

Phylogenetic analysis of mitochondrial DNA sequences reveals polyphyly in the goitred gazelle (*Gazella subgutturosa*)

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Abstract Goitred gazelle (*Gazella subgutturosa*) rank among the most endangered mammals on the Arabian Peninsula and the Asian steppes. Past conservation efforts have been plagued by confusion about the phylogenetic relationship among various—phenotypically discernable—populations, and even the question of species boundaries was far from being certain. This lack of knowledge had a direct impact on conservation measures, especially ex situ breeding programmes, hampering the assignment of captive stocks to potential conservation units. Here, we provide a phylogenetic framework, based on the analysis of mtDNA sequences of a number of individuals collected from the wild and captivity throughout the species' natural range. Our analyses revealed a polyphyly within the presumed species of *G. subgutturosa* resulting in two distinct

clades: one on the Arabian Peninsula, Iraq, Jordan, Syria and Turkey (*Gazella marica*; sand gazelle) and one genetically diverse larger clade from the rest of its Asian range (*G. subgutturosa*; goitred gazelle). Additionally, we provide a quick method (PCR-RFLP) to analyse the taxonomic affiliation of captive gazelles that will be used for re-introductions into the wild.

Keywords Arabian Peninsula · Cryptic species · *Gazella subgutturosa* · Phylogeny · Conservation units

Introduction

Conservation genetics has major implications for the conservation of biodiversity by clarifying taxonomic

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relationships (Awise 1989), and determining stocks or individuals from captive breeding programs for future reintroductions (Vogler and DeSalle 1994). Gazelles are a very diverse group of mammals, with phylogenetic relationships within this group being largely unexplored (Groves 1989, 1997), a fact that has hampered and complicated conservation efforts (Ryder 1987; Hammond et al. 2001). Here, we ask whether or not goitred gazelles (*Gazella subgutturosa*) consist of more than one species, and second, we investigate the taxonomic affiliation of a large number of captive individuals that have been bred for reintroduction.

The conventional view is that *G. subgutturosa* occurs over a very wide range from Oman across the Arabian Peninsula to southern Turkey (Mallon and Kingswood 2001), following the steppes of central Asia eastwards into central Mongolia (Kingswood and Blank 1996; Mallon and Kingswood 2001). Several subspecies have been described across this range, with two occurring in the Middle East:

- (1) The sand gazelle or 'reem' (currently recognized as *G. s. marica*; Groves and Harrison 1967), is found in open habitats of the Arabian Peninsula, ranging through Iraq, Jordan and Syria into southern Turkey (Mallon and Kingswood 2001). Sand gazelles are characterized by pale body colour and a white face. Females bear long slender horns, which can be also much reduced. Adult males are heavier-bodied, with a noticeably thickened neck in breeding condition (though typically with a less obviously developed goitre than in continental Asian forms) and much longer, lyrate horns (Groves and Harrison 1967; Kingswood and Blank 1996).
- (2) The Persian goitred gazelle (*G. s. subgutturosa*) differs from *G. s. marica* by having a larger body weight (Kingswood and Blank 1996), and by adult females usually lacking horns or sometimes growing short horns. Adult males develop a prominent swelling on the larynx during the rutting season, the so-called 'goitre'.

The sand gazelle was first described as a full species (*G. marica*; Thomas 1897) and later synonymized with the closely related slender-horned gazelle (*Gazella leptoceros*) of the sand dune systems of northern Africa (Ellerman and Morrison-Scott 1951). More recently, it has been considered a subspecies of *G. subgutturosa* based on morphological and karyological similarity (Groves and Harrison 1967; Kingswood et al. 1996, 1997). The disappearance of the sand gazelle from large parts of its range in Arabia can be attributed to a combination of over-hunting and intense over-grazing by domestic livestock (Thouless et al. 1997). The modern distribution of wild sand gazelles in Saudi Arabia is limited to the two original populations in the northern protected areas (Seddon et al. 1997; Thouless

et al. 1997), and two reintroduced populations at Mahazat as-Sayd and Uruq Bani Ma'arid (Haque and Smith 1996; Cunningham and Wacher 2009).

A primary objective of our present study was to analyse the phylogenetic relationships between *G. s. subgutturosa* from east of the Euphrates-Tigris basin and those from the Arabian Peninsula (*G. s. marica*). To do so, we analysed sequence variation of a mitochondrial marker (cytochrome *b*) of samples obtained from wild sand (*G. s. marica*) and Persian goitred gazelles (*G. s. subgutturosa*) of known origin, from museum specimens, as well as from captive-breeding stocks. In addition, we used PCR-RFLP analysis to investigate the taxonomic affiliation of a large number of individuals from a series of captive groups of gazelles.

