities in central Iran including Kalmand-Bahadoran Protected Area, Mouteh Wildlife Refuge, and Ghamishlou National Park and wildlife Refuge (Fig. 1). In 2012, Mouteh WR harboured approximately 3000–4000, and Ghamishlou NP&WR about 3000 Persian gazelles (unpublished data). Hence the center of Iran can be considered as an important area for this species in the country. Fresh faeces where collected in the field, after having observed the animal from a distance to ensure species identification. This noninvasive sampling helped avoid the capturing of the animals, hence reducing the risk of injuries and disturbing social groups. For each area, 35 samples were collected and preserved using ethanol 96% methods. Sequences from other gazelle taxa were obtained from GenBank (Table 1).

DNA extraction: Whole genomic DNA was extracted from fecal samples using Bioneer DNA extraction kit (Takapozist) following the manufacturer's instructions. The 5′-region of the cytochrome *b* (Cyt *b*) gene was PCR-amplified using the versatile primers L14724 and H15149 [3, 8-10, 17, 18].

Sequencing: The PCR reactions were performed in a final volume 25 µl containing 1 µl of DNA, 1 µl of each primer, and 22 µl water using the Bioneer PCR kit. Amplifications of markers were performed under the following conditions: initial denaturation (30 s at 94°C); then for 30 cycles, denaturation, 94°C, 30 s; annealing, 55°C, 30 s; extension, 72°C, 45 s; final extension, 10 min, 72°C. PCR products were purified using the Bioneer kit (Takapozist) following the manufacturer's instructions. 5 samples of Purified PCR products

for each area were analysed on an ABI 3130 automated sequencer. Sequences were edited for correction with SeqScape v2.6 software (Applied biosystems). All sequences were deposited in GenBank (Accession Number KF790602-16, Table 1).

Sequences from GenBank and those from our dataset were aligned with Mega version 5 [19] and adjusted by eye. A Maximum likelihood (ML) tree [20] of the Cyt *b* sequences using Close-Neighbor-Interchange algorithm was constructed to display a graphical view of the Gazelle species studied here [21, 22]. The robustness of the ML tree was assessed by performing bootstrapping analyses with 1000 replicates.

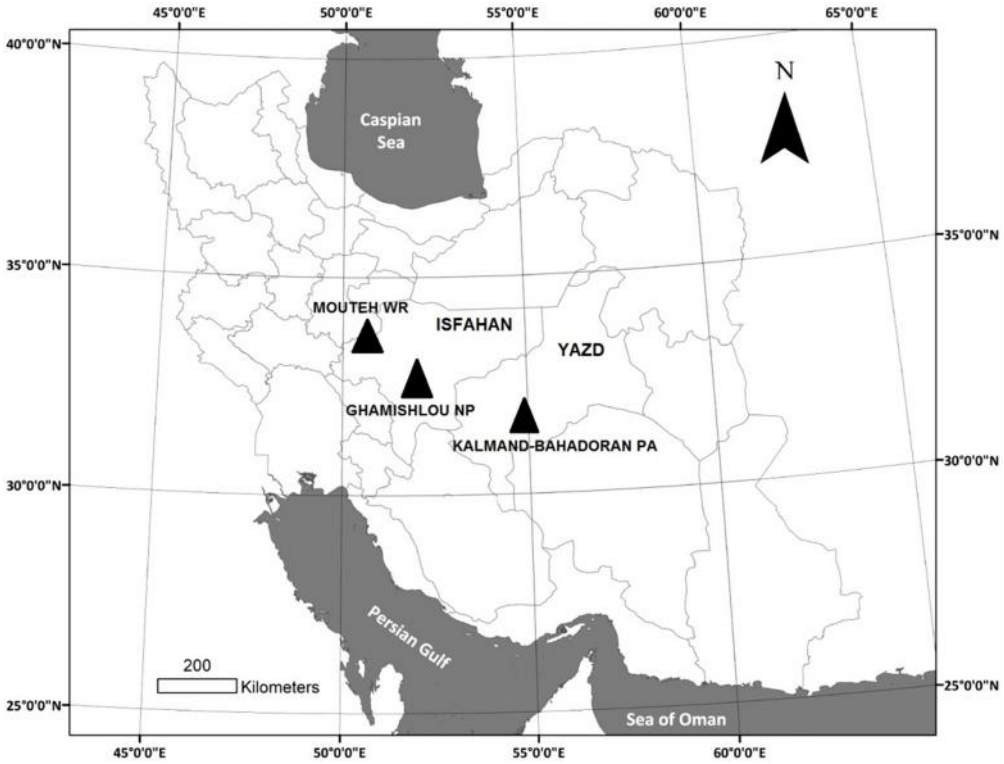

Figure 1: Map of Iran and geological location of the sampling area (black triangles).

