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Mitochondrial diversity of European pond turtles (*Emys orbicularis*) in Anatolia and the Ponto-Caspian Region: Multiple old refuges, hotspot of extant diversification and critically endangered endemics

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Abstract

The European pond turtle, *Emys orbicularis* (Linnaeus, 1758), is one of the world's most widely distributed chelonian species. We investigated its mitochondrial phylogeography and demography using ~1300 cyt *b* sequences from the entire range, with a focus on the eastern part, in particular on Turkey, where the species currently suffers massive losses. Coloration data from >1450 turtles were compared with mtDNA differentiation to assess the validity of the currently accepted subspecies from Turkey, the Black Sea Region, the Transcaucasus, and Iran. Our study region harbors considerable part of the mtDNA diversity of *Emys*, including a newly discovered lineage and 16 new haplotypes. In this area corresponding to approximately one-third of the entire distribution range, six out of the ten mitochondrial lineages and about half of all 72 haplotypes occur. Two mitochondrial lineages (VIII, X) are confined to small ranges along the southern coast of Turkey, another lineage (I) occupies the remainder of Turkey, the entire Black Sea Region, and the north-eastern part of the species' range. In the south-western corner of the Black Sea and in the Aegean Region, two lineages (II, IV) occur that have their main distribution areas farther west. In the Transcaucasus and northern Iran, another endemic lineage (VII) is found. Lineage I is the largest and most diverse of all lineages and has its greatest diversity in Anatolia. Phylogeographic and demographic data suggest Anatolia as an ancient glacial refuge for turtles harboring mitochondrial lineages I, VIII and X, and that Anatolia and the Black Sea coasts constitute a hotspot for a younger burst of diversification within lineage I. These two regions correspond to the glacial refuge from which lineage I turtles recolonized more northerly parts of the range in the Holocene; lineage II represents an off-shoot of lineage I that became isolated in a westward-located refuge in the south-eastern Balkans during a

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previous Pleistocene glacial. Our data on coloration indicate that such characters have only limited value for delineating evolutionarily significant units. We propose to reduce the number of subspecies using mtDNA lineages as arbiter, and to recognize three subspecies as valid in Turkey, the Black Sea Region, the Transcaucasus and Iran: *Emys orbicularis orbicularis* (mtDNA lineage I); *E. o. eiselti* Fritz et al., 1998 (X); and *E. o. persica* Eichwald, 1831 (VII). However, the southern Turkish lineage VIII most probably represents an additional undescribed subspecies. Both southern Turkish endemics are critically endangered, with only three surviving populations of fewer than 30 adults each. We recommend establishing sanctuaries for them, and including them in the IUCN Red List.

Introduction

The European pond turtle, *Emys orbicularis* (Linnaeus, 1758), is one of the world's most widely distributed chelonian species, ranging over more than 6000 km from the North African Atlantic coast in the west to the Aral Sea in the east, and over approximately 2000 km from the region of Moscow in the north to the Turkish-Syrian border in the south (Fritz 2003). Within this huge range, a deep phylogeographic structure has been discovered, in part matching morphologically defined subspecies (Lenk et al. 1999; Fritz et al. 2005a, 2007). The extant distribution of mitochondrial lineages is the result of repeated Pleistocene constrictions and a Holocene expansion of the turtle's range (Lenk et al. 1999; Fritz et al. 2007; Sommer et al. 2007, 2009), although most of the lineages are thought to predate the Pleistocene (Lenk et al. 1999). In previous studies, we identified eight mitochondrial lineages within *E. orbicularis* (Lenk et al. 1999; Fritz et al. 2005a, 2007); a closely allied ninth lineage corresponds to the recently described *E. trinacris* Fritz et al., 2005, endemic to Sicily. Populations in the north of the range of *E. orbicularis* harbor only three haplotypes belonging to two distinct lineages, while a much higher diversity, 51 haplotypes in eight lineages, was found in the southern parts of its range (Lenk et al. 1999; Fritz et al. 2007). This pattern agrees well with the paradigm of southern genetic richness and northern purity (Hewitt 1996, 2000) and reflects rapid Holocene dispersal from only two distinct refuges, one in the south-eastern Balkans and another one somewhere farther east (Lenk et al. 1999; Fritz et al. 2007; Sommer et al. 2007, 2009).

With respect to phylogeography, Turkey is a little-studied region in the south of the species' range, but four morphologically defined subspecies are thought to occur there (*E. o. colchica*, *E. o. eiselti*, *E. o. hellenica*, *E. o. luteofusca*; Fritz 2003), suggesting considerable genetic variation. Due to large-scale alterations of landscape and water resources development (Harmancioğlu et al. 2001; Kiliç et al. 2005, 2006), populations of the European pond turtle currently suffer massive losses in Turkey (Taşkavak and Reimann 1998; Ayaz et al. 2007, 2008; Ayaz unpubl. observ.). Previous investigations focused either on other regions of the species' range (Germany: Fritz et al. 2004; Italy: Fritz et al. 2005a; France: Fritz et al. 2005b; Ukraine: Kotenko et al. 2005; Iberian Peninsula: Velo Antón et al. 2008; extinct Central European and Scandinavian populations: Sommer et al. 2009) or included only few Turkish samples (Lenk et al. 1999; Fritz et al. 2007), so that the genetic structure of Turkish populations remains largely unknown. However, such data are urgently needed in order to develop any future management strategy.

Previous genetic work found most of the few Turkish samples studied (from northern and central Anatolia) to represent a mitochondrial lineage distributed all around the Black Sea and in the north-eastern part of the species' range. However, two samples from the Turkish southern coast turned out to belong to a highly distinct, endemic lineage (Fritz et al. 2007), underscoring that Turkey might harbor an important part of the species' genetic diversity.

In the present paper, we analyze the mitochondrial diversity of *E. orbicularis* throughout its Turkish range, correlate this dataset with coloration data as used for subspecies delineation, and compare our findings with earlier-published information on neighboring Balkan and Caucasian populations (Fritz et al. 2007) as well as with new data from the northern Black Sea Region (Ukraine), in order to examine the phylogeography and demography of pond turtles and to assess whether the currently accepted subspecies in our study region are confirmed by mtDNA differentiation. Finally, we use our results to derive recommendations for conservation.

Material and methods

Study region, sampling, and chosen marker gene

In the course of our investigations, more than 3000 European pond turtles were trapped or hand-collected

in Turkey and the Ukraine. The sex and basic morphological data (size, coloration and pattern) were recorded for each adult individual. After taking blood or tissue samples (tail tips), the turtles were released at the capture site. Morphological data from other Turkish, Balkan, Black Sea and Caucasian populations were acquired using the holdings of all major European natural history museums (cf. Fritz 1992, 1994; Fritz et al. 1998).