Material and methods

The origin of samples, provenance, collector and the kind of material sampled (skin, blood, hairs) are summarized in Table 1. Sequences from other gazelle taxa were obtained from GenBank (7 sequences, Rebholz and Harley 1999; Hassanin and Douzery 1999; Hammond et al. 2001), the Estación Experimental de Zonas Áridas in Almeria, Spain (*G. cuvieri*), Wadi al-Safa Wildlife Centre, UAE (*G. bennetti*), Prince Ahmed bin Abdulaziz Farm, Saudi Arabia (*G. leptoceros loderi*; reported to originate from Tunisia), or from the wild (*G. leptoceros leptoceros*: Hatiyat Umm Ghuzlan, western Egypt; *G. cuvieri*: Chambi N.P., Tunisia). Sequences from '*G. gazella*' were reanalysed from another study (Wronski et al. 2010).

DNA extraction of hair and blood samples was carried out as described in Wronski et al. (2010). The 5'-region of the cytochrome *b* gene was PCR-amplified using the versatile primers L14724 and H15149 (Kocher et al. 1989). Double stranded PCR products were sequenced with a Thermosequenase-based cycle-sequencing kit using an end-labelled primer protocol using γ P³³ as a label (Amersham-Pharmacia Biotech). Both L14724 and H15149 were used as sequencing primers. Sequence reactions were run on standard polyacrylamide sequencing gels and visualised by autoradiography. For museum samples, each sample was amplified and sequenced from at least two separate DNA extractions. Autoradiographs were scored by eye, with each individual sequence being read at least twice. All unique haplotypes have been deposited in GenBank (accession numbers HQ316150–HQ316165).

Sequences were aligned using MAFFT version 6 (Katoh et al. 2005) using the iterative refinement method E-INS-i. A χ^2 -test as implemented in PAUP 4.0b10 (Swofford 2002) was used to test for compositional heterogeneity of base frequencies. Maximum likelihood (ML) analysis of the dataset was conducted using RAxML version 7.0.3

Table 1 List of specimens (wild and captive) of *G. s. subgutturosa* and *G. s. marica* included in the phylogenetic analyses, their collectors, accession numbers, and sample types

Species	Origin	Wild/captive	Collector/accession number	Sample type
<i>G. s. subgutturosa</i>	Aksu, Chinese Turkistan	Wild	Cumberland, BMNH London/HQ316159	Tissue
<i>G. s. subgutturosa</i>	Al-Areen, Bahrain*	Captive	Hundertmark, Mohammed, KKWRC	Blood, hairs
<i>G. s. subgutturosa</i>	Kabul Zoo, Afghanistan	Captive	Lindsay, ZSL	Hairs
<i>G. s. subgutturosa</i>	Samarra, Iraq	Wild	Pitman, BMNH London	Tissue
<i>G. s. subgutturosa</i>	Unknown*	?	MNHN Paris/AF036282	Sequence
<i>G. s. subgutturosa</i>	San Diego Zoo, originally Teheran Zoo, Iran (2)	Captive	Hammond, KKWRC/HQ316157	DNA
<i>G. s. subgutturosa</i>	Azerbaijan	Wild	Lindsay, ZSL/HQ316158	Tissue
<i>G. s. subgutturosa</i>	Warsan Farm, Abu Dhabi, UAE (2)*	Captive	Hoy, Warsan Farm/HQ316156	Hairs, tissue
<i>G. s. marica</i>	Harrat Al Harrah/Iraqi border*	?	Rangers, SWC	Tissue
<i>G. s. marica</i>	Ramlat Fasad, Oman	Wild	Butler, Harrison Museum/HQ316160	Tissue
<i>G. s. marica</i>	Abu Al Jir, Iraq	Wild	Harrison, Harrison Museum/HQ316162	Tissue
<i>G. s. marica</i>	Al Khunfah Protected Area, Saudi Arabia (3)	Wild	Rangers, SWC	Blood, tissue
<i>G. s. marica</i>	King Khalid Wildlife Research Centre, Saudi Arabia*	Captive	KKWRC staff members	Blood, tissue
<i>G. s. marica</i>	Harrat Al Harrah, Saudi Arabia	Wild	Wacher, KKWRC	Hairs, tissue
<i>G. s. marica</i>	Harrat Al Harrah, Saudi Arabia (4)	Wild	Rangers, SWC/HQ316164	Tissue
<i>G. s. marica</i>	Syria	Wild	Ginani	Tissue
<i>G. s. marica</i>	Wadi Al-Safa Wildlife Centre, Sharjah, UAE (6)*	Captive	Nader, KKWRC/HQ316161	Tissue
<i>G. s. marica</i>	Warsan Farm, Abu Dhabi, UAE (2)*	Captive	Hoy, Warsan Farm	Skin
<i>G. s. marica</i>	Syria/Jordan	Captive	Williamson, KKWRC	Hairs
<i>G. s. marica</i>	Qarn Sahma, Oman	Wild	Harrison, Harrison Museum	Tissue
<i>G. s. marica</i>	Warsan Farm, Abu Dhabi, UAE (4)*	Captive	Hoy, Warsan Farm/HQ316163	Hairs, tissue
?	Rutba region, Iraq (5)*	Captive	Hammond, Al-Aqeel, Mubarak, KKWRC/HQ316165	Tissue

