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Genetic Evidence for Premature Taxonomic Inflation in Middle Eastern Tortoises

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The wide-ranging spur-thighed tortoise (*Testudo graeca*) has recently been split into a complex of species (nine in the Middle East). Because *T. graeca* is a taxon of conservation concern, an accurate understanding of major evolutionary lineages in this taxon is important for guiding conservation strategies. We sequenced a rapidly evolving mitochondrial marker for 30 specimens of *Testudo graeca* from localities tied to the newly proposed morphology-based names in the Middle East. Our data reveal major inconsistencies between morphological and molecular groupings and demonstrate that taxonomic schemes based solely on either data set are problematic. Given the extremely low variation (as little as 0%) between newly recognized species, we consider *T. graeca* in the Middle East as a single species pending further study. We strongly recommend that future systematic studies should strive to compare different data types to avoid misleading taxonomic changes.

Taxonomic inflation, when many existing subspecies are raised to species level, is a common trend in modern systematics as phylogenetic studies revise traditional infraspecific taxonomies (Isaac et al. 2004). This phenomenon of fluctuating taxonomy can have profound and sometimes deleterious implications for management efforts that rely on species lists for guidance (Isaac et al. 2004; Mace 2004). Nevertheless, there are numerous examples of systematic studies showing that a single widespread species actually represents multiple species having restricted ranges. In these cases, despite the problems taxonomic changes may cause, taxonomic inflation is desirable. However, when taxonomic inflation is premature it can reintroduce many unfamiliar, ephemeral species names into the literature and obscure important evolutionary lineages.

A notable example of rapid, and potentially premature, taxonomic inflation within a group of conservation concern (TFTSG 1996) is the spur-thighed tortoises (*Testudo graeca* Linnaeus, 1758). *Testudo graeca* ranges from North Africa east to the Iranian plateau, but is considered vulnerable

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due to habitat degradation and commercial exploitation. Throughout its wide distribution, there is substantial morphological variation, especially among Middle Eastern populations (Fig. 1). Although traditionally considered a single polymorphic species, some authors have asserted that Middle Eastern *T. graeca* should be split into as many as nine separate species (Table 1), most of which were previously considered subspecies. Because this taxonomic inflation is based on a single line of evidence (morphology) and a liberal (diagnostic) species concept, the possibility of premature taxonomic inflation is high.

In contrast to the rampant splitting done by morphologists, molecular studies of Middle Eastern *T. graeca* have emphasized the close genetic similarity among morphologically divergent populations (van der Kuyl et al. 2002, 2005). This pattern of low genetic variation led these authors to explicitly question the recognition of any *Testudo* morphotypes as distinct lineages. In a response to van der Kuyl et al. (2002), Perälä (2004) suggests their result of low genetic variation is biased by the slowly evolving segment of the mitochondrial genome they studied (*rrnS*). Perälä (2004) also noted that the specimens in the study by van der Kuyl et al. (2002) lacked good geographic provenance. This second problem, a paucity of well-documented reference material for genetic studies, is a recurring theme in turtle systematics (Parham et al. 2001; Stuart and Parham 2004; Parham et al. 2004).

The purpose of this study is to provide a genetic test for the newly recognized morphology-based taxonomy in Middle Eastern *T. graeca* using a rapidly evolving molecular marker and specimens with well-documented locality data. By emphasizing specimens from the type locality of newly recognized species, we can provide direct evidence for their genetic distinctiveness. In this way our taxonomic recommendations can consider both the molecular and morphological perspectives. We would consider congruence between the distinctiveness of morphological and molecular groups to be strong evidence that the newly recognized species represent independent evolutionary lineages (e.g., see Stuart and Parham 2004). On the other hand, incongruent molecular and morphological variation or overall low sequence divergence would argue against recognizing newly proposed names pending additional study.

Institutional abbreviations.— CAS, The California Academy of Sciences, San Francisco, California, USA; MVZ, Museum of Vertebrate Zoology, Berkeley, California; ZIN, Zoological Institute of St. Petersburg, Russia.

