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Mitochondrial and nuclear DNA analyses reveal pronounced genetic structuring in Tunisian wild boar *Sus scrofa*

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Abstract We analysed 74 wild boars from Tunisia with respect to patterns of genetic differentiation and diversity based on sequences of the mitochondrial control region and genotypes at eight nuclear microsatellite loci. Analysis of molecular variance for both marker systems and Bayesian structure analysis of our microsatellite data revealed a clear break between northern and southern populations. Southern wild boar were monomorphic for one of three mtDNA haplotypes; the other two (one of which only occurred in three individuals) were confined to the north. A comparison with published sequences showed all three haplotypes to belong to the major European clade E1. Microsatellite diversity was similar to that found in earlier studies of wild boar (expected heterozygosity of 0.695 and 0.597 for the north and south, respectively). Contrary to the mtDNA results, we did not find unequivocal evidence of a bottleneck in Tunisian wild boar based on our microsatellite data. The clear distinction between northern and southern populations may be due to an Algerian origin of the southern animals.

Keywords Genetic diversity · Microsatellites · mtDNA · *Sus scrofa* · Tunisia

Introduction

North Africa harbours a great variety of ungulate species, among them threatened taxa like gazelles, oryx, addax, aoudad and Barbary deer. However, it has not received as

much attention by population geneticists and conservationists as Eurasia, the Americas or sub-Saharan Africa where large herbivores have been studied in great detail. The present study is dedicated to closing this gap by providing the first in-depth analysis of North African wild boar in Tunisia.

The wild boar is one of the most widely distributed terrestrial mammals and has by far the largest range of all suid species. It occurs from Western Europe to the Russian Far East, extending southwards as far as North Africa, the Mediterranean Basin and the Middle East, through India, Indo-China, Japan, Taiwan and the Greater Sunda Islands of Southeast Asia (Groves and Grubb 1993). The occurrence of wild boar in North Africa ranged from Morocco eastward through Egypt, but today it is almost extinct in most of the region (Gharaibeh 1997). Its history is unknown and controversial. Archaeological evidence indicates that pigs were bred in Ancient Egypt in the fourth millennium BC and that they were widespread in North Africa (Ramirez et al. 2009). According to palaeontological records, *Sus scrofa* has been a component of the Maghreb fauna from the Pleistocene and throughout the Holocene (Dobson 1998). Recent genetic studies dealing with wild boar diversity included only few specimens of North African *S. scrofa* (four animals in Ojeda et al. (2006) and nine in Ramirez et al. (2009)) and showed genetic similarity, based on mitochondrial (cytochrome b) and autosomal markers (microsatellites and coding genes), between European and North African wild boar populations. This similarity would suggest that there might have been interchanges between North Africa and the European populations. While European wild boar have been studied widely with respect to genetic structure, hybridisation and phylogeography (Ferreira et al. 2009; Koutsogiannouli et al. 2010; Nikolov et al. 2009; Scandura et al. 2008; Vernesi et al. 2003), there is as yet no study dealing with the North African part of the

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distribution range at the population level. In Tunisia, the wild boar is very common, widespread and the most important large game species. Populations are increasing, and distribution ranges are expanding throughout the country (Direction Générale des Forêts, hereafter abbreviated as DGF, personal communication). In the northern part of Tunisia, the current total population of wild boar is estimated to be in the range of approximately 100,000 individuals, and it is still increasing and expanding. This expansion started in the 1980s from Tabarka/Ain-draham in the northwest of the country, and today, wild boar are very common in most of the northern districts (less so in the Cap-bon and the Sahel regions; Gharaibeh 1997). In the South, wild boar have been present since the early 1980s when wildlife managers reported that some individuals, supposedly from southern Algeria, crossed Jebel Brikis in Tamarza near the border and occupied Tozeur (Fig. 1). After approximately 10 years, the population had

