

# Phylogenetic Relationships among the Species of the Genus *Testudo* (Testudines: Testudinidae) Inferred from Mitochondrial 12S rRNA Gene Sequences

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**To test phylogenetic relationships within the genus *Testudo* (Testudines: Testudinidae), we have sequenced a fragment of the mitochondrial (mt) 12S rRNA gene of 98 tortoise specimens belonging to the genera *Testudo*, *Indotestudo*, and *Geochelone*. Maximum likelihood and neighbor-joining methods identify two main clades of Mediterranean tortoises, one composed of the species *Testudo graeca*, *Testudo marginata*, and *Testudo kleinmanni* and a second of *Testudo hermanni*, *Testudo horsfieldii*, and *Indotestudo elongata*. The first clade, but not the second, was also supported by maximum parsimony analysis. Together with the genus *Geochelone*, a star-like radiation of these clades was suggested, as a sister-group relationship between the two *Testudo* clades could not be confirmed. The intraspecies genetic variation was examined by sequencing the mt 12S rRNA fragment from 28 specimens of *T. graeca* and 49 specimens of *T. hermanni* from various geographic locations. Haplotype diversity was found to be significantly larger in *T. graeca* compared with *T. hermanni*, suggestive of reduced genetic diversity in the latter species, perhaps due to Pleistocene glaciations affecting northern and middle Europe or other sources of lineage reduction. No ancient mt 12S rRNA gene haplotypes were identified in *T. graeca* and/or *T. hermanni* originating from islands in the Mediterranean Sea, suggesting that these islands harbor tortoise populations introduced from the European and African mainland.** © 2002 Elsevier Science (USA)

**Key Words:** Testudinidae; Mediterranean tortoises; 12S rRNA gene; phylogeny.

## INTRODUCTION

Testudinids are found on every continent except Australia and Antarctica. Few species live in the Amer-

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icas at present, but the fossil record is extensive in North America, dating back to the early Eocene (Williams, 1950; Auffenberg, 1974; Bramble, 1982; Ernst and Barbour, 1989a; Alderton, 1993). Tortoise fossils of considerable age have also been found in Africa and Asia (Crumly, 1983). At present, a large diversity, about half of the recognized tortoise species, is found in Africa and the Mediterranean region. Of these, six species are currently recognized in the genus *Testudo*. The spur-thighed tortoise *Testudo graeca* is most prominent in northern Africa, but is also present in southeast Europe and has been introduced at several other locations, including Greece and southern Spain. Four subspecies are recognized, of which *T. g. graeca* of North Africa; *T. g. iberica* of the Balkans, Greece, Turkey, Iran, and Russia; and *T. g. terrestris* of Libya, Israel, Egypt, and Syria are the best described. Little is known about the fourth subspecies, *T. g. zarudnyi*, which is restricted to the Central Iranian Plateau and Afghanistan. A morphological study suggested that the first three subspecies should be elevated to full species level (Gmira, 1993). Furthermore, it was recently hypothesized that *T. graeca* of Algeria is a separate species, *Testudo whitei*, or should even be classified as a separate genus, *Furculachelys whitei* (Highfield and Martin, 1989). Coloration and patterning vary within *T. graeca* subspecies and are not reliable for identification (Lambert, 1995). It has been postulated that the Egyptian tortoise *Testudo kleinmanni* is related to *T. graeca* (Loveridge and Williams, 1957). This very small tortoise species is found in northern Africa (Libya, Egypt, and Israel), where it is severely endangered. Of the European tortoise *T. hermanni*, two subspecies and recognized: *T. h. hermanni*, which is endemic in Italy, France, and Spain, and *T. h. boettgeri* of the Balkans and Greece. Differences in type can easily be observed among *T. hermanni* subspecies (Guyot and Devaux, 1997). *Testudo horsfieldii*, the four-toed or Russian tortoise, sometimes known as *Agrionemys horsfieldii*

(Khozatsky and Mlynarski, 1966), ranges more eastward into central Asia (southeastern Russia, Iran, Afghanistan, and Pakistan). *Testudo marginata* [Greece and probably introduced by man into Italy around 200 BC (Ballasina, 1995)] and *Testudo weissingeri* (Bour, 1996) from Greece are species with restricted habitats and doubtful phylogenetic placement. It has been postulated that *T. weissingeri* is a dwarf form of *T. marginata*, the largest European tortoise species. *Indotestudo elongata*, a species ranging from India to Malaysia, is regarded as being only distantly related to European and African tortoises. Formerly it was included into the genus *Geochelone*, but has now been elevated to full genus level (Ernst and Barbour, 1989b).