For genetic investigations, we chose the phylogeographically highly informative mitochondrial cytochrome *b* (cyt *b*) gene, a marker used in previous investigations on pond turtles; cyt *b* sequences of 184 samples from Turkey and the Ukraine were produced for this study. Using data published earlier (reviewed in Fritz et al. 2007), we considered a total of 1291 mtDNA sequences of European pond turtles (*Emys orbicularis*, *E. trinacris*), representing the entire range of the genus. A special focus was set on the eastern part of the range of *E. orbicularis*, corresponding to Anatolia and the Ponto-Caspian Region, an area including the eastern Balkans as well as the regions of the Black, Caspian, and Aral Seas (Fig. 1, top). From these regions 397 sequences were available, including the sequences from Turkey and the Ukraine (see Appendix A in the online edition of this paper).

Laboratory techniques and haplotype identification

Total genomic DNA of most samples was extracted by overnight incubation at 55 °C in lysis buffer (6% DTAB, 1.125 M NaCl, 75 mM Tris-HCl, 37.5 mM EDTA, pH 8.0), including 0.5 mg of proteinase K (Merck; Whitehouse Station, NJ), and by subsequent purification following the DTAB method (Gustincich et al. 1991). DNA was precipitated from the supernatant with 0.2 volumes of 4 M LiCl and 0.8 volumes of isopropanol, centrifuged, washed, dried, and resuspended in TE buffer. Alternatively, total genomic DNA of a few samples was isolated with the Nucleospin Tissue Kit (Macherey Nagel; Düren, Germany).

Polymerase chain reaction (PCR) was used to amplify an mtDNA fragment containing the complete cyt *b* gene and approximately 20 bp of the adjacent tRNA-Thr gene. For PCR and sequencing the primers mt-a-neu3, mt-c-For2, mt-f-na3, and mt-E-Rev2 (Praschag et al. 2007) were used. PCR product purification and sequencing also followed Praschag et al. (2007). None of the sequences contained internal stop codons, and nucleotide frequencies corresponded to those of coding mtDNA; we therefore conclude to have amplified and sequenced mtDNA and not nuclear copies of mitochondrial genes.

Using TCS 1.21 (Clement et al. 2000), we collapsed individual sequences into haplotypes and compared them to the previously identified 51 haplotypes of *Emys orbicularis* and 5 haplotypes of *E. trinacris* (Fritz et al.

2007). Haplotype nomenclature follows Lenk et al. (1999) and Fritz et al. (2007) in that Roman numerals designate major clades of haplotypes as revealed by phylogenetic analyses (= mtDNA lineages); individual haplotypes within each lineage bear consecutive letters. GenBank accession numbers of the newly identified haplotypes are FM991957–FM991972.

Phylogenetic and haplotype network analyses

Phylogenetic relationships for all haplotypes of *Emys orbicularis* and *E. trinacris* were estimated using the six outgroup sequences of Lenk et al. (1999), representing all genera of the subfamily Emydinae (*Actinemys marmorata*, *Clemmys guttata*, *Emydoidea blandingii*, *Glyptemys insculpta*, *G. muhlenbergii*, *Terrapene ornata*). Maximum Parsimony (MP) calculations were run using PAUP* 4.0b10 (Swofford 2002; equal weighting, command: hs add = cl); support for the resulting topology was calculated using the bootstrap under the same settings (1000 replicates). 774 of 1031 aligned sites (cyt *b*) were constant, and 140 characters were variable and parsimony-informative; 117 variable characters were parsimony-uninformative. For the ingroup, 941 characters were constant, 51 variable characters were parsimony-informative, and 39 variable characters were parsimony-uninformative.

MULTIPHYL (Keane et al. 2007) was used for Maximum Likelihood (ML) estimation and model selection. Hierarchical ML ratio tests resulted in the selection of the Kimura-3-parameter model with unequal base frequencies and gamma rate heterogeneity among sites, regardless of which model selection criterion was used (AIC1, AIC2, or BIC; Keane et al. 2006). ML optimization was performed using the subtree pruning and regrafting (SPR) algorithm with five rearrangement levels starting from a neighbor joining tree. Bootstrap support for ML was obtained using GARLI 0.95 (Zwickl 2006), employing 1000 replicates (settings: genthreshfortopoterm = 5000 ratematrix = 6 rate statefrequencies = estimate ratehetmodel = gamma numratecats = 4 invariantsites = none). The Bayesian approach to reconstructing the evolutionary relationships and their associated uncertainty was performed with MrBAYES 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) using two parallel runs, each with four MCMC chains (heating parameter λ set to 0.1 to obtain convergence). The chains ran for 7.5 million generations, with every 500th generation sampled. The first 2000 sampled trees were discarded as burn-in. The remaining trees were used for generating a 50% majority rule consensus tree. The posterior probability of any individual clade in this consensus tree corresponds to the sum of the posterior probabilities of
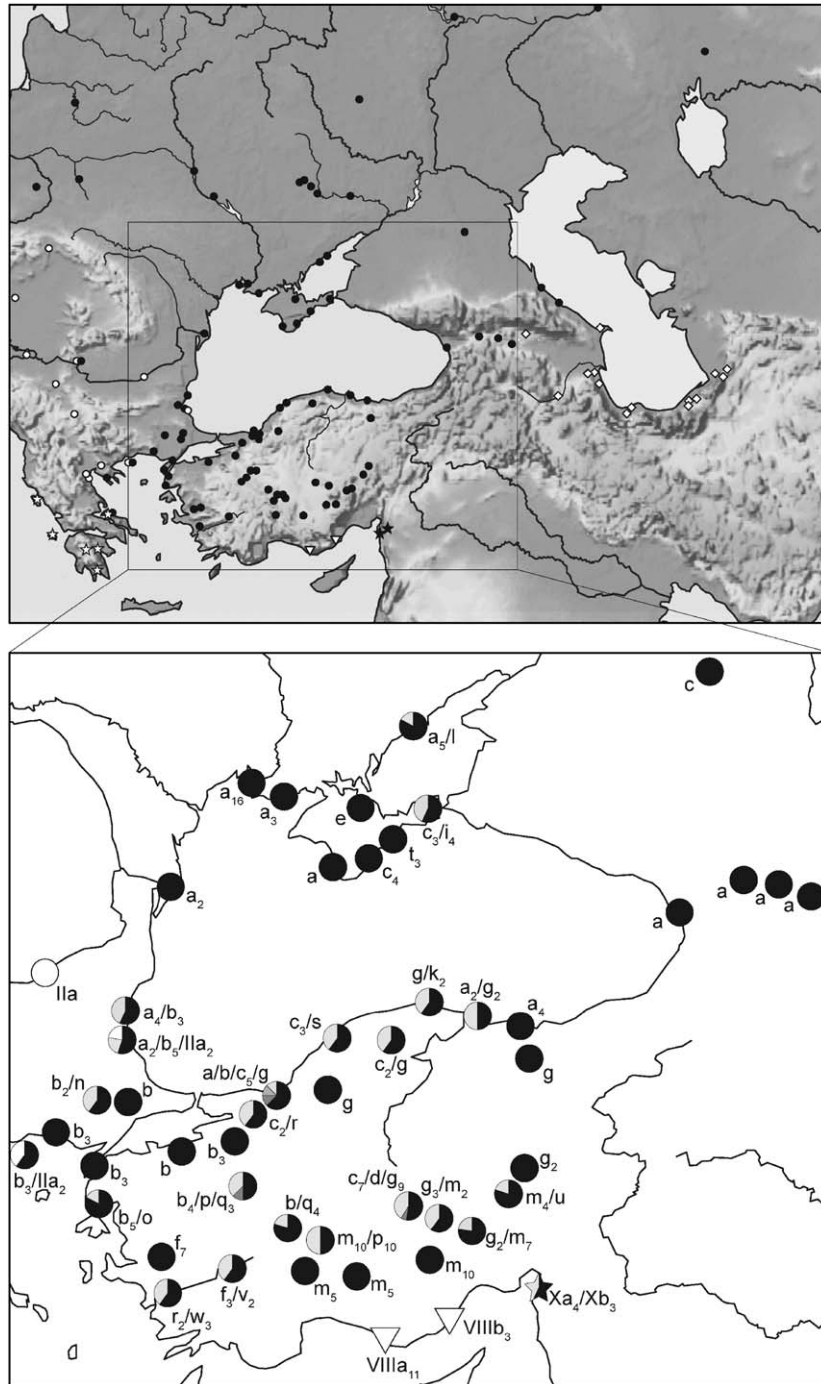
Fig. 1. (Top) Distribution of mtDNA lineages of *Emys orbicularis* in the eastern part of its range. Lineage symbols: black circles = lineage I; white circles = II; white stars = IV; diamonds = VII; triangles = VIII; black stars = X (newly discovered). (Bottom) Distribution of individual haplotypes in Anatolia and the Black Sea Region. Pie charts indicate syntopic haplotypes and their approximate relative frequencies. Letters without Roman numerals designate lineage I haplotypes; for other lineages the Roman numerals are given. All lineage I haplotypes north of the enlarged map sector represent haplotype Ia, except for one record of haplotype Ih in north-eastern Ukraine. Subscripts indicate individual numbers of haplotypes greater than 1.