grown to about 1,000 individuals. Today, the wild boar is found in reed (roselières) habitats of the southern districts (Gabes, Kebilli, Gafsa and Tozeur). Wild boar are numerous in some districts, causing serious crop damages. As a consequence, the DGF organised “battues administratives” in order to reduce the wild boar population. In Ain Draham and the governorate of Jendouba in general, wild boar hunts are very important economically: Hunting is regulated by the DGF, and tourists pay for venison export permits. This system was also established in the South, but due to the smaller population, the hunting season is shorter. However, despite many thousands of wild boar being culled, it is clear this has not had a great effect on their growing potential, and further conflicts seem unavoidable. The present study aims at elucidating the genetic patterns of wild boar in Tunisia and relating them to results recently published for European wild boar. The molecular markers chosen are nuclear microsatellite loci and sequences of the mitochondrial control region, both known to be powerful tools in population genetics that have previously been analysed in other studies on game species including wild boar (Ferreira et al. 2009; Nikolov et al. 2009; Scandura et al. 2008; Vernesi et al. 2003).

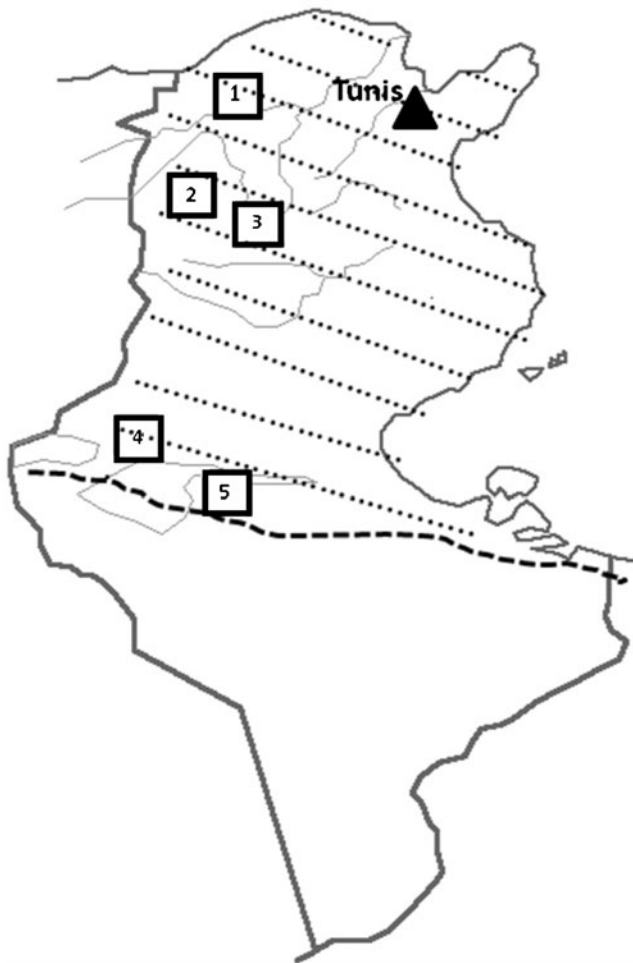


Fig. 1 Sampling localities of wild boar (*Sus scrofa*) in Tunisia: north (1: Béja, 2: Kef and 3: Séliana) and south (4: Tozeur and 5: Kebilli). The spotted area refers to the distribution range of *S. scrofa* in Tunisia. Within this range, wild boar are not distributed continuously, but detailed information is as yet not available

Materials and methods

Sampling and molecular analysis

A total of 74 wild boar tissue samples were collected at five sites in Tunisia during the hunting seasons in 2007/2008 (Béja, $n=14$; Séliana, $n=9$) and 2008/2009 (Kef, $n=15$; Tozeur, $n=25$; Kebilli, $n=11$). Our sampling scheme covered large parts of the distribution area of wild boar in Tunisia (Fig. 1).

Total DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer’s protocol. A fragment of the mitochondrial control region was amplified with the primers CTR 22L and CTR 515H (Fickel and Hohmann 2006). Polymerase chain reaction (PCR) amplification conditions comprised initial denaturation at 95°C for 5 min and 40 cycles of 94°C for 90 s, 55°C for 75 s and 72°C for 90 s with a final extension step at 72°C for 10 min. Amplicons were subsequently sequenced using the forward primer CTR 22L.

