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Morphological and mitochondrial variation of spur-thighed tortoises, *Testudo graeca*, in Turkey

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Testudo graeca has a wide distribution under different geographic, climatic and ecological conditions, and shows high morphological differences especially in the Asian (Middle Eastern and Caucasian) parts of the range. This study investigates morphometric and genetic differentiation in the *T. graeca* complex in Turkey using the densest sampling to date. We sequenced two mt-DNA loci (ND4 and cyt *b*) of 199 samples and combined them with previously published data. Bayesian analysis yielded six well-supported clades, four of which occur in Turkey (*ibera*, *terrestris*, *armeniaca* and *buxtoni*). The *armeniaca* mtDNA clade locally represents a morphometrically distinct burrowing ecomorph. However, previous studies have shown that individuals outside Turkey possessing *armeniaca* mtDNA lack the distinctive *armeniaca* morphotype we observed, precluding taxonomic conclusions.

Key words: mtDNA, morphometry, Testudinidae, *Testudo*, Turkey

INTRODUCTION

Spur-thighed tortoises (*Testudo graeca* Linnaeus 1758) occur on three continents (Europe, Africa, and Asia). Such a wide distribution under different geographic, climatic, and ecological conditions causes high morphological differences in shell shape, coloration and pattern, most notable in the Asian (Middle Eastern and Caucasian) parts of the range (Parham et al., 2006; Fritz et al., 2007; Türkozan et al., 2010). Based on these morphological differences, nine Asian taxa were recognised (Guyot, 2004), whereas molecular studies revealed discordance between morphological variation, with six mitochondrial clades (Parham et al., 2006; Fritz et al., 2007). Subsequently, Mikulíček et al. (2013, using nuclear AFLP), recovered four geographically well-defined units, which combine one or more of the mtDNA clades, and Mashkaryan et al. (2013, using nuclear microsatellites) revealed weak differences and extensive gene flow between the six mtDNA clades. These conflicting findings reflect the different levels of variation inherent to each marker, and a complex evolutionary history likely involving both vicariance and gene flow.

In addition to conflicting genetic evidence, contrasting patterns of morphological variation are also reported. Perälä (2002) divided *T. graeca* populations along the Mediterranean (southern) coast of Turkey into several species or subspecies based on morphometric data. Türkozan et al. (2010) showed that the populations along the Mediterranean coast of Turkey are morphometrically homogenous, but that some inland Turkish populations are morphometrically distinct, reflecting some of the genetic clade assignments of Parham et al. (2006) and Fritz et al. (2007).

The current study expands on Türkozan et al. (2010) and provides the most comprehensive and geographically dense comparison of genetic and morphological variation for the *T. graeca* complex in the Middle East and Caucasus to date. The higher genetic coverage in the region where the deepest mitochondrial clades and most divergent phenotypes come into contact allows us to compare geographically referenced morphological data with genetic information without the a priori assumption of subspecies based on mtDNA clades sensu Fritz et al. (2007).

MATERIALS AND METHODS

Sampling and DNA extraction

A total of 199 tissue samples (nail clippings) were collected across Turkey between 2002 and 2006, and used for the analyses of two mtDNA loci (194 and 199 samples were used for the amplification of cytochrome b (CytB) and NADH dehydrogenase (ND4), respectively). We combined our data with previously published ND4 sequences from Parham et al. (2006), and previously published CytB sequences from Fritz et al. (2007) and Mashkaryan et al. (2013). Genbank accession numbers

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Table 1. Primers used in this study

ND4				
Primer	**Primers**	**PCR**	**Sequence**	**Tm (˚C)**
L-ND4-TG	5´ GTA GAG GCC CCA ATT GCA G 3´ (Parham et al., 2006)	✓	✓	64.5
H-Leu-TG	5´ TGT ACT TTT ACT TGG AAT TGC ACC A 3´ (Parham et al., 2006)	✓	✓	65.1
CytB				
mt-A1	5´ CCC CCT ACC AAC ATC TCA GCA TGA TGA AAC TTC G 3´ (Fritz et al., 2007)	✓	✓	70.7
mt-a-neu2	5´ CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC 3´ (Fritz et al., 2007)	✓	✓	72.7
mt-Fr	5´ CTA AGA AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA 3´ (Fritz et al., 2007)	✓	✓	67.0
mt-c2	5´ TGA GGA CAA ATA TCA TTC TGA GG 3´ (Fritz et al., 2007)		✓	61.7
mt-E-Rev	5´ GCA AAT AGG AAG TAT CAT TCT GG 3´ (Fritz et al., 2007)		✓	60.4

Table 2. Uncorrected p distances (percentages) between clades of *T. graeca*

	ibera	***terrestris***	***armenica***	***buxtoni***	***zarudnyi***
ibera	---				
terrestris	0.022	---			
armenica	0.048	0.044	---		
buxtoni	0.04	0.039	0.042	---	
zarudnyi	0.035	0.036	0.037	0.03	---
African	0.039	0.04	0.042	0.041	0.038

and sample locations are shown in Appendix 1. Total genomic DNA was extracted from ethanol preserved nails with the standard phenol-chloroform protocol (Sambrook et al., 1989). The primers used in this study are shown in Table 1.

