



Reconstructions of the past distribution of *Testudo graeca* mitochondrial lineages in the Middle East and Transcaucasia support multiple refugia since the Last Glacial Maximum

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A cycle of glacial and interglacial periods in the Quaternary caused species' ranges to expand and contract in response to climatic and environmental changes. During interglacial periods, many species expanded their distribution ranges from refugia into higher elevations and latitudes. In the present work, we projected the responses of the five lineages of *Testudo graeca* in the Middle East and Transcaucasia as the climate shifted from the Last Glacial Maximum (LGM, Mid – Holocene), to the present. Under the past LGM and Mid-Holocene bioclimatic conditions, models predicted relatively more suitable habitats for some of the lineages. The most significant bioclimatic variables in predicting the present and past potential distribution of clades are the precipitation of the warmest quarter for *T. g. armeniaca* (95.8 %), precipitation seasonality for *T. g. buxtoni* (85.0 %), minimum temperature of the coldest month for *T. g. ibera* (75.4 %), precipitation of the coldest quarter for *T. g. terrestris* (34.1 %), and the mean temperature of the driest quarter for *T. g. zarudnyi* (88.8 %). Since the LGM, we hypothesise that the ranges of lineages have either expanded (*T. g. ibera*), contracted (*T. g. zarudnyi*) or remained stable (*T. g. terrestris*), and for other two taxa (*T. g. armeniaca* and *T. g. buxtoni*) the pattern remains unclear. Our analysis predicts multiple refugia for *Testudo* during the LGM and supports previous hypotheses about high lineage richness in Anatolia resulting from secondary contact.

Keywords: *Testudo graeca*, niche modeling, Last Glacial Maximum, Middle East, Transcaucasia

INTRODUCTION

The spur-thighed tortoise, *Testudo graeca* complex (sensu Parham et al., 2006), has a wide distribution (Fig. 1) across a highly diverse landscape (e.g. from semi deserts, grasslands, traditional agricultural areas, shrublands and mixed forest). Previous studies have shown that, across this broad range, Pleistocene climate fluctuations and ecological conditions created profound morphological differences, especially in the Asian (Middle Eastern and Caucasian) parts of the range (Turkozan et al., 2010, 2018). Molecular studies revealed discordance between morphological variation and six mitochondrial clades (Parham et al., 2006; Fritz et al., 2007; Turkozan et al., 2010; Mikulíček et al., 2013; Turkozan et al., 2018). The matter is complicated by the fact that specimens assigned to some mitochondrial DNA (mt hereafter) clades are morphologically distinct within some areas and not others (e.g., the mt clade that corresponds to “*T. g. armeniaca*” includes individuals that are phenotypically highly specialised only in Anatolia) and also that some mt lineages (e.g. those corresponding to “*T. g. zarudnyi*” of eastern Iran) require additional phenotypic study.

Following Turkozan et al. (2010, 2018), we do not subscribe to the mitochondrial subspecies of Fritz et al. (2007). Still, for comparability purposes, we do continue to refer to the mt clades that are associated with the different mt lineages by their subspecific epithet (e.g. *armeniaca*, *buxtoni*). Meanwhile, one of the few analyses of nuclear markers (Mikulíček et al., 2013) shows some concordance with the mtDNA clades, suggesting that the mt lineages represent actual evolutionary lineages. Despite these unresolved taxonomic issues, comparisons of genetic variation and the application of ecological niche modeling to mitochondrial lineages have helped develop hypotheses about the paleobiogeographic history of the *T. graeca* complex in different parts of its range (Anadón et al., 2015; Graciá et al., 2017; Javanbakht et al., 2017).

There is clear evidence that the western range of the *T. graeca* complex has been contracting and expanding through time, driven by climate with the modifying effect of lithology and topography (Anadón et al., 2007; Graciá et al., 2017). Anadón et al. (2015) found apparent niche differences among five mitochondrial lineages of *T. graeca* in Africa (sublineages of the *graeca* mt clade in our terminology), with rainfall playing a primary role in

