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Is the imperilled spur-thighed tortoise (*Testudo graeca*) native in Sardinia? Implications from population genetics and for conservation

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Abstract. Using mtDNA sequences and 12 microsatellite loci, we compare populations of *Testudo graeca* from Sardinia and North Africa. The observed pattern of almost no differentiation combined with reduced variation in the Sardinian population is consistent with introduction in prehistoric or historic times from what is now Tunisia and neighbouring Algeria. Furthermore, in the light of the recently published recommendation to eradicate the non-native *T. graeca* from Italy, we review recent studies on the archaeological and fossil record, on the phylogeography and population genetics of the three other chelonian species occurring in Sardinia (*Emys orbicularis*, *T. hermanni*, *T. marginata*). We conclude that the extant Sardinian populations of all four species are not native. However, they are and should be safeguarded under EC law (Council Regulation No 338/97 on the Protection of Species of Wild Fauna and Flora; Flora Fauna Habitat Directive: Appendix IV, Art. 12) because they serve as a back-up for the declining mainland populations. Moreover, these populations constitute an important part of the human-shaped natural heritage of the Mediterranean.

Keywords: conservation, *Emys orbicularis*, management, microsatellites, mtDNA, phylogeography, *Testudo hermanni*, *Testudo marginata*.

Introduction

The populations of most Western Palaearctic tortoise species are declining throughout their ranges, with four of the five species placed in the IUCN Red List categories Near Threatened (*Testudo hermanni*), Vulnerable (*T. graeca*, *T. horsfieldii*), and Critically Endangered (*T. kleinmanni*). Only populations of *T. marginata* are considered to be stable (Red List category Least Concern; IUCN, 2010). Traditionally, Sardinian populations of the spur-thighed tortoise (*T. graeca*) and the marginated tortoise (*T. marginata*) have been regarded as introduced (Angelini, 1899; Mertens and Wermuth, 1960; Bringsøe et al., 2001; Buskirk et al., 2001; Carpaneto, 2006a, 2006b). This view is supported by recent genetic investigations using mitochondrial DNA sequences and nuclear genomic fingerprinting (Fritz et al., 2005a, 2009), even though the possibility of natural oversea dispersal was acknowledged for *T. graeca*. By contrast, Sardinian Hermann's tortoises (*T. hermanni*) and the sole freshwater turtle species occurring in Sardinia, the European pond turtle (*Emys orbicularis*), are traditionally considered native (Dürigen, 1897; Mertens and Wermuth, 1960; Cheylan, 2001; Fritz, 2001; Mazzotti, 2006; Mazzotti and Zuffi, 2006). While Carpaneto (2006a, 2006b) suggested in his influential tortoise chapters in the 'Atlas of Italian Amphibians and Reptiles' that the Sardinian population of *T. marginata* should be protected as a back-up for the Greek populations, he recommended that *T. graeca* should be eradicated in Italy, at least from sites where the native *T. hermanni* occurs in sympatry. Although the latter suggestion clearly contradicts legislation, as *T. graeca* is a protected species under EC Law (Council Regulation No 338/97 on the Protection of Species of Wild Fauna and Flora; Flora Fauna Habitat Directive: Appendix IV, Art. 12), it caused major concern among Italian conservationists. This concern was reinforced

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by the recent discovery that Sardinian *T. graeca* represent well-established, reproducing populations and not only scattered individuals, implying that *T. graeca* might have occurred for a very long time on Sardinia. Spur-thighed tortoises are widely distributed along the central west coast of Sardinia and particularly abundant on the nearby islet Mal di Ventre (Corti et al., 2004, 2007; C. Corti, pers. observ.).

In the present study, we re-examine the origin of Sardinian *T. graeca* and discuss our results in the light of legislation and recent findings on the phylogeography of other Mediterranean chelonians. In doing so, we expand the sampling of Sardinian tortoises of Fritz et al. (2009) considerably and correlate sequence variation of the mitochondrial cytochrome *b* gene with information from 12 microsatellite loci. For comparison with Sardinian *T. graeca*, we use the genetically most closely related tortoises from northern Africa (Tunisia, neighbouring Algeria; Fritz et al., 2009).

Materials and methods

Sampling, DNA extraction, PCR, fragment analysis and sequencing

Blood, tissue (skin, muscle) and salivary samples were obtained from different localities of Sardinia, Tunisia and neighbouring Algeria (fig. 1; table 1), preserved in 96% ethanol and stored at −80°C until processing. The samples from North Africa and four Sardinian samples were the same as in Fritz et al. (2009). Total DNA was isolated using either standard proteinase K and phenol chloroform protocols (Sambrook and Russell, 2001), the peqGOLD Tissue DNA Mini Kit (protocol for tissue samples; PEQLAB, Erlangen, Germany), or the InnuPREP DNA Mini Kit (protocol for isolating buccal swabs or tissue; Analytik Jena, Germany).

Twelve microsatellite loci were analysed for each tortoise following Salinas et al. (2010): Goag5, Goag6 (designed for *Gopherus agassizii*; Edwards et al., 2003), GmuB08, GmuD16, GmuD51 (designed for *Glyptemys muhlenbergii*; King and Julian, 2004), GP61, GP81 (designed for *Gopherus polyphemus*; Schwartz et al., 2003), Test10, Test21, Test56, Test71, and Test76 (designed for *Testudo hermanni*; Forlani et al., 2005). Microsatellite loci were individually PCR-amplified in a final volume of 25 μl using 1 unit *Taq* polymerase (Bioron, Ludwigshafen, Germany) with the buffer recommended by the supplier and a final concentration of 1.6 mM $MgCl_2$ (Bioron), 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 0.4 μM of each primer (forward primer fluorescent-labelled; table 2) and approximately 10-40 ng of total DNA. In cases of PCR failure, the reaction was repeated using the same conditions but with 0.25-0.5 μg of Bovine Serum Albumin (BSA, Fermentas) added. The PCR cycling conditions were as follows: 43 cycles with denaturation at 94°C for 60 s but for 11 min for the first cycle, annealing at 58°C for 45 s during the first 3 cycles, followed by annealing at 55°C for 45 s during the remaining 40 cycles, and extension at 72°C for 45 s but 30 min for the final cycle. Fragment lengths were determined on an ABI 3130xl genetic analyser using the GeneScan^{TM}-600 LIZ® Size Standard and the software GENEMAPPER (Applied Biosystems, Foster City, USA). For each tortoise, the loci Goag5, Goag6, GmuB08, Gmu16, GmuD51, and Test76 were combined in one sequencer run and GP61, GP81, Test10, Test21, and Test71 in another; Test56 was processed alone.