Numbers in *brackets* indicate the number of samples obtained from that location, while *asterisks* indicate unknown provenance and ? unknown whether an individual was wild or captive

(Stamatakis 2006), using the GTR+ Γ +I model. Parsimony analysis was performed in PAUP*v.4.0b10 (Swofford 2002) using a heuristic search with the TBR (tree bisection-reconnection) option and 1,000 random sequence additions. Bootstrap support was estimated by 1,000 replicates. Hypothesis testing using the Approximately Unbiased (AU)-test as implemented in CONSEL (Shimodaira and Hasegawa 2001) was performed under the ML-criterion to compare constrained monophyly of *G. s. subgutturosa* against the best tree.

To rapidly assess the cytochrome *b* haplotype of a large number of captive *G. s. marica* and *G. s. subgutturosa* the

same PCR product used for sequencing (see above) was digested with HaeII, HinfI, NlaIII, RsaI and DdeI. These restriction enzymes identified 5 nucleotides that diagnosed membership of either the *G. s. marica*-clade or *G. s. subgutturosa*-clade (Table 2). PCR products were digested following manufacturer’s instructions (New England Biolabs), separated on 1% agarose gels, and bands were visualized under UV after ethidium bromide staining. In total, 894 samples were RFLP typed from the following captive populations: Prince Mohammed Al Sudairy Centre, Qassim, Saudi Arabia ($n = 424$), KKWRC ($n = 253$), KKWRC animals released in the Urug Bani Ma’arid

Table 2 PCR-RFLP haplotypes of *G. s. marica* and *G. s. subgutturosa*

Taxon	HaeIII 193	HaeIII 352	HinfI 163	HinfI 280	NlaIII 139	NlaIII 300	NlaIII 342	NlaIII 388	RsaI 369	RsaI 277	DdeI 377
<i>G. s. marica</i>	0	1	0	1	1	1	1	1	1	1	1
<i>G. s. subgutturosa</i>	1	1	1	1	0	1	1	1	0	0	0

The presence of a restriction site is indicated by 1, while absence of a is indicated by 0. *Numbers* given indicate the position of the restriction site in base pairs. This applies to a region of the cytochrome *b* gene amplified using the primers L14724 and H15149

Protected Area, Saudi Arabia ($n = 105$), Al Areen Wildlife Park, Bahrain ($n = 99$), and gazelles confiscated at a Saudi Arabia/Iraq border post, which were said to have originated from the Rutba region of Iraq ($n = 13$).

Results and discussion

Our final dataset included 27 Operational Taxonomic Units (OTUs) and 333 aligned nucleotide positions with 46 parsimony-informative sites. A χ^2 -test showed no significant deviation from stationarity for the nucleotide composition.

Maximum likelihood (ML) and maximum parsimony (MP) analyses resulted in congruent topologies (Fig. 1). Both analyses recovered high bootstrap support for the monophyly of the OTUs assigned to *G. s. marica* [ML-bootstrap (MLB): 92%; MP-bootstrap (MPB): 94%] and *G. s. subgutturosa* (MLB: 97%; MPB: 97%). However, these taxa do not show a sister group relationship, as *G. s.*

marica shows a closer relationship to individuals from *G. s. cuvieri* and *G. leptoceros* (Fig. 1). Monophyly of a clade comprising the latter three taxa is strongly supported (MLB: 94%; MPB: 97%).

Crucially, a monophyletic clade comprising both *G. s. subgutturosa*-subtaxa was significantly rejected by hypothesis testing using an AU-test ($P = 0.001$). OTUs from wild caught animals belonging to the *G. s. marica*-clade originated from Saudi Arabia, Jordan, Syria, and Oman. All animals with known provenance in the *G. s. subgutturosa*-clade are from Iraq, Iran, Afghanistan, Azerbaijan and Chinese Turkistan. Our data, therefore, reinforce the observation made by Hammond et al. (2001) that the two “subspecies” *G. s. subgutturosa* and *G. s. marica* are more distantly related than previously thought. The Arabian subspecies (*G. s. marica*) is clearly more closely related to the north-African species *G. leptoceros* and *G. cuvieri*, suggesting that *G. s. marica* and *G. s. subgutturosa* have evolved independently. Our findings support the earlier interpretation of Ellerman and Morrison-Scott (1951) while conflicting with the grouping pattern inferred from a comparison of skull structure and horn conformation (Groves and Harrison 1967).