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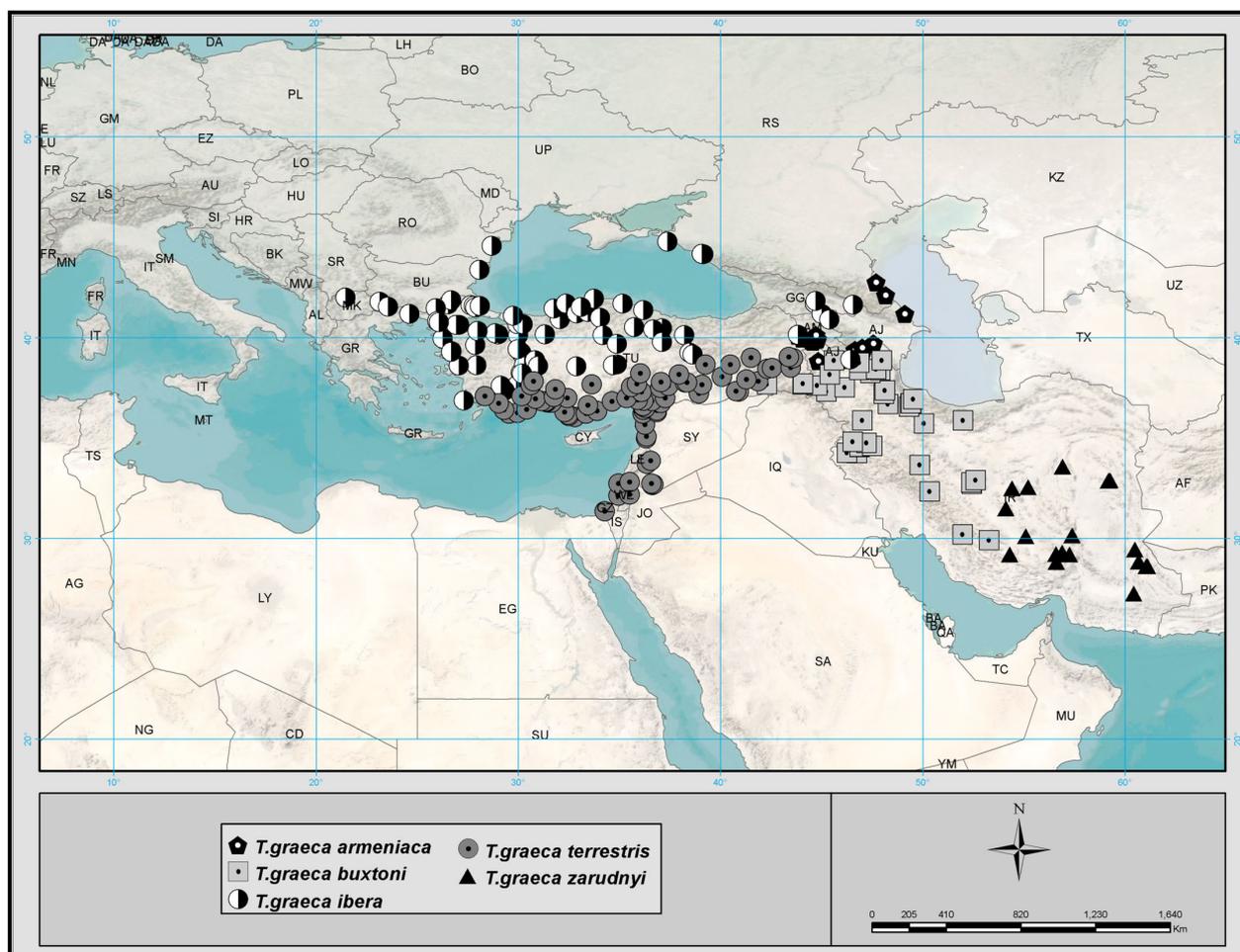


Figure 1. Occurrence records used for modeling *T. graeca* mt lineages in Maxent. Data were compiled from previously published papers (Parham et al., 2006; Fritz et al., 2007; Parham et al., 2012; Mashkaryan et al., 2013; Javanbakht et al., 2017; Türkozan et al., 2018)

shaping their distribution. In contrast, a comparison of the current distribution patterns and the reconstructed historical ranges of *T. graeca* hypothesised that the distribution ranges of the three mitochondrial lineages in Iran and Transcaucasia had not changed substantially since the Last Glacial Maximum (LGM; Javanbakht et al., 2017). In this work, we used distribution records of previous studies and our records to reassess past range dynamics in the eastern part of the range of the *T. graeca* complex (Middle East, Anatolia, and Transcaucasia). Our study differs from previous studies by including a large missing distribution of *T. graeca* from Anatolia.