Using samples obtained from six tortoise species, including several subspecies, of the genus *Testudo*, and from *I. elongata*, *Geochelone sulcata*, *Geochelone pardalis*, *Geochelone (Chelonoides) carbonaria*, *Geochelone (Chelonoides) denticulata*, *Cuora flavomarginata*, *Emys orbicularis*, and *Trachemys scripta elegans*, we sequenced part of the mitochondrial (mt) 12S rRNA gene to analyze phylogenetic relationships in this subset of the family Testudinidae. This gene has previously been used to elucidate relationships of chelid turtles (Seddon *et al.*, 1997), turtle lineages in general (Shaffer *et al.*, 1997), and of Madagascan tortoises (Caccone *et al.*, 1999a).

## MATERIALS AND METHODS

### *Amplification and Sequencing*

Blood or saliva was obtained from 98 specimens belonging to 16 species or subspecies of tortoises (Testudines: Testudinidae), a yellow-margined box turtle [*Cuora flavomarginata* (Testudines: Bataguridae)], three individuals of the European terrapin [*Emys orbicularis* (Testudines: Emydidae)], and nine individuals of the American red-eared slider turtle [*Trachemys scripta elegans* (Testudines: Emydidae)] (Table 1). DNA was extracted by a procedure using silica and guanidine thiocyanate (Boom *et al.*, 1990). Amplification of approximately 400 nucleotides of the mt 12S rRNA gene was done with the primer set 12S-L01091/12S-H01478 described by Kocher *et al.* (1989). PCR primers were extended with T7 and SP6 promoter sequences, respectively, to facilitate direct sequencing of the PCR product. Sequencing was performed in both directions using a PE-Applied Biosystems 373 automated sequencer, using the Dyanamic direct cycle sequencing kit and the Dyanamic energy transfer dye primer set from Amersham Int. (UK), following the manufacturer's protocols. Sequences used in the analyses were deposited with GenBank (Accession Nos. AF175326–AF175341).

### *Phylogenetic Analysis*

Obtained sequences were aligned with Clustal-W (Thompson *et al.*, 1994), and the alignment (Fig. 1) was checked by eye. Maximum parsimony (MP) analysis was performed with PAUP\* 4.0 (version 4.0.0d55 for Unix) (Swofford, 1998), using a heuristic search with simple step-wise sequence addition and tree bisection reconnection branch swapping (TBR) and saving all optimal trees for subsequent branch-swapping steps (MULPARS). Random addition of sequences did not change tree topology. Gaps were treated as uninformative and excluded from the analysis, as were areas with difficult alignment. ACCTRAN character state optimization was always used, and 10,000 bootstrap replicates were performed. Branches with bootstrap values less than 50% were collapsed.

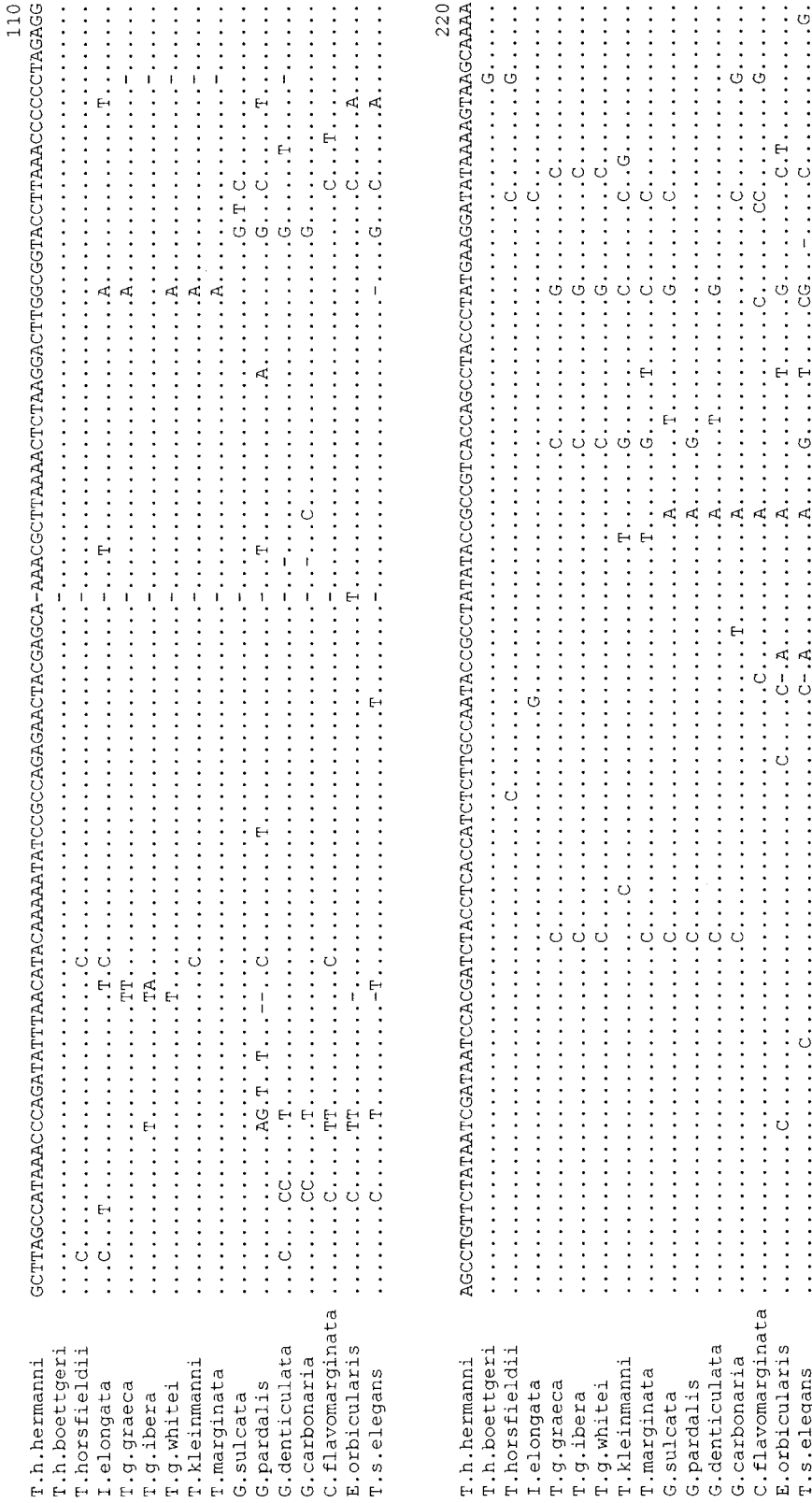
Neighbor-joining (NJ) trees (Saitou and Nei, 1987) of the sequences and reference sequences were constructed using the NJ option in the MEGA package (Kumar *et al.*, 1993). The distance matrix was based upon the two-parameter method of Kimura (1980). In distance analysis, gaps introduced for optimal alignment were treated as additional information and used in pair-wise comparison, except for areas with ambiguous alignment, which were excluded from the analysis. Treating gaps as uninformative did not significantly alter the NJ trees. Finally, the data were analyzed using the maximum-likelihood (ML) method as implemented in PHYLIP (Felsenstein, 1994). The option FASTDNAML version 1.1.1a was used with the transition/transversion ratio set at 2.0.