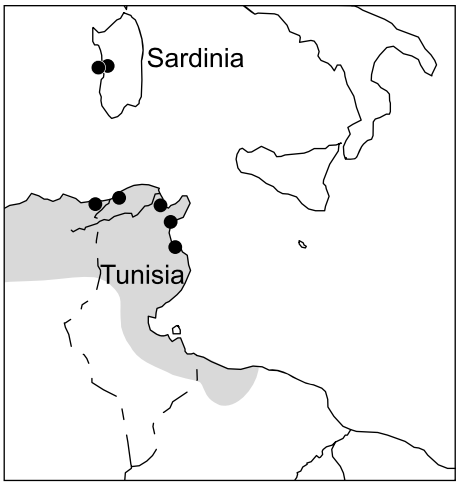

Figure 1. Collection sites of *Testudo graeca* samples (closed circles) used in the present study. Distribution range in North Africa shaded.

In addition, the nearly complete mitochondrial cytochrome *b* gene (cyt *b*) and approximately 20 bp of the adjacent DNA segment encoding the transfer RNA for Threonine (tRNA-Thr) were sequenced when these data were not available for the same samples from earlier studies (Fritz et al., 2007, 2009). Two samples that previously yielded highly distinct haplotypes (A5, GenBank accession number AM230972; A9, AJ888343) were re-sequenced to test for possible sequencing errors. Two overlapping mtDNA fragments were amplified using the primer pairs CytbG plus mt-E-Rev2 (Spinks et al., 2004; Fritz et al., 2006) and mt-c-For2 plus mt-f-na (Fritz et al., 2006; annealing temperature for both primer pairs, 55°C). PCR was performed in a final volume of 20 μl using 1 unit *Taq* polymerase (Bioron) with the buffer recommended by the supplier and a final concentration of 0.25 mM of each dNTP (Fermentas), 0.5 μM of forward and reverse primer and approximately 10-40 ng of total DNA. When PCR failure occurred, the reaction was repeated with 0.25-0.5 μg BSA (Fermentas) added.

Table 1. *Testudo graeca* samples used in the present study. MTD = Museum of Zoology, Senckenberg Dresden. Sample codes starting with D, complete specimen in herpetological collection; sample codes starting with T, tissue collection. Superscripts following the sample type refer to the method of DNA extraction using (1) standard proteinase K and phenol chloroform protocols, (2) the peqGOLD Tissue DNA Mini Kit, or (3) the InnuPREP DNA Mini Kit. Accession numbers for haplotypes are A1: AM230971, A2: FM162020, A3: FM162021, A4: FM162022, A6: FM162023, A7: FM162024, A8: FM162025, A10: FR686466.

MTD	Site	Latitude	Longitude	Sample type	mtDNA haplotype	Microsatellites
T 3250	Algeria: El Kala	36.857253N	8.511464E	blood[1]	A7: Fritz et al. (2009)	n/a
T 800	Tunisia: vicinity of Nabeul	36.456480N	10.735085E	blood[1]	A4: Fritz et al. (2009)	This study
T 4151	Tunisia: Djebel Boukornine near Hammam Lif	36.726500N	10.321617E	tissue[2]	A2: Fritz et al. (2009)	This study
D 41852	Tunisia: set to between Tamra and Sidi Ferdjani*	37.141359N	9.164795E	tissue[2]	A4: Fritz et al. (2009)	This study
D 42883	Tunisia: set to between Tamra and Sidi Ferdjani*	37.141359N	9.164795E	tissue[2]	A2: Fritz et al. (2009)	This study
D 42893	Tunisia: set to between Tamra and Sidi Ferdjani*	37.141359N	9.164795E	tissue[2]	A4: This study**	This study
D 44857	Tunisia: set to between Tamra and Sidi Ferdjani*	37.141359N	9.164795E	tissue[2]	A6: Fritz et al. (2009)	This study
D 44865	Tunisia: set to between Tamra and Sidi Ferdjani*	37.141359N	9.164795E	tissue[2]	A2: Fritz et al. (2009)	This study
D 46397	Tunisia: set to between Tamra and Sidi Ferdjani*	37.141359N	9.164795E	tissue[2]	A3: Fritz et al. (2009)	This study
D 46588	Tunisia: set to between Tamra and Sidi Ferdjani*	37.141359N	9.164795E	tissue[2]	A6: Fritz et al. (2009)	This study
T 149	Tunisia: Sousse	35.816845N	10.602523E	blood[1]	A1: Fritz et al. (2007, 2009)	n/a
T 5002	Tunisia: Tabarka	36.959000N	8.752000E	tissue[2]	A6: Fritz et al. (2009)	This study
T 5003	Tunisia: Tabarka	36.959000N	8.752000E	tissue[2]	A6: Fritz et al. (2009)	This study
T 5004	Tunisia: Tabarka	36.959000N	8.752000E	tissue[2]	A8: Fritz et al. (2009)	This study
T 5005	Tunisia: Tabarka	36.959000N	8.752000E	tissue[2]	A6: Fritz et al. (2009)	This study
T 5710	Sardinia: Is Arenas	40.055160N	8.472965E	oral swab[3]/tissue[1]	A6: This study	This study
T 5700	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study
T 5701	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study

Table 1. (Continued).