MATERIAL & METHODS

We used the maximum entropy machine-learning algorithm Maxent version 3.4.1 (Phillips et al., 2006; Phillips & Dudík, 2008; Elith et al., 2011) to predict the geographically suitable habitats for *T. graeca* mt lineages in the present, LGM and Mid-Holocene. All GIS operations were conducted using ArcGIS 10.6 (www.arcgis.com) and SDMtoolbox 2.4 (Brown, 2014). Presence data for *T. graeca* lineages were compiled from previously published papers (Parham et al., 2006; Fritz et al., 2007; Parham et al., 2012; Mashkaryan et al., 2013; Javanbakht et al., 2017; Türkozan et al., 2018). However, we omitted

Table 1. Bioclimatic variables, bold variables are the subset used in ENMs

Bioclimatic Variable	Description
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

the record of *T. terrestris* from Sicily in our analyses since this population has been introduced (Fritz et al., 2007). A total of 247 occurrence records were used for modeling across the distribution range of each mt clade (85 *ibera*; 41 *buxtoni*; 81 *terrestris*; 22 *armeniaca*; 18 *zarudnyi*). The genetic assignment of each sample was confirmed by Turkozan et al. (2018). Data consisting of 19 bioclimatic variables for present (1950-2000), LGM (~22000 years ago) and Mid-Holocene (~6000 years ago) were downloaded from WorldClim database (Hijmans et al., 2005; www.worldclim.org) at a resolution of 2.5 arc minutes (approx. 4.5 km at the equator). The 19 bioclimatic variables derived from monthly temperature and precipitation values (for detailed information, see www.worldclim.org/bioclim). To identify and remove highly correlated variables, the Remove Highly Correlated Variables option in SDMtoolbox version 2.4 was used (Brown, 2014), and a maximum 0.8 correlation coefficient was allowed (Feldman et al., 2017). We used the subset of bioclimatic variables in ENMs (Table 1). We used the permutation importance to evaluate the relative contributions of bioclimatic variables.

To predict the suitable distribution habitats of lineages during the LGM and Mid-Holocene, CCSM4, MIROC-ESM, and MPI-ESM-P general circulation models (GCM) (see WorldClim database for further information) were used. The logistic values of three different GCM simulations were averaged to summarise predictions for the past. All bioclimatic variables were masked to cover the range of distribution of the species (as in Soberón & Peterson, 2005) for ENM.

As described before (Merow et al., 2013), MaxEnt contrasts presence data against background data where presence and absence are not measured. The issues related to MaxEnt background selection during model application are described before (Brown et al., 2016; Elith et al., 2011; Merow et al., 2013). To reduce the issues related to background selection, Minimum Convex Polygons (MCP) with presence data were created for background selection for each *T. graeca* lineage (by Background Selection via Bias File option in SDMtoolbox). The home range of *T. graeca* is 3 to 10 hectares (Cobo & Andreu, 1998; Attum et al., 2011; Anadón et al., 2012) and so a 1 km buffer distance was selected for MCPs.

Identifying the optimum model parameters for model performance of MaxEnt (Elith et al., 2011; Merow et al., 2013) was conducted by spatial jackknifing (k-fold cross-validation, k = 3) in SDMtoolbox. Multiple models with different feature class (FC) combinations (1=linear; 2=linear, quadratic; 3=hinge; 4=linear, quadratic, and hinge; 5=linear, quadratic, hinge, product, threshold) and regularisation multipliers (RM) (from 0.5 to 5 with 0.5 increments) were tested. The “minimum training presence” threshold was used during analysis. The best model criteria were the Omission error rate, then AUC in Spatial Jackknifing. After optimising Maxent models for each lineage, final models with optimised parameters were created. To quantify the niche overlap of *T. graeca* mt lineages, ENMTools 1.4.4 (Warren et al., 2010) software was used. The output ascii files of ENMs were used for niche overlap analysis. Niche overlap in

ENMTools is calculated via Schoener's D (D); ranging from 0 (no similarity) to 1 (complete overlap) (Warren et al., 2008). An identity test was also performed, which is an indicator that the ENMs of the two species are more different from expected by chance. Identity test results show the degree of niche overlap when samples are from the same distribution. Comparisons of the D values of identity tests with the D values of Niche overlap analysis of actual data demonstrate whether populations are different. The accuracy of the ENM's was performed by Area Under the Curve (AUC) of the Receiver Operating characteristic Curve (ROC) (Fielding & Bell, 1997). An AUC > 0.5 indicates that the model performs better than a random prediction (Gassó et al., 2012). AUC is a reliable assessment method because it is not affected by choice of threshold (Fois et al., 2018; Yi et al., 2016).