Additionally, we genotyped our samples at eight polymorphic microsatellite loci that have recently been used in other wild boar studies: S0001, S0005, S0007, S0062, S0070 (Fredholm et al. 1993), S0097 (Ellegren et al. 1993), S0121 and S0228 (Robic et al. 1994). The forward primer of each pair was labelled with an ABI fluorescent dye (6-FAM or HEX). The amplification profile comprised an initial step of denaturation at 95°C for 5 min, followed by

40 cycles of 92°C for 30 s, annealing temperature (55–58°C) for 30 s and 72°C for 30 s. A final extension step at 72°C for 10 min concluded the reaction. PCR-amplified microsatellite alleles were sized using a 96-capillary MegaBACE automatic sequencer with an internal Rox-500 size standard (Applied Biosystems) and the Genetic Profiler software (Amersham Biosciences).

Statistical analysis

The control region sequences were aligned using BioEdit 7.0 (Hall 1999). Haplotype and nucleotide diversities were calculated with DnaSP 5.00 (Rozas and Rozas 1999). Haplotype diversity is the probability that two randomly drawn individuals in a population show a different haplotype. In contrast to nucleotide diversity, which is the probability that two randomly drawn sequences differ at a given site, it does not consider the amount of sequence divergence between haplotypes. Population structure between the north and south was quantified by means of an analysis of molecular variance (AMOVA) as implemented in the program Arlequin version 3.11 (Excoffier et al. 2005). The AMOVA was based on traditional F -statistics and φ -statistics, which takes into account haplotype frequencies and the genetic distance between haplotypes. φ -statistics was calculated under a Tamura and Nei (1993) model of nucleotide substitution, which was the most similar available model in Arlequin to the HKY model which was found to best describe our data set based on the Modeltest algorithm (Posada and Crandall 1998)

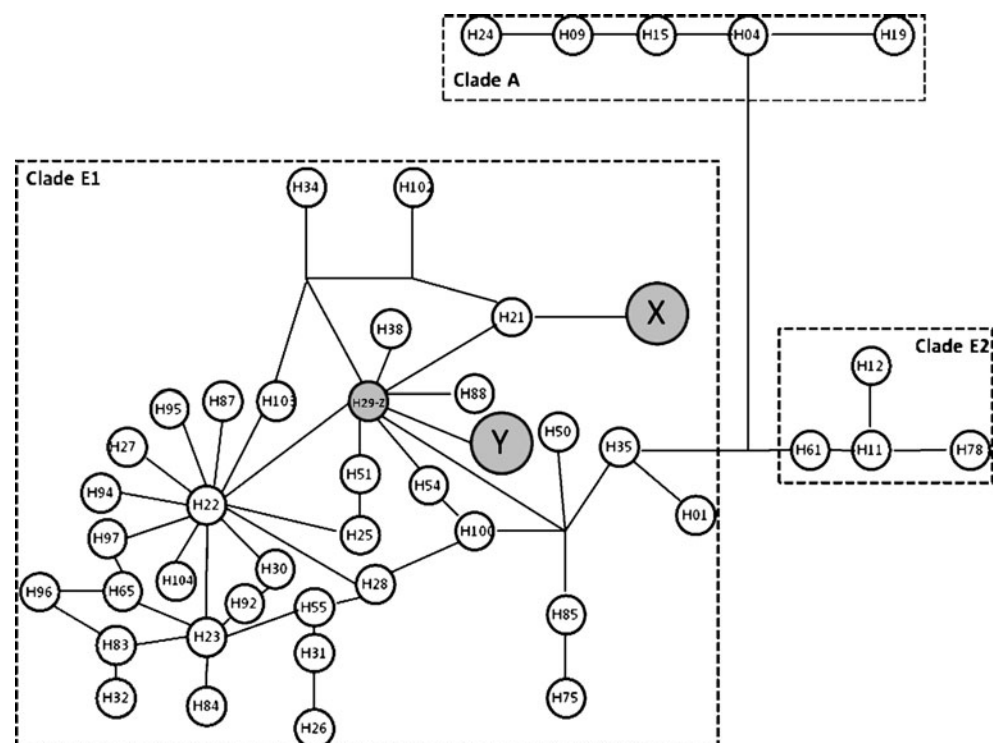
implemented in FindModel (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>).