Materials and Methods

Our study includes DNA sequence data from 34 museum vouchers from nine countries (Appendix 1; Algeria, Armenia, Bulgaria, Georgia, Iran, Israel, Russia, Tunisia, Turkey) with precise locality data. Vouchers are deposited at CAS, MVZ, and ZIN. Detailed locality information is available from these institutions. The following is a list of the voucher information (CAS/MVZ/ZIN) and Genbank (DQ) numbers for 34 tortoise samples (four outgroups, 30 *Testudo graeca*):

Outgroups: *Agrionemys horsfieldii* (CAS 184468/DQ080045); "*Testudo*" *hermanni* (MVZ 238087/EF100728), *Testudo kleinmanni* (MVZ 230361/DQ080048), *Testudo marginata* (MVZ 247484/DQ080047). Ingroup (*Testudo graeca*, numbers refer to Figure 2): 1) Tunisia (MVZ 235707/ DQ080049); 2) Algeria (MVZ 235706/EF100729); 3) Israel (MVZ 247481/EF100730); 4) SE Turkey (CAS 218245/DQ080050); 5) SE Turkey (MVZ 244865/EF100731); 6) S Turkey (CAS 218279/EF100732); 7) S Turkey (CAS 217879/EF100733); 8) E Turkey (CAS 218130/EF100734); 9) S Turkey (CAS 217708/ EF100735); 10) Bulgaria (MVZ 238086/EF100736); 11) Georgia (ZIN 23028/EF100737); 12) Russia (ZIN 23030/EF100738); 13) NW Turkey (CAS 217485/EF100739); 14) NW Turkey (CAS 217676/EF100740); 15) E Iran (MVZ 234282/EF100741); 16) E Iran (MVZ 234284/EF100742); 17) E Iran (MVZ 234285/EF100743); 18) E