RESULTS

The best model parameters determined by Spatial Jackknifing for each lineage were as follows; *armeniaca* FC=linear – RM=3.5, *buxtoni* FC=linear - RM=5, *ibera* FC=linear – RM=4, *terrestris* FC=linear, quadratic – RM=5, *zarudnyi* FC=linear – RM=5. According to Area Under the Curve (AUC) of the Receiver Operating characteristic Curve (ROC) values of ENMs, all models performed better than a random prediction (*armeniaca*: 0.83, *buxtoni*: 0.65, *ibera*: 0.73, *terrestris*: 0.73, *zarudnyi*: 0.75). The most significant bioclimatic variables in limiting the potential distribution of clades are Precipitation of Warmest Quarter (BIO 18) for *armeniaca* (95.8 %), Precipitation Seasonality (Coefficient of Variation) (BIO 15) for *buxtoni* (85 %), Min Temperature of Coldest Month (BIO 6) for *ibera* (75.4 %), Precipitation of Coldest Quarter (BIO 19) for *terrestris* (34.1 %) and Mean Temperature of Driest Quarter (BIO9) for *zarudnyi* (88.8 %). The predictions for the reconstructed past (mid-Holocene and LGM) and present bioclimatic conditions (Fig. 2) suggested that the *armeniaca* clade survived in a potential refugium in the Caucasus and Central Anatolia and expanded its range from this refugium. Its ultimate possible range included north-eastern Anatolia and some parts of the Black Sea region during the mid-Holocene before the range retracted its distribution to Caucasus again as at present. The model also suggests additional suitable areas in central and northeastern Anatolia where these areas are currently occupied by *ibera* clade. In contrast, *buxtoni* may have survived the LGM in the Zagros, Caucasus, Elburz Mountains, Anatolia, and coastal parts of Greece and Bulgaria, but after the LGM, suitable areas included almost all of Anatolia, Greece, part of Macedonia and Albania during the mid-Holocene and contracted its range to south-eastern Anatolia and the Zagros mountains. The model suggests additional suitable areas in north-eastern Turkey and central Anatolia, European Turkey, the coastal belt of Greece, and some parts of Macedonia and Montenegro. Exceptionally, the potentially suitable areas for *ibera* during the LGM include the coastal belt of central and eastern Black Sea coast of Anatolia, some parts of the Levant, Northern Cyprus, Aegean Islands including Crete, and Greece. After the LGM, during the mid-Holocene,