all trees that contain that clade, and thus is a measure for its frequency and credibility.

Within the same species or between closely related species, relationships of haplotypes are likely to be reticulate and ancestral haplotypes may persist, which is why intraspecific gene evolution may be only imperfectly reflected by dichotomous trees (Posada and Crandall 2001). Thus, reconstruction methods enforcing bifurcations might not adequately represent all (conflicting) information. To explore this ambiguity, we also applied

the Median Joining (MJ) network reconstruction algorithm (Bandelt et al. 1999) as implemented in NETWORK 4.5.0.0 (sparse network; equal weighting; ε = 0). A further advantage of network analyses is that information about the age of haplotypes may be obtained. Interiorly located haplotypes, having more than one mutational connection, are thought to be ancestral to and older than tip haplotypes (Posada and Crandall 2001).

Analyses of population structure

In phylogenetic analyses haplotypes cluster in usually well-supported clades (= mtDNA lineages) that are strongly correlated with geographical distribution. Accordingly, our chosen population structure corresponds to the six genetically and geographically distinct mtDNA lineages (I, II, IV, VII, VIII, and X) present in the study region. The significance and extent of this putative population structure was tested using global and pairwise AMOVAs (Excoffier et al. 1992) and exact Fisher tests (Raymond and Rousset 1995; Goudet et al. 1996) as implemented in ARLEQUIN 3.1 (Excoffier et al. 2005).

Population diversity and divergence

To assess population diversity and divergence parameters, the number of polymorphic sites and haplotypes, polymorphism patterns, haplotype diversity H (Nei 1987; equation 8.4, gene diversity, in which 2n is replaced by n), as well as nucleotide diversity π (Nei 1987; equations 10.5 and 10.6) and population mutation parameter per site θ (Nei 1987; equation 10.3) were calculated. In addition, the number of silent mutations and the K_a/K_s ratio ω were computed, using *Actinemys marmorata* (GenBank accession number AJ131430) and *Emydoidea blandingii* (AJ131432) for interspecific comparison as required for ω. The extent of population divergence as reflected in DNA polymorphism patterns between the six lineages was investigated using the number of fixed and shared polymorphisms as well as the number of mutations polymorphic in one population but not in the other (Hey 1991).

Tests of neutrality and population demography

Analyses of population diversity and divergence, population size changes, and tests of neutrality were conducted using DNASP 4.20 (Rozas et al. 2003). Deviations from a neutrally evolving, constant-sized population model were tested based on Tajima's D (Tajima 1989), Fu and Li's D* and F* (Fu and Li 1993), Fu's F_s (Fu 1997), and Ramos-Onsins and Rozas' R_2 (Ramos-Onsins and Rozas 2002).
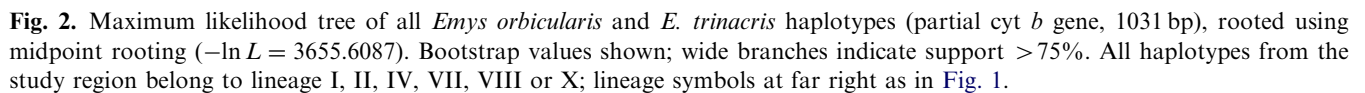
Analysis of isolation-by-distance

For lineage I, a differentiation process of isolation-by-distance was tested. The analysis was conducted at the individual level for two data partitions, for the study region south of the 47th parallel and for samples north of this latitude. Genetic distances between individuals were estimated under ML using PAUP* 4.0b10 (Swofford 2002). The substitution model employed was the HKY model with base frequencies for A = 0.31433, C = 0.30453, and G = 0.11707, and with a transition-transversion rate ratio of 19.86. Geographic distances were computed as great circle distances using the R package PBS Mapping 2.55 (Schnute et al. 2008). Subsequently, geographic distances were transformed to log-distances as suggested by Rousset (1997). A Mantel test of the distance matrices was conducted as implemented in the R package APE (Paradis et al. 2004).