PCR amplification was carried out using Taq DNA polymerase (Fermentas) and performed in a 50ul volume (1X Taq Buffer, 0.2mM dNTPs, 0.4mM each primer, 1.5mM $MgCl_2$, and 1ul Taq polymerase). The thermal profile for ND4 consisted of an initial 4 min denaturation step at 94 °C, 35 cycles of denaturation at 94 °C for 45 sec, annealing at 57 °C for 30 sec, and extension at 72 °C for 80 sec, with a final 10 min extension step at 72 °C. The thermal profile for CytB consisted of an initial 4 min denaturation step at 94 °C, 35 cycle of denaturation at 94 °C for 45 sec, annealing at 57 °C for 1 min, extension at 72 °C for 2 min, and a final 10 min extension step at 72 °C. PCR products were purified using the SIGMA GenElute PCR Clean-Up Kit. Each DNA fragment was sequenced in both directions, using the BigDye Terminator (Applied Biosystem) kit. Sequencing products were analysed on an AB3730xl automated DNA sequencer (Macrogen, Seoul, South Korea).

Data Analysis

Partial sequences of ND4 (849bp) and partial CytB (986 bp) mitochondrial DNA sequences were aligned using Clustal X (Thompson et al., 1997) with default parameters and ambiguous bases resolved by eye using BioEdit (Hall, 1999).

A Maximum likelihood analysis (ML) was performed using RAxML Version 8.1.11 (Stamatakis, 2014). The dataset was partitioned by codon position for protein coding regions and by whether a region consisted of protein coding sequences or not. Due to limitations of model selection in RAxML, all partitions were set to GTRGAMMA. Automatic settings were used and all ML analyses were run for 1000 bootstraps. Bayesian inference were conducted in Mr. Bayes 3.2.3 (Ronquist et al., 2012). The alignment was partitioned as in the RAxML analysis, and the partitions were model tested in jModeltest2 (Darriba et al., 2012, Guindon & Gascuel, 2003), using the best-fitting model under the Akaine Information Criterion (AIC, CytB: HKY + I + G for codon position 1, GTR + G for codon position 2, GTR + G for codon position 3, K80 for the tRNA partition; ND4: GTR for the first and third codon positions, HKY + I + G for the second position, and HKY + G for the tRNA partition). The models used for the concatenated analysis were the same, except that tRNA from both fragments was dealt with as a single partition with the model HKY + G. All Bayesian inference analyses were run with one cold chain and three hot chains for 10 million generations and sampled every 1000 generations with 25% of samples discarded as burn-in. Tracer 1.6 (Rambaut et al., 2014) was used to determine that the analysis had converged, and analyses were rerun with different number seeds for a total of four times to determine that the analysis converged on the same answer. All Maximum-likelihood and Bayesian inference analyses were conducted on the CIPRES Science Gateway (Miller et al., 2010). Intergroup and intragroup uncorrected genetic distances were calculated under default settings in MEGA7 (Kumar et al., 2016).

Population genetic statistics were calculated in DnaSP Version 5.10.1 (Librado and Rozas, 2009). Default parameters were used after manually setting the datasets to mitochondrial and haploid data, as well as annotating coding and noncoding codon positions and assigning codon positions to protein coding regions. CytB and ND4 regions were examined separately due to missing sites for some samples. In order to identify new haplotypes, similar haplotypes were collapsed in DnaSP. A list was

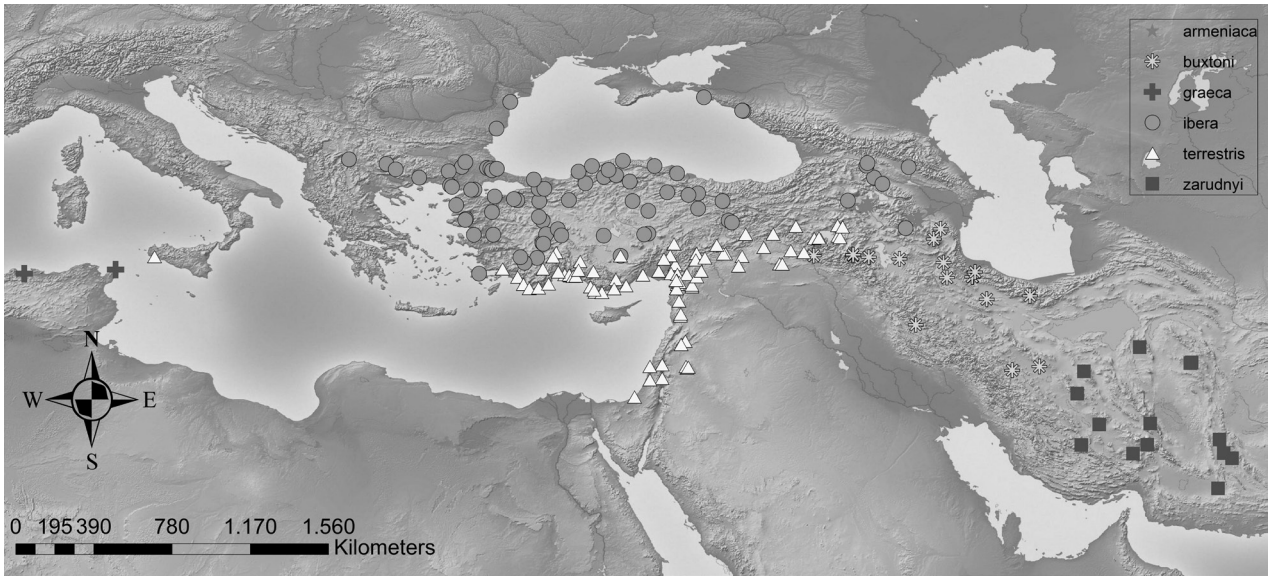

Figure 1. Map showing the localities of *T. graeca* samples used in this study with coloured symbols representing the different mitochondrial clades.

generated and manually checked for haplotypes that did not contain previous Genbank sequence. The CytB dataset was pruned of samples that did not meet a threshold of less than 5% missing data in the alignment. Intergroup and intragroup uncorrected genetic distances were calculated under default settings in MEGA7 version 7 (Kumar et al., 2016).