MTD	Site	Latitude	Longitude	Sample type	mtDNA haplotype	Microsatellites
T 5702	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study
T 5703	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A10: This study	This study
T 5704	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study
T 5705	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study
T 5706	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study
T 5707	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study
T 5708	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study
T 5709	Sardinia: Mal di Ventre Island	39.989453N	8.307643E	oral swab[3]/tissue[1]	A6: This study	This study
T 5695	Sardinia: Narbolia, Montiferru	40.102056N	8.556338E	oral swab[3]/tissue[1]	A6: This study	This study
T 5696	Sardinia: Narbolia, Montiferru	40.102056N	8.556338E	oral swab[3]/tissue[1]	A6: This study	This study
T 5697	Sardinia: Narbolia, Montiferru	40.102056N	8.556338E	oral swab[3]/tissue[1]	A6: This study	This study
T 1113	Sardinia: Putzu Idu	40.024757N	8.410569E	blood[1]	A6: This study***	This study
T 5698	Sardinia: Seu	39.928512N	8.540952E	oral swab[3]/tissue[1]	A6: This study	This study
T 5699	Sardinia: Seu	39.928512N	8.540952E	oral swab[3]/tissue[1]	A6: This study	This study
T 5044	Sardinia: Sinis Peninsula	40.023585N	8.499499E	blood[1]	A6: Fritz et al. (2009)	This study
T 5045	Sardinia: Sinis Peninsula	40.023585N	8.499499E	blood[1]	A6: Fritz et al. (2009)	This study
T 1112	Sardinia: Sinis Peninsula: S. Giovanni di Sinis	39.886080N	8.435814E	blood[1]	A6: Fritz et al. (2009)	This study

*Tortoise confiscated at German airport from flight arriving from Tunisia.
**Misidentified as haplotype A5 (AM230972) in Fritz et al. (2007, 2009).
***Misidentified as haplotype A9 (AJ888343) in Fritz et al. (2005a, 2009).

Table 2. Locus-specific molecular diversity indices for Tunisian vs. Sardinian spur-thighed tortoises based on microsatellite data. N_A = number of alleles, A_R = allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficient, p = probability for F_{IS} (statistically significant values asterisked). Subscripts of loci indicate fluorescent dyes (biomers.net, Ulm, Germany).

Locus	Total allele size range ($n = 33$)	Tunisia ($n = 13$)								Sardinia ($n = 20$)							
		N_A	Allele size range	Null alleles	A_R	H_O	H_E	F_{IS}	p	N_A	Allele size range	Null alleles	A_R	H_O	H_E	F_{IS}	p
$Goag5_{Atto550}$	258-267	2	258-261	no	2.00	0.85	0.51	−0.71	1.000	3	258-267	no	2.47	0.89	0.53	−0.72	1.000
$Goag6_{6\text{-}Fam}$	374-390	6	376-390	no	6.00	0.56	0.72	0.24	0.227	3	374-382	no	2.50	0.56	0.54	−0.03	0.613
$GmuB08_{Atto565}$	213-255	11	222-255	no	9.81	0.75	0.92	0.20	0.067	7	213-243	no	5.77	0.74	0.77	0.05	0.433
$GmuD16_{Hex}$	273-325	10	273-325	no	9.48	0.70	0.90	0.23	0.033	8	273-313	no	6.96	0.88	0.85	−0.04	0.754
$GmuD51_{Atto550}$	144-224	10	144-220	yes	9.04	0.55	0.90	0.40	0.006	11	144-224	yes	8.14	0.58	0.86	0.33	0.002*
$Test76_{Hex}$	114	1	114	no	1.00		monomorphic			1	114	no	1.00		monomorphic		
$GP61_{6\text{-}Fam}$	177-193	4	177-193	no	3.50	0.67	0.54	−0.24	0.896	2	185-193	no	2.00	0.55	0.45	−0.23	0.952
$GP81_{Hex}$	362-368	3	362-368	no	2.99	0.42	0.62	0.34	0.106	2	362-364	no	1.82	0	0.12	1.00	0.052
$Test10_{Hex}$	222-250	10	222-250	yes	8.55	0.62	0.87	0.30	0.006	8	226-240	yes	6.87	0.41	0.85	0.53	0.002*
$Test21_{Atto565}$	204-208	2	204-206	no	2.00	0.31	0.37	0.17	0.513	3	204-208	no	2.93	0.63	0.53	−0.19	0.898
$Test71_{6\text{-}Fam}$	128-130	2	128-130	no	1.75	0.08	0.08	–	–	1	128	no	1.00		monomorphic		
$Test56_{Hex}$	247-312	17	247-312	yes	13.82	0.75	0.97	0.23	0.002*	13	255-299	yes	10.06	0.59	0.91	0.36	0.002*
Overall	–	6.5	–	–	5.83	0.52	0.61	0.16	0.002	5.2	–	–	4.29	0.48	0.53	0.10	0.017

The PCR cycling conditions were as follows: 36 cycles with denaturation at 94°C for 45 s but for 3 min for the first cycle, annealing at 55°C for 20 s, and extension at 72°C for 90 s but 10 min for the final cycle. PCR products were purified using the ExoSAP-IT enzymatic cleanup (USB Europe GmbH, Staufen, Germany; 1 : 20 dilution; modified protocol: 30 min at 37°C, 15 min at 80°C) and sequenced on an ABI 3130xl using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the primers mt-E-Rev2 and mt-c-For2. When the sequences were too short, the primers CytbG and mt-f-na were additionally used to gain the full length of approximately 1160 bp. Individual sequences were aligned in BIOEDIT (Hall, 1999), manually collapsed into haplotypes and compared with previously published data of North African and Sardinian *T. graeca* (Fritz et al., 2005a, 2007, 2009). Of two samples representing previously published, distinct mtDNA haplotypes (A1, A7; Fritz et al., 2009; table 1), no microsatellite data could be produced. However, their mitochondrial sequences were included in all calculations.

