

Low Genetic Diversity in the Goitered Gazelle *Gazella subgutturosa* (Güldenstädt, 1780) (Artiodactyla: Bovidae) in North-western China as Revealed by the Mitochondrial Cytochrome *b* Gene

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Abstract: The goitered gazelle (*Gazella subgutturosa*) is a vulnerable, protected species in China. To gain insight into the current genetic diversity of the goitered gazelle in the Xinjiang Uyghur Autonomous Region (XUAR), north-western China, we collected samples from wild individuals from across the species' natural range within XUAR and sequenced 995 bp of the mitochondrial cytochrome *b* gene from 113 samples. Among these samples, we detected 19 haplotypes, of which 14 were novel. The studied goitered gazelles exhibited high haplotype diversity (0.804) but low nucleotide diversity (0.001) as compared to other antelope species. In a Bayesian phylogenetic tree, haplotypes formed only one clade, with no apparent geographical population structure. Neutrality test and mismatch distribution analyses indicated a past demographic bottleneck event. Urgent protective measures must be planned to retain and recover genetic diversity in this species in order to avoid extinction.

Key words: *Gazella subgutturosa*, bottleneck event, urgent protective measure, vulnerable species, Xinjiang

Introduction

The goitered gazelle *Gazella subgutturosa* (Güldenstädt, 1780), also called the Persian gazelle, is an important ungulate inhabiting desert and semi-desert areas. Historically this species was distributed from the Arabian Peninsula northward to Kazakhstan across the Middle East and from Syria westward to Mongolia and north-eastern China (Fig 1). It might be extinct in some countries, such as Yemen, Afghanistan, Pakistan and Kyrgyzstan (DURMUŞ 2010) and is already extinct in the wild in Georgia, Armenia, Kuwait and Qatar. Ranked as a class-II key protected wildlife species in China (WANG 1998), it is now classified as "Vulnerable" by the International Union for Conservation of Nature (IUCN 2017). Its

distributional area and population size have sharply decreased in recent decades due to grazing interference, overhunting and poaching, agricultural encroachment, as well as roads and railways imposing physical barriers and resulting in population fragmentation (WANG 1998, IUCN RED LIST 2017).

Genetic diversity is essential for the long-term survival of any population or species and knowledge of diversity levels is extremely important in conservation and evolutionary biology (FREELAND et al. 2011). Some relevant studies exist for the goitered gazelle. A single population in Iran exhibited very low genetic diversity in the mitochondrial control region (481 bp) and five out of seven microsatellite loci

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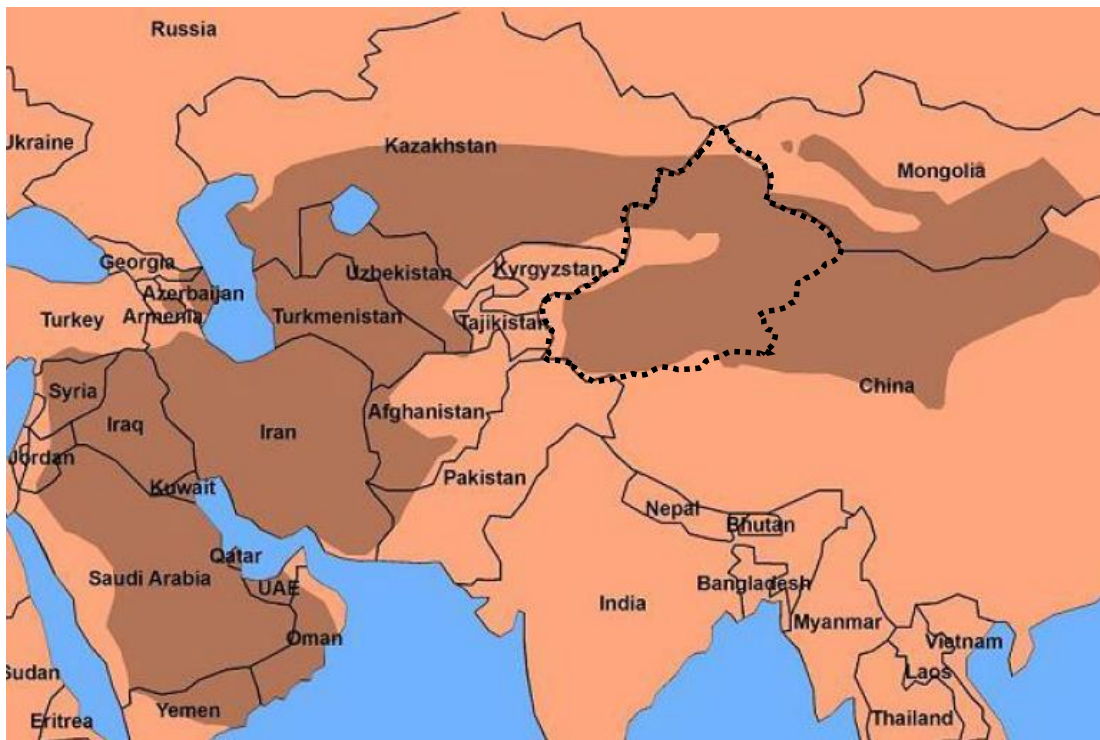