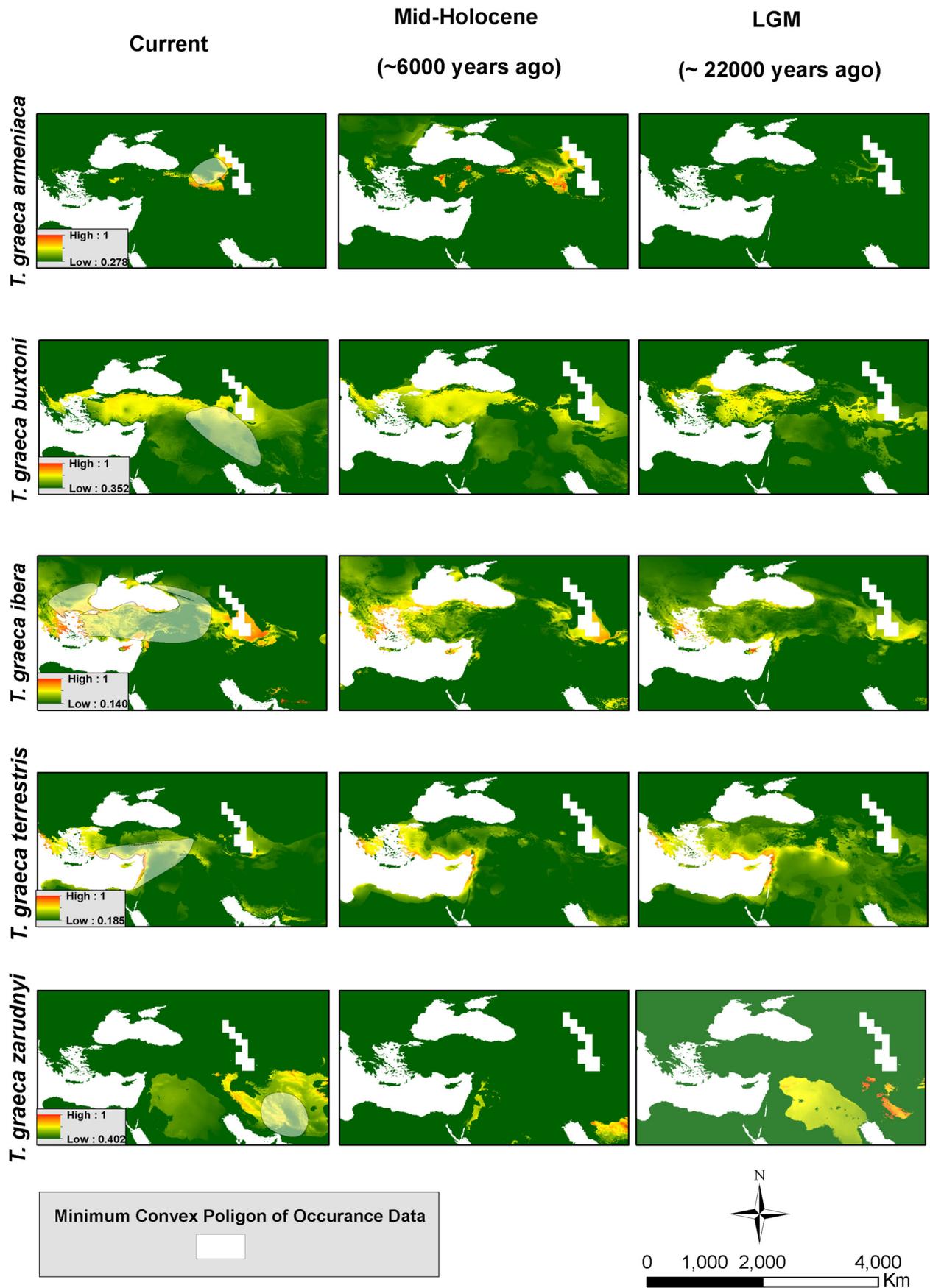


Table 2. Predicted habitat suitability maps of *T. graeca* lineages for Current, Mid- Holocene, and LGM

Table 2. Permutation contribution percentage of biological variables to the distribution of *T. graeca* mt lineages.

Variable	Permutation Contribution (%)				
	<i>ibera</i>	<i>terrestris</i>	<i>buxtoni</i>	<i>armeniaca</i>	<i>zarudnyi</i>
BIO2	-	18.5	-	-	-
BIO3	0.4	-	-	-	-
BIO4	-	-	-	4.2	-
BIO5	-	-	-	-	11.2
BIO6	75.4	-	-	-	-
BIO8	9.5	2.7	-	-	-
BIO9	-	29.6	15.0	-	88.8
BIO15	1.7	-	85.0	-	-
BIO18	-	12.2	-	95.8	-
BIO19	13.0	34.1	-	-	-

Table 3. Schoener's D values of niche overlap scores.

Lineages	<i>armeniaca</i>	<i>ibera</i>	<i>terrestris</i>	<i>zarudnyi</i>	<i>buxtoni</i>
<i>armeniaca</i>	1.0000	0.2529	0.0882	0.0580	0.1618
<i>ibera</i>	x	1.0000	0.3138	0.0874	0.3115
<i>terrestris</i>	x	x	1.0000	0.0515	0.3473
<i>zarudnyi</i>	x	x	x	1.0000	0.2712
<i>buxtoni</i>	x	x	x	x	1.0000

Table 4. Minimum and maximum values of Schoener's D scores of identity tests.