In order to analyse our sequences in a European framework, we aligned them with 44 wild boar haplotypes published by Scandura et al. (2008; in particular, see their Fig. 2) available in GenBank. A median-joining (MJ) network of haplotypes was created with the software NETWORK 4.5.1.0 (Bandelt et al. 1999).

In order to verify if stutter bands, large allele dropout and null alleles have distorted microsatellite allele frequencies and heterozygosity calculations (Pemberton et al. 1995; Wattier et al. 1998; Ewen et al. 2000), the programme MICRO-CHECKER (van Oosterhout et al. 2004) was used to test our data set for these potential error sources. Our final data set was tested for linkage disequilibrium between each pair of loci with Genepop (Raymond and Rousset 1995).

Population determination is classically based upon the geographical origin of samples. However, the genetic structure of populations is not always reflected by the geographical proximity of individuals. Therefore, we analysed population structure using the Bayesian approach implemented in the STRUCTURE software (Pritchard et al. 2000) which aims at delineating clusters of individuals on the basis of their multi-locus genotypes. The software was run under admixture conditions and correlated allele frequencies with numbers of clusters to be simulated (K) varying from one to five. Ten replicate runs were performed for each K value without a priori information on the origin

Fig. 2 Median-joining network based on mtDNA haplotypes (present study and Scandura et al. 2008). *White circles* indicate sequences observed in European wild boar or domestic pigs; *grey circles* denote Tunisian wild boar sequences; X+Z, north; Y, south. The size of *grey circles* is proportional to the haplotype frequency. Clade A refers to Asia; clade E1 refers to Europe and E2 to Italy



of the individuals. The simulation of K with the highest log-likelihood value was chosen as representing our data set best. In a second approach, based on the prior information on the number of clusters present, we ran the programme with our two groups (north and south, see below) and calculated the probability of assignment to these clusters. We carried out these calculations twice: once with all loci and once with only those loci in Hardy–Weinberg equilibrium (see below). The results were the same, and in the following, we present the statistics based on all loci.

Genetic structure was also analysed with a factorial correspondence analysis (FCA) with GENETIX (Belkhir et al. 2004). This approach clusters individuals along axes based on their multi-locus genotypes and is useful in delineating different genetic entities.

Arlequin was used to calculate observed (H_O) and expected (H_E) heterozygosities and deviations from Hardy–Weinberg equilibrium and to assess differentiation among sample sites and inferred populations by means of AMOVAs. In the latter, F_{ST} values (Wright 1951) and R_{ST} values (Slatkin 1995) were calculated. Both parameters yield the proportion of the total genetic variance that is found among populations (as opposed to variation within populations), but F -statistics is based on allele frequencies and the infinite alleles model, whereas R -statistics is based on differences in allele size and the stepwise mutation model. The number of alleles per population was calculated with FSTAT 2.9.3 (Goudet 1995).

The available information on wild boar in Tunisia does not contain data on bottlenecks. To gain further insight into the species' demographic history, we used the programme Bottleneck (Cornuet and Luikart 1996) employing, as recommended for microsatellite data by the authors, the Wilcoxon test of deviation from mutation-drift equilibrium under the two-phase model of microsatellite evolution with 90% of the mutations following the strict stepwise mutation model and 10% being multistep changes. The rationale behind this is that in recently bottlenecked populations, gene diversity at neutral loci is higher than the equilibrium heterozygosity estimated from the number of alleles under the assumption of mutation-drift equilibrium because allele diversity is lost more quickly than heterozygosity (Cornuet and Luikart 1996). Additionally, we calculated Garza and Williamson's (2001) M , which is the ratio of allele number to the range in allele size at a given microsatellite locus. The mean value across loci can be used to detect bottlenecks because a reduction in population size is expected to reduce the number of alleles faster than the range in allele size (the loss of any allele will decrease the former, but the latter will only be reduced if the shortest or longest allele gets lost). Significance values were Bonferroni-corrected whenever multiple tests were carried out.