Fig. 1. Partial map of Asia showing the historical distribution of the goitered gazelle (after DURMUŞ 2010). The species is now locally extinct in Georgia, Armenia, Kuwait, and Qatar. The approximate study area is shown with black dashed line.

deviated from the Hardy-Weinberg equilibrium, implying possible inbreeding (ZACHOS et al. 2010). Data from the mitochondrial control region (985 bp) indicate strict isolation of the goitered gazelle population in Azerbaijan from other, genetically similar populations in Uzbekistan and Turkmenistan (SOROKIN et al. 2011). A study of 15 microsatellite loci from six geographic populations (KHOSRAVI et al. 2018) have detected severe bottleneck events across a long period, which results in decreased genetic diversity (NYSTROM et al. 2006) and isolation by distance for the goitered gazelle population in Iran.

The goitered gazelle is widely distributed in the Xinjiang Uyghur Autonomous Region (XUAR), north-western China. In XUAR, the population size in the 1990s was estimated to be about 13,000–24,000 individuals in Changji Prefecture and 7000–18,000 in the Kalamaili Ungulate Nature Reserve (KUNR); around 5580 individuals were reported elsewhere (SU et al. 1999). The average density was 0.71 ± 0.17 individual/km² based on data from 19 transects in northern XUAR and 0.57 ± 0.26 individual/km², based on eight transects in the Humul Prefecture, Southern XUAR (reviewed in SUN et al. 2002, XU et al. 2008). Nonetheless, the high gazelle densities in protected areas were considered likely due to increasing mining activities and human interference around the species' habitat (CHU et al. 2009). The population size in the Ebnur Lake National

Wetland Nature Reserve (ELNWR) was estimated at no more than 314 individuals in 2015 (NABI et al. 2015). Current population data are lacking for other parts of the species' range in XUAR.

Although several genetic studies (DONG et al. 2016, LV et al. 2015, TURAP et al. 2016, TURSUN et al. 2016,) have recently been conducted on the goitered gazelle in north-western China, these studies examined only samples from captive animals in zoos, wild animals in protected areas such as national nature reserves, or were restricted to a few samples. The goal of our study was to examine the genetic diversity of the goitered gazelle throughout its natural range in XUAR, by analysing mitochondrial cytochrome *b* (CYT *b*) sequences from samples taken from wild individuals. Our study provides crucial data for future studies across the range of the species, as well as for breeding programs and conservation.