RESULTS

All 15 Persian gazelle samples (Table 1) yielded DNA of sufficient quality and quantity to allow PCR amplification of the 425 bp fragment of Cyt *b* (L14724-H15149). All samples were successfully sequenced. Nucleotide frequencies were 32.1% A, 26.7% C, 14.4% G, and 26.8% T.

Table 1: List of specimens of *Gazella subgutturosa* and other species included in the phylogenetic analysis, accession numbers and sample types.

Taxon	Origin	Individual ID	Tree ID	Accession number	Sample type
G. subgutturosa	Yazd, Kalmand-Bahadoran PA Lat 31/48, long 54/47	1YAZD900916	KAL1	KF790602	Feces
		1YAZD900918	KAL2	KF790603	Feces
		1YAZD900919	KAL3	KF790604	Feces
		1YAZD900922	KAL4	KF790605	Feces
		1YAZD900924	KAL5	KF790606	Feces
G. subgutturosa	Isfahan, Mouteh WR Lat 33/62, long 50/63	1SFHN900913	MOT1	KF790607	Feces
		1SFHN900914	MOT2	KF790608	Feces
		1SFHN900915	MOT3	KF790609	Feces
		1SFHN900916	MOT4	KF790610	Feces
		1SFHN900917	MOT5	KF790611	Feces
G. subgutturosa	Isfahan, Ghamishlou NP Lat 32/86, long 51/26	2SFHN900904	QAM1	KF790612	Feces
		2SFHN900905	QAM2	KF790613	Feces
		2SFHN900906	QAM3	KF790614	Feces
		2SFHN900907	QAM4	KF790615	Feces
		2SFHN900908	QAM5	KF790616	Feces
G. bennettii				JN632635	GenBank
G. cuvieri				JN632636	GenBank
G. dorcas				JN632638	GenBank
G. erlangeri				JN632639	GenBank
G. gazella				JN632640	GenBank
G. leptoceros				JN632641	GenBank
G. spekei				JN632642	GenBank
G. subgutturosa				JN632644	GenBank
G. arabica				KC188765	GenBank
Saiga tatarica				JN632700	GenBank

The best tree based on Cyt *b* sequences obtained from the ML analyses is depicted in Figure 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [23]. *Saiga tatarica* was used as an out-group. The ML tree was obtained using a Close-Neighbor-Interchange algorithm [21, 24]. Accession numbers are given for sequences obtained from GenBank.

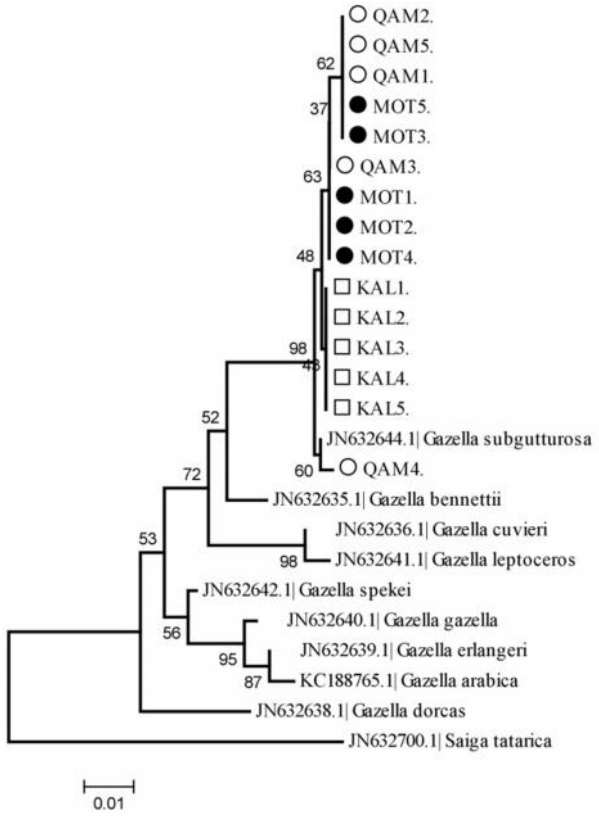

Figure 2: Maximum likelihood tree of the cytochrome *b* dataset

The numbers of base substitutions per site from analysis between sequences are shown at the lower-left side of Table 2 [25]. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. The number of base differences per sequence from analysis between sequences is shown at the upper-right side of Table 2.