To calculate divergence times between tortoise clades based upon the 12S rRNA gene, we first checked rate constancy by the method of Takezaki *et al.* (1995). Substitution rates of 0.25%/my (Avise *et al.*, 1992), 1%/my, 1.63%/my (Schubart *et al.*, 1998), and 2%/my were used to date divergence events in tortoises (Table 2).

## RESULTS

### *Phylogenetic Reconstitutions*

The 12S rRNA gene data set consisted of 404 total characters, 282 of which were constant and 71 of which were parsimony informative. MP, ML, and NJ trees for the tortoise 12S data set are shown in Fig. 2. Irrespective of the method used, tortoises always formed a monophyletic clade, as node A is present in all three trees. Also, there is support for the batagurids as their sister group in two of the three trees (Gaffney and Meylan, 1988). Several nodes appear in all trees, e.g., node B (clustering *T. graeca*, *T. kleinmanni*, and *T. marginata*). Differences between the trees will be discussed in more detail below.



**FIG. 1.** Alignment of 16 tortoise and turtle 12S rRNA gene fragments amplified with primers 12S-L01091 and 12S-H01478 (Kocher et al., 1989); numbering of the primers refers to their location in the human mtDNA. The T. h. hermanni and T. h. boettgeri sequences are from animals originating from Italy and Albania, respectively.