Materials and Methods

Pieces of muscle tissue from poached individuals or as fecal samples were collected from wild populations of the goitered gazelle (Fig. 2). Muscle-tissue samples were obtained from Aksu, Bay, Bugur, Lopnur, Altuntagh and Hoten, as well as a few from Atush, Qakilik and Qarqan. Fresh feces were collected in the field from across most of the species' range in XUAR. The fecal individual identifications were carried out

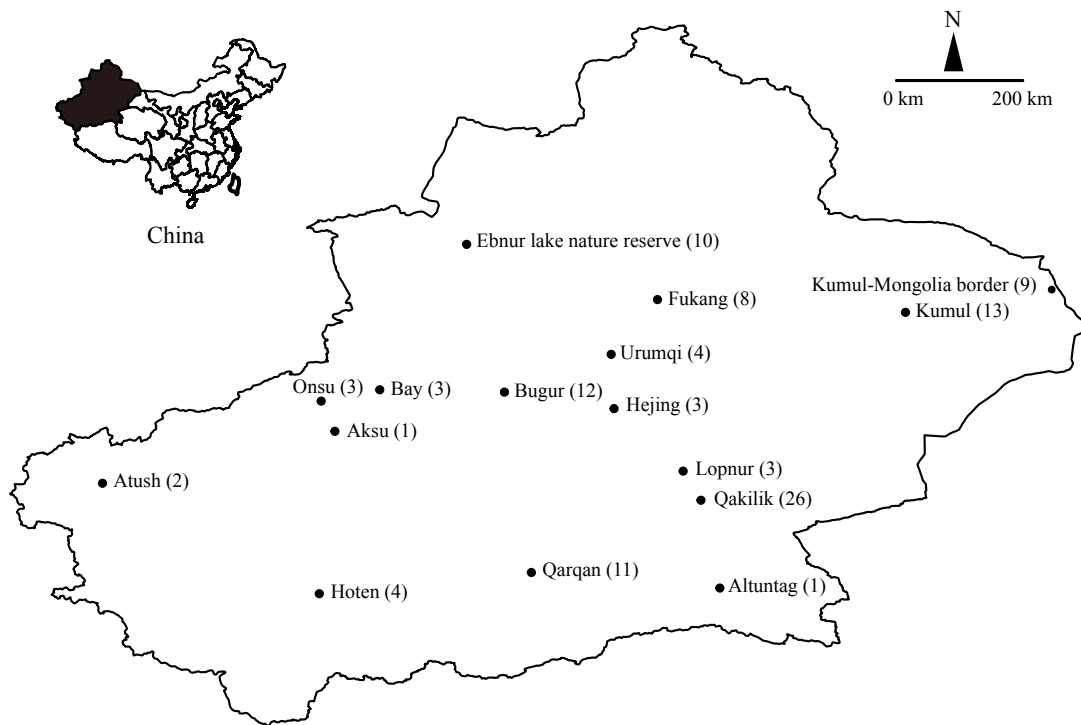


Fig. 2. Map of the Xinjiang Uyghur Autonomous Region (XUAR), China showing the localities of muscle or fecal samples collected from goitered gazelles (*Gazella subgutturosa*) in this study, with the sample size at each locality indicated in parentheses.

as described in ABDURIYIM et al. (2018). This non-invasive sampling avoided the capturing of animals, hence reducing the risk of injuries and disturbing social groups. A total of 113 samples (32 muscle tissues and 81 fecal samples) were collected and preserved in 96% ethanol at -80°C until DNA extraction.

Total genomic DNA was extracted from fecal samples by using the method of RISALAT et al. (2012) and from muscle samples by using the method described in ZHAO (2006). DNA was extracted into 30 μl of TE buffer and preserved at -4°C until use. The *CYT b* gene was PCR-amplified using the universal primers L14724 and H15149 (L14724: 5'-TGACTA ATG ATA TGA AAA ACC ATC GTT G-3'; H15149: 5'-TGC TCT CCT TCT CTG GTT TAC AAG AC-3') (KOCHER et al. 1989, IRWIN et al. 1991). Reactions were performed in 50 μl reaction volumes each containing 0.5 units of *Taq* polymerase, 1.5 mM MgCl_2 , 40 μM each dNTP and 200 nM each primer. Amplification conditions were 5 min at 95°C ; 32 cycles of 60s at 94°C , 60s at 54°C and 90s at 72°C ; and 10 min at 72°C . Purification of PCR products and sequencing were done by Dingguo Changsheng Biotech (Beijing, China), with L14724 and H15149 used as sequencing primers. Chromatograms were analysed with Seqman 6.1 (SWINDELL & PLASTERER 1997) in the Lasergene v. 6 package (DNASTAR), and sequences were aligned by using ClustelW 1.6 imple-

mented in MEGA v. 6 software (TAMURA et al. 2013).