Morphology

For this study, specimens were classified into taxa (subspecies of Fritz et al., 2007) according to mitochondrial lineages determined in this study. The same data set from Türkozan et al. (2010) was used to test for morphological differences among molecular clades, only using samples collected at sites where mtDNA clades were identified. Of the 171 (81♂, 90♀) samples included in the analysis, 26 (15♂, 11♀) were *armeniaca*, 11 (3♂, 8♀) were *buxtoni* 80 (36♂, 44♀) were *ibera* and 54 (27♂, 27♀) were *terrestris*.

Body measurements were taken with either wooden (accuracy ± 1mm) or dial calipers (accuracy ± 0.02mm). All individuals were photographed, measured, and subsequently released. The morphometric measurements taken are the same as Türkozan et al. (2010): straight carapace length (SCL), from the outermost projection of the cervical scale to the outermost projection of the posteriors marginals; plastron length (PL), from the outermost projection of the gulars to the posterior end of the anals; median carapace width (CW), at the center of the carapace; maximum carapace width (MCW), at posterior marginals 7-9; carapace height (CH), the vertical measurement between the most dorsal point of carapace and the most ventral point of plastron; length of bridge (LB), between axillary and inguinal scales; maximum (not midline) gular scale length (MGSL); maximum (combined left plus right) gular scale width (MGSW); maximum humeral scale width (CHSW); maximum pectoral scale width (CPSW); maximum abdominal scale width (CAbSW); maximum femoral scale width (CFSW); maximum anal scale width (CSAW); gular suture length (GSL), Humeral suture length (HSL); pectoral suture length (PSL); abdominal suture length (AbSL); femoral suture length (FSL); anal suture length (ASL); nuchal length (NL), straight-line measurement from the anterior edge of cervical scale to posterior end; nuchal width (NW), the width of cervical at the posterior end; maximum width of first vertebral scale (VW1); maximum width of second vertebral scale (VW2); maximum width of third vertebral scale (VW3); maximum width of fourth vertebral scale (VW4); maximum width of fifth vertebral scale (VW5); maximum median length of first vertebral scale (VL1); maximum median length of second vertebral scale (VL2); maximum median length of third vertebral scale (VL3); maximum median length of fourth vertebral scale (VL4); maximum median length of fifth vertebral scale (VL5); maximum dorsal width of supracaudal scale (DSW); maximum ventral width of supracaudal (VSW); maximum median length of supracaudal length (SL); first pleural scale along length as the minimum straight line distance between the anteriormost and posteriormost contact points with adjacent (normally first and fifth) marginal scales (CL1); length of second pleural scale along the marginal (CL2); length of third pleural scale along the marginal (CL3); length of fourth pleural scale along the marginal (CL4); and maximum inner height of anterior shell opening parallel to median axis (IHASO).

We performed discriminant function analysis (DFA) with stepwise selection (*F* for entry: 3,84; for removal: 2,71) to obtain a subset of variables that provided best discrimination. Since the DFA is sensitive to number of samples only females were analyzed. Measurements used in DFA were standardised for CL. Furthermore, principal component analysis (PCA) were performed to raw measurements to further support the morphological differences among lineages. Significance levels for all tests were set at $p < 0.05$. All statistical analyses were performed using STATISTICA 7.0.

Table 3. Genetic diversity within *T. graeca*. n = number of sequences, S = number of polymorphic sites, k = average number of pairwise nucleotide differences, π = nucleotide diversity (Standard Deviation in parenthesis), h = number of haplotypes, Hd = haplotype diversity (Standard Deviation in parenthesis), - = test could not be run or statistic could not be calculated due to low sequence or haplotype number, NS = Not significant.