Results

Diversity, phylogeny and geographic distribution of haplotypes

In the course of this study, we identified 16 new haplotypes of *Emys orbicularis* from Turkey and the Ukraine, resulting in a total of 67 haplotypes of *E. orbicularis* and five haplotypes of *E. trinacris* when previously published data are considered (Lenk et al. 1999, Fritz et al. 2005a, 2007). These 72 *Emys* haplotypes constitute a well-supported clade under all tree-building methods; haplotypes of *E. trinacris* (lineage III) appear as basal in all analyses (Figs. 2, 3). However, the monophyly of lineage III is well-supported under MP only; the weak support in ML and Bayesian analyses is due to homoplasy introduced into the dataset by the genetically remote outgroup taxa. In an ML search excluding outgroups (not shown), the monophyly of the haplotypes of lineage III was well-supported by a bootstrap value of 98%.

Within *E. orbicularis*, haplotypes form nine mostly well-supported lineages arising from an unresolved backbone. One of these (lineage X) was previously unknown and includes two new haplotypes (Xa, Xb) from the southern coast of Turkey (east of Amanus Mountains; Fig. 1). Other samples from our study region belong to lineage I (Turkey plus northern Black Sea Region and north-eastern part of the species' range, with 13 new haplotypes; Ik–Iw), lineage II (north-west of study region), lineage IV (south-west of study region), lineage VII (Transcaucasus and northern Iran), and lineage VIII (southern coast of Turkey, with one new haplotype; VIIIb). Lineages II and IV have wide

Fig. 2. Maximum likelihood tree of all *Emys orbicularis* and *E. trinacris* haplotypes (partial cyt *b* gene, 1031 bp), rooted using midpoint rooting ($-\ln L = 3655.6087$). Bootstrap values shown; wide branches indicate support $> 75\%$. All haplotypes from the study region belong to lineage I, II, IV, VII, VIII or X; lineage symbols at far right as in Fig. 1.

distributions beyond our study region (lineage II: mainly Danube Region and France; lineage IV: circum-Adriatic). Lineage I reaches its north-western range border in Poland (Lenk et al. 1999; Fritz et al. 2007), slightly beyond our study area, whereas lineages VII, VIII and X are confined to the study region.

Fig. 3. Fifty-percent majority rule consensus of 234 equally parsimonious trees for *Emys* haplotypes and outgroups (CI = 0.6817, RI = 0.8275; 421 steps). Numbers above nodes are MP bootstrap values; below nodes, Bayesian posterior probabilities. *Actinemys*-*Emydoidea* node not found under BA. For further explanation, see Fig. 2.

Lineage I is monophyletic in all analyses, despite weak support (<50% in ML analyses with and without outgroups as well as under MP; posterior probability 56%). Lineage I forms a well-supported clade with lineage II (94% and 97% bootstrap support in ML analyses with and without outgroups, respectively; 93% in MP analysis; posterior probability 100%).

Monophyly of the other lineages from our study region is well-supported (Figs. 2, 3).

In the MJ network (Fig. 4), lineages I and II are not clearly distinct, while all other lineages correspond to well-defined haplotype clusters. Lineage II appears to be derived from the ancestral node haplotype of lineage I (Im).
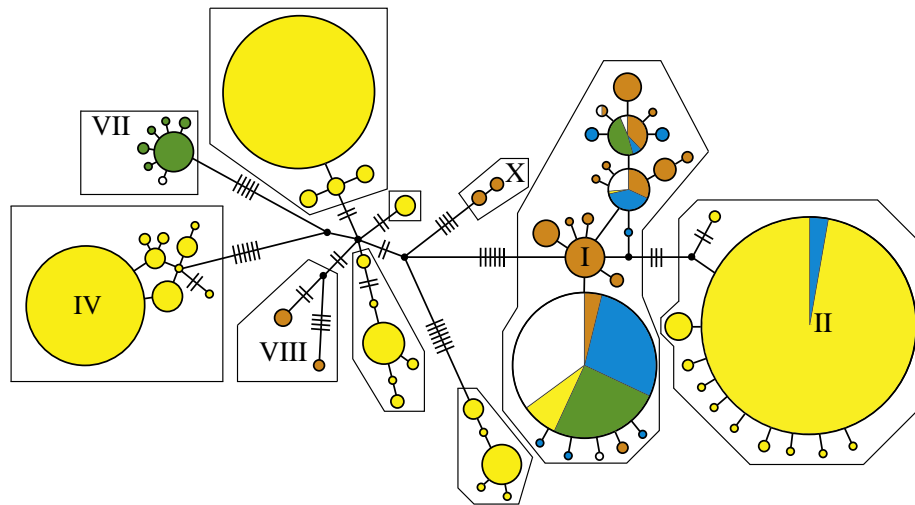
Fig. 4. Median Joining network of all 72 identified *Emys* haplotypes (partial cyt *b* gene, 1031 bp), based on range-wide sampling (1291 pond turtles). Symbol size corresponds to approximate haplotype frequency. Boxes denote haplotypes representing the same mtDNA lineage; only lineages occurring in our study region are numbered. Colors indicate geographic origin: Anatolia = orange; northern Black Sea Region = blue; Caspian Sea Region = green; farther western parts of range = yellow; unknown origin (captive or allochthonous pond turtles) = white. Northern Black Sea Region includes European Turkey, adjacent easternmost Greece, Bulgaria, Romania, eastern Serbia, Ukraine, and Georgia. Missing node haplotypes black. Each line joining haplotypes indicates one nucleotide substitution except when hashmarks across lines are present; then each hashmark indicates one step. Central node haplotype of lineage I (with Roman numeral) is Im.

Table 1. Patterns of polymorphism and genetic diversity within the six mitochondrial lineages present in the study area.

Lineage	n	S	η	Type	H	h	π (per site)	θ (per site)	η$_{Sil}$	ω
All	397	59	59	8-51-0-0-0-0	38	0.886	0.00561	0.00872	39	0.046
I	300	21	21	9-12-0-0-0-0	22	0.818	0.00176	0.00324	13	0.047
II	32	3	3	3-0-0-0-0-0	2	0.063	0.00018	0.00072	1	0.045
IV	13	6	6	2-4-0-0-0-0	4	0.756	0.00206	0.00188	4	0.052
VII	31	5	5	3-2-0-0-0-0	6	0.402	0.00043	0.00121	4	0.043
VIII	14	6	6	0-6-0-0-0-0	2	0.363	0.00211	0.00183	3	0.046
X	7	1	1	0-1-0-0-0-0	2	0.571	0.00055	0.00040	0	0.056

n: number of individuals sampled. S: total number of polymorphisms. η, η$_{Sil}$: numbers of total and silent mutations, respectively. Type of polymorphism: number of sites with singletons (2 variants) – parsimony-informative (PI) polymorphisms (2 variants) – singletons (3 variants) – PI polymorphisms (3 variants) – singletons (4 variants) – PI polymorphisms (4 variants). H: number of haplotypes. h: haplotype diversity. π: nucleotide diversity per site. θ: Watterson's population mutation parameter per site. ω: K$_a$/K$_s$ ratio using *Actinemys marmorata* (GenBank accession number AJ131430) and *Emydoidea blandingii* (AJ131432) for interspecific comparison.