The nucleotide composition and number of transitions and transversions were calculated with MEGA v. 6. Nucleotide diversity (π) and haplotype diversity (h) values were calculated in DnaSP v. 5 (LIBRADO & ROZAS 2009) and interpreted using GRANT & BOWEN (1998). DnaSP was also used to conduct a neutrality test and mismatch distribution analysis. Phylogeny was reconstructed using Bayesian analyses performed in MrBayes version 3.1.2 (RONQUIST & HUELSENBECK 2003), using a partition model in which different substitution models were applied to different gene partitions. The best-fit substitution model was determined for each partition by using the Bayesian information criterion (BIC) implemented in KAKUSAN v. 4 (TANABE 2011). The Bayesian analyses began with random starting trees and ran for 3 million generations, with Markov chains sampled every 100 generations, after a burn-in phase of 25% generations. Analyses were checked with Tracer v1.6.0 (RAMBAUT et al. 2014) for convergence of parameter values sampled from the chains. The Bayesian tree was visualised using TREEFINDER v. March 2011 (JOBBERG et al. 2004). A median-joining (MJ) network (BANDELIT et al. 1999) was constructed with Network v. 4 (www.fluxus-engineering.com) as an alternative way of visualising the relationships among haplotypes. Our data set for the phylogenetic analyses

contained the following CYT *b* sequences obtained from GenBank: 12 sequences from goitered gazelles from KUNR (Genbank: KM978960 - KM978991); two from *G. s. subgutturosa* from Turkmenistan (JN632644 and NC020710); one from *G. subgutturosa* of unknown origin (AF036282); one from *G.*

marica from the Arabian Region (JN632643) and one sequence each from *G. cuvieri* (JN632636), *G. leptoceros* (JN632641), and *G. bennettii* (JN632635). One sequence from *Saiga tatarica* (JN632635) was included as the outgroup taxon.

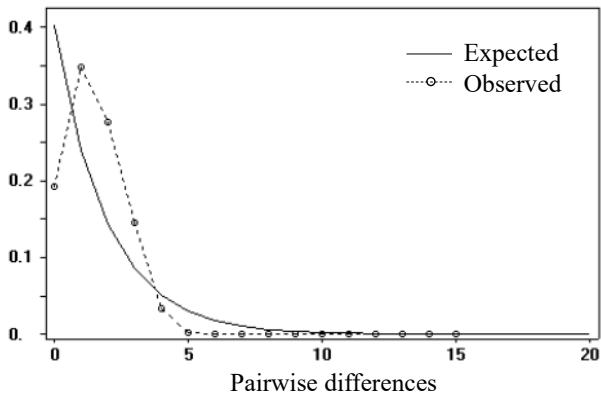


Fig. 3. Mismatch distribution for CYT *b* sequences from goitered gazelles in the XUAR population.

Results

In total, 113 sequences (length of the alignment, 995 bp) were obtained for CYT *b*. The average nucleotide frequencies were A=31.4%, G=13.2%, C=28.8% and T=26.6% overall, while A=47.8%, C=36.1%, G=1.8%, and T=14.6% for the third position. Based on 21 polymorphic sites (20 transitions and one transversion, of which 13 were potentially phylogenetically informative), 19 haplotypes were observed. Fourteen (GenBank accession numbers: LC333582-LC333595) of the 19 haplotypes were novel, with the other five previously detected in individuals from the Kalamaili Ungulate Nature Reserve (KUNR) in XUAR: HAP01 (GenBank accession number: KM978960), HAP02 (KM978961),