Genetic diversity and divergence of Sardinian and North African tortoises

Mutational relationships of mitochondrial sequences were examined by a parsimony network analysis as implemented in TCS 1.21 (Clement et al., 2000). For mitochondrial and microsatellite data, diversity and divergence parameters were estimated for Sardinian and North African tortoises separately. The number and size of microsatellite alleles was compared using a frequency table produced with the software CONVERT (Glaubitz, 2004). Possible linkage among microsatellite loci was tested using ARLEQUIN 3.11 (Excoffier et al., 2005) and 1000 dememorisation steps followed by additional 10 000 steps of the Markov Chain; the resulting p values were Bonferroni-corrected for multiple comparisons (Rice, 1989). ARLEQUIN was further used to estimate locus-specific observed (H_O) and expected heterozygosity (H_E) and to perform a locus-by-locus analysis of molecular variance (AMOVA; 10 000 permutations). Deviations from Hardy-Weinberg equilibrium and the locus-specific excess or deficiency of heterozygotes (inbreeding coefficient F_{IS}; Weir and Cockerham, 1984) were assessed using GENEPOP 4.0 (Rousset, 2008). Exact p values for inbreeding coefficients were estimated by the Markov Chain method (1000 dememorisation steps followed by 100 000 iterations) and resulting values were again Bonferroni-corrected. The locus-specific allelic richness was estimated with the software FSTAT (Goudet, 1995). Frequencies of mitochondrial haplotypes were assessed in ARLEQUIN and used for an AMOVA (10 000 permutations). The same software was also used to determine nucleotide diversities and net-nucleotide diversity between Sardinian and North African tortoises.

Bayesian inference of population structure

For inferring population structuring based on unlinked genetic markers, several Bayesian algorithms exist. However, different methods may obtain conflicting results with respect to the number of genetic clusters (K) and the degree of admixture between clusters, and none of the methods is clearly superior to the others. Therefore, the parallel application of different algorithms for exploring the robustness of the inferred population structure is advisable (Pearse and Crandall, 2004; Chen et al., 2007; Frantz et al., 2009). Consequently, our microsatellite data were subjected to two different clustering algorithms, a spatially explicit and a spatially non-explicit method that differed also in their ability to cope with null alleles.

The non-explicit analysis was performed using the software STRUCTURE 2.3.2 (Pritchard et al., 2000; Falush et al., 2003; Hubisz et al., 2009). Here, the main criterion for delimiting clusters is the search for groups in Hardy-Weinberg equilibrium and linkage equilibrium. This search is conducted for each locus separately, which allows detecting genetic clusters of admixed origin. The number of clusters was estimated using posterior probabilities [highest ln P(D)] for $K = 1, \ldots, 10$. Four different scenarios were analysed for two data sets, one including all 12 microsatellite loci and the other only the nine loci without null alleles (table 2): (i) an admixture scenario in which individuals are allowed to have mixed ancestry with allele frequencies correlated and (ii) an admixture scenario with allele frequencies not correlated; (iii) a no-admixture scenario with allele frequencies correlated, or (iv) not correlated. The admixture model assumes recent or current gene flow, so that individuals can have ancestors from more than one population. By contrast, the no-admixture model assumes no or only negligible gene flow with the consequence that ancestry from only one population is favoured. In this context, correlated allele frequencies are expected in populations with a common origin, resulting in similar allele frequencies. On the other hand, uncorrelated allele frequencies are expected for populations that are isolated for a long time, so that allele frequencies can be considered independent. The burn-in was set to 10^5 and the number of further MCMC runs to 5×10^4. Calculations were repeated 10 times for each K; convergence of likelihood values was reached after the burn-in. Clusters and individual admixture were visualized with barplots.

As spatially explicit Bayesian clustering method, GENELAND 3.1.4 (Guillot et al., 2005) was used. Similar to STRUCTURE, GENELAND estimates the number of K by searching for units in Hardy-Weinberg equilibrium and linkage equilibrium, but with using geographical coordinates of each sample as prior information. For this purpose, the geographical coordinates of samples from confiscated Tunisian tortoises were set to the central Tunisian coast (between Tamra and Sidi Ferdjani), the region where the tortoises originated from (table 1). An advantage of GENELAND is that this software allows for correcting the clustering results for null alleles (Guillot et al., 2008), whereas STRUCTURE results may be biased by null alleles (Falush et al., 2003, 2007). K was set to range from 1 to 10, using the options allele frequencies correlated or not correlated, and for both of these settings null alleles existent (as suggested by MICRO-CHECKER 2.2.3; van Oosterhout et al., 2004), 10^6 MCMC steps, and burn-in of 10^5. Calculations were repeated 10 times each and the following parameters were obtained:

number of clusters (K), cluster assignment of each individual, inbreeding coefficient F_{IS} of each cluster, and pairwise F_{ST} values between clusters. Standard diversity and divergence parameters estimated by GENELAND (Hardy-Weinberg equilibrium, linkage equilibrium, F_{IS}, F_{ST}) were subsequently checked using ARLEQUIN, as recommended by Guillot et al. (2005).