Table 2: Estimates of evolutionary divergence between sequences

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	JN632644 G. subgutturosa		16	20	19	14	18	22	23	12	1	1	1	1	1	2	2	3	2	3	3	3	2	1	3	40
2	JN632636 G. cuvieri	0/041		19	18	14	2	21	19	10	17	17	17	17	17	17	17	18	17	18	18	18	17	17	18	46
3	JN632639 G. erlangeri	0/051	0/048		3	7	19	2	16	16	21	21	21	21	21	21	21	22	21	22	22	22	21	21	22	38
4	JN632640 G. gazella	0/049	0/046	0/007		6	18	5	15	15	20	20	20	20	20	20	20	21	20	21	21	21	20	20	21	39
5	JN632642 G. spekei	0/035	0/035	0/017	0/015		14	9	11	12	15	15	15	15	15	15	15	16	15	16	16	16	15	15	16	37
6	JN632641 G. leptoceros	0/046	0/005	0/048	0/046	0/035		21	19	12	19	19	19	19	19	19	19	20	19	20	20	20	19	19	20	48
7	KC188765 G. arabica	0/057	0/054	0/005	0/012	0/022	0/054		18	18	23	23	23	23	23	23	23	24	23	24	24	24	23	23	24	40
8	JN632638 G. dorcas	0/059	0/048	0/040	0/038	0/027	0/048	0/046		18	24	24	24	24	24	24	24	25	24	25	25	25	24	24	25	36
9	JN632635 G. bennetti	0/030	0/025	0/041	0/038	0/030	0/030	0/046	0/046		11	11	11	11	11	11	11	12	11	12	12	12	11	13	12	41
10	KAL1.	0/002	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028		0	0	0	0	1	1	2	1	2	2	2	1	2	2	39
11	KAL2.	0/002	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028	0/000		0	0	0	1	1	2	1	2	2	2	1	2	2	39
12	KAL3.	0/002	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028	0/000	0/000		0	0	1	1	2	1	2	2	2	1	2	2	39
13	KAL4.	0/002	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028	0/000	0/000	0/000		0	1	1	2	1	2	2	2	1	2	2	39
14	KAL5.	0/002	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028	0/000	0/000	0/000	0/000		1	1	2	1	2	2	2	1	2	2	39
15	MOT1.	0/005	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028	0/002	0/002	0/002	0/002	0/002		0	1	0	1	1	1	0	3	1	39
16	MOT2.	0/005	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028	0/002	0/002	0/002	0/002	0/002	0/000		1	0	1	1	1	0	3	1	39
17	MOT3.	0/007	0/046	0/057	0/054	0/041	0/052	0/062	0/065	0/030	0/005	0/005	0/005	0/005	0/005	0/002	0/002		1	0	0	0	1	4	0	40
18	MOT4.	0/005	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028	0/002	0/002	0/002	0/002	0/002	0/000	0/000	0/002		1	1	1	0	3	1	39
19	MOT5.	0/007	0/046	0/057	0/054	0/041	0/052	0/062	0/065	0/030	0/005	0/005	0/005	0/005	0/005	0/002	0/002	0/000	0/002		0	0	1	4	0	40
20	QAM1.	0/007	0/046	0/057	0/054	0/041	0/052	0/062	0/065	0/030	0/005	0/005	0/005	0/005	0/005	0/002	0/002	0/000	0/002	0/000		0	1	4	0	40
21	QAM2.	0/007	0/046	0/057	0/054	0/041	0/052	0/062	0/065	0/030	0/005	0/005	0/005	0/005	0/005	0/002	0/002	0/000	0/002	0/000	0/000		1	4	0	40
22	QAM3.	0/005	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/028	0/002	0/002	0/002	0/002	0/002	0/000	0/000	0/002	0/000	0/002	0/002	0/002		3	1	39
23	QAM4.	0/002	0/043	0/054	0/051	0/038	0/049	0/060	0/062	0/033	0/005	0/005	0/005	0/005	0/005	0/007	0/007	0/010	0/007	0/010	0/010	0/010	0/007		4	41
24	QAM5.	0/007	0/046	0/057	0/054	0/041	0/052	0/062	0/065	0/030	0/005	0/005	0/005	0/005	0/005	0/002	0/002	0/000	0/002	0/000	0/000	0/000	0/002	0/010		40
25	JN632700 Saiga_tatarica	0/107	0/124	0/101	0/104	0/098	0/129	0/107	0/095	0/109	0/105	0/105	0/105	0/105	0/105	0/105	0/105	0/108	0/105	0/108	0/108	0/108	0/105	0/110	0/108	

DISCUSSION

Information about phylogenetic relationships among and within threatened groups of animals is essential for conservation efforts [14, 15]. There are more than 4000 Persian gazelles in Mooteh Wildlife Refuge which make this refuge an important genetic pool for gazelles in Iran. However, poaching and loss of suitable habitats due to road constructions for rural and urban development, as well as extensive agriculture and industrial activities have all reduced the numbers of gazelles. Likewise, man-made barriers have prohibited gazelles to disperse and immigrate to other habitats like the Haftadgholle Protected Area in Markazi province which can lead to the isolation of the populations.