Population structure, diversity and divergence

A global AMOVA testing population structure for the six lineages in our study region is highly significant (p < 0.001); among-population variation explains 86.46% of the total variation. Support for the structuring is also obtained by an Exact Fisher Test (p < 0.001). Pairwise F$_{st}$ values range from 0.76030 (lineages I and II) to 0.98129 (lineages II and X). All pairwise AMOVAs and tests of differentiation are significant.

Lineage I is the largest and most diverse of all lineages, comprising 23 haplotypes (one-third of all 72 haplotypes of *Emys orbicularis* and *E. trinacris*; Fig. 4). We identified haplotypes of this lineage in 300 turtles

from the study region. Lineage I has the highest population mutation value θ, the highest haplotype diversity, and a relatively high nucleotide diversity. Haplotypes of lineage I are characterized by 21 polymorphic sites, nine of which are singletons (Table 1). Most of the haplotypes of lineage I occur only in the south of its range, in particular in Anatolia (Fig. 1; Appendix A). A Mantel test considering all individuals of lineage I from south of the 47th parallel revealed a highly significant correlation between genetic and geographic distances (p ⩽ 0.001), whereas north of that line isolation-by-distance breaks down because there occurs only one haplotype, Ia (with one exception, see caption to Fig. 1).

Lineages IV (13 samples) and VIII (14 samples) are the most diverse groups with respect to nucleotide diversity and possess intermediate θ and haplotype diversity values. Lineage VIII includes two haplotypes that are differentiated by six sites. Due to this rather pronounced difference, the diversity estimates within this group are comparatively high. Lineages II, VII and X, comprising 32, 31 and seven individuals, respectively, are generally less diverse, in particular lineages II and VII, which are represented by relatively large samples. Lineage II, with only two haplotypes, is the least diverse group in the study area. Lineage VII, despite its six haplotypes, has relatively low diversity estimates. As expected for the protein-coding cyt *b* gene, a high number of polymorphisms are silent mutations and the K_a/K_s ratio is clearly smaller than 1 in all partitions, suggesting purifying selection.

All six lineages in our study region possess a large number of fixed differences and no or only few shared polymorphisms (Table 2), which suggests advanced lineage sorting. Shared polymorphisms are found between lineage I and lineages II, IV and VII; other shared polymorphisms occur only between lineages II and VII. Lineages VIII and X do not share any polymorphisms with any of the other lineages.

Table 2. Genetic differentiation between the six mitochondrial lineages of pond turtle.

	I	II	IV	VII	VIII	X
I	–	3/1	11/1	11/1	8/0	9/0
II	20/2	–	15/0	14/2	11/0	12/0
IV	20/5	3/6	–	11/0	9/0	12/0
VII	20/4	1/3	6/5	–	8/0	12/0
VIII	21/6	3/6	6/6	5/6	–	7/0
X	21/1	3/1	6/1	5/1	6/1	–

Entries above matrix diagonal: number of fixed differences/number of shared mutations. Below diagonal: number of sites polymorphic only in first lineage (column)/number of sites polymorphic only in second lineage (row).

Tests of neutrality and population demography

Tests of the neutral population model suggest reduced genetic variation for lineages IV, VIII and X (Table 3). Tajima's D, as well as Fu and Li's D* and F* statistics, are positive though non-significant. Fu's F_s statistic, however, shows a significant deficit of rare mutations in lineages VIII and X. In contrast, lineages I, II and VII are characterized by an excess of rare mutations (singletons). This excess is especially significant for lineage I (Fu and Li's D* and F* statistics and Fu's F_s test). Values of D* and F* are significant in lineage II, F_s is highly significant in lineage VII; the R_2 statistic is only significant for lineage VII. The reduction of genetic variation could be an artefact in the case of lineage IV, however, because in this group the greatest diversity occurs outside of the study area (in southern Italy; Fritz et al. 2005a), whereas the ranges of lineages VIII and X lie entirely within the study region.

Geographic variation in shell coloration

Shell coloration is an important character used for subspecies delineation in previous studies. The Central Anatolian subspecies *Emys orbicularis luteofusca* was characterized as having a mainly to entirely yellowish brown carapace coloration, whereas all other Anatolian subspecies were described as possessing a dark-colored carapace (Fig. 5). Pond turtles from the northern part of the species' range are known to be exclusively dark-colored (Fritz 1998, 2003). Our coloration and pattern data for more than 1450 pond turtles from Anatolia and the Black Sea Region provide evidence that dark-colored pond turtles also occur in the range of *E. o. luteofusca*. However, a high percentage of Central Anatolian pond turtles do have a light-colored, yellowish brown carapace with or without dark radiating pattern, as previously described for *E. o. luteofusca*. On the other hand, similarly light-colored individuals also occur in the south-eastern Balkans and the southern Crimea (Fig. 6).

Table 3. Results of neutrality tests: Tajima's D, Fu and Li's D*, Fu and Li's F*, Fu's F_s, and Ramos-Onsins and Rozas' R_2.

Lineage	D	D*	F*	F_s	p ($F_s \leqslant$ obs F_s)	R_2	p ($R_2 \leqslant$ obs R_2)
I	−1.18318	−2.97584**	−2.71545**	−9.281	0.021	0.0426	0.101
II	−1.72954	−2.73255**	−2.83037**	0.071	0.603	0.1740	0.822
IV	0.37190	0.05865	0.15945	1.221	0.757	0.1758	0.662
VII	−1.74781	−1.50538	−1.83494	−4.331	0.002	0.0642	0.016
VIII	0.55008	1.28811	1.24784	4.962	0.993	0.1813	0.798
X	1.34164	0.95346	1.10146	0.856	1.000	0.2857	0.290