Results

Genetic diversity and divergence of Sardinian and North African tortoises

With one exception, all newly sequenced samples from Sardinia yielded the previously identified haplotype A6; the remaining sample contained a new haplotype (A10, GenBank accession number FR686466; table 1). A10 differs in one mutational step from A6. Tortoises from Tunisia and adjacent Algeria yielded seven distinct haplotypes (A1-A4, A6-A8) that differ by a maximum of six mutational steps (fig. 2). The

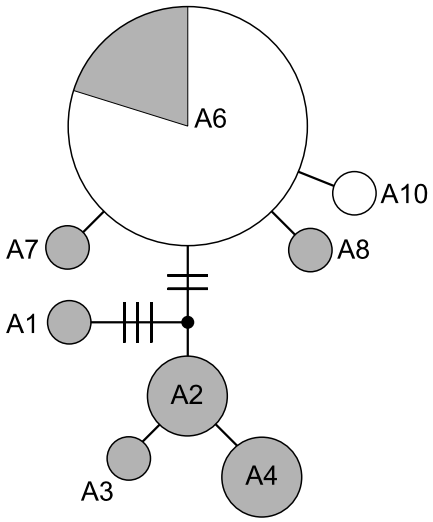

Figure 2. Parsimony network for mtDNA haplotypes of Sardinian and North African *Testudo graeca* based on an alignment of 1164 bp. Symbol size corresponds to haplotype frequency. Grey symbols or slices refer to North African tortoises; white, Sardinian tortoises. Small black circle, missing node haplotype. Each line connecting two haplotypes represents one substitution, except when hashmarks are present. Then, each hashmark is one mutational step. Haplotype frequencies are for A6 = 24, A2 = 3, A4 = 3, all other haplotypes were found only once. Greatest outgroup probability has the most frequent haplotype A6 (0.7105).

previously published highly distinct haplotypes A5 (Tunisia) and A9 (Sardinia), characterized by 11 or nine singletons, respectively, turned out to be sequencing errors (table 1). Our resequencing of the same samples resulted in an unambiguous assignment of the Tunisian sample to haplotype A4 and of the Sardinian sample to haplotype A6.

All twelve nuclear microsatellite markers were found to be in linkage equilibrium for Sardinian and Tunisian tortoises each, although there is evidence for non-random association between different pairs of loci in Sardinian (Goag5-Test21, GmuD16-Test56) and Tunisian tortoises (Goag5-GP61, GmuD51-Test10, Test10-Test21). For Sardinian and Tunisian tortoises, null alleles are present at loci GmuD51, Test10, and Test56. A significant excess of homozygosity was found for Sardinian tortoises at loci GmuD51 and Test10, and for Sardinian and Tunisian tortoises at locus Test56 (table 2). Despite smaller sample size, diversity indices of microsatellites are higher for Tunisian tortoises (average estimates for Tunisia: N_A: 6.5, A_R: 5.83, H_O: 0.52, H_E: 0.61; for Sardinia: N_A: 5.2, A_R: 4.29, H_O: 0.48, H_E: 0.53). This is also mirrored by mtDNA with distinctly higher haplotype and nucleotide diversities in North Africa (table 3).

Statistically significant fixation indices for both microsatellites (F_{ST}: 0.049, $p < 0.0001$) and mtDNA (F_{ST}: 0.39, $p = 0.0001$) suggest that Sardinian and North African tortoises are genetically divergent. However, for microsatellites only 4.89% of the observed variation occurs between Sardinia and Tunisia and 95.11% within these two groups, while 39.01% of the mitochondrial variation is observed between and 60.99% within the groups. The clearly higher between-group percentage of mtDNA and its higher F_{ST} value are caused by the different haplotype frequencies in Sardinia and North Africa (table 3). Yet, the low net-nucleotide divergence (0.00065, $p = 0.0002$) reveals weak differentiation at the nucleotide level, and this pattern of negligible differentiation of Sardinian

Table 3. Frequencies of mitochondrial haplotypes and indices for mtDNA diversity. Standard deviations of diversity indices in parentheses. North African tortoises are from Tunisia and adjacent Algeria.

Group	n	Haplotype frequencies								Haplotype diversity	Nucleotide diversity
		A1	A2	A3	A4	A6	A7	A8	A10		
North Africa	15	0.07	0.20	0.07	0.20	0.32	0.07	0.07	0	0.85 (0.06)	0.00236 (0.00149)
Sardinia	20	0	0	0	0	0.95	0	0	0.05	0.10 (0.09)	0.00009 (0.00017)

tortoises is supported by haplotype network analysis (fig. 2; see also above). When it is considered that the weakly differentiated haplotype A10 could be found in North Africa when more tortoises will be studied, this suggests that the haplotypes of Sardinian tortoises are part of the variation occurring in Tunisia and neighbouring Algeria.

Bayesian inference of population structure

Population structure and the number of genetic clusters (K) were inferred without prior assignment of samples using two different Bayesian methods (STRUCTURE, GENELAND). The STRUCTURE results differed depending on the model and the number of used microsatellite loci. Either all tortoises were placed in one cluster or Sardinian and Tunisian tortoises were assigned to two clusters (table 4). Yet, when two clusters were inferred, their demarcation was weak and for many individuals an admixed ancestry was suggested (fig. 3). Correspondingly, locus-by-locus AMOVAs resulted in a very low F_{ST} value of 0.05 ($p < 0.001$), irrespective of whether all microsatellite loci were processed or when the three loci with null alleles (GmuD51, Test10, Test56) were excluded.

By contrast, the spatially explicit GENELAND analysis corrected for null alleles revealed $K = 3$ under the assumption of correlated allele frequencies. One cluster corresponded to Sardinian tortoises, while Tunisian specimens were placed in two distinct clusters, one for the tortoises from the vicinity of Tabarka and another one for all other Tunisian tortoises (table 5). However, the fixation indices (F_{ST}) among these clusters were again very low and amounted to only 0.042-0.058 (estimated by GENELAND), indicating negligible genetic divergence. In all three populations occurs an excess of homozygotes (F_{IS} for Sardinia: 0.24, for Tabarka: 0.31, for other Tunisian tortoises: 0.26), perhaps due to a Wahlund effect. Subsequent ARLEQUIN analyses substantiated low divergences among clusters (locus-by-locus AMOVA: F_{ST} 0.082, $p < 0.001$). Homozygous excess was confirmed for all three clusters ($p < 0.001$ for all clusters), but there was no pattern of linkage disequilibrium.