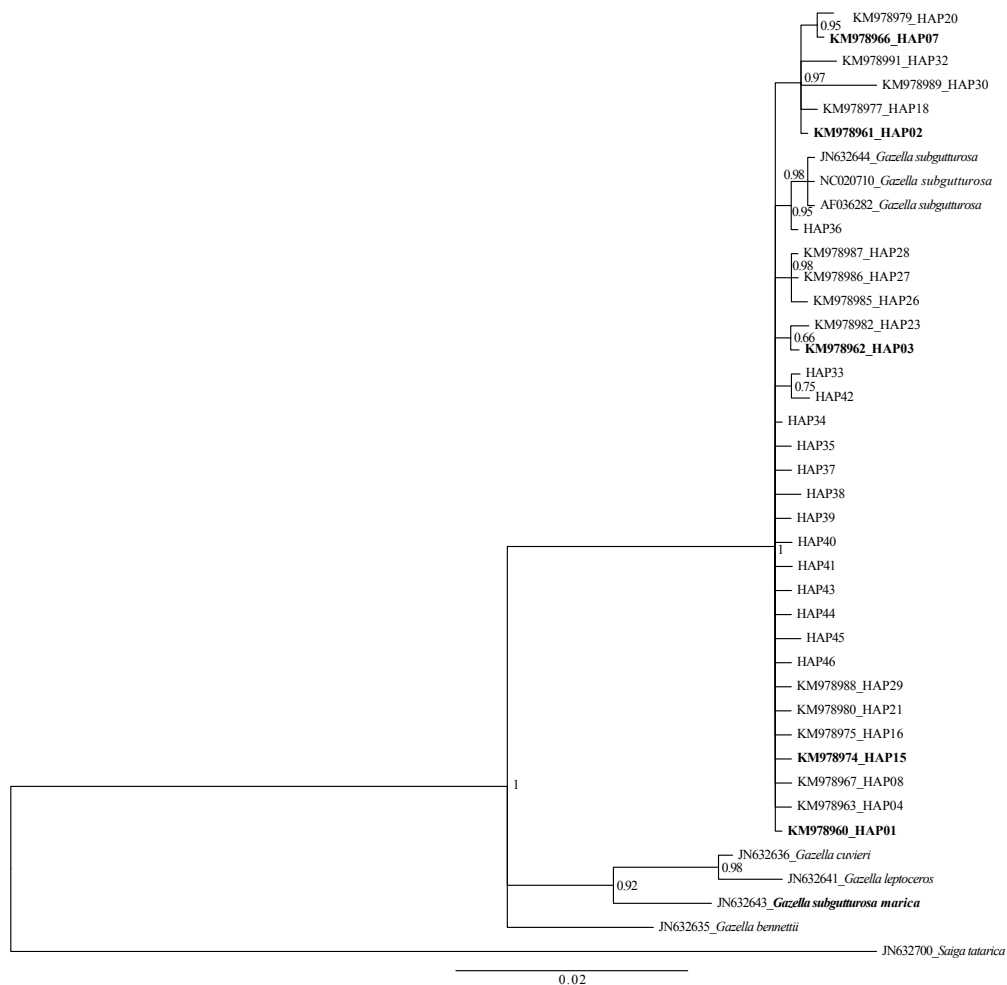


Fig. 4. Bayesian phylogenetic tree based on 995 bp of the mitochondrial cytochrome *b* gene. Previously detected haplotypes also observed in this study are in bold; HAP33 to HAP46 are new haplotypes detected in this study. Haplotypes preceded by GenBank accession numbers were obtained from GenBank. Numbers near nodes are posterior probability values.

Table 1. Variable sites among cytochrome *b* haplotypes detected in the goitered gazelle in Xinjiang Uyghur Autonomous Region, China, haplotype frequencies among 113 individuals and haplotype locations. Hap stands for haplotype, freq for frequency. Abbreviations for haplotype locations: AB—Ebnur Lake Wetlan National Nature Reserve; FK—Fukang; TL—Urumqi; KM*—Komul-Mongolia border; KM—Kumul; LP—Lopnur; QK—Qakilik; QQ—Qarqan; HJ—Hejing; BU—Bugur; HT—Hoten; AT—Atush. @ indicates sample locations including AB, HJ, BU, Onsu, Aksu, Bay, Altuntag, LP, FK, KM, KM*, HT, QQ, and Kalamaili Angulate Nature Reserve (DONG et al. 2016).