**: significant at $p \leqslant 0.05$. Confidence intervals for F_s and the R_2 statistic for a constant-sized, neutrally evolving population were obtained by coalescence simulations run with 1000 replicates and conditioned on the number of polymorphic sites in the sample. Simulation-derived probabilities (p) for the observed (obs) F_s and R_2 values are given.
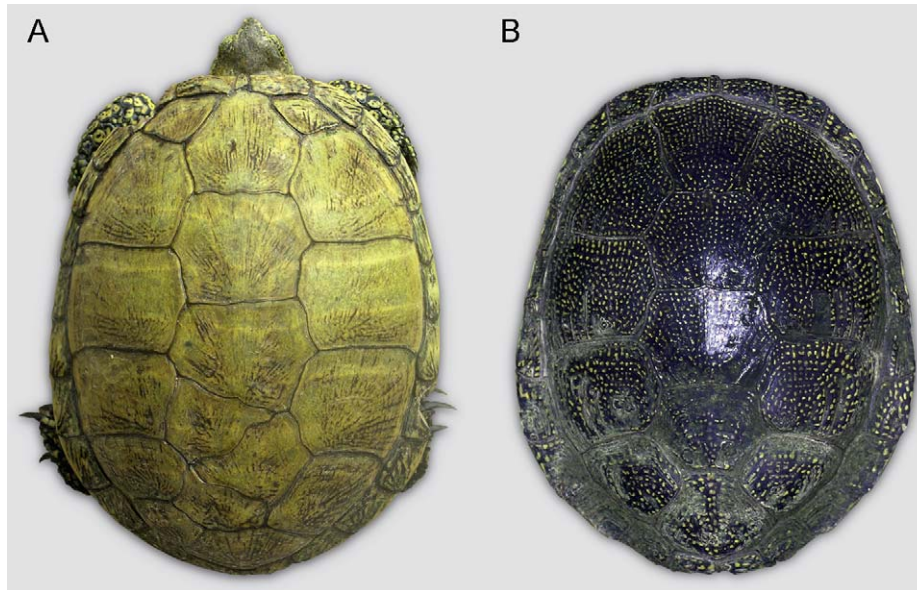
Fig. 5. Carapace coloration types of Anatolian pond turtles. (A) Light coloration type as described as characteristic for *Emys orbicularis luteofusca*. (B) Dark coloration type as described as characteristic for *E. o. orbicularis*, *E. o. colchica*, *E. o. hellenica*, and *E. o. eiselti* (Fritz et al. 1998).

Discussion

Mitochondrial phylogeography

The present investigation has revealed for *Emys orbicularis* a strikingly high level of phylogeographic structuring in our study region, especially in Anatolia which harbors considerable parts of the species' mitogenetic diversity. Although our study region corresponds to no more than about one-third of the entire distribution range of the genus *Emys*, six out of the ten mitochondrial lineages and approximately half of all 72 identified haplotypes occur there (Fig. 4). Anatolia alone harbors three mtDNA lineages and 20 haplotypes (28%); lineages VIII and X and 16–17 individual haplotypes are endemic here. The high number of haplotypes of lineage I and their high degree of endemism are remarkable. Of 23 lineage I haplotypes, only one identified from a captive individual of unknown origin is not recorded from our study region. More than two-thirds of the lineage I haplotypes (16) occur in Anatolia, and 12 or 13 (one haplotype also recorded in another turtle of unknown geographic origin) of these are known only from here. Endemic lineage I haplotypes are confined to geographically well-delimited, relatively small regions, especially in Anatolia and the Crimea. Three haplotypes (Ia, Ib, Ic) are shared between Anatolia and other regions. Haplotype Ib occurs in western Anatolia and the neighboring south-eastern Balkan Peninsula; haplotype Ic is shared between Anatolia, the Crimea and the region between

the Black and Caspian Seas. Haplotype Ia, with a wide distribution occupying the north-east of the species' range from Poland to the Aral Sea, occurs in northern Anatolia and all around the northern Black Sea, including the west Caucasian Colchis Region (Fig. 1; Appendix A).

According to its interior position in the network, haplotype Im, endemic to the southern Anatolian Plateau, is ancestral to all other lineage I haplotypes as well as to lineage II. This is in accord with the hypothesis that the split between lineages I and II is distinctly younger than the divergence between the other mitochondrial lineages of *Emys* (Pleistocene vs. Pliocene; Lenk et al. 1999), and it suggests that lineage II branched off from lineage I in a refuge in the south-eastern Balkans during a previous Pleistocene glacial.

As for lineage I, all pond turtles from north of the 47th degree of latitude harbor only haplotype Ia (with a single exception, a closely related unique tip haplotype). South of that line, haplotype Ia and at least 20 of the 22 other haplotypes of this lineage occur and show here a highly significant correlation between genetic and geographic distances. North of the 47th parallel such correlation does not exist, which suggests recently established populations resulting from rapid Holocene range expansion and genetic pruning due to founder effects. By contrast, the high southern diversity of lineage I is likely to be caused either by fragmentation of Anatolia and the Black Sea coasts into microrefuges or by reduced glacial gene flow between local populations
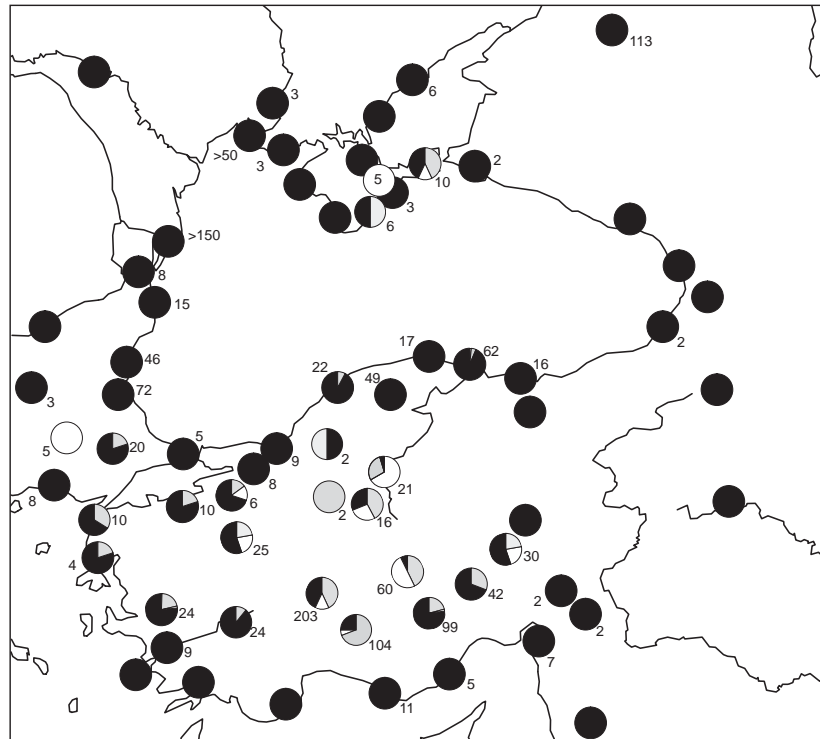
Fig. 6. Distribution of carapace coloration types among >1450 pond turtles in Anatolia and the Black Sea Region; white symbol = coloration yellowish brown (Fig. 5A), black = mainly black (Fig. 5B), grey = intermediate. Pie charts indicate approximate local frequency distribution; numbers, sample sizes greater than 1. Not all samples correspond geographically to Fig. 2, because more populations were studied for shell coloration. Pond turtles occurring north of the region shown exhibit only mainly black carapace coloration.