Under the assumption of not correlated allele frequencies, GENELAND suggested only two clusters. Sardinian tortoises were grouped now together with Tunisian tortoises from Tabarka, and the second cluster comprised all other Tunisian specimens (table 5). The fixation index (F_{ST}) between these two clusters was 0.053, indicating again only insignificant divergence. In both clusters occurs an excess of homozygotes (F_{IS} for each: 0.26). Subsequent analyses of the clusters with ARLEQUIN (locus-by-locus AMOVA: F_{ST} 0.077, $p < 0.001$) confirmed this pattern; for the two clusters a significant excess of homozygotes is evident ($p < 0.001$ each), but no obvious pattern of linkage disequilibrium.

In summary, population structures inferred from GENELAND differed when allele frequencies were set correlated or not correlated. Regardless of the allele frequency model, the overall population structure is decidedly weak and all inferred clusters show an excess of homozygotes.

Discussion

Our data provide strong evidence that Sardinian *Testudo graeca* belong not only to the same subspecies as in Tunisia and neighbouring Alge-

Table 4. Mean ln P(D) values and their standard deviations (SD) for different Ks of non-spatial Bayesian inference of population structuring (STRUCTURE; arithmetic mean of 10 independent runs for each K). The first value in each column refers to an analysis using all 12 loci; the second value is based on an analysis of nine loci (loci with null alleles excluded). Maximum values indicating the most likely number of clusters in bold and asterisked.

K	Admixture model Allele frequencies correlated		Admixture model Allele frequencies not correlated		No-admixture model Allele frequencies correlated		No-admixture model Allele frequencies not correlated	
	Mean ln P(D)	SD	Mean ln P(D)	SD	Mean ln P(D)	SD	Mean ln P(D)	SD
1	**−1006.07*/−527.79***	1.07/1.13	−1007.32/**−528.00***	0.09/0.05	−1004.82/**−527.43***	1.41/1.25	−1007.41/−528.00	0.15/0.05
2	−1012.21/−557.78	3.49/15.92	**−997.44***/−559.53	2.86/25.37	**−997.55***/−528.77	1.53/4.99	**−979.43*/−519.85***	0.57/0.20
3	−1035.12/−564.78	6.25/32.48	−1193.17/−558.67	167.82/24.99	−1020.01/−591.22	3.15/29.38	−1025.87/−550.00	3.27/1.46
4	−1065.06/−580.44	15.71/35.55	−1300.29/−553.67	99.55/14.17	−1056.93/−592.80	47.22/14.59	−1077.28/−590.91	11.71/3.95
5	−1120.41/−581.72	27.14/24.31	−1312.12/−577.27	145.28/45.12	−1096.57/−597.74	28.75/7.78	−1110.62/−579.06	17.81/1.56
6	−1207.97/−570.13	25.88/29.11	−1174.00/−573.39	13.27/17.41	−1101.79/−603.78	32.96/11.26	−1099.74/−567.51	9.94/1.21
7	−1234.89/−564.36	30.36/28.68	−1121.16/−578.30	5.17/6.56	−1114.92/−602.27	53.77/19.23	−1082.45/−563.77	4.16/1.28
8	−1307.24/−562.00	45.99/33.10	−1104.10/−610.01	4.06/76.79	−1096.12/−598.80	43.49/8.67	−1072.77/−560.56	4.79/0.78
9	−1311.94/−563.64	40.49/38.94	−1099.98/−587.25	4.68/5.58	−1085.99/−597.93	11.95/11.55	−1062.92/−558.75	3.17/1.08
10	−1372.85/−558.81	43.90/28.66	−1096.31/−676.22	4.41/218.73	−1087.71/−602.50	48.71/12.68	−1065.02/−558.88	4.51/1.35