	Clade	N	s	k	π (SD)	h	Hd (SD)	Fu's F	Fu & Li's D	Fu & Li's F	Tajimas's D
CytB	CytB	299	192	21.683	0.0257 (0.0010)	74	0.935 (0.007)	-4.555	-4.6482 (P<0.02)	-3.3691 (P<0.02)	-1.1023 (P>0.10) NS
	African	1	-	-	-	1	-	-	-	-	-
	armeniaca	33	17	2.917	0.0031 (0.0004)	9	0.703(0.056)	-0.572	-2.4588 (0.10>P<0.05) NS	-2.34919 (0.10>P>0.05) NS	-1.0196 (P>0.10) NS
	buxtoni	22	70	14.416	0.0151 (0.0018)	13	0.931 (0.036)	0.899	-1.2321 (P>0.10) NS	-1.3874 (P>0.10) NS	-1.08001 (P>0.10) NS
	ibera	115	54	2.846	0.0030 (0.0005)	21	0.714 (0.035)	-6.849	-6.4613(P<0.02)	-5.6736 (P<0.02)	-2.3026 (P<0.01)
	terrestris	121	74	4.255	0.0049 (0.0004)	33	0.896(0.016)	-14.428	-4.1561 (P<0.02)	-3.9998 (P<0.02)	-2.2340 (P<0.01)
	zarudnyi	7	4	1.714	0.0016 (0.0004)	4	0.810 (0.130)	-0.428	-0.0686 (P>0.10) NS	0.00000 (P>0.10) NS	0.2390 (P>0.10) NS
ND4	ND4	229	109	12.011	0.0147(0.0008)	50	0.858 (0.019)	-5.155	-3.6783(P<0.02)	-2.9461(P<0.05)	-1.1299(P>0.10) NS
	African	2	10	10.000	0.0149 (0.0074)	2	1.000 (0.500)	-	-	-	-
	armeniaca	7	5	2.952	0.0035 (0.0007)	5	0.905 (0.103)	-0.737	0.5967 (P>0.10) NS	0.5479 (P>0.10) NS	0.1726 (P>0.10) NS
	buxtoni	18	12	5.124	0.0063 (0.0008)	4	0.725 (0.055)	5.316	0.7402 (P>0.10) NS	1.0485 (P>0.10) NS	1.32699 (P>0.10) NS
	ibera	94	23	0.793	0.0001 (0.0002)	12	0.311 (0.063)	-8.259	-5.3457 (P<0.02)	-5.0810 (P<0.02)	-2.4484 (P<0.01)
	terrestris	97	44	2.939	0.0035 (0.0004)	28	0.886 (0.018)	-16.284	-5.2281(P<0.02)	-4.7522 (P<0.02)	-2.0729 (P<0.05)
	zarudnyi	11	0	0.000	0.00000	1	0.000	-	-	-	-

Table 4. Classification matrix from discriminant function analysis for morphologic populations (rows: observed classifications, columns: predicted classifications) ARM: *armeniaca*, BUX: *buxtoni*, TER: *terrestris*, IBE: *ibera*

	Percent Correct	ARM	BUX	IBE	TER
ARM	100	11	0	0	0
BUX	0	0	0	5	3
IBE	76	0	1	29	8
TER	43	0	0	13	10
Total	62,5	11	1	47	21

RESULTS

All phylogenetic methods used in this study supported similar tree topologies with regard to the major clades, and therefore only the BI tree is shown. Bayesian analysis yielded six well supported clades (see Figure 1) corresponding to the mtDNA clades of Parham et al. (2006; Figs. 2-5). Of these clades, four occurred in Turkey (Fig. 1). The *ibera* and *terrestris* mtDNA clades are sister groups, and the *armeniaca* mtDNA clade is sister to the *graeca* mtDNA clade. The *buxtoni* mtDNA clade is sister to the *zarudyni* mtDNA clade. The *ibera* mtDNA clade includes tortoises from the Caucasus, Bulgaria, Greece, Republic of Macedonia, Romania, Russia, and Anatolia together with European Turkey. The *terrestris* mtDNA clade includes tortoises from southeastern Turkey, the Levant, Syria, Israel, and the Mediterranean coast of Turkey. The westernmost specimen of the *terrestris* mtDNA clade is from Kızılağaç village, Province Muğla, Turkey; the easternmost specimen is from Sağlarca village near Eruh. The northern distribution of the *terrestris* mtDNA clade is limited by the Taurus Mountain range. Seven samples from Karapınar (Meke Lake), central Anatolia, were assigned to two different mtDNA clades (*terrestris* and *ibera*). The *buxtoni* mtDNA clade includes tortoises from Şırnak, Turkey (westernmost point and 40 km away from the nearest *terrestris* record) and western Iran. The *zarudyni* mtDNA clade includes tortoises only from east Iran. The *armeniaca* mtDNA clade contained individuals from eastern Turkey, Armenia, Nagorno Karabag and Azerbaijan. The *greaca* mtDNA clade includes tortoises from Algeria and Tunisia. The uncorrected distances among clades ranged from 0.022 (*ibera-terrestris*) to 0.048 (*ibera-armeniaca*) (Table 2). The intra clade variation was between 0.0006 (*zarudyni*) and 0.015 (African).

Of the 50 ND4 haplotypes found, 34 were previously unsampled. Twenty-one of those haplotypes were assigned to the *terrestris* mtDNA clade, nine were assigned to the *ibera* mtDNA clade, and two were assigned to the *buxtoni*, and *armeniaca* mtDNA clades each. For CytB, after thirty-six sequences from Genbank were removed for missing more than 5% of the sequence used in the alignment (See Online appendices, Appendix 2), the alignment consisted of 299 samples with 1059 sites, 1042 which were protein coding and the rest consisted of non-coding flanking tRNA. Of the 74 CytB haplotypes found, 32 were previously unrecorded. Sixteen of those haplotypes were assigned to the *terrestris* mtDNA clade, eight were assigned to the *ibera* mtDNA clade, and eight were assigned to the *buxtoni* mtDNA clade. Low nucleotide diversity, high haplotype diversity, negative Tajima'D and Fu's F suggest a recent population expansion (Table 3).