Lineages	<i>armeniaca</i>	<i>ibera</i>	<i>terrestris</i>	<i>zarudnyi</i>	<i>buxtoni</i>
<i>armeniaca</i>	1.0000	0.5666 – 0.7842	0.5460 – 0.7870	0.4669 – 0.7457	0.5460 – 0.7870
<i>ibera</i>	x	1.0000	0.6639 – 0.8397	0.4309 – 0.7748	0.6531 – 0.8341
<i>terrestris</i>	x	x	1.0000	0.3839 – 0.7661	0.6120 – 0.8220
<i>zarudnyi</i>	x	x	x	1.0000	0.5379 – 0.7908
<i>buxtoni</i>	x	x	x	x	1.0000

suitable areas included the Caucasus, Aegean coasts of Anatolia, southern Cyprus, and the Balkans. The current distribution of *ibera* includes most of the suitable mid-Holocene range except the north-eastern Black Sea coast and Cyprus where no *Testudo* currently occur. The model suggests additional appropriate areas along the coastal belt of eastern Mediterranean and Levant where *terrestris* occurs presently. The models indicate that the *terrestris* clade may have survived along the Mediterranean coastal belt of Anatolia, including the Levant region and island of Cyprus, western coast of Greece, and Albania. The hypothesised suitable areas remained stable during the mid-Holocene and then retracted. The model suggests additional appropriate areas on the west coast of Anatolia, the west coast of Greece and Albania, Caucasus, and the island of Cyprus. The *zarudnyi* clade may have survived in the Zagros Mountains and north-west Syria. The hypothesised suitable range contracted to the southern Zagros Mountains and the Dasht-e Lut during the mid-Holocene and remained in that region until recently. The model suggests additional appropriate areas at Kopet

Mountains and Afghanistan where they do not occur.

Schoener's D Values for niche overlap scores between lineages are given in Table 3. Niche overlap scores appear to be compatible with the known distribution of species. The highest overlap values ($D > 0.3$) were observed between the pairs of *ibera* – *terrestris*, *ibera* – *buxtoni*, and *terrestris* – *buxtoni* clades. The lowest overlap score was 0.08 between *armeniaca* and *terrestris* clades. Clearly, *zarudnyi* occupy a differentiated niche, among others (Table 3).

Minimum and maximum Schoener's D values of 100 replicates of identity test for *T. graeca* mt lineages are given in Table 4. The results indicate that ENMs of the species are more different than expected by chance because, for any pair of species, the Schoener's D values are higher than niche overlap values (Schoener's D value of Niche overlap analysis for *ibera-terrestris* pair is 0.3138 and Schoener's D value of identity test for the same pair is between 0.6639 and 0.8397). The potential distributions of clades at present is generally in line with the known distribution ranges of the clades.

DISCUSSION

The ecological niches of mt clades were similar, with temperature extremes and precipitation related variables playing the most crucial role in determining suitable habitats (Table 2). Similar results were presented for the western range of *T. graeca* lineages in Africa (Anadón et al., 2015). In west Asia, Javanbakht et al., (2017) found that *armeniaca*, *buxtoni*, and *zarudyni* mt clades did not significantly expand their distribution after the LGM, with precipitation delimiting the distribution. However, it seems that the authors erroneously interpreted their results by not understanding that in principal component analyses, both negative and positive principal components loadings have the same power, and the negative value shows only the direction of the relationship. As shown in Javanbakht et al. (2017: Table 3), temperature related factors (maximum temperature of warmest month BIO 5 and mean temperature of the coldest quarter BIO 10) were more responsible for delimiting the distribution of the lineages than precipitation. Therefore, the combination of temperature extremes and precipitation appears to shape the distribution pattern of *T. graeca*. However, it is not the only factor shaping the distribution of the species but also the biotic factors, accessibility of the region, and evolutionary capacity of the population to adapt to new conditions (Soberón & Peterson, 2005). This explains why some clades are missing from suitable niches during the mid-Holocene and the present time. Extensive deserts in Iran with arid climate (Javanbakht et al., 2017) and the Anatolian Diagonal with a steep environmental gradient associated with temperature seasonality (Gür, 2016) seem to be shaping the distribution of *T. graeca* in the east and mainland Anatolia. The range of *T. graeca* covers areas with rainfall values of 800-1200 mm (north-western Africa; Anadón et al., 2015) to arid conditions in semideserts in Iran, which is a sign of ecological plasticity (Javanbakht et al., 2017). Such plasticity was recently represented for *T. armeniaca* populations (Arakelyan et al., 2018) with a low domed shell shape living in the burrows, and high domed tortoises living in the steppes. The climate was humid and cold during LGM in Anatolia, and forest vegetation covered 80-90 % of the land cover in north-western Anatolia and the Black Sea coast and 50 % of the Mediterranean coast. Similarly, eastern Anatolia and western Iran were cold and arid during the LGM (Şenkul & Dogan, 2013). During the Pleistocene, glacier development within Anatolia was limited to higher mountain peaks (Atalay, 1996) while the lowlands remained open, developing steppe communities (Michaux et al., 2004). This provided suitable habitats for temperate species to survive the LGM (Rokas et al., 2003; Fritz et al., 2009). During the Holocene, humidity and temperature increased, and present day climatic conditions in eastern Turkey and Lake Van region probably started 8200 years ago (Wick et al., 2003). It is therefore probable that low genetic distance among *Testudo* clades (Turkozan et al., 2018) may be the result of a very recent distribution pattern.

