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THE INTER- AND INTRASPECIFIC STATUS OF AEGEAN *MAUREMYS RIVULATA* (CHELONIA, BATAGURIDAE) AS INFERRED BY MITOCHONDRIAL DNA SEQUENCES

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The genus *Mauremys* (Chelonia, Bataguridae) is widely distributed throughout Asia, Europe and NW Africa. Three species (*Mauremys caspica*, *Mauremys rivulata* and *Mauremys leprosa*) are discontinuously distributed around the Mediterranean region. Present distributions are much smaller than those documented within the fossil record of *Mauremys* in the Mediterranean region. All three extant species are identified on the basis of morphology. In the present study we compare partial mitochondrial DNA sequences of cyt-*b* from 16 populations of *Mauremys rivulata* from Greece, one from Jordan (*M. rivulata*), two from Syria (*M. caspica*) and one from Morocco (*M. leprosa*). Comparison of cyt-*b* partial sequences supports the monophyly of the three species considered, as well as their proposed taxonomic status (i.e. separation at the species level). *Mauremys leprosa* is the most differentiated of the three, *M. caspica* and *M. rivulata* being more closely related. Climatic changes during the Pleistocene influenced the distribution of *M. rivulata* and resulted in a latitudinal oscillation of the populations in a north – south direction in Greece, and consequently in a mixing of their genetic material. This hypothesis is confirmed by the absence of correlation between genetic distances and geographical origin of the specimens studied.

Key words: Aegean region, cytochrome *b*, Mediterranean, phylogeography

INTRODUCTION

Mauremys caspica (Gmelin, 1774), *Mauremys rivulata* (Valenciennes, 1833) and *Mauremys leprosa* (Schweigger, 1812) are the sole European representatives of the diverse family Bataguridae. The family consists of 23 genera, distributed in the Palearctic region, with the exception of the genus *Rhinoclemmys*, which is distributed in Central and South America (Pough *et al.*, 1998; Fritz, 2001). The systematic position of the family is very problematic (McDowell, 1964; Gaffney & Meylan, 1988; Pough *et al.*, 1998; Fritz, 2001). It has been traditionally considered to be a subfamily of Emydidae (Hirayama, 1984; Iverson, 1992), or a separate paraphyletic family (Gaffney & Meylan, 1988; Pough *et al.*, 1998), but nowadays the opinion of other authors (Shaffer *et al.*, 1997; Fritz, 2001) - who consider Bataguridae as a separate monophyletic family - prevails, although they all agree that to resolve this issue, additional data from more complete sampling are required. The family Bataguridae is known at least from the Eocene in the Old World and the Nearctic. It is possible that the Paleocene "Emydidae", described from China, actually belong to the Bataguridae family (Fritz, 2001). The genus *Mauremys* is known in the Western Palearctic from the Oligocene (Fritz, 2001), but Melentis (1966) places its origin earlier in the Eocene. Prior to the Pleistocene, it was widely distributed in Europe, North Africa and the Arabian Peninsula.

Until very recently *M. rivulata* and *M. leprosa* were treated as subspecies of *M. caspica*. *Mauremys leprosa* was the first to be raised to species level. According to Fritz (2001), this elevation had been considered by Boulenger (1889) and later by Siebenrock (1909), but was not widely acknowledged until after 1980 (Busack & Ernst, 1980). It was based on morphometric studies as well as on the biochemical studies of Merkle (1975). *Mauremys leprosa* is geographically separated from the other two species by a gap in the present distribution of the genus (Fig. 1). According to Busack & Ernst (1980), the geographic isolation, which led to cessation of gene flow, started in the Pliocene.

Mauremys rivulata was raised to the species level more recently (Fritz & Wischuf, 1997). The authors stated that morphometric features could not separate the two species, but suggested species status on the basis of the colour pattern of the carapace and plastron. Although the two species are separated by geographic barriers (Fritz & Wischuf, 1997) and occupy ecologically different habitats (Busack & Ernst, 1980), a narrow contact zone exists, from which Fritz & Wischuf (1997) report two hybrids.

Apart from the morphological studies (Busack & Ernst, 1980; Fritz & Wischuf, 1997; Tok, 1999), Merkle (1975) examined these species by protein electrophoresis. He examined 17 proteins and found that *M. caspica*

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and *M. rivulata* were identical; *M. leprosa* shared only 13 of these proteins with the other two taxa.

Fritz and Wischuf (1997) noted that the coloration of specimens preserved in ethanol degrades and is not as clear in old animals as it is in young animals. Moreover, turtles of the genus *Mauremys* exhibit great intraspecific variation in colour patterns (Schleich *et al.*, 1996). There are no molecular studies on the Mediterranean *Mauremys* species. We therefore considered that the use of a molecular approach to independently test the present taxonomy would be of great interest. In the present study we investigate the intraspecific relationships of Aegean *M. rivulata* and their relation to the other two Mediterranean *Mauremys* species. We address questions on genetic distances among the species and attempt a reconstruction of their phylogenetic relationships.

We use partial sequences of cytochrome-*b* (cyt-*b*) of the mitochondrial DNA (mtDNA), a gene already used in several similar studies (Lamb *et al.*, 1994; Lamb & Lydeard, 1994; Lenk *et al.*, 1998; Lenk *et al.*, 1999). Mitochondrial DNA is a very useful tool in detecting genetic differences and phylogeographic patterns at the intraspecific level, or in closely related species, due to its non-recombining mode of inheritance, rapid pace of evolution and extensive intraspecific polymorphism (Avise *et al.*, 1987). Mitochondrial DNA evolution rate appears significantly slower in Testudines, relative to other groups of vertebrates (Avise *et al.*, 1992; Lamb *et al.*, 1994; Lenk *et al.*, 1999). Nevertheless, mtDNA can be very informative in cases where morphological data seem to be inconclusive (Lenk *et al.*, 1998), which is the case with *Mauremys* species (Fritz & Wischuf, 1997).