(cf. Irwin 2002 for isolation-by-distance leading to phylogeographic structure without strict barriers). When it is considered that most of Central Anatolia is covered by steppe and semi-desert today, habitats unsuitable for freshwater turtles, it seems likely that this diversification process continues and that the Black Sea coasts and Anatolia have served not only as glacial refuges to lineages I, VIII, and X, but also represent a hotspot of extant diversification of lineage I.

The observed syntopic occurrence of some frequent lineage I haplotypes at certain sites (Fig. 1; Appendix A) could be the result of either Holocene range expansions or an old pattern of incomplete sorting, whereas the allopatric distribution of the two haplotypes of lineage VIII, separated by a distribution gap of 100 km, suggests that this lineage survived the last glaciation in two distinct microrefuges along the southern Turkish coast and has achieved complete lineage sorting already.

Demography

Analyses of the mitochondrial diversity in animals generally are interpreted as providing information about population structure and history rather than about selection (but see Bazin et al. 2006). Assuming that the mitochondrial lineages represent homogenous populations and that the relatively small sample sizes for lineages IV, VIII and X adequately characterize these populations, distinct patterns of population size changes emerge. Whilst lineage IV shows no significant deviations from a stable, constant-sized population, the excess of internal polymorphisms and low genetic diversities in lineages VIII and X indicate that both have experienced a reduction in population size. Indeed, *Emys orbicularis* is extremely rare along the Turkish southern coast (Fritz 2003; see also below under "Conservation considerations and recommendations"). In contrast, for lineage I the excess of singletons and haplotypes clearly suggests population expansion. Two other haplotype groups within the present study also show patterns of population growth, though to a somewhat lower extent. Significant population growth was indicated for lineages II and VII at least by some test statistics, and population expansion is also suggested by the star-like network configuration of both lineages (Fig. 4). Thus, lineage VII, endemic to the Transcaucasus and northern Iran, obviously experienced a different history compared to lineages VIII and X, endemic to southern Turkey. We suppose that ecological constraints (limited suitable habitats, mountain barriers) and the competition with another

codistributed terrapin species, the extremely abundant *Mauremys rivulata*, have prevented a Holocene population expansion of *E. orbicularis* in southern Turkey. In Central Anatolia, the Caucasus Region and Iran, *M. rivulata* is replaced by *M. caspica,* and *E. orbicularis* seems to be able to cope better with that species (Fritz 2003). The demography of lineage II, although only marginally represented in the very west of our study region, reflects a major Holocene population and range expansion, leading to the colonization of Central and Western Europe and of Scandinavia (Lenk et al. 1999; Fritz et al. 2007; Sommer et al. 2009).

In conclusion, our phylogeographic and demographic data indicate that (i) Anatolia is an ancient glacial refuge for pond turtles harboring mitochondrial lineages I, VIII and X; (ii) Anatolia and the Black Sea coasts are a hotspot for a younger burst of diversification within lineage I; (iii) these regions correspond to the glacial refuge from which lineage I turtles recolonized more northerly parts of the species' range in the Holocene; and (iv) lineage II represents an off-shoot of lineage I that became isolated in a farther westward-located refuge in the south-eastern Balkans during a previous Pleistocene glacial.

Implications for subspecies delineation

Traditionally, much emphasis was given to shell coloration for subspecies delineation in pond turtles (reviews in Fritz 1998, 2003). However, such characters have been shown to be variable in the south of the range and prone to selective pressure (Fritz et al. 2006). Consequently, a comparison of coloration with mtDNA data seems promising to gain new insights concerning taxon limits, even though recently concerns have been voiced about basing evolutionary interpretations on mtDNA data alone (Ballard and Whitlock 2004; Edwards et al. 2005; Bazin et al. 2006; but see Zink and Barrowclough 2008).

Currently, four subspecies of European pond turtles are thought to occur in Turkey (Fritz 2003): *Emys orbicularis luteofusca* Fritz, 1989, endemic to Central Anatolia; *E. o. colchica* Fritz, 1994, northern Turkey and Turkish Black Sea coast (also adjacent Bulgaria, Greece, Black Sea coast of Russia, Georgia, and Crimea); *E. o. eiselti* Fritz et al., 1998, endemic to south-eastern Turkey east of the Amanus Mountains; and *E. o. hellenica* (Valenciennes, 1832), south-western Anatolia (otherwise of circum-Adriatic distribution; Fritz et al. 2005a). Two further subspecies are recognized from the eastern Transcaucasus (*E. o. iberica* Eichwald, 1831) and northern Iran (*E. o. persica* Eichwald, 1831), respectively; in the northern part of

the species' range occurs *E. o. orbicularis* (Linnaeus, 1758).

While the Central Anatolian subspecies *E. o. luteofusca* and the Transcaucasian *E. o. iberica* were characterized as having a mainly or entirely yellowish brown carapace coloration, the other subspecies were thought to be dark-colored (Fritz 2003). From the core distribution area of *E. o. luteofusca*, the southern Anatolian Plateau, no genetic data were previously available, but eight specimens from a more northerly locality in Central Anatolia yielded haplotypes of lineage I (Ic, Id; Lenk et al. 1999). The subspecies *E. o. colchica* is morphologically weakly defined, differing from *E. o. orbicularis* only by its slightly smaller size (Fritz 1998, 2003). Pond turtles from the range of *E. o. colchica* yielded haplotypes of lineage I (Ia, Ib, Ic, Ii; Lenk et al. 1999; Fritz et al. 2004, 2007; Kotenko et al. 2005; haplotype It added by present study). The description of the endemic subspecies *E. o. eiselti*, also dark-colored, was based on only four specimens (Fritz et al. 1998). Morphologically it also resembles *E. o. orbicularis*, but is distinctly smaller (reaching only approximately 13 cm maximum shell length compared to 23 cm in *E. o. orbicularis*) and differs from all other known subspecies of *E. orbicularis* by its extremely small gular scutes (Fritz et al. 1998; Fritz 2003). Genetic data were previously not available for *E. o. eiselti*. The fourth subspecies from Turkey, *E. o. hellenica*, otherwise has a circum-Adriatic distribution (Fritz et al. 2005a). Due to their small size and soft part coloration, pond turtles from western Anatolia were tentatively assigned to this subspecies. However, this assessment was based on only 11 adult museum specimens (Fritz 1994; Fritz et al. 1998), and a later study perpetuating the provisional subspecies assignment (Taşkavak and Ayaz 2006) did not compare Anatolian turtles with specimens from the circum-Adriatic region. Genetically, circum-Adriatic populations of *E. o. hellenica* are well-differentiated; their range perfectly agrees with the distribution of mitochondrial lineage IV (Fritz et al. 2005a). Genetic data from the putative Anatolian range of *E. o. hellenica* were previously not available. The Transcaucasian and Iranian subspecies, *E. o. iberica* and *E. o. persica*, share a peculiar soft part coloration and differ mainly by their shell coloration in that adult *E. o. iberica* have a pale yellow-brown carapace, somewhat resembling *E. o. luteofusca*, whereas *E. o. persica* has a dark-colored carapace (Fritz 1998, 2003). Previously published cyt *b* data demonstrated a perfect agreement of the distribution of mtDNA lineage VII with the ranges of both subspecies (Fritz et al. 2007).