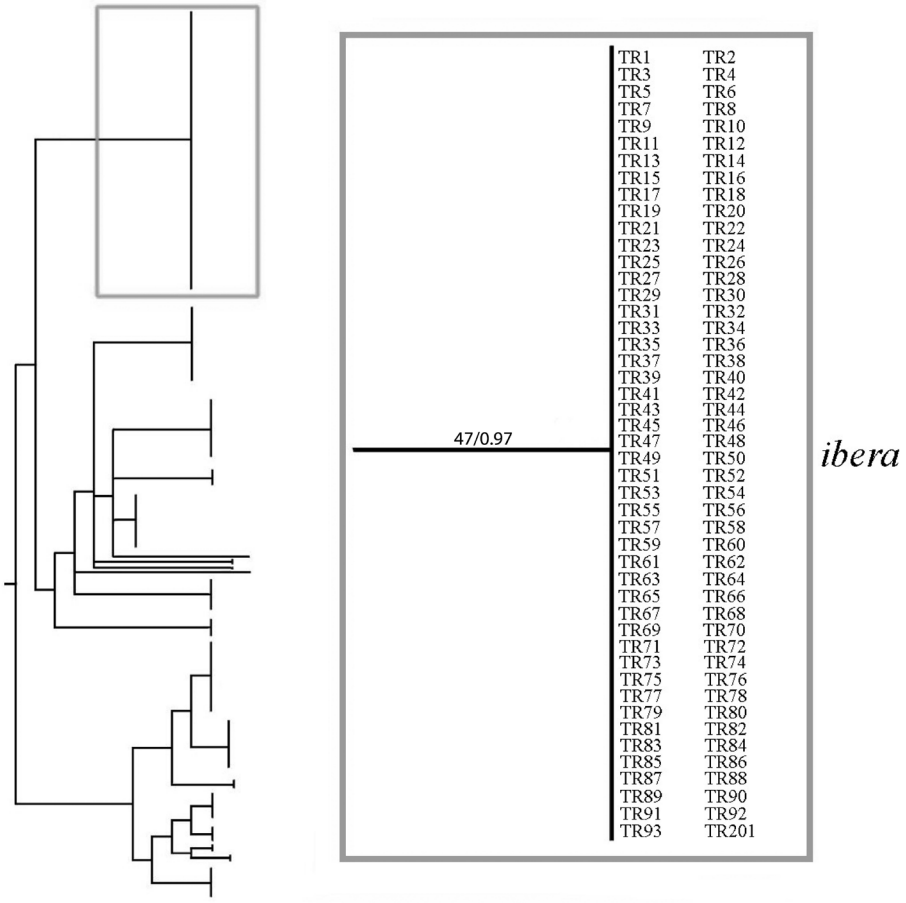

Figure 2. Extended Bayesian tree of samples of the *ibera* mtDNA clade of *T. graeca* used in this study. The overall mitochondrial tree showing location of each clade is shown on the left.

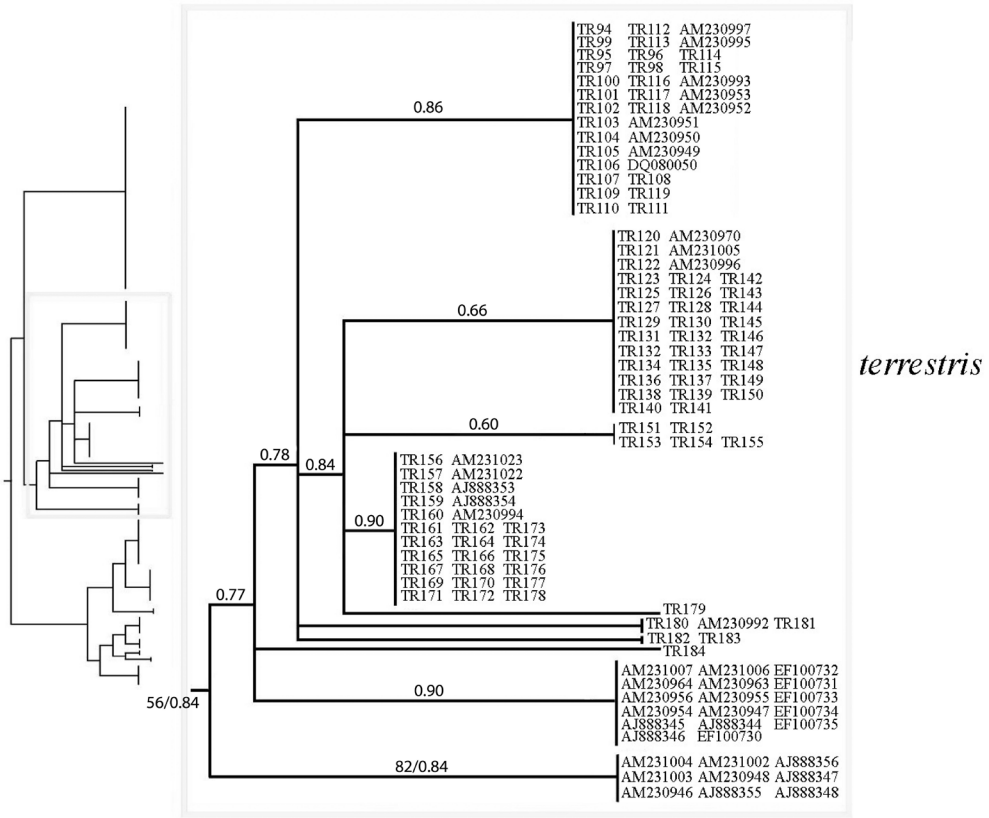

Figure 3. Extended Bayesian tree of the *terrestris* mtDNA clade of *T. graeca* used in this study. The overall mitochondrial tree showing location of each clade is shown on the left.