We are aware of the limitations of analysing mtDNA data only, but based on the marker examined here, the sand gazelle appears to form a more distinct conservation unit than might have been expected; in this sense restoration to full species status with name *G. marica* (Thomas 1897) may be the most helpful solution with respect to future conservation management and legislation for the *marica*-, *leptoceros*- and *subgutturosa*-grouping. It remains to be studied if other (i.e. nuclear) markers support our claim of polyphyly. However, Zink and Barrowclough (2008) demonstrated for birds that mitochondrial markers proved to be robust indicators of population histories and species boundaries. Theoretically, *G. s. subgutturosa* samples of unknown provenance, i.e. captive specimens, might have had a misleading effect on our interpretation, but our discussion of polyphyly is based primarily on individuals of known provenance.

Female *G. s. marica* show consistently long horns unlike generally hornless *G. s. subgutturosa* females (Groves and Harrison 1967), while specimens from the region between the Euphrates-Tigris basin and the Zagros Mountains of Iran were found to present a mix of characters. Consequently, this zone has been considered a hybrid zone (Groves and Harrison 1967; Kingswood and Kumamoto 1988; Groves 1997; Al-Robae and Kingswood 2001). Sampling from the putative hybrid zone is very limited (Rutba region, Iraq in Table 1) but samples clustered with specimens from Chinese Turkistan and Afghanistan (Table 1) suggesting that either no overlap zone exists, or only male-biased introgression occurs.

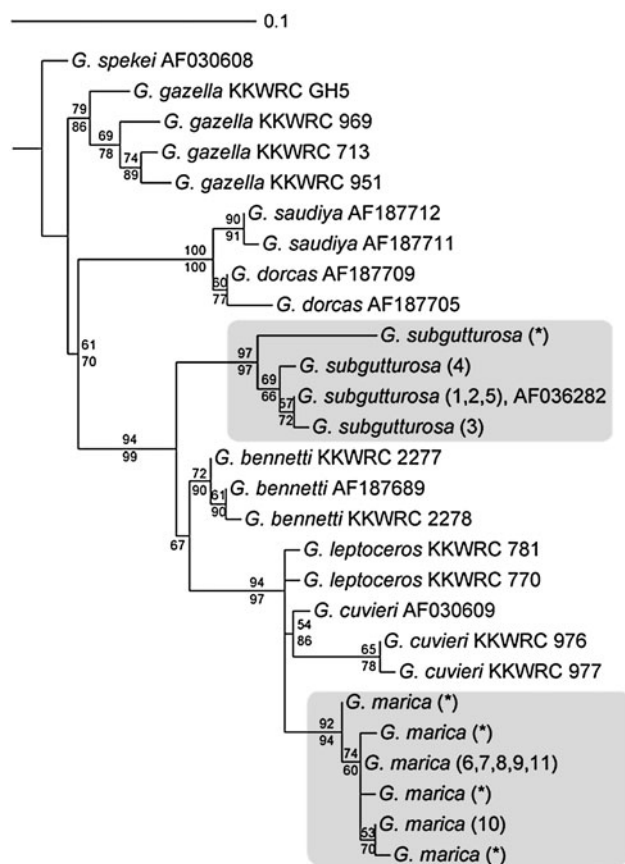


Fig. 1 Maximum likelihood tree of the cytochrome *b*-dataset with the GTR+ Γ +I model. Likelihood bootstrap-support (*below*) and parsimony bootstrap support (*above*) were estimated from 1,000 replicates and are given at the branches. The numbers given in brackets correspond with the known provenances as shown in Table 1; asterisks indicate unknown provenance. For sequences obtained from GenBank accession numbers are given; for sequences obtained from KKWRC, sample numbers are provided

The RFLP-analysis confirmed the biogeographical patterns we found from our sequence analysis as all captive animals from Saudi Arabia and Bahrain showed *G. s. marica* haplotypes, whilst gazelles of probable Iraqi origin were largely *G. s. subgutturosa* (see Table 1) with a minority of *G. s. marica* haplotypes (3 out of 13). All KKWRC animals, including those released in the Uruq Bani Ma'arid Protected Area, had *G. s. marica* mtDNA.

The genetic differences described here show that the original concern (Greth et al. 1996) to avoid mixing these putative 'subspecies' in captivity was fully justified. Further enlargement of the genetic sample base, especially of Asian *G. s. subgutturosa* and African *G. leptoceros*, and re-examination of the morphology of the entire group, is certainly indicated.

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