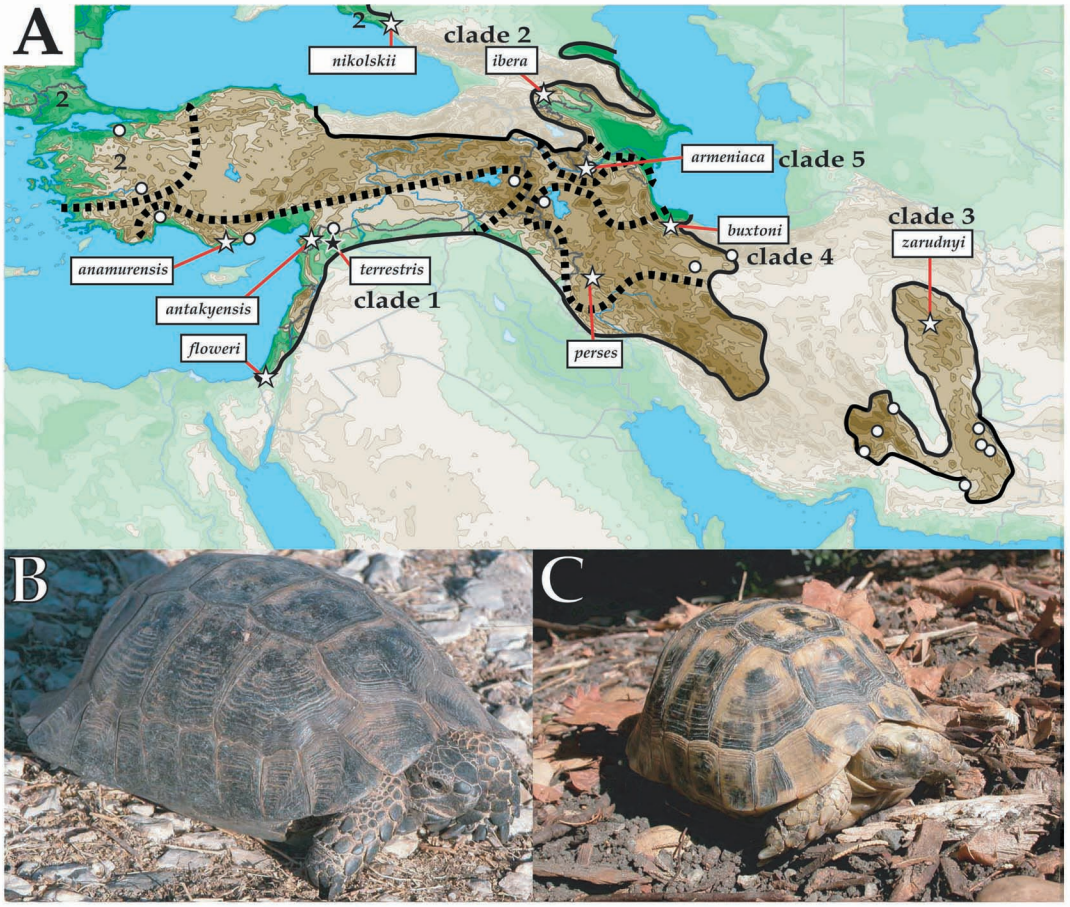


FIGURE 1. A): Map showing localities of sequenced museum specimens (white circles and stars) from the eastern part of the range of *T. graeca*. Stars represent type localities. Sample 10 (from Bulgaria) is not shown. Non-faded areas within the solid black line represent an estimate of the eastern distribution of *T. graeca* in Asia. The dashed black line represents estimates of the haplotype distribution. B-C) An example of significant morphological variation within genetically similar *T. graeca*. Some populations from Turkey are large (>25 cm), brown, and have posteriorly flared shell margins (B, *anamurensis*) whereas nearby populations are characteristically small (<20 cm) and yellowish (C, *antakyensis*). Despite these obvious morphological differences, these populations are just 0.2% divergent for *nad4*.

Iran (MVZ 234291/EF100744); 19) E Iran (MVZ 234292/EF100745); 20) E Iran (MVZ 234509/EF100746); 21) E Iran (MVZ 243423/EF100747); 22) E Iran (MVZ 243879/EF100748); 23) NW Iran (MVZ 245923/EF100749); 24) NW Iran (MVZ 245922/EF100750); 25) NW Iran (MVZ 234290/EF100751); 26) NW Iran (MVZ 236881/EF100752); 27) NW Iran (MVZ 236882/EF100753); 28) NW Iran (MVZ 245921/EF100754); 29) Armenia (ZIN 23026/EF100755); 30) Armenia (ZIN 23025/EF100756). The sequences for five samples (1, 4, *A. horsfieldii*, *T. kleinmanni*, *T. marginata*) were taken from Parham et al. (2006).

The outgroups *A. horsfieldii*, "*Testudo*" *hermanni*, *T. kleinmanni*, and *T. marginata* were chosen based on Parham et al. (2006). Although our study emphasized Middle Eastern *T. graeca*, we sequenced two samples from the African part of their range. One of these samples is from Algeria, within the restricted range of *T. graeca* sensu stricto (see Guyot 2004). We sequenced specimens from the type region (i.e., topotypic specimens) of all nine proposed Middle Eastern species and one nearby taxon from Europe (*nikolskii* from Russia). We can verify that our eastern Iranian spec-

imens are *zarudnyi* because they are identical to a sequence from the type specimen (JFP, BLS, and NB Ananjeva, in prep.). A sample from the 10th type locality (for *terrestris*, Aleppo in Syria) could not be acquired, but we provide a sample from adjacent Turkey (<100 km north; Fig. 1) that is well within the range of *terrestris* fide Perälä (2002:92). Even though we are confident in this referral, we label the latter haplotype as "*terrestris*" with quotes (see Fig. 2). In addition to the above specimens we also include one *T. graeca* from Bulgaria.