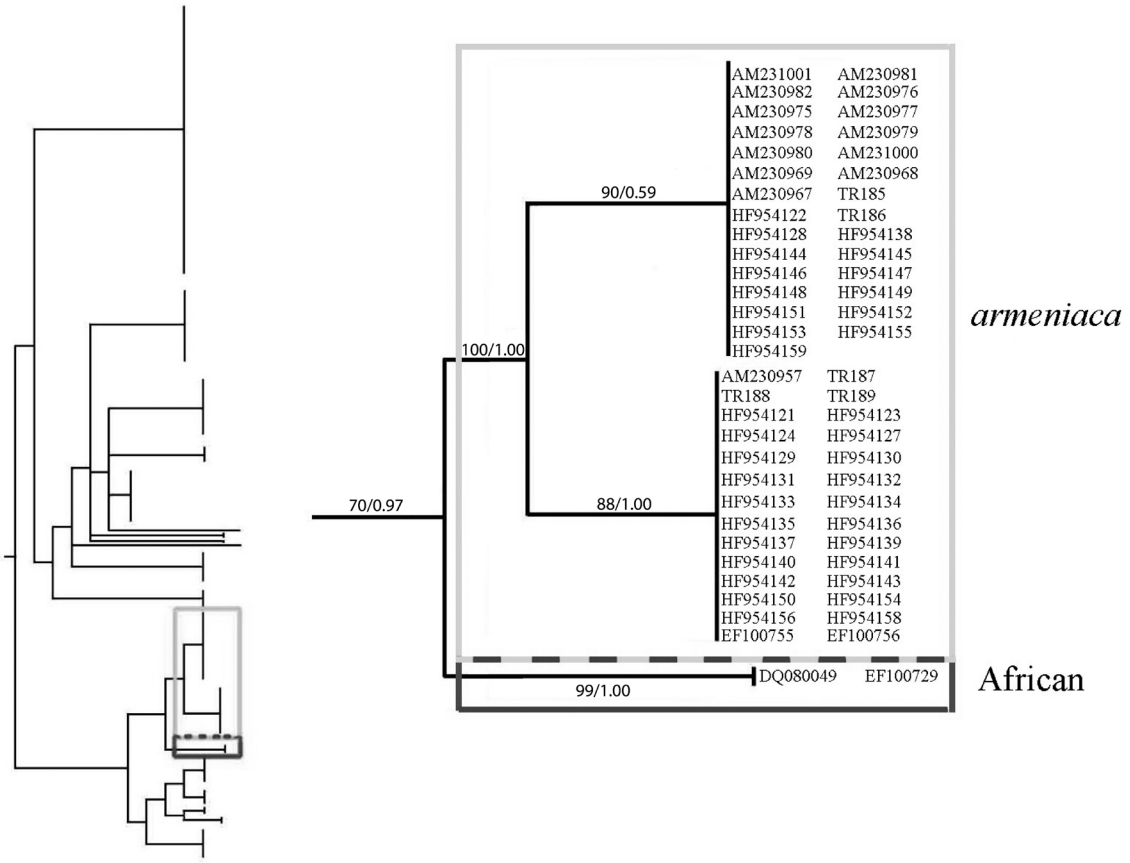

Figure 4. Extended Bayesian tree of the *armeniaca* mtDNA clade of *T. graeca* used in this study. The overall mitochondrial tree showing location of each clade is shown on the left.