The distribution model of *T. graeca* clades in the present work are in line with the classical glacial range

contraction and interglacial range expansion model (Stewart et al., 2010) except the *zarudyni* clade which contracted during the interglacial period. Our analysis supports multiple potential refugia during LGM, namely Caucasus, Anatolia, and Balkans. This is in line with the concept that temperate adapted taxa are confined to southern refugia (Stewart et al., 2010). These refugia are well defined for other species in the Balkans, Anatolia, and the Caucasus during glacials (Hewitt, 2000; Joger et al., 2007; Wielstra et al., 2013).

During the Quaternary ice ages, Anatolia is known to have served as a vital refugium for species that later expanded their distributions during interglacial periods to Europe and Caucasus (Hewitt, 2000; Gür, 2013; Korkmaz et al., 2014). Anatolia is the region where three of the world's 35 biodiversity hotspots meet and interact, namely the Caucasus, Irano-Anatolian, and Mediterranean hotspots (Mittermeier et al., 2004). The north-east and south-west orientation of the Anatolian Diagonal provided a continuous mountain chain connection between the Mediterranean and the Caspian Sea that is linking the southern Taurus diversity hotspot to the nearby Caucasus and Near East mountain system. This connection was used as a corridor for the expansion out of Anatolia during a favorable glacial period by other species such as the Anatolian mountain frogs (*Rana macrocnemis* group) and oriental green lizards (*Lacerta trilineata* group) (Veith et al., 2003; Ahmadzadeh et al., 2013). Similarly, high lineage diversity in Anatolia is clearly due to secondary contact after range expansion, and Anatolia is likely an essential corridor for this. On the other hand, the Anatolian diagonal can also be an environmental barrier with a steep ecological gradient associated with temperature seasonality (Gür, 2016).

The south-eastern Taurus Mountains are located on the junction between Turkey's Taurus Mountain and Iran's Zagros mountains, where the *buxtoni* mtDNA clade is limited by forest steppe. This clade comes into close contact with the *terrestris* mtDNA clade (Turkozan et al., 2018) in southern Anatolia. Together, the Taurus-Zagros range separates the Anatolian-Iranian plateau from the Mesopotamian Lowlands (Sarıkaya et al., 2011). These physical barriers, together with precipitation and temperature related factors, help to delimit the distribution of *Testudo graeca*.

The models predict range expansion for most clades, except *terrestris* and *zarudyni*, in the mid-Holocene, which is typical for other species of vertebrates in Anatolia and the European Peninsula (Taberlet et al., 1998; Hewitt, 2000; Schmitt, 2007). Based on our model, *T. graeca* retreated to glacial refugia during the LGM and expanded its range during the mid-Holocene. However, this is not the case for *zarudyni* clade, which occupied a broader distribution range during LGM and contracted during the mid-Holocene. Javanbakht et al. (2017) also showed a range contradiction in *zarudyni* clade. In conclusion, our study supports multiple refugia for *Testudo* during LGM and that Anatolia was an important corridor for the range expansion of *Testudo graeca*.

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