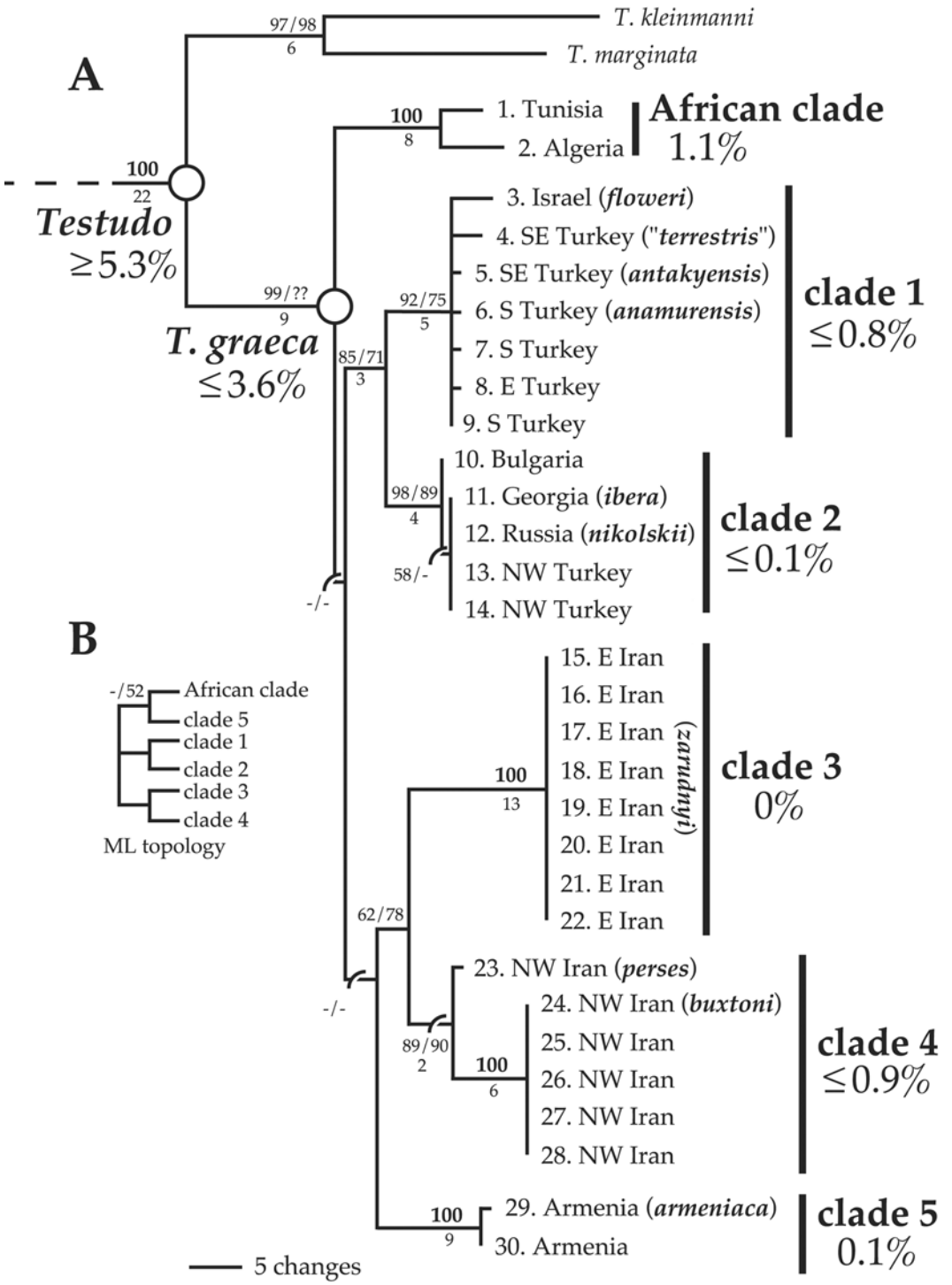
TABLE 1. List of Middle Eastern taxa proposed as new species (e.g., Lapparent de Broin 2001; Perälä 2002; Guyot 2004) also including *T. nikolskii* from adjacent Russia. The taxa are listed in chronological order, starting with the oldest name. The mitochondrial clade (see Fig. 2) is indicated on the right (in bold if the taxon is the oldest available name for that clade). Note that *T. graeca* is not listed because the type locality of *T. graeca* sensu stricto is in Africa (Mertens and Müller 1928).

terrestris	Forsskål 1775	**1**
ibera	Pallas 1814	**2**
zarudnyi	Nikolskii 1896	**3**
buxtoni	Boulenger 1915	**4**
floweri	Boddenheimer 1935	1
anamurensis	Weissinger 1987	1
armeniaca	Chlhikvadze and Bakradze 1991	**5**
nikolskii	Chlhikvadze and Bakradze 1991	2
antakyensis	Perälä 1996	1
perses	Perälä 2002	4

Total genomic DNA was extracted from frozen muscle or liver tissues (Appendix) except for two samples (the outgroup "*Testudo*" *hermanni* and *T. graeca* sample 10) that were donated with tissues preserved in ethanol. To provide a fair test of genetic distinctiveness, we sequenced rapidly evolving regions of the mt genome, *nad4* and adjacent tRNAs (*trn-H-S-L*) (simply "*nad4*" hereon). This marker has proven useful for distinguishing between closely related populations of other turtles (Stuart and Parham 2004; Spinks and Shaffer 2005). Spinks and Shaffer (2005) found that *nad4* shows more genetic structure for closely related populations than the widely used *cob*-based markers. If there is any taxonomically significant genetic structure within *T. graeca*, we feel that it should be recoverable by studying variation and in *nad4*. DNA was amplified with the primers L-ND4-TG (5'-GTAGAGGCCCCAATTGCAG-3') and H-Leu-TG (5'-TGTACTTTTACTTGGAATTGCACCA-3'). The amplifying primers and two internal primers, L-ND4nt-TG (5'- ACCCATACACGAGAACATCTCCT -3') and H-ND4nt-TG (5'- TGTTAACTCTCCTATTAGGTTAAT -3'), were used in the sequencing reactions. The resulting sequences were edited and aligned by eye.