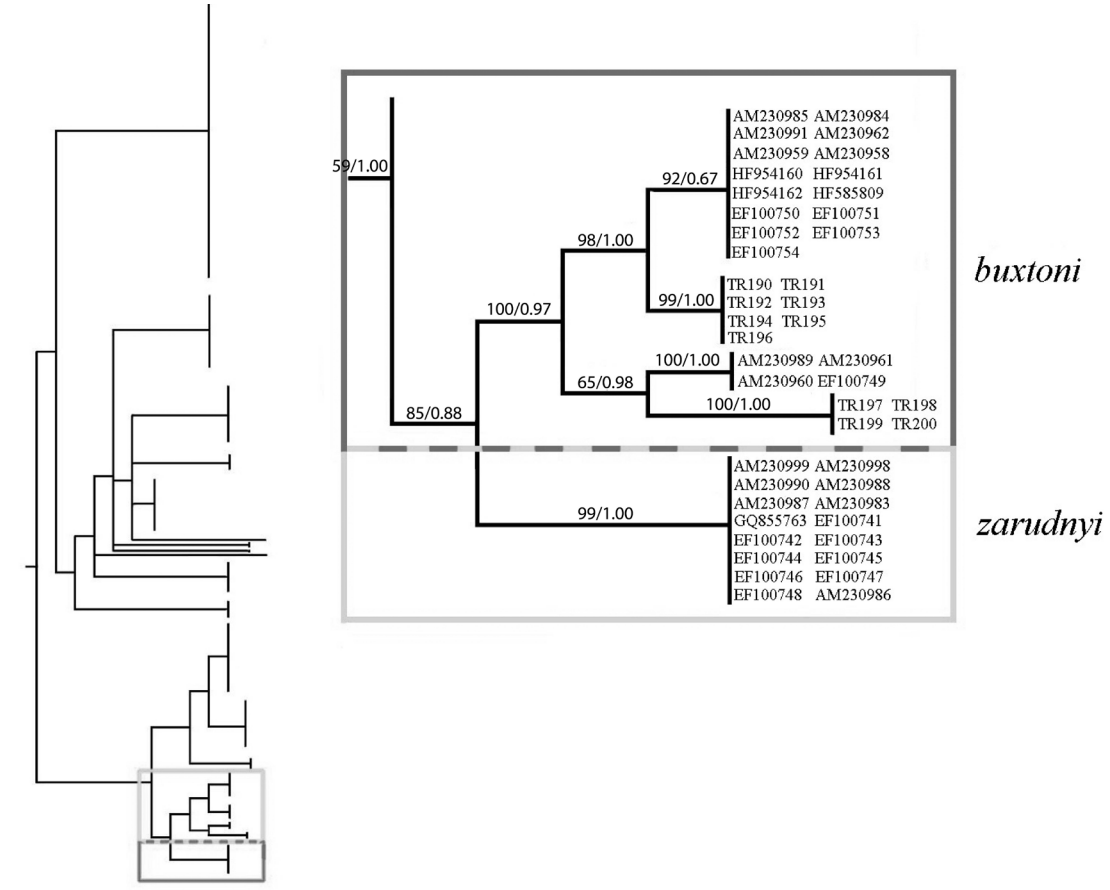

Figure 5. Extended Bayesian tree of the *buxtoni* mtDNA and the *zarudyni* mtDNA clade of *T. graeca* used in this study. The overall mitochondrial tree showing location of each clade is shown on the left.

Table 5. Principal component loadings of morphometric characters.

Variable	Factor 1	Factor 2	Factor 3
CL	-0,98527	0,056033	0,005615
PL	-0,98558	0,071031	-0,009379
CW	-0,98154	0,067987	-0,061550
MCW	-0,94933	0,040551	-0,063126
CH	-0,97242	0,066687	0,100431
LB	-0,92397	-0,429031	0,059865
MGSL	-0,89300	0,026598	-0,300681
CGSW	-0,87002	0,082015	0,300361
CHSW	-0,91160	-0,424941	-0,093412
CPSW	-0,95597	0,134011	-0,161781
CabSW	-0,94377	0,107487	-0,228006
CFSW	-0,96614	0,035017	-0,036663
CASW	-0,94322	0,090684	-0,067086
GSL	-0,81353	-0,001996	-0,386924
HSL	-0,72475	0,064444	0,535875
PSL	-0,16531	0,980750	-0,096608
AbSL	-0,92265	-0,333960	-0,036133
FSL	-0,71413	0,253066	0,386709
ASL	-0,75506	-0,213520	-0,421773
NL	-0,84634	-0,044661	0,150913
NW	-0,42599	-0,228330	0,311541
VW1	-0,86649	-0,050379	0,190464
VW2	-0,92513	-0,020171	0,194272
VW3	-0,96004	-0,009998	0,147988
VW4	-0,97307	0,006054	0,044585
VW5	-0,88770	0,115711	-0,207919
VL1	-0,95869	0,034584	0,085344
VL2	-0,96272	-0,019458	0,117758
VL3	-0,95731	0,026555	0,196068
VL4	-0,92217	-0,003989	0,090103
VL5	-0,93449	0,067368	0,019106
DSW	-0,79902	-0,021963	-0,325218
VSW	-0,91959	0,075979	-0,202497
SL	-0,91183	0,121743	0,013802
CL1	-0,96673	0,028291	-0,065187
CL2	-0,95450	-0,022425	-0,078485
CL3	-0,95929	-0,015095	0,007065
CL4	-0,90012	-0,016643	0,197550
IHASO	-0,91842	-0,015981	-0,166726

Morphology

Three discriminant functions were produced, and only the first two made significant contributions to distinguishing morphologically based taxa ($p < 0.001$; CGSW contributes most, followed by HSL, CPSW and GSL). Plastral measurements are major variables that allow us to discriminate between tortoise clades. The first discriminant function (DF) accounted for 94% of between-group variability. The first DF is weighted most heavily by CGSW and CPSW. The second function is shaped by CGSW and to a lesser extent HSL and GSL. The first and second DF discriminate mostly between *armeniaca* and the other clades (Fig. 6). Overall, the DFA resulted in 62.5% of classification corresponding to their clade (Table 4). Out of eight *buxtoni* samples, five classified as *ibera* and three as *terrestris*, while of the 38 *ibera* eight were classified as *terrestris*, one as *buxtoni*, and the rest as *ibera* (see Table 4).

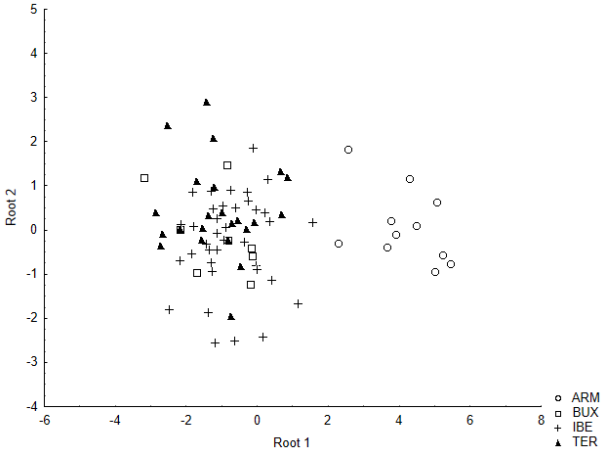

Figure 6. Specimens of females from the *armeniaca*, *buxtoni*, *ibera*, and *terrestris* mtDNA clades plotted in canonical variate space number of variables in the model: 4. Wilk's λ = 0.16175, F(12.148) = 12.257, P < 0.00001

