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

Antoinette C. van der Kuyl,*† Donato L. Ph. Ballasina,† John T. Dekker,* Jolanda Maas,* Ronald E. Willemsen,† and Jaap Goudsmit*

*Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands; and †Centro CARAPAX, CP 34, 58024 Massa Marittima (GR), Italy

Received January 4, 2001, revised August 6, 2001

INTRODUCTION

Testudinids are found on every continent except Australia and Antarctica. Few species live in the Americas 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. ibera of the Balkans, Greece, Turkey, Iran, and Russia; and T. g. terestris 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 Dynemic direct cycle sequencing kit and the Dynemic 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 Reconstructions

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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. h. hermanni</td>
<td>GCTTAGGCACTAATAACCCAGATATTTAATATCTCCGTGACAGAATAGCACGCTAAGAGGCTTAAATGAGGG</td>
</tr>
<tr>
<td>T. h. boettgeri</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>T. horsfieldii</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>L. elongata</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>T. g. graeca</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>T. g. iberia</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>T. g. whitiei</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>T. kleinmanni</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>T. marginata</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>G. sulcata</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>G. pardalis</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>G. dentichilata</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>G. carbonaria</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>C. flavomarginata</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>E. orbicularis</td>
<td>..................................................................................................</td>
</tr>
<tr>
<td>T. s. elegans</td>
<td>..................................................................................................</td>
</tr>
</tbody>
</table>

VAN DER KUYL ET AL.
Fig. 1—Continued
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.
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 Eurafri-Can 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 Eurafri-Can 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 Majorca 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 (Alvarez et al., 2000). In T. g. ibera from Turkey and Bulgaria, three haplotypes were found, differing by 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 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 (Alvarez et al., 2000). This finding suggests separation at the subspecies

### TABLE 1

<table>
<thead>
<tr>
<th>Species, Origin, and Number of Individual Tortoises Sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
</tr>
<tr>
<td>Testudo hermanni hermanni</td>
</tr>
<tr>
<td>Testudo hermanni boettgeri</td>
</tr>
<tr>
<td>Testudo horsfieldii</td>
</tr>
<tr>
<td>Testudo kleinnmanni</td>
</tr>
<tr>
<td>Testudo margnata</td>
</tr>
<tr>
<td>Testudo weissingeri</td>
</tr>
<tr>
<td>Testudo graeca graeca</td>
</tr>
<tr>
<td>Testudo graeca ibera</td>
</tr>
<tr>
<td>Testudo graeca nabeulensis</td>
</tr>
<tr>
<td>Testudo graeca Sardinia</td>
</tr>
<tr>
<td>Testudo (graeca) white</td>
</tr>
<tr>
<td>Indotestudo elongata</td>
</tr>
<tr>
<td>Geochelone sulcata</td>
</tr>
<tr>
<td>Geochelone pardalis</td>
</tr>
<tr>
<td>Geochelone carbonaria</td>
</tr>
<tr>
<td>Geochelone (chelonoides) denticulata</td>
</tr>
<tr>
<td>Cuora flavomarginata (Emydidae: Batagurinae)</td>
</tr>
<tr>
<td>Emys orbicularis (Emydidae: Emydinae)</td>
</tr>
<tr>
<td>Trachemys scripta elegans (Emydidae: Emydinae)</td>
</tr>
</tbody>
</table>

* Proposed new species.
* Recently described as Furcululashes minimaralis or Testudo flavominalaralis (Highfield and Martin, 1990).
* Proposed new subspecies or species, Furcululashes nabeulensis (Highfield, 1990).
* Proposed new species, formerly T. graeca.
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 mt12S 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 mt16S 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 mt12S 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 J anzen (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 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., 2000).

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.

ACKNOWLEDGMENTS

The authors thank Lucy Phillips for editorial review, Mr. G. Handrinos of the Greek Forestry Department (Athens, Greece), and Kacem Salaheddine, Karem, Mounir of the Forestry Department and the National Parks of Tunisia.

REFERENCES