Phylogenies were reconstructed using the maximum parsimony and maximum likelihood optimality criteria implemented in PAUP* 4.0b10 (Swofford 2002). Maximum parsimony analyses were performed with equal weighting of nucleotide substitutions using the branch-and-bound search option. The model of sequence evolution that best described the data was inferred using the Akaike Information Criterion as implemented in Modeltest 3.7 (Posada and Crandall 1998). The selected model was HKY + G, with base frequencies A = 0.3657, C = 0.2630, G = 0.1190, and T = 0.2522, ti/tv ratio = 10.1348, and gamma distribution shape parameter = 0.1963. Maximum likelihood analyses were performed under this model using the heuristic search option, stepwise addition with 500 random addition replicates, and TBR branch swapping. Nodal support was evaluated with 1000 nonparametric bootstrapping pseudoreplications (Felsenstein 1985) for the MP analysis and

FIGURE 2 (right). A. One of the two shortest trees recovered by maximum parsimony. Numbers above the nodes refer to MP and ML bootstraps respectively. Numbers below the nodes refer to decay indices. Sequence divergences within some clades for the *nad4* marker are shown (rounded to the nearest tenth percent) including the minimum divergence among traditional *Testudo* species, the maximum divergence among *T. graeca* populations sampled here, and the maximum divergence within the major haplotype clades. Note that for clades with one or two haplotypes no range of values is implied. All ingroup samples are listed by their voucher number and region of origin with topotypic taxa listed where appropriate. B. The alternative maximum likelihood topology shown as an inset.


A
T. kleinmanni
T. marginata
97/98
6
100
8
1. Tunisia
2. Algeria
African clade
1.1%
100
22
Testudo
≥5.3%
3. Israel (floweri)
4. SE Turkey ("terrestris")
5. SE Turkey (antakyensis)
6. S Turkey (anamurensis)
7. S Turkey
8. E Turkey
9. S Turkey
clade 1
≤0.8%
99/??
9
T. graeca
≤3.6%
92/75
5
85/71
3
10. Bulgaria
98/89
4
11. Georgia (ibera)
12. Russia (nikolskii)
58/-
13. NW Turkey
14. NW Turkey
clade 2
≤0.1%
-/-
B
-/52
African clade
clade 5
clade 1
clade 2
clade 3
clade 4
ML topology
15. E Iran
16. E Iran
17. E Iran
18. E Iran
19. E Iran
20. E Iran
21. E Iran
22. E Iran
(zarudnyi)
100
13
clade 3
0%
62/78
23. NW Iran (perses)
24. NW Iran (buxtoni)
25. NW Iran
26. NW Iran
27. NW Iran
28. NW Iran
-/-
89/90
2
100
6
clade 4
≤0.9%
100
9
29. Armenia (armeniaca)
30. Armenia
clade 5
0.1%
5 changes

500 for the ML analysis. We also obtained decay indices (="branch support'' of Bremer 1994) for all nodes. All reported sequence divergence percentages are uncorrected pairwise distances.

RESULTS

All phylogenetic analyses yielded the same major groupings of closely related haplotypes. *Testudo graeca* is found to be monophyletic with respect to other *Testudo* species (*kleinmanni* and *marginata*). Within *T. graeca* there are six well-supported mt clades, one in Africa and five in the Middle East (clades 1–5 labelled on Fig. 2). Parsimony analyses yield two shortest trees (e.g., Fig. 2A), differing only in the placement of sample 3 (*floweri*) as basal to or else within the polytomy including the rest of clade 1. The ML tree is identical to the figured parsimony tree except that clade 5 (including *armeniaca*) is closer to the African clade in the ML tree (Fig. 2B). Although the basal relationships among *T. graeca* clades are not well supported, the sister relationship between some clades (e.g., 1+2 or 3+4) are supported by both analyses. Clade 1+2 is primarily centered on Turkey whereas our samples for clade 3+4 are wholly Iranian. The geographic intersection of clades 1+2, 3+4, and 5 is at the juncture of the Anatolian and Iranian geomorphic provinces. The genetic divergences within each of the five Middle Eastern clades is extremely low (~1% or less) even though some of these clades include two or more topotypic specimens.