Hap	Variable sites																		Hap freq	Hap locations			
	2	1	1	2	2	3	4	4	4	5	5	5	6	7	8	8	8	9			9	9	9
	2	6	6	1	3	3	0	1	6	0	3	7	3	3	2	2	6	3			6	8	9
HAP33	C	T	A	A	A	T	A	A	C	T	C	T	C	C	T	A	T	C	C	T	T	12	KM,AT,BU,HT
HAP34	C	.	.	T	12	AB,HJ,QQ,TL
HAP35	G	T	2	BU
HAP36	T	G	12	HJ,QK,LP
HAP37	G	.	T	1	KM
HAP38	.	C	T	.	T	3	KM
HAP39	.	.	.	G	.	C	.	.	T	1	QQ
HAP40	C	.	.	T	.	.	.	T	1	QQ
HAP41	A	C	.	.	T	1	QQ
HAP42	.	.	T	2	QQ,QK
HAP43	T	C	1	QK
HAP44	T	T	.	.	.	2	KM*
HAP45	T	C	T	.	.	1	KM*
HAP46	T	T	1	KM*
HAP1	T	45	@
HAP2	T	.	.	C	C	10	AB,BU,FK,TL
HAP3	T	C	2	FK
HAP7	G	.	.	.	T	.	.	C	C	2	FK,TL
HAP15	T	C	.	2	KM*

HAP03 (KM978962), HAP07 (KM978966) and HAP15 (KM978974). Interestingly, HAP01 was detected in more than one-third (40%) of the gazelle individuals in our study (Table 1), suggesting that it was the ancestral haplotype sequence. Haplotype and nucleotide diversity were 0.804 ± 0.031 and 0.00149 ± 0.00087 , respectively. Both Tajima's D (-1.7976) and Fu's FS (-11.9442) values for the examined population were negative, but only Fu's FS value was statistically significant ($p < 0.0001$), indicating that the goitered gazelle in XUAR has undergone population expansion, perhaps following a reduction in size but not a recent bottleneck. The unimodal mismatch distribution (Fig. 3) indicated a single demographic expansion in the past, again not recently.

Our Bayesian tree (Fig. 4) showed one major *G. subgutturosa* clade containing all of the haplotypes from XUAR, the sequence from Turkmenistan and the sequence of unknown origin. The clade was poorly resolved, consisting of a large polytomy, with only a few evident sub-clades. Nodes for the gazelle

and goitered gazelle clades were well supported, with posterior probability values of 1. Within the phylogenetic tree, there was no correlation between geographical location (sampling site) and phylogenetic relationship (Table 1 and Fig. 4). An MJ haplotype network (Fig. 5) including our sequences and representative goitered gazelle sequences from GenBank showed a star-like topology centred around HAP01, the most frequent haplotype, with most of the other haplotypes divergent by one or two mutational steps. Interestingly, the goitered gazelle haplotypes from Turkmenistan and of unknown origin were related through HAP36 to haplotype HAP01, which was present in most individuals from the Tarim Basin.

Discussion

Genetic variation is generally a measure of a population's evolutionary potential. Because mitochondrial genes have a higher rate of evolution than nuclear genes in vertebrates (BIRKY et al. 1983), they