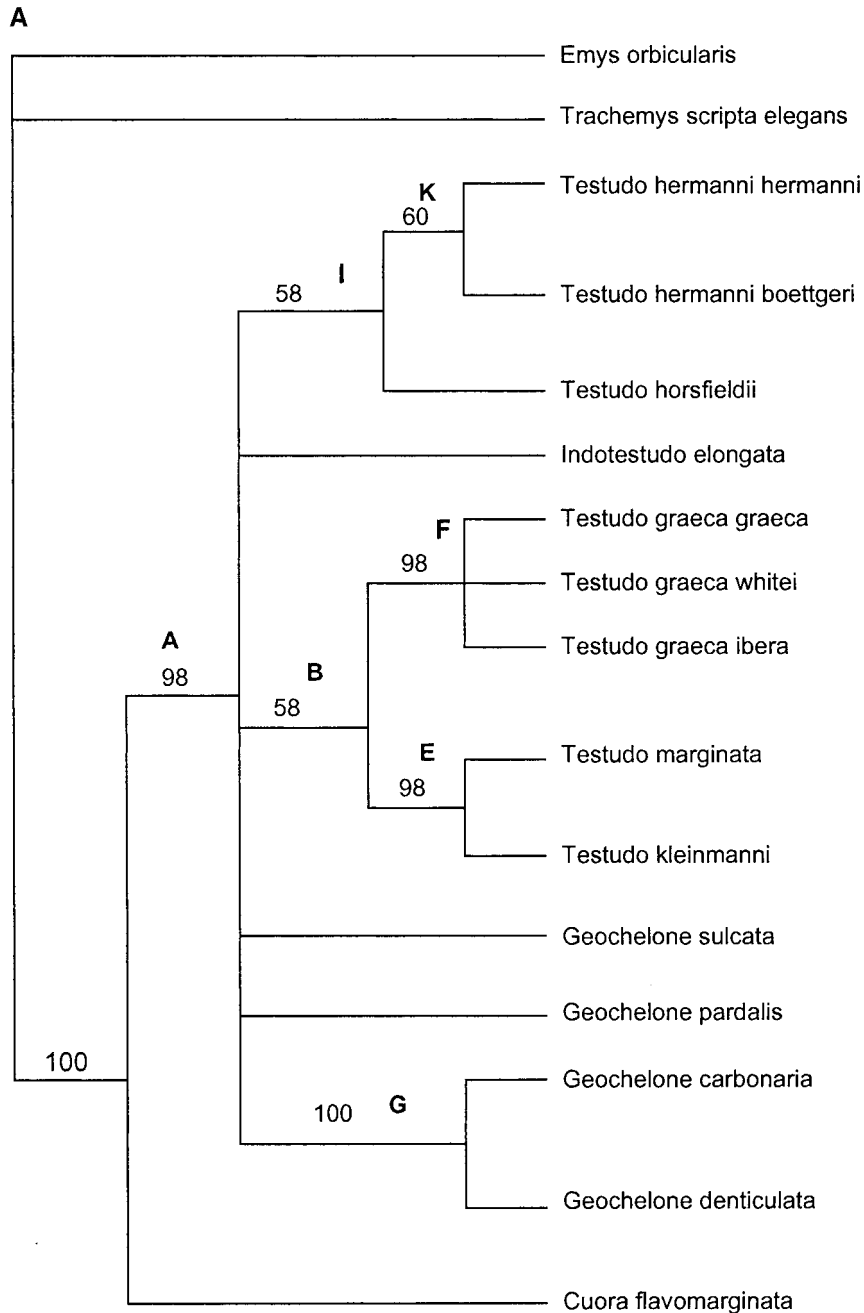
330

TAGCACAAACA-----GCTAACAAAGTCAGGTCAGGTCAGCTAGCTTACTGAGATGGAGAAATGGGCTACATTTTCTAAAC-TAGAAATCAATT-CACGGAAAAG-AACTATGA  
 .C.....G.....T.....-T.....-T.....  
 C.A.A.....A..T.....T.....-T.....-T.....  
 C.T.....A..T.....T.....T.....-T.....-T.....  
 T.....A..T.....T.....T.....-T.....-T.....  
 T.kleinmanni  
 T.marginata  
 G.sulcata  
 G.pardalis  
 G.denticulata  
 G.carbonaria  
 C.flavomarginata  
 E.orbicularis  
 T.s.eiegans

404

AACAAGTCCTATAAGTAGGATTTAGCAGTAATAATAGGGATCAGAAATGCCCAATTTAAGCCCGTCTTAAGGTACGC  
 .....C.....CT...A.....A.....T.....C.....C.....  
 .....T.....A.....T.....C.....C.....  
 .....T...C...C.....A..T.....C.....C.....T  
 .....T...C...C.....A..T.....C.....C.....T  
 .....T...C...C.....A..T.....C.....C.....T  
 .....T...C...C.....T...C.....A..T  
 .....T...C...C.....G.....T...C.....A..T  
 .....T...C...C.....T...C.....C.....C.....  
 .....T...C...C.....A.....C.....C.....C.....C.T  
 .....GA.T.C.C.C.....A.A.....T.T.....T.A.TC...C...  
 .....G.T.C...C.....A.C.....T.....ATTC...C...  
 .....T...C...C.....T.....GCT...G.....G.....G...  
 .....T...A..C.....CT.T.A...GA...G...A.....G...G.T  
 .....T...AGC.....CT...A...GA.....A.....G...G...G...

FIG. 1—Continued



**FIG. 2.** (A) MP tree of tortoise 12S rRNA sequences. The 50% majority-rule consensus tree of 215 equally parsimonious trees, obtained from the heuristic search option in PAUP4\* and rooted using the *Emys orbicularis* sequence [tree length = 225, consistency index (CI) = 0.6000, homoplasy index (HI) = 0.5408, retention index (RI) = 0.5408, rescaled consistency index (RC) = 0.3245] is shown. Values are bootstrap percentages (10,000 replicates were performed). *Cuora* represents the Batagurinae, *Emys* and *Trachemys* represent the Emydinae (B) ML tree of tortoise 12S rRNA sequences. A total of 468 trees were examined, the Ln likelihood of the tree shown = -1630.70. (C) NJ tree of tortoise 12S rRNA sequences. One hundred bootstrap replicates were analyzed and numbers given are bootstrap confidence levels (BCL).