Results

mtDNA

Sequencing of 399 bp of the mitochondrial control region was successful in 67 individuals and yielded eight polymorphic positions defining three haplotypes with the two major haplotypes X (in the north) and Y (in the south) differing by seven transitions and one indel. Each of these two haplotypes occurred 32 times. A third haplotype, separated from Y by a single transition, was found in three individuals sampled in Kef (north), resulting in an overall haplotype diversity of 0.550 and a nucleotide diversity of 1.016%. The AMOVA for north vs. south revealed identical ϕ_{ST} and F_{ST} values of 0.920 ($P=0.000$), indicating that 92% of the total genetic variation were located between the two groups. The mean sequence divergence between north and south was 1.8%.

The complete alignment with GenBank sequences comprised 323 bp and 46 different haplotypes. The network (Fig. 2) shows that all three Tunisian haplotypes belong to the European haplogroup E1 found by Scandura et al. (2008), which encompasses wild boar from large parts of Europe. Haplotype Y characteristic of the southern part of Tunisia only differs by one mutation from the European haplotype H29 identified in Spain (Alves et al. (2003) in Scandura et al. (2008)); this latter is identical to the rare Tunisian haplotype Z. Haplotype X from northern Tunisia is closest to haplotype H21, which is very common throughout Europe. GenBank comparisons confirmed that X and Y are new haplotypes and have not hitherto been detected in wild boar.

Microsatellites

The eight microsatellite loci did not show significant linkage disequilibrium, and consequently, they were all included in the multi-locus analyses. The MICRO-CHECKER software did not find any distortion caused by large allele dropout but found potential evidence of null alleles at four loci (S0005, S0097, S0121 and S0228) and stutter bands at two loci (S0005 and S0121). The latter, however, could be ruled out after careful inspection of our electropherograms. When north and south were considered separately, null alleles and stutter bands were suggested at two loci (S0121 and S0005). All loci were polymorphic, and a total of 64 alleles were found with the number of alleles per locus ranging from 3 to 11. Descriptive statistics, heterozygosity values and numbers of private alleles (found only either in the north or south) are given in Table 1. We found one deviation from H-W equilibrium (S0121) in the northern group and two (S0005 and S0121) in the southern one, all due to a heterozygosity deficit.

Table 1 Allele number, size range, private alleles, and observed and expected heterozygosities (H_O , H_E) for each sub-population

Loci	Na		Size range		A_{priv}		H_O		H_E		P value	
	N (38)	S (36)	N	S	N	S	N	S	N	S	N	S
S0001	6	8	199–209	199–217	–	2	0.710	0.694	0.772	0.668	0.709	0.944
S0005	11	8	216–258	216–238	6	3	0.631	0.388	0.769	0.602	0.043	0.000*
S0007	6	6	161–183	161–185	1	1	0.842	0.777	0.752	0.804	0.643	0.562
S0062	5	3	184–206	188–204	2	–	0.675	0.628	0.699	0.561	0.641	0.540
S070	7	4	272–284	274–284	3	–	0.552	0.361	0.588	0.383	0.244	0.402
S0097	10	6	219–247	219–247	4	–	0.657	0.527	0.771	0.621	0.004	0.010
S0121	9	7	162–188	166–190	3	1	0.416	0.416	0.717	0.570	0.000*	0.000*
S0228	3	3	221–227	221–227	–	–	0.263	0.500	0.489	0.597	0.004	0.605
Average							0.593	0.536	0.695	0.597		
SD							0.181	0.151	0.103	0.117		
Total	57	45			19	7						

Size range is the smallest and largest allele for each group of samples

Na number of alleles, A_{priv} private alleles, N north, S south

* P value for deviation from Hardy–Weinberg equilibrium (Bonferroni-corrected significance level $P=0.003125$)