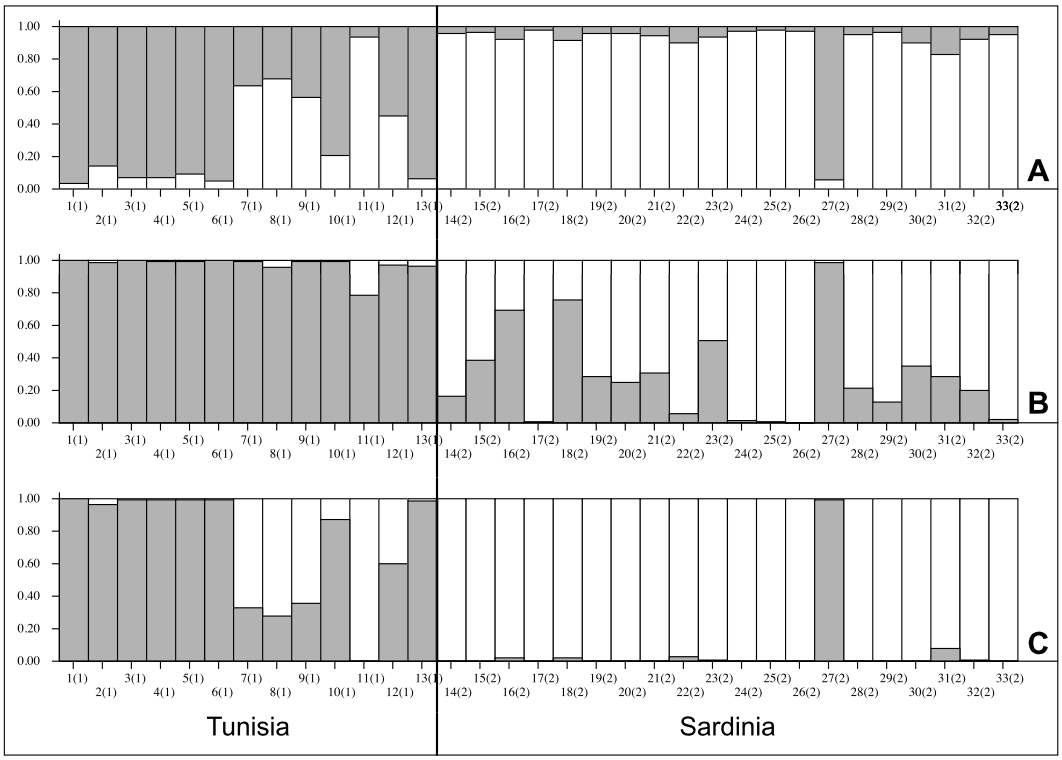

Figure 3. Barplots indicating the number of genetic clusters ($K = 2$) and individual population assignment as inferred by STRUCTURE using all 12 loci. (A) Admixture model with allele frequencies not correlated, (B) no-admixture model with allele frequencies correlated, (C) no-admixture model with allele frequencies not correlated. Membership proportions from the runs ($n = 10$ each) with the highest likelihood value. Individuals 1-13: Tunisia; 14-33: Sardinia. Under the admixture model with allele frequencies correlated only one cluster is suggested for Tunisian and Sardinian tortoises.

ria (*T. g. nabeulensis*; Fritz et al., 2009), but fall within the genetic variation occurring in North Africa. Population genetic analyses using quickly evolving microsatellite loci indicate only negligible differentiation of Sardinian tortoises, and one spatially explicit analysis corrected for null alleles even placed Sardinian tortoises together with certain Tunisian specimens (from Tabarka) in one and the same cluster. With respect to mitochondrial haplotypes, nearly all Sardinian tortoises possess a haplotype that was also recorded in North Africa, and the sole distinct Sardinian haplotype differs by only one mutational step from the others. By contrast, up to six mutational steps occur among haplotypes of Tunisian and Algerian tortoises (fig. 2). This situation suggests that Sardinian tortoises contain only part of the genetic variation found in North Africa. Such a pattern is expected after a founder effect. This implies that the Sardinian population is derived from North African spur-thighed tortoises that were either introduced by man or reached Sardinia by recent transoceanic dispersal.

For tortoises there are several cases of such-like oversea dispersal known. The most prominent example are the Galápagos tortoises (*Chelonoidis nigra* complex) that must have reached this volcanic archipelago via the Pacific Ocean from the 1000 km distant South American mainland (Caccone et al., 1999). Moreover, a live Aldabra tortoise (*Aldabrachelys gigantea*) encrusted with barnacles (Lepadidae) was recently washed ashore on the East African coast (Gerlach et al., 2006), providing for the first time direct evidence that tortoises may sur-

Table 5. Probability of population membership of Tunisian and Sardinian tortoises estimated by GENELAND (spatially explicit Bayesian inference, corrected for null alleles). Results from run ($n = 10$) with highest likelihood value. Tunisian tortoises from Tabarka asterisked. The population assignment of each tortoise is derived from the greatest probability for population membership (in bold). The existence of three weakly differentiated populations is the most likely scenario under the assumption that null alleles exist and that allele frequencies are correlated between populations; when allele frequencies are not correlated, two discrete populations are suggested.

No	Specimen ID	Origin	Allele frequencies correlated				Allele frequencies not correlated		
			Probability of population membership			Population assignment	Probability of population membership		Population assignment
			Sardinia (1)	Tunisia (2)	Tabarka (3)		Tunisia (1)	Sardinia, Tabarka (2)	
1	MTD T 800	Tunisia	0.148	**0.576**	0.276	2	**0.972**	0.028	1
2	MTD T 4151	Tunisia	0.145	**0.545**	0.310	2	**0.747**	0.253	1
3	MTD D 41852	Tunisia	0.147	**0.590**	0.262	2	**1.000**	0	1
4	MTD D 42883	Tunisia	0.147	**0.590**	0.262	2	**1.000**	0	1
5	MTD D 42893	Tunisia	0.147	**0.590**	0.262	2	**1.000**	0	1
6	MTD D 44857	Tunisia	0.147	**0.590**	0.262	2	**1.000**	0	1
7	MTD D 44865	Tunisia	0.147	**0.590**	0.262	2	**1.000**	0	1
8	MTD D 46397	Tunisia	0.147	**0.590**	0.262	2	**1.000**	0	1
9	MTD D 46588	Tunisia	0.147	**0.590**	0.262	2	**1.000**	0	1
10*	MTD T 5002	Tunisia	0.169	0.311	**0.519**	3	0	**1.000**	2
11*	MTD T 5003	Tunisia	0.169	0.311	**0.519**	3	0	**1.000**	2
12*	MTD T 5004	Tunisia	0.169	0.311	**0.519**	3	0	**1.000**	2
13*	MTD T 5005	Tunisia	0.169	0.311	**0.519**	3	0	**1.000**	2
14	MTD T 5710	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
15	MTD T 5700	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
16	MTD T 5701	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
17	MTD T 5702	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2

Table 5. (Continued).

No	Specimen ID	Origin	Allele frequencies correlated				Allele frequencies not correlated		
			Probability of population membership			Population assignment	Probability of population membership		Population assignment
			Sardinia (1)	Tunisia (2)	Tabarka (3)		Tunisia (1)	Sardinia, Tabarka (2)	
18	MTD T 5703	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
19	MTD T 5704	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
20	MTD T 5705	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
21	MTD T 5706	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
22	MTD T 5707	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
23	MTD T 5708	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
24	MTD T 5709	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
25	MTD T 5695	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
26	MTD T 5696	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
27	MTD T 5697	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
28	MTD T 5698	Sardinia	**0.654**	0.181	0.164	1	0	**1.000**	2
29	MTD T 5699	Sardinia	**0.654**	0.181	0.164	1	0	**1.000**	2
30	MTD T 5044	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
31	MTD T 5045	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2
32	MTD T 1112	Sardinia	**0.658**	0.178	0.164	1	0	**1.000**	2
33	MTD T 1113	Sardinia	**0.659**	0.177	0.163	1	0	**1.000**	2

vive long exposure to sea water. Biogeography and genetics argue for further examples (Le et al., 2006), so that a natural colonization of Sardinia from North African *T. graeca* has to be taken into account (Fritz et al., 2009). However, the counter-running major Mediterranean surface currents (Razouls et al., 2009) do not favour the dispersal of Tunisian tortoises to the western Sardinian coast. Consequently, introduction by man is more likely, especially when it is considered that another tortoise species, *T. marginata*, was evidently introduced to Sardinia (Bringsøe et al., 2001), and all extant chelonians occurring on the Balearic Islands (*Emys orbicularis*, *T. graeca*, *T. hermanni*) were also introduced in prehistoric or historic times (Buskirk et al., 2001; Cheylan, 2001; Fritz, 2001; Fritz et al., 2006).