#### Maximum Parsimony

Although the MP tree supports monophyly of the tortoises in this study (node A), it cannot very well resolve relationships within the Testudinidae, except for some species and subspecies (see nodes B, E, G, F, and K, all of which appear in the other trees as well).

Node I, joining *T. horsfieldii* and *T. hermanni*, is present in both MP and NJ trees, but not in the ML tree, which recognizes *I. elongata* as the sister species of *T. horsfieldii*. However, the position of *I. elongata* could also be the result of long branch attraction due to inadequate taxon sampling.

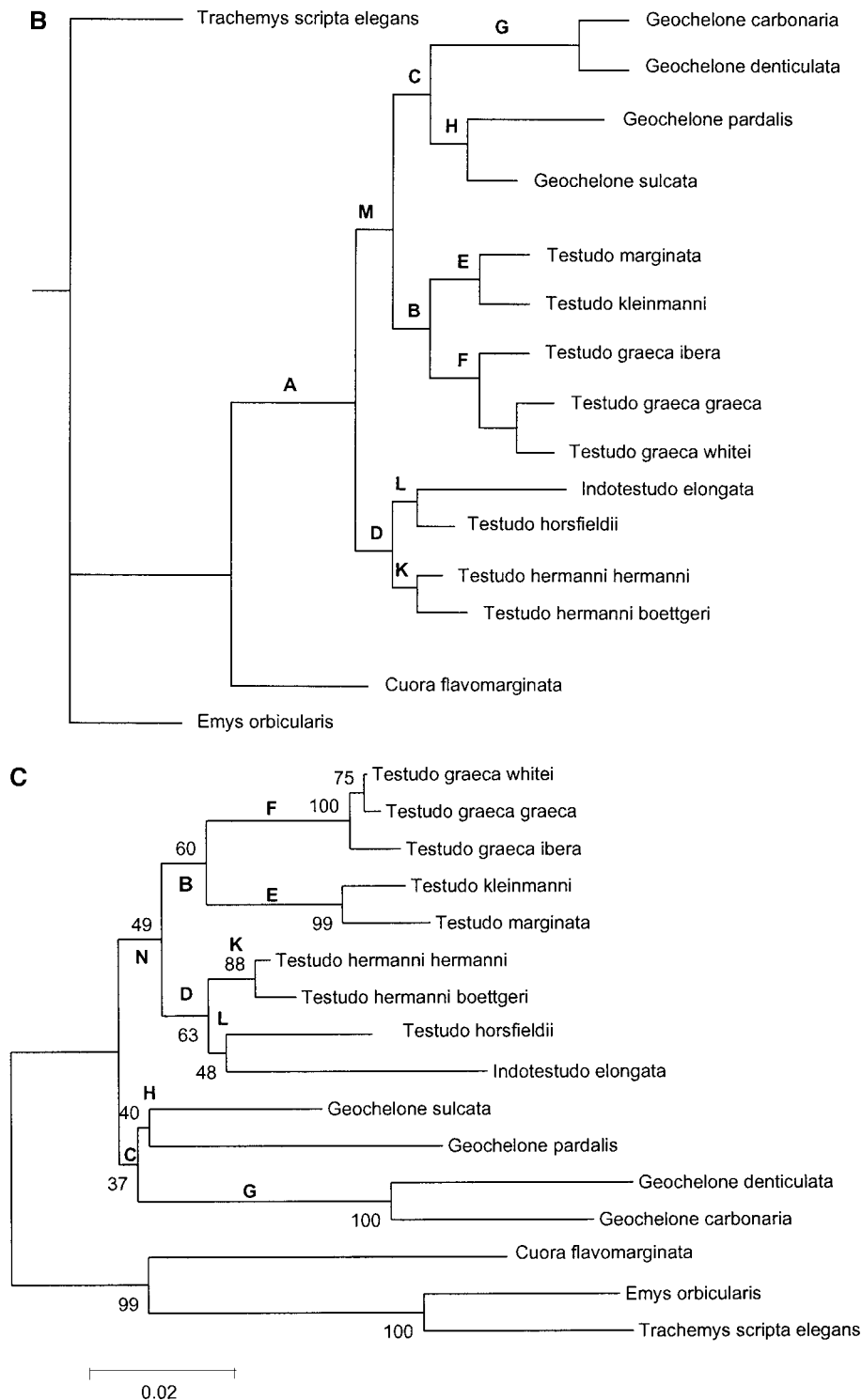


FIG. 2—Continued

*ML and NJ*

The ML and NJ trees display similarity in interclade relationships and will be discussed together. Nodes B, F, E, G, and K from the MP tree are also present in the ML and NJ tree. Both trees resolve relationships

within *Geochelone* and Eurasian *Testudo* in an identical way (nodes C, H, and G and nodes D, L, and K, respectively). The major difference between the trees is the resolution of the three major tortoise clades (node M versus node N). In the ML tree, the African tortoise

species *T. kleinmanni*, the Eurafrikan species *T. graeca*, and the European species *T. marginata* are recognized as the sister clade to the *Geochelone* complex (node M). In contrast, in the NJ tree the Eurasian species of *Testudo* (*T. hermanni*, *T. horsfieldii*) and of *I. elongata* are the sister clade to Eurafrikan *Testudo* (Fig. 2C, node N).