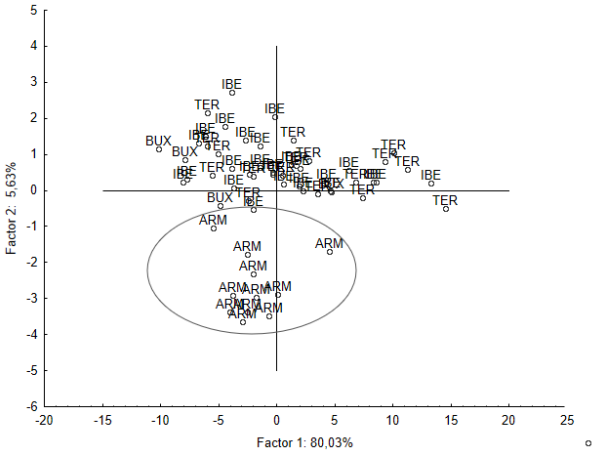

Figure 7. Scattered diagram of PCA along PC1 and PC2 based on 39 morphometric characters (names of clades abbreviated as in Tables 3 and 4)

The PCA results further support the morphological difference of *armeniaca* (80.3%) from other lineages (Fig. 7). The factor loadings of each variable are presented in Table 5.

DISCUSSION

The mitochondrial distance was highest between *ibera* and *armeniaca*, which are syntopic in Caucasus where they hybridise with each other (Mashkaryan et al., 2013). Nuclear AFLP data, on the other hand, found the highest genetic differentiation between the western Mediterranean (north Africa) and central-eastern Iranian clusters (*zarudnyi* mtDNA clade, Mikulíček et al., 2013), a difference likely linked to different evolutionary history of genetic markers and extensive gene flow among mtDNA clades.

Compared to the other mtDNA clades, *ibera* has a

range wide distribution from west to east, whereas the *terrestris* mtDNA follows the Taurus range across Turkey (except one locality out of this range where *terrestris* and *ibera* are syntopic). This clade comes into close contact with the *buxtoni* mtDNA clade at the Anatolian Diagonal in the east, one of the most effective physical and ecological barriers for many species (reviewed in Gür, 2016). The distribution of the *buxtoni* mt clade is limited by the Zagros Mountain forest steppe, one of the five ecoregions in the Irano-Anatolian hotspots. The *armeniaca* mtDNA clade in Turkey is limited to lowlands of the Araxes Valley characterised by Mediterranean climate. However, syntopic occurrences of *ibera* and *terrestris* (this study), *ibera* and *armeniaca* (Mashkaryan et al., 2013), and *armeniaca* and *buxtoni* (Javanbakht et al., 2017) combined extensive gene flow among mtDNA clades (Mashkaryan et al., 2013; Mikulíček et al., 2013), and suggests the existence of hybrid zones and parapatric distributions. Fritz et al. (2007) found the highest genetic diversity in Transcaucasia, which is considered to be a radiation center of *Testudo*, while the present study shows that overall genetic diversity is higher in Levantine and western Iran (Table 3).

Türkozan et al. (2010) previously reported the distinctiveness of three mt clades based on only morphology, namely *ibera*, *buxtoni* (i.e., perses), and *armeniaca* in Turkey. However, they expressed their results with caution due to a lack of genetic sampling within Turkey and morphometric studies outside of Turkey. In this current study, the only morphometrically distinct mtDNA clade is *armeniaca*, which is represented a highly specialised burrowing ecomorph in Turkey and parts of Armenia. The recovery of a distinct morphology for turtles assigned to the *armeniaca* mtDNA clade, one of the most divergent mtDNA lineages of the *T. gracea* complex in Asia, has raised the possibility of a distinct species based on Turkish samples alone (Türkozan et al., 2010). Other studies (Parham, et al., 2006; Mashkaryan et al., 2013) show that the *armeniaca* mtDNA haplotype extends beyond the geographic range of the morphologically specialised *armeniaca* morphotype, to the western Caspian Sea region where they lack the burrowing ecotype. For the remainder of the mtDNA clades, it appears that morphology cannot discriminate them from each other (Parham et al., 2006; Fritz et al., 2007, Türkozan et al., 2010). We therefore recommend that the subspecies status of *Testudo* clades in west Asia should not be used, given that they are based conflicting evidence derived from mtDNA differentiation alone (Mashkaryan et al, 2013; Mikulíček et al., 2013).

There is a clear evidence that the ranges of *T. graeca* in Asia have been contracting and expanding through time. Similarly, Anadon et al. (2015) found clear niche differences among five subspecies of *T. graeca* in Africa, with rainfall playing a primary role in shaping the distribution of clades, as in Iran and Transcaucasia (Javanbakht et al., 2017). The presence of glacial refugia (Atalay, 2002), refugia within refugia (Schmitt, 2007), wide elevational range (0-5137 m), and various macro and microclimates and vegetation types (Atalay, 2002) have impacted on the distribution of *T. graeca* complex in Turkey. The extent to which these factors resulted in the currently observed genetic clades, however, remains unresolved. Future studies should focus on geographically dense genomic-scale markers such as RADSeq in combination with phenotypic data.

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