are useful for analysing genetic diversity at the genus and species levels (ARIF & KHAN 2009). The CYT *b* gene applied to assess the genetic diversity of the goitered gazelle in XUAR in our study exhibited 19 haplotypes, with mean haplotype diversity 0.804 and mean nucleotide diversity 0.00149. Mitochondrial DNA has been used to assess genetic diversity in other antelope species or subspecies, such as the saiga antelope *Saiga tatarica* (KHOLODOVA et al. 2006), Grant's gazelle *Gazella granti* (ARCTANDER et al. 1996), Przewalski's gazelle *Procapra przewalskii* (LEI et al. 2003), the Tibetan antelope *Procapra picticaudata* (ZHANG & JIANG 2006) and the goitered gazelle in Iran *G. s. subgutturosa* (FADAKAR et al. 2013, ZACHOS et al. 2010). Compared to the haplotype (0.467–0.932) and nucleotide diversity (0.0039–0.1004) in these species, the XUAR goitered gazelle population seems to show high haplotype but low nucleotide diversity. Surprisingly, the goitered gazelle in XUAR exhibited a considerably lower level of genetic variation than some of the endangered species like the Tibetan and saiga antelopes (KHOLODOVA et al. 2006, ZHANG & JIANG 2006) and the critically endangered Przewalski's gazelle (LEI et al. 2003). This low level of genetic diversity has likely resulted from bottleneck events (FREELAND et al. 2011) or founder effects (HAMNER et al. 2007). The neutrality (Tajima's *D* and Fu's *FS* tests) and mismatch distribution analyses (Fig. 3) suggest a past demographic bottleneck event, as the unimodal distribution is generally found in the populations that have gone through a bottleneck (ROGERS & HARPENDING 1992). Intriguingly, a study of ten microsatellite loci from the Kumul and Hejing goitered gazelle populations in XUAR showed a high level of genetic diversity, with an average of 5.7 alleles (TURSUN et al. 2016). NING et al. (2016) found ten highly polymorphic loci among 20 microsatellite loci in *Bos taurus* and *Ovis aries*. The most plausible reason for such a discrepancy is that mtDNA has a fourfold shorter coalescence time than microsatellites and loss of diversity in mtDNA should be faster in bottlenecked/declining populations (BIRKY et al. 1983, NYSTROM et al. 2006).

However, a study of the goitered gazelle in northern XUAR by DONG et al. (2016) detected higher nucleotide diversity for CYT *b* (30 haplotypes, of which we detected only five) than our study and individuals in the ELNWR (northern XUAR) also exhibited high genetic diversity in the mitochondrial DNA control region (TURAP et al. 2016). This higher diversity may be reflected in our haplotype network (Fig 5); while most individuals group in a star-like topology around haplotype HAP01, HAP02 and its descendants form a group relatively highly

divergent from HAP01. Populations in contact with other populations, e.g. by migration or dispersal, have been found to possess higher proportion of genetic variation than those of small and isolated populations (FRANKHAM et al. 2010, RICO et al. 2016). This is more likely due to the contact of gazelles in northern XUAR with an adjacent population such as the Mongolian population. The gazelles regularly migrate from Mongolia into China from October onwards and return to Mongolia in late February (WANG et al. 1997). This migration may have led to a larger population in KUNR, which is near Mongolia, in winter (19,677 individuals) than in other seasons (6000–14,000 individuals; CHU et al. 2009). This may also be due to the higher possibility of contacts among populations in northern relative to southern XUAR. The mtDNA D-loop analysis of gazelles in XUAR have not shown sharing of haplotypes among geographic populations either, especially within southern XUAR, indicating lack of migration or dispersal in southern compared to northern XUAR populations (ABDURIYIM et al. 2018). RAHMUTULLA (2015) also has shown a substantially fragmented potential distributions for gazelles in southern XUAR, which might lead to genetic differentiation.

Interestingly, HAP01, which DONG et al. (2016) found in 28 individuals, occurred in 45 of the individuals we examined (Table 1). It was positioned at the centre of our haplotype network (Fig. 5) and also that of DONG et al. (2016), surrounded by haplotypes divergent by 1–2 mutational steps. Individuals having HAP01 were broadly geographically distributed (Table 1) across ELNWR, Fukang, Komul, Hejing, Bugur, Onsu, Qakilik, Qarqan and Altuntagh (locations in this study) and KUNR (in DONG et al. 2016). HAP01 thus shows characteristics of an ancestral haplotype (FREELAND et al. 2011).

The complete CYT *b* gene, or even 360–425 bp toward the 5' end (WRONSKI et al. 2010, WACHER et al. 2011, KANKILIÇ et al. 2012), has proven highly effective in resolving phylogenetic relationships among gazelles at the species level (LERP et al. 2011). All haplotypes from our study and from GenBank formed one major clade, demonstrating that the individuals included were goitered gazelles (*Gazella subgutturosa*). However, FADAKAR et al. (2013) have found a 425 bp fragment at the 5'-end CYT *b* not to delineate the goitered gazelle population in Iran. Similarly even the 995 bp region may not be sufficient for delineating subspecies or examining geographical population structure within the goitered gazelle and for these purposes additional markers such as the control region (ZACHOS et al. 2010), microsatellites, and/or paternally inherited genes on the Y chromosome gene may need to be analysed.