The east Asian species *I. elongata* was consistently included in the European *Testudo* subgroup by both ML and NJ methods, showing an affiliation to the Eurasian species *T. horsfieldii* (node L).

#### 12S rRNA Gene Variation in *Testudo hermanni*

Of the 40 individual *T. h. hermanni* examined, 38 contained an identical 12S haplotype, although they were from different geographic origins (Table 1). A second haplotype, differing by two nucleotides, was found in the two specimens originating from the mainland of France (Provence). All *T. h. hermanni* from the Mediterranean islands of Sardinia, Corsica, or Mallorca possessed a 12S gene haplotype identical to the 29 *T. h. hermanni* from mainland Italy and Spain, suggesting recent introductions. Three 12S haplotypes were found in the eastern subspecies *T. h. boettgeri*, which differed from each other by 1–3 nt. *T. h. boettgeri* from the Pelopponesus (Greece) carried the most divergent 12S haplotype, differing from the other two by one transversion and one (or two) transitions. In summary, two 12S haplotypes were found in 40 animals originating from 18 locations for *T. h. hermanni*, while for *T. h. boettgeri* three haplotypes were present in 9 specimens from 5 locations.

#### 12S rRNA Gene Variation in *Testudo graeca*

The species *T. graeca* ranges in northern Africa from Morocco to the Middle East into Turkey and the Balkans. All *T. graeca* 12S rRNA gene sequences from different locations were found to group into a single clade, suggesting they represent a single species (Fig. 2). However, there was variation in 12S haplotypes from separate geographic locations and in individuals assigned morphologically to different subspecies. Together, a total of 10 12S haplotypes were detected in 28 *T. graeca* specimens originating from nine locations. In *T. g. graeca* from northern Africa, three haplotypes were found, which differed by 1–2 nt transitions/1 nt deletion. Another study also detected one of these 12S haplotypes in eastern Moroccan *T. graeca* (Álvarez *et al.*, 2000). In *T. g. ibera* from Turkey and Bulgaria, three haplotypes were found, differing by a 1-nt transition or a 1-nt transversion at an identical position in the 12S sequence. Sequencing of five specimens representing a proposed new species with distinct morphological features, *T. whitei* (Highfield and Martin, 1989), revealed that they contained four closely related 12S haplotypes. The *whitei* haplotypes (differing from each other by one to three transitions) formed a separate

TABLE 1

### Species, Origin, and Number of Individual Tortoises Sequenced

Species	Origin	No. of individuals
<i>Testudo hermanni hermanni</i>	Spain (mainland: Albera)	4
	Spain: Mallorca	2
	Italy (mainland, 12 regions)	25
	Italy: Sardinia/Sicily	3/2
	France (mainland: Provence)	2
	France: Corsica	2
<i>Testudo hermanni boettgeri</i>	Greece	2
	Greece: Korfou	2
	Greece (Pelopponesus) <sup>a</sup>	2
	Romania/Bulgaria	2
	Albania	1
<i>Testudo horsfieldii</i>	Kazakhstan/Uzbekistan	3
<i>Testudo kleinmanni</i>	Libya	2
<i>Testudo marginata</i>	Italy (Sardinia)	1
	Italy (mainland: Tuscany)	1
<i>Testudo weissingeri</i>	Greece (SW Pelopponesus)	2
<i>Testudo graeca graeca</i>	Tunisia <sup>b</sup>	6
	Morocco	5
	Italy (Sardinia)	2
<i>Testudo graeca ibera</i>	Bulgaria	2
	Turkey	4
	Border Libya/Tunisia	1
<i>Testudo graeca nabeulensis</i> <sup>c</sup>		
<i>Testudo graeca Sardinia</i> <sup>a</sup>	Italy (Sardinia)	3
<i>Testudo (graeca) whitei</i> <sup>d</sup>	Algeria	4
	Morocco	1
<i>Indotestudo elongata</i>	Nepal	4
<i>Geochelone sulcata</i>	Sudan	2
	Mali/Niger	2
<i>Geochelone pardalis</i>	Mozambique	1
<i>Geochelone (chelonoides) carbonaria</i>	Colombia	1
<i>Geochelone (chelonoides) denticulata</i>	South America	2
<i>Cuora flavomarginata</i> (Emydidae: Batagurinae)	Malaysia/China	1
<i>Emys orbicularis</i> (Emydidae: Emydinae)	Tunisia	2
	Unknown	1
<i>Trachemys scripta elegans</i> (Emydidae: Emydinae)	U.S.A.	9

<sup>a</sup> Proposed new subspecies.