In contrast to *T. graeca* and *T. marginata* (Angelini, 1899; Mertens and Wermuth, 1960; Bringsøe et al., 2001; Buskirk et al., 2001; Carpaneto, 2006a, 2006b), two other chelonian species, *T. hermanni* and *E. orbicularis*, are traditionally regarded native in Sardinia (Dürigen, 1897; Mertens and Wermuth, 1960; Cheylan, 2001; Fritz, 2001; Mazzotti, 2006; Mazzotti and Zuffi, 2006). The fossil record seems, at first glance, to corroborate this view with Sardinian finds of *T*. cf. *hermanni* (Abbazzi et al., 2004: Plio-Pleistocene border) and *E. orbicularis* (Chesi et al., 2008: several Late Pleistocene and Holocene finds), but none for *T. graeca* and *T. marginata*. However, genetic data indicate a totally lacking mitochondrial differentiation of Sardinian (and Corsican) *T. hermanni* compared to Sicilian tortoises (Fritz et al., 2006; Giacalone et al., 2009). Furthermore, Sardinian (and Corsican) *E. orbicularis* are genetically not differentiated from the widely distributed continental subspecies *E. o. galloitalica* (Lenk et al., 1999; Fritz et al., 2005b), even in quickly evolving microsatellite markers, suggestive of extinction of a native population and later reintroduction by man (Pedall et al., 2010). Moreover, a recent meta-analysis of archaeological and palaeontological records provides evidence that most Western Mediterranean populations of *T. hermanni* were driven extinct during a pronounced cold event of the last glacial (Heinrich event 4, approx. 39 500-38 000 years ago; Morales-Pérez and Sanchis-Serra, 2009), implying that, as in *E. orbicularis*, the tortoises were later reintroduced in Sardinia and Corsica.

The Mediterranean fauna has been heavily impacted and altered by humans for thousands of years, and this is especially true for Corsica and Sardinia (e.g., Corti et al., 1999a, 1999b; Masseti, 2009). We could imagine that turtles and tortoises, as 'living cans', were well-suited live provision for prehistoric settlers and seafarers and that some surplus individuals were abandoned after the arrival on the islands or even intentionally introduced there as a later food resource. A similar case was recently unravelled for Madagascar, where a continental African turtle species was introduced long ago (Vargas-Ramírez et al., 2010). More examples are expected to be discovered in future. The large-scale usage of chelonians as live provision is also well-known from sailors in the 17th to 19th Centuries. The reason making these animals so attractive for provision is that they could be stored aboard for long periods without needing to be fed or watered (Chambers, 2004). When it is considered that prehistoric societies in the Western Mediterranean appreciated chelonians as food (Cheylan, 1998, 2001; Stiner et al., 1999, 2000; Morales-Pérez and Sanchis-Serra, 2009), such an introduction scenario seems very likely to explain the occurrence of the four non-native chelonian species of Sardinia.

Populations of *T. graeca* are confined in Sardinia to the region of Oristano and the islet Mal di Ventre, about 8 km off the central west coast. Mal di Ventre is a small, uninhabited island that is separated by 17-20 m deep sea from Sardinia (Corti et al., 2007). According to this water depth (Antonioli et al., 2007), Mal di Ventre was connected to Sardinia until approximately 8000-7500 years ago, and it could be speculated that the tortoises should have been present both

on Sardinia and Mal di Ventre before the land connection was flooded by the raising Holocene sea level. However, it is known that the islet was repeatedly inhabited by man from prehistoric to medieval times (Casu, 2004) and it cannot be excluded that the tortoises were introduced later. For the region of Oristano, human occupation is known since prehistoric times (Webster, 1996) and one major Phoenician town, Tharros, is located within the extant range of *T. graeca*. Tharros was founded in the 7th Century BC (Pirazzoli, 2005). Together with our genetic data, this situation suggests that *T. graeca* was introduced in western Sardinia by prehistoric or early historic settlers, perhaps by Phoenicians coming from what is now Tunisia and neighbouring Algeria in North Africa.

Although we conclude that *T. graeca* was introduced in Sardinia long ago, we strongly disagree with Carpaneto (2006a) that the species should be eradicated there. Such action would be in conflict with the legal situation since *T. graeca* is protected by EC law (Council Regulation No 338/97 on the Protection of Species of Wild Fauna and Flora; Flora Fauna Habitat Directive: Appendix IV, Art. 12). According to recent genetic investigations and as outlined above, none of the four chelonian species of Sardinia is native and their occurrence on Sardinia resembles the situation of the mouflon (*Ovis orientalis*). This species was introduced in prehistoric times (Masseti, 1997, 2003) and is protected by the same EC regulations as *T. graeca*. Nobody would recommend eradication of the mouflon in Sardinia because it is not native there. All of these species, mouflon and chelonians, represent part of the rich natural heritage of the Western Mediterranean, resulting from the millennia-old interaction of man and nature, and deserve the same protection. Moreover, since all of the Sardinian tortoise species are not native, none endangers an autochthonous species by outcompeting. Rather, all Sardinian tortoises serve as a back-up for the mainland populations in Europe and Africa most of which are declining (IUCN, 2010), whereas the tortoise habitats in Sardinia are relatively secure due to traditional pasture farming (C. Corti, pers. observ.).

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