According to our new data, there is no evidence for the occurrence of *E. o. hellenica* in Anatolia (Fig. 1; Appendix A). All studied western Anatolian populations harbor haplotypes of lineage I, suggesting that the earlier morphological assignment of these populations

to *E. o. hellenica* was based on superficial similarity and small sample size. We also found no genetic support for the validity of the morphologically weakly defined subspecies *E. o. colchica*. If this subspecies represented a distinct evolutionarily significant lineage, it should be expected to correspond to a distinct mitochondrial lineage or at least distinct haplotypes. This is not the case. Notably, turtles from the vicinity of the type locality of *E. o. colchica* (Colchis Region) yielded only haplotype Ia, the most frequent haplotype occurring in the range of *E. o. orbicularis*. Therefore, we propose to relegate the name *E. o. colchica* to synonymy with *E. o. orbicularis*.

With respect to *E. o. luteofusca* the situation is a bit more complicated. The core part of its range harbors the endemic haplotype Im, the only haplotype identified in some populations there. However, our morphological data demonstrate that the shell coloration of *E. o. luteofusca* is more variable than previously assumed, and that turtles with a similar yellowish brown carapace also occur in other parts of our study region (Fig. 6). Considering the many other endemic lineage I haplotypes, this suggests that *E. o. luteofusca* merely represents part of the rich genetic diversity encountered in the south of the range of lineage I. Moreover, when the distribution of haplotypes is compared with coloration (Figs. 1 and 6), it is obvious that there is no strict association of yellowish brown carapace coloration with any haplotype. Therefore, we propose to also place *E. o. luteofusca* into the synonymy of *E. o. orbicularis*. This results in the somewhat uncomfortable situation that *E. o. orbicularis* comprises then various populations with very differently colored turtles: In the north of the subspecies' range only dark-colored turtles occur, whereas some of the southern populations comprise very light-colored, yellow-brownish individuals. However, this does not contradict the new subspecies delineation, especially when it is taken into account that such coloration polymorphisms also occur in other subspecies (Fritz 2003) and that dark coloration is favored by selection in the north of the range (Fritz et al. 2006).

In contrast to *E. o. luteofusca*, the subspecies *E. o. eiselti* was confirmed as representing a distinct evolutionarily significant lineage, perfectly corresponding to the newly identified mtDNA lineage X. Moreover, our data provide evidence for the existence of another evolutionary lineage along the southern Mediterranean coast (corresponding to lineage VIII) that most likely represents an undescribed further subspecies. A morphological characterization and comparison with other subspecies is underway and will be published elsewhere.

Although we have no new quantitative data about coloration for *E. o. iberica* and *E. o. persica*, it is obvious that the combined ranges of both subspecies perfectly agree with the distribution of mtDNA lineage VII. This suggests that both together constitute one and the same evolutionary lineage and that their purported coloration differences reflect at best population-specific differences. Therefore, we propose to lump these two simultaneously described subspecies (Eichwald 1831) under the name of *E. o. persica*, according to the first reviser principle of the International Code of Zoological Nomenclature (ICZN 1999: Art. 24.2).

In summary, we suggest recognizing the following three subspecies for Turkey, the Black Sea Region, the Transcaucasus and Iran: *Emys orbicularis orbicularis* (Linnaeus, 1758), corresponding to mtDNA lineage I; *E. o. eiselti* Fritz et al., 1998, corresponding to lineage X; and *E. o. persica* Eichwald, 1831, corresponding to lineage VII. The southern Turkish mtDNA lineage VIII most probably represents a fourth, undescribed subspecies.

Conservation considerations and recommendations

If mtDNA data are accepted as a proxy for genetic diversity, it is obvious that Turkey harbors a substantial part of the extant diversity within *Emys orbicularis*. The endemic subspecies *E. o. eiselti* and another putative subspecies from the central Mediterranean coast of Turkey (mtDNA lineage VIII) represent distinct, old evolutionary lineages. Both are critically endangered and deserve the highest conservation priority. The subspecies *E. o. eiselti* is endemic to the Amik-Maraş rift valley, and once also occurred in the Asi (Orontes) River along the Turkish-Syrian border and in the now entirely drained Lake Amik. During three excursions in spring 2006, 2007, and 2008, we were only able to confirm the presence of *E. o. eiselti* at two neighboring sites, while all other localities reported in the literature (Fritz et al. 1998) have been destroyed. We estimate the only known surviving population of *E. o. eiselti* to consist of fewer than 30 adults. The two known populations of turtles harboring mtDNA lineage VIII most probably are of similar size.

The continuing alterations to the landscape in the Amik Region (Kiliç et al. 2006) pose serious threats to the survival of *E. o. eiselti*; the situation on the central Mediterranean coast of Turkey is equally serious (Ayaz unpubl. observ.). We propose to establish sanctuaries in both regions for the three known relic populations. Considering the genetic differentiation of the two lineage VIII populations, both should be treated as distinct management units. To increase public and political awareness, we further recommend inclusion of *E. o. eiselti* and the putative subspecies from the central Mediterranean coast of Turkey in the IUCN Red List of Threatened Animals.

Although other Turkish pond turtle populations are also declining throughout the range, their situation is by

far not as critical as in the case of the two southern endemics, and populations comprising several hundreds to thousands of individuals still exist. Nevertheless, to preserve for the long term as much of their considerable genetic variation as possible, we also recommend to establish several sanctuaries that should encompass populations harboring all of the identified endemic haplotypes.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this study (Appendix 1) can be found in the online edition at: doi:10.1016/j.ode.2009.02.002.

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