According to the phylogenetic tree (Fig. 2) all populations from the three habitats share the same branch, therefore, this region of Cyt *b* is appropriate for the species-level recognition of gazelles. Kalmand's Gazelles are separated from the two other populations of Isfahan province. This separation is the result of different evolutionary events or genetic distances among this and other populations in this survey. The populations of Isfahan province (Mooteh and Ghamishlou) are close to each other, thus allowing the exchange of a few individuals. This gene flow is possible because they are located in close geographic proximity and some migration corridors join them together. Also, it could probably be said that the division of these populations has taken place recently since there is no remarkable genetic difference between them. Meanwhile, a substantial result is that an individual belonging to Ghamishlou could be separated from the others by relatively large distances (Fig. 2), which shows the connection of this population with others in Isfahan or adjacent provinces by means of immigrations of individuals. However, this interpretation needs to be confirmed by studying more Persian gazelle populations. Our phylogenetic tree (Fig. 2) shows that Persian gazelles have closer relationships to individuals from *G. bennetti*, *G. cuvieri* and *G. leptoceros*, a finding which is congruent with Wacher et al, 2010 [8], but against the results reported by Nasiri and Mahdavi, 2012 [26].

As shown in the tree, Jeebir (*G. bennetti*) is a sister group to the Persian gazelle, suggesting that these species might have had similar evolutionary paths (Fig. 2). Most previous studies were based on 425 base pair sequences of Cyt *b*. Our findings show that this region of Cyt *b* gene is not capable of distinguishing between populations of the same species, thus being suitable only for clustering the species of this genus.

According to the results of haplotype diversity, all Kalmand individuals belong to one haplotype. Three individuals of Mooteh belonged to the same haplotype as one Ghamishlou individual. Also two Mooteh individuals formed another haplotype with three Ghamishlou individuals. In addition, one Ghamishlou individual was recognized as an independent haplotype. It is clear that the least haplotype diversity exists in Kalmand and the highest haplotype diversity in Ghamishlou. Such differences in haplotype diversity among habitats are caused by spatial situations of Kalmand which is geographically isolated from adjacent habitats and has less gene flow with other populations consequently. In contrast, Ghamishlou, which is located at the junction of several immigration pathways for gazelles from different habitats, has more haplotype diversity than other investigated populations.

The important matter to be considered is that our segment was not large enough to segregate the gazelles properly. In addition, our results regarding diversity were based on this segment with 425 bp length. Therefore, other regions of genome or the complete Cyt *b* must be analyzed to evaluate our results. In addition, using mtDNA markers only shows maternal relationships, and to investigate paternal ancestry, at least one marker from the male's sexual chromosome is necessary.

In our study, the gazelles were classified as Persian gazelles (*Gazella subgutturosa*). However, this short marker is not capable of identifying species properly. We suggest performing sequencing on the complete Cyt *b* to yield more reliable results, similar to what was carried out by Lerp et al, 2011 [6]. Accordingly, for a more detailed study, longer regions of Cyt *b* or whole Cyt *b* should be used. Lerp et al, 2011 [6] have also shown longer regions of Cyt *b* to be highly effective in resolving phylogenetic relationships among gazelles [17].

The Persian gazelle is a widely distributed species, especially in central parts of Iran. It is monophyletic in central Iran, and the results of its phylogenetic tree demonstrate that the populations are still in contact, and that gene flows among populations of Mooteh and Ghamishlou are taking place. Unfortunately all populations have decreased, and are being threatened by habitat degradation and fragmentation as a result of road construction, industrial development and the urbanization of Ghamishlou National Park and Wildlife Refuge. Road developments have fragmented habitats into two separate parts preventing the gazelles' movement among habitats of the Kalmand-Bahadoran Protected Area [11].

Urgent protective measures and conservation plans are needed to preserve the Gazelle populations' genetic diversity. Otherwise, habitat degradation and other threatening factors can result in shrinking and isolated populations. These could result in its susceptibility to genetic drift, bottle neck, demographic problems, and unbalanced sex ratio, hence pushing it towards the edge of extinction.

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