<sup>b</sup> Recently described as *Furchulachelys minimaralis* or *Testudo flavominimaralis* (Highfield and Martin, 1990).

<sup>c</sup> Proposed new subspecies or species, *Furchulachelys nabeulensis* (Highfield, 1990).

<sup>d</sup> Proposed new species, formerly *T. graeca*.

sister group to the *T. g. graeca* sequences. Two of our *T. g. whitei* 12S haplotypes have been detected in *T. graeca* from western Morocco (Álvarez *et al.*, 2000). This finding suggests separation at the subspecies

TABLE 2

**Divergence Times of *Testudo* Species Determined Using Four Estimations of the mtDNA Evolution Rate (% Sequence Divergence/my)**

	0.25% (mya)	1% (mya)	1.63% (mya)	2% (mya)
Two main <i>Testudo</i> clades	21.4	5.7	3.3	2.6
<i>T. hermanni</i> / <i>T. horsfieldii</i>	11.3	2.8	1.7	1.4
<i>T. g. graeca</i> / <i>T. g. iberica</i>	2.3	0.6	0.4	0.3
<i>T. kleinmanni</i> / <i>T. marginata</i>	9.0	2.3	1.4	1.1

level, but not at the species level. We named this subspecies tentatively *T. graeca whitei*, awaiting formal classification. Thus, 12S rRNA gene sequences do support the existence of at least three subspecies of *T. graeca* but do not support the claims of the existence of additional *T. graeca* subspecies as suggested in Table 1.

#### Divergence Times

The method of Takezaki *et al.* (1995) was used to check any deviation from linearity in the evolution rate of the tortoise mt 12S sequence. Only *I. elongata* showed an aberrant rate, which can probably be attributed to inadequate taxon sampling, and the sequence was subsequently removed from the data set used to date divergence events in *Testudo*.

Generally, evolution rates for mtDNA are estimated to be in the range of 1–2% sequence divergence per million years. However, Avise *et al.* (1992) estimated turtle mtDNA to evolve at an approximately eightfold slower rate (around 0.25%/my). Schubart *et al.* (1998) calculated a value of 1.63% for the mt 16S rRNA gene of crab species adapted to terrestrial habitats. As both the 16S and the 12S rRNA genes have similar evolution rates, we also applied this latter rate to our tortoise tree (Table 2).

## DISCUSSION

Phylogenetic trees based upon a mt 12S rRNA gene fragment confirmed monophyly of the Testudinidae, regardless of the tree-building method (MP, ML, and NJ). The MP method was more conservative and thus less able to resolve phylogenetic relationships among tortoises. Mediterranean tortoises were grouped together only in the NJ tree, albeit with a low bootstrap value. Monophyly of the genus *Testudo* is further corroborated in this tree by the presence of the genus *Indotestudo*. If the position of *I. elongata* in this tree is due to long branch attraction, *Testudo* could still be monophyletic. However, the ML tree also does not support monophyly of *Testudo*, as the genus *Geochelone* is the sister group of a subclade of *Testudo* (*T. marginata*, *T. graeca*, and *T. kleinmanni*) in this analysis. The

controversy between the methods could possibly be solved by including additional species in the analyses, such as sequences of the tortoise genera *Malacochersus* and *Chersina*. Morphological analysis of *Testudo* does not support monophyly of the genus (Gmira, 1993), in line with the results from the 12S rRNA gene sequencing presented here.

Two clades of *Testudo* were consistently supported by ML plus NJ trees, one consisting of the "northern" species *T. hermanni*, *T. horsfieldii*, and *I. elongata* and another encompassing the "southern" species *T. graeca*, *T. kleinmanni*, and *T. marginata*. Considering the consistent clustering of *T. hermanni* with *T. horsfieldii*, but not with other species of *Testudo*, the earlier suggestion (Khozatsky and Mlynarski, 1966) to include *T. horsfieldii* in a new genus, *Agrionemys*, is supported by our mitochondrial sequence analysis. According to this analysis, *Agrionemys* should include *T. hermanni*, which was also suggested by Gmira (1993). The position in the trees of *I. elongata* can possibly be attributed to the phenomenon of long branch attraction, due to inadequate taxon sampling. However, Ross and Crumly (1983) noted that the distribution of *I. elongata* comes very close to *T. horsfieldii*, which would be predicted by close phyletic affinity. *I. elongata* was originally named *Testudo elongata* (Blyth, 1853).