The STRUCTURE analysis resulted in the highest log-likelihood for two groups (Fig. 3), which matched well with an overall differentiation pattern of north vs. south. The AMOVA (north/south) revealed significant F_{ST} and R_{ST} values with respective values of 0.078 and 0.0253 ($P=0.000$). The overall F_{ST} value among sample localities was also significant ($F_{ST}=0.076$, $P=0.000$). This, however, was due only to the differentiation between north and south, as the pairwise F_{ST} values between northern and southern sample sites were the only significant values with the exception of Béja and Séliana in the north whose F_{ST} was just below the corrected significance threshold ($P=0.0048$). When we ran a separate STRUCTURE analysis for the north, however, the highest likelihood value was yielded for $K=1$, suggesting a panmictic northern population.

FCA was applied to assess the degree of structuring, and it also separated the north and south with little overlap (not shown). The Bottleneck test failed to identify the genetic signature of a demographic decline irrespective of whether the total population, the five sample sites or the two populations, north and south, were analysed. By contrast, the M ratio showed very low values. The average values of M across loci were 0.36 and 0.31 in the northern and the

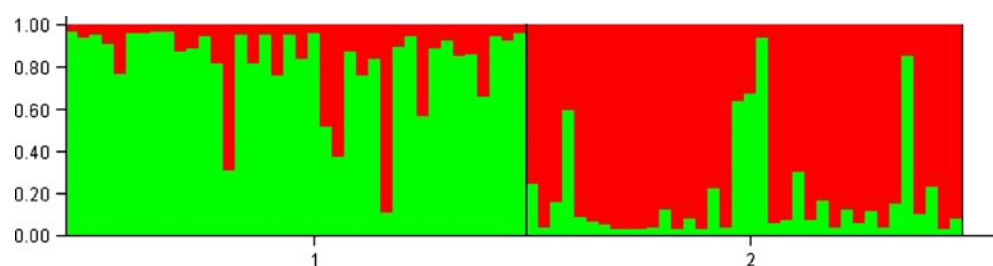
southern group and 0.36 for the total population. Garza and Williamson (2001) typically found M values larger than about 0.7 in non-bottlenecked populations, so our M values indicate a decrease in population size.

Discussion

We analysed 74 wild boar from five different sample sites across Tunisia as a contribution to our knowledge on the largely neglected North African ungulate fauna. Both mtDNA sequences and nuclear microsatellites revealed a genetic break between the north and south of the country but no further substructuring.

Microsatellite-based differentiation between north and south ($F_{ST}=0.078$) was similar to the overall genetic divergence among four populations in Italy and Hungary ($F_{ST}=0.08$, $P<0.05$; Vernesi et al. 2003) but lower than among Portuguese populations with pairwise F_{ST} values ranging from 0.138 to 0.178 (north/centre/south) and a global F_{ST} of 0.155 (Ferreira et al. 2009). Differentiation estimates obtained with mtDNA were higher than those obtained with microsatellite data in our study area.

Fig. 3 Bar plot based on Bayesian STRUCTURE analysis. Each column refers to one individual. Colours refer to the two inferred clusters. Population 1: north, population 2: south



Mutually non-exclusive explanations that might account for this discrepancy are (1) differential dispersal between the sexes—as a polygynous mammal, the wild boar is characterised by strong female philopatry and male-biased dispersal (Poteaux et al. 2009); (2) differences in effective population size between the two marker systems; and (3) F_{ST} values obtained with microsatellite data might be an underestimate because of the high polymorphism of microsatellites leading to a large amount of genetic diversity within populations (Hedrick 1999; Balloux and Lugon-Moulin 2002).

The STRUCTURE analysis yielded a clear split between the indigenous northern population and the recently established southern one, but also suggested a certain amount of admixture. This result is confirmed by a small zone of overlap between north and south in the FCA. This is due probably to natural migration since wild boar have a very high and fast expansion capacity. Due to its quick growing potential, this species occupied new areas where it had formerly been absent. Figure 1 shows the potential distribution of wild boar in Tunisia (Gharaibeh 1997) based on observations, but the species almost certainly has a discontinuous distribution in this area. Unreported translocations among hunting grounds, however, cannot be ruled out as an alternative explanation for the admixed animals, but no translocations from Europe have been recorded. Finally, the genetic overlap between the north and south might also be due to independent immigration of Algerian wild boar into both areas.