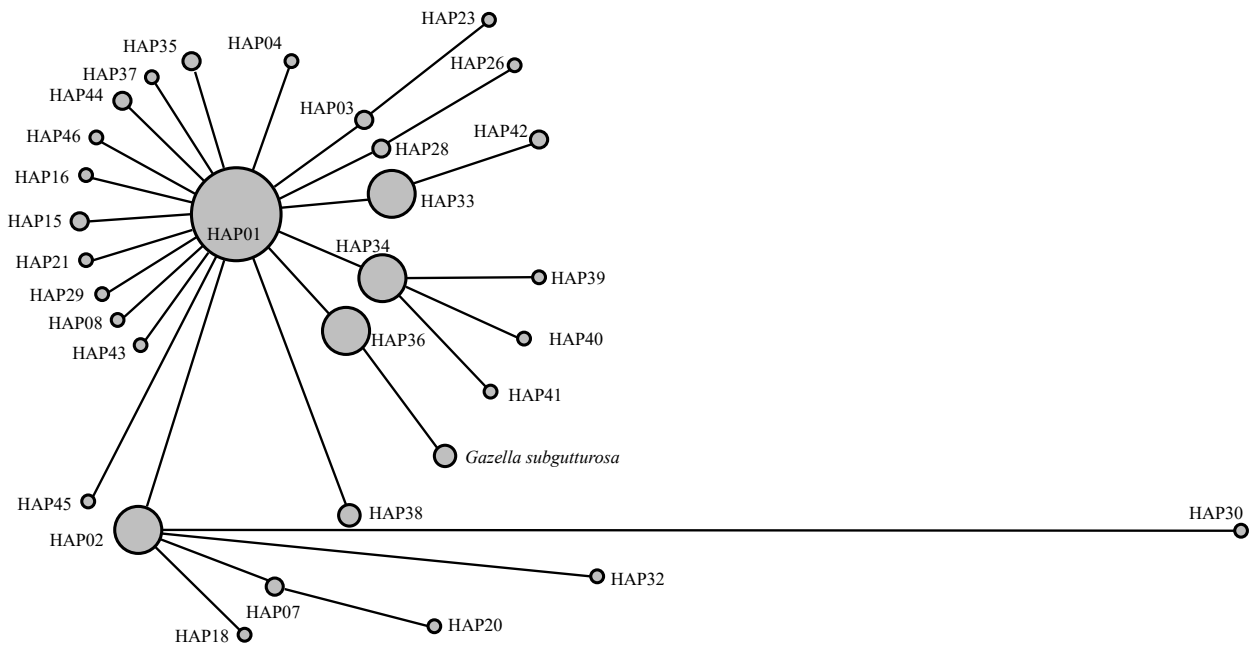


Fig. 5. Median-joining network based on 995 bp of the cytochrome *b* gene from 128 goitered gazelle individuals. Each circle indicates a different haplotype, with the size of circles proportional to the haplotype frequency. The length of connecting lines corresponds to the number of mutational steps.

The populations of the goitered gazelle are threatened by poaching, habitat fragmentation and loss due to road or railway construction, industrial development and urbanisation, both throughout the range of the species (FADAKAR et al. 2013, IUCN 2017) and in XUAR (DONG et al. 2016, SUN et al. 2002, XU et al. 2008). Present-day populations have proven to be fragile: they are affected by landscape composition and anthropogenic factors (KHOSRAVI et al. 2018), as well as by extreme winter weather (DONG et al. 2016). The low genetic diversity we observed in XUAR calls for urgent protective measures and conservation plans to prevent further genetic loss. Otherwise, the threats mentioned above could lead to shrinking and isolated populations, resulting in inbreeding and susceptibility to genetic drift, bottleneck effects (observed in this study) and an imbalanced sex ratio, finally forcing the goitered gazelle towards the edge of extinction.

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