Interestingly, the smallest (*T. kleinmanni*) and the largest (*T. marginata*) of Mediterranean tortoises were found to cluster together in all analyses. The close affinity between *T. kleinmanni* and *T. marginata* was also observed by Gmira (1993), who suggested a new genus, *Chersus*, for these two species, with *T. graeca* as its sister group, similar to our observations. The Greek peninsular form *T. weissingeri* (Bour, 1996) most likely represents a recent dwarf form of *T. marginata*, since both contain an identical 12S haplotype. Suggestive of this hypothesis is that *T. weissingeri* hatchlings are comparatively large and are the same size as *T. marginata* offspring (D. Ballasina, personal observation). Artner (1996) already questioned the validity of the species status of *T. weissingeri*, as he could detect all of its characteristics in *T. marginata*. He attributed the small size of *T. weissingeri* to the poor feeding conditions in its natural habitat or suggested it to represent at most a subspecies of *T. marginata*.

Extant *T. h. hermanni* may represent a recent radiation from a single Pleistocene refuge, as suggested by the limited 12S rRNA gene variation found. This refuge could have been located in the south of Italy (Sicily), as has been suggested for *E. orbicularis* (Lenk *et al.*, 1998). Molecular data obtained for different taxa, both plant and animal, also indicate that the southern peninsulas of Europe, and the Balkans, acted as major Pleistocene Ice Age refugia (Taberlet *et al.*, 1998; Hewitt, 1999). Taberlet *et al.* (1998), studying postglacial colonization routes, noted that Italian lineages of plants and animals were often isolated due to the pres-



ence of the Alpine barrier. Tortoises of the Balkans had several refuges, which could explain the relatively larger mt haplotype divergence in *T. h. boettgeri* compared with that in *T. h. hermanni* as a founder effect. It is possible that *T. h. hermanni* from southern France represents a different lineage from a second refuge, a finding warranting further research. Animals from Spain contained a 12S haplotype identical to Italian *T. h. hermanni*. It is likely that they were introduced by man, as has been observed for Spanish *E. orbicularis* (Lenk *et al.*, 1998) and for Spanish *T. graeca* (Álvarez *et al.*, 2000).

The method of Takezaki *et al.* (1995) showed that the tortoise 12S gene has evolved in a linear fashion and could thus be used to calculate divergence times. Caccone *et al.* (1999a) showed earlier that tortoise mtDNA, including the rRNA genes, has been evolving linearly. Emergence of *Testudo* species was estimated using different rates of evolution, e.g., "normal rates," varying between 1 and 2%, and an approximately eightfold slower turtle rate determined before (Avise *et al.*, 1992; Bowen *et al.*, 1993). Caccone *et al.* (1999a,b) used a rate of 0.4–0.6%/my for both the mt cyt *b* and 16S rRNA genes in evolution studies of Madagascar and Galápagos tortoises. However, Seddon *et al.* (1998) showed that the evolution rate has not slowed in the turtle mt 12S rRNA gene, compared with the rate in mammals. Weisrock and Janzen (2000), studying mt cytochrome *b* sequences in softshell turtles, also found that the evolution rate of mtDNA is not slower in turtles. When using evolution rates of 1–1.63% (Table 2), the radiation of the two extant *Testudo* clades was found to coincide with worldwide climatic changes. A rate of 1% placed this event at the Miocene/Pliocene border (around 5.6 my). A faster rate of 1.63% would imply that the divergence of *Testudo* clades dated to the mid-Pliocene (4–3 my). Radiation of extant species of the genus *Testudo* was dated to the Middle Pliocene/Pleistocene (3–1.4 my) using these two rates, while recognized subspecies of *Testudo* are much younger and radiated in the late Pleistocene (1.2–0.5 my). Assuming that tortoise mtDNA evolves at only 0.25% sequence divergence/million years (Avise *et al.*, 1992), the emergence of modern *Testudo* clades species is pushed far back into the Miocene, a finding less compatible with the fossil record. In the Pliocene, tortoises resembling *T. graeca* and *T. hermanni* were abundant in Central Europe (Mlynarski, 1962). Mlynarski dates the emergence of *T. cf. hermanni* and *T. cf. graeca* to the Pleistocene, with the modern subspecies arising only in the Holocene, although he recognized the occurrence of primitive tortoises of the genus *Testudo* as early as the Tertiary. However, Crumly (1983) mentions *Testudo* taxa from the Miocene in Asia. Fossils of *T. marginata* resembling modern marginated tortoises have been described from the Pleistocene (Bachmayer *et al.*, 1975).

Our mitochondrial sequences suggested that no ancient tortoise species are autochthonous on any island (all 12S sequences were identical to those of mainland species). On the Italian island of Sardinia, tortoises representing both African and European subspecies of *T. graeca* were detected by sequencing. For centuries, tortoises were used as a source of meat aboard ships (until the invention of tinned food), which could explain how Mediterranean islands became populated with mainland tortoise species.

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