In line with previous wild boar studies, the expected heterozygosity (H_E) over all loci in the north and south (0.695 and 0.597) was low compared to other ungulates (for red and roe deer, see e.g. Hmwe et al. 2006a, b; Zachos et al. 2006), and even the seriously bottlenecked Tunisian Barbary red deer was more variable ($H_E=0.78$, Hajji et al. 2007). However, it was within the range of European wild boar populations (Nikolov et al. (2009) for Bulgaria, $H_E=0.67$; Scandura et al. (2008) for large parts of continental Europe, $H_E=0.66$). Haplotype diversity was low, and nucleotide diversity was rather high (Scandura et al. (2008) for Europe (excl. Italy): haplotype diversity=0.829 and nucleotide diversity=0.5). This finding is typical of transient bottlenecks where many haplotypes are lost without reducing nucleotide diversity to the same extent because the remaining haplotypes are not closely related (Nei et al. 1975). Only one haplotype was found in the southern population, while in the northern group, two were detected, one of which was rare and identical to haplotype H29 in European studies (Alves et al. 2003; Scandura et al. 2008). This mitochondrial pattern is very similar to that found in Tunisian Barbary red deer, which harbours only two non-related haplotypes of similar frequency (Hajji et al. 2007). Further studies on other ungulate species will have

to show if this mitochondrial genetic depletion is characteristic across taxa in the region. Contrary to mtDNA, our microsatellite results do not show similarly unambiguous signs of genetic depletion. The bottleneck test was negative, but the low M ratio was indicative of a bottleneck event. The test carried out with the Bottleneck software is based on a relative excess of heterozygosity, which is a transient phenomenon, while low M ratios can persist for very long (Garza and Williamson 2001). Therefore, our results may perhaps best be explained by a bottleneck in the more distant past. A similar discrepancy between the two approaches was also found in Amur tigers (*Panthera tigris altaica*) and explained along the same lines by the authors of that study (Henry et al. 2009). Intermediate microsatellite variability and, at the same time, lack of mitochondrial variation are not uncommon and have been found before, e.g. in Serbian red deer (Feulner et al. 2004). Potential reasons for this discrepancy include the higher mutation rates at microsatellite loci (leading to a relatively quicker generation of new variants after a bottleneck) and the fourfold higher effective population size for the nuclear genome compared to mitochondrial loci, which results in genetic drift being four times weaker for nuclear loci.

In Tunisia, genetic variability is somewhat higher in the northern group, and this also holds for the mtDNA (two haplotypes found vs. only one in the south). The reason for this is probably that the two groups have different histories and sizes. The northern group currently contains around 100,000 individuals and has been undergoing a permanent demographic expansion. The southern group does not exceed 1,000 individuals (including all districts) and was probably founded via natural dispersal from South Algeria through Jebel Brikis in the 1970s and 1980s as reported by the DGF. These separate histories are also reflected by the relatively large amount of private northern and southern alleles. The analysis of Algerian wild boar, however, is needed to corroborate this colonisation scenario for the southern part of Tunisia.

Tunisia is home to many ungulate species, but these animals have only rarely been studied by population geneticists. We have shown that wild boar at the southern margin of their distribution harbour genetic diversity of almost the same magnitude as the large continuous Eurasian population but display pronounced structuring. Wild boar are the only non-threatened ungulate species in Tunisia and play an important economic role as game animals that attract tourist hunters from abroad. In some mountain regions (Ain Draham), the ecosystem service provided by wild boar hunting is the only major source of income. Further genetic studies combining our data with those from other North African countries, in particular Algeria, and studies on distribution patterns and behaviour of wild boar are needed to gain a deeper insight into the

biology of this ecologically and economically important North African ungulate species.

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