DISCUSSION

Our results support previous reports of low genetic diversity among newly recognized species within *T. graeca* (van der Kuyl et al. 2002, 2005). For example, three clades that include topotypes from two or more newly recognized species (clades 1, 2, 4) show variation of 1% or less. Specimens referable to three of the newly recognized species (*anamurensis*, *antakyensis*, and *terrestris*) have haplotypes that are nearly identical (<0.5% different) with the two most different-looking taxa in this group (*anamurensis* and *antakyensis*; Fig. 1B–C) just 0.2% different. Our samples of the newly recognized species *ibera* and *nikolskii* have identical (0% different) *nad4* haplotypes. All of these examples are considerably less than the minimum distance found between other unambiguously well-established species of testudinoid turtles using the same marker (4.4–8.8%; Feldman and Parham 2004; Stuart and Parham 2004; Parham et al. 2004). Another example of incongruence is the two samples of clade 5 (topotypic *armeniaca* [sample 29] and a nearby sample [sample 30]). Despite occurring within 11 km of one another and sharing a close genetic similarity (0.1%), the phenotypes of these turtles are extremely different. Sample 29 is typical of the diagnostic *armeniaca* morph with a low-domed shell and a rigid plastron (diagnostic characters that distinguish the taxon *armeniaca* from all other *T. graeca*). In contrast, sample 30 is a typical of other *T. graeca* with a high-domed shell and kinetic plastron. Finally, most of the samples of clade 4 are within the range of *perses* (Perälä 2002:91–92; Guyot 2004), but our mtDNA show that these populations have haplotypes that are identical to our sample of topotypic *buxtoni*. Thus, in almost every clade (but see comments on clade 3 below), our data strongly refute the assumption that morphology is an accurate indicator of significant genetic groupings.

How do we explain extreme morphological variation with so little genetic change? The possibility that *T. graeca* populations can evolve diagnostic morphological differences over short time intervals cannot be refuted and would explain our data. In fact, Fritz et al. (2005) provide an excellent example of such rapid environmentally driven adaptive evolution in a close relative of *T. graeca*, *Testudo marginata*. Future studies of species diversity within the *Testudo* should be designed to test for the presence of high morphological plasticity before taxonomic revisions are suggested.

Although genetic diversity within the *T. graeca* samples studied here is low, the rapidly evolving *nad4* marker did recover more detailed structure to the mitochondrial variation of the *T. graeca* complex than did *rrnS* (van der Kuyl et al. 2002, 2005). The borders between these geographically restricted mitochondrial lineages highlight potential contact zones for future systematic study. Further attempts to define species-level taxa within *T. graeca* should be aimed at the distinctiveness of these five clades. The logic is that if clade 1 is not a distinct species from clade 2, then the four named species with clade 2 haplotypes can not be considered valid. Meanwhile, only one of the five mt clades (clade 3, a single haplotype) corresponds to potentially diagnostic lineage (*zarudnyi*) that is geographically isolated from the rest of *T. graeca*. Whether these eastern Iranian populations should be considered a distinct species is debatable, especially since it would render *T. graeca* paraphyletic. Therefore we recommend that the entire *T. graeca* complex be considered as a single species pending further study.

The premature taxonomic inflation of *T. graeca* in the Middle East is similar to the description of many rare Southeast Asian turtles in the late 1990s. Both clades are from poorly studied regions and experienced rapid taxonomic growth based on pioneering morphological studies. In the case of the newly described turtle species from Southeast Asia, subsequent genetic work led to the invalidation of some species (Parham et al. 2001; Spinks et al. 2004; Stuart and Parham, in press) whereas others proved to be distinct evolutionary lineages worthy of recognition (McCord et al. 2000; Engstrom et al. 2002; Stuart and Parham 2004; Parham et al. 2004). Such taxonomic uncertainty is an inevitability because species concepts differ. However, the lessons learned from the *Testudo* and other Asian turtle examples are that systematists can help reduce premature taxonomic changes by withholding recommendations based on a single data set such as mtDNA or morphology.

Conclusions

Molecular data sets can explicitly test taxonomic schemes based on morphology by utilizing topotypic specimens. Our results for *T. graeca* show that the newly proposed species (Table 1, Fig. 2) do not accurately reflect diversity and so we recommend against using the newly proposed names. Nevertheless, we did uncover genetic structure within *T. graeca* that should provide a framework for future investigations. It is conceivable that additional study will revalidate some of newly proposed taxa. In this respect, our overall prospectus for Middle Eastern *T. graeca* is intermediate between the views in that it comprises one or ten species.

Evaluating species-level taxonomies will always be subjective and depend on the species concept or data type employed. But systematists can simplify the situation by implementing species-level taxonomic changes more conservatively. If a minimum criterion for such changes was concordance between two or more independent data sets (mtDNA, morphology, or nuDNA), it would greatly diminish the proliferation of ephemeral taxa and other inappropriate changes to species lists. This is especially important for taxa of conservation concern. We understand that this cautious approach may lead to a temporary underestimation of biodiversity in some cases, but feel that the ultimate taxonomic changes will be more likely to accurately reflect diversity and so gain credibility with conservation agencies and systematists alike.

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Appendix

Detailed localities for sequenced specimens (see Materials and Methods for museum voucher and GenBank numbers). Outgroups: *Agrionemys horsfieldii*, within 2 km of Khiveabad along the Turkmenistan-Iran border, foothills of the Kopet Dag Mts; "*Testudo*" *hermanni*, 7 km north of Kresna; *Testudo marginata*, Mount Hymettus, Attica, Greece. Ingroup: 1) 3 km northwest (by road) El Haoueria, Nabul, Tunisia; 2) Annaba, Skikda Wilayat, Algeria; 3) Near Newe Deqalim, southern Gaza Strip, Israel; 4) Kilis-Gozkaya road, 8 km northwest of Kilis, Turkey; 5) Antakya, Hatay Province, Turkey; 6) Anamur, Icel Province, Turkey; 7) Bolacalikoyuncu, on the east side of Tasuca, Icel Province, Turkey; 8) Coravanis Village, Bostanici Municipality, Van Province, Turkey; 9) 4 km NW of Korkuteli, approximately 70 km west-northwest of Antalya, Antalya Province, Turkey; 10) 8 km north of Kresna, Bulgaria; 11) Tbilisi, Georgia; 12) Black Sea coast, Russia; 13) 9.8 km south of Bursa on the Bursa-Uludag road, Bursa Province, Turkey; 14) Kiziloren, approximately 70 km south-southwest of Afyon, Afyon Province, Turkey; 15) 93 km north-northwest of Khast (by road to Zahedan), Sistan and Baluchistan Province, Iran; 16) Jam Chin Valley, Kuh-e-Taftan, 25 km north (by air) of Khast, Sistan and Baluchistan Province, Iran; 17) Cheshmeh Ziarat, 30 km west (by road) of Zahedan, Sistan and Baluchistan Province, Iran; 18) Desert between Khabr Mountain and Mazr Mountain, 30 km west of Khabr, Kerman Province, Iran; 19) Desert between Khabr Mountain and Mazr Mountain, 30 km west of Khabr, Kerman Province, Iran; 20) Garm-bit, Sistan and Baluchistan Province, Iran; 21) West side of Sirch Tunnel, on the road from the Kerman-Mahan road to Shahdad, Kerman Province, Iran; 22) 36.25 miles south-southwest of Qariat al Arab (by air), Kerman Province, Iran; 23) 5 km (by air) west of Lalabad Village, 40 km (by air) northwest of Kermanshah, Kermanshah Province, Iran; 24) Harzevil ("Big tree") Village in old Manjil, on the road from Qazvin to rasht, Gilan Province, Iran; 25) Lar Dam, Tehran Province, Iran; 26) 15 km south (by road to Dizaj) at junction with Orumiye to Turkey border highway, East Azarbaijan Province; 27) 3 km (by air) south of Buin, which is 55 km south of Qazvin, Zanjan Province, Iran; 28) Harzevil ("Big tree") Village in old Manjil, on the road from Qazvin to Rasht, Gilan Province, Iran; 29) Meghri, Syunik Region, Armenia; 30) 11 km east of Meghri, Shvani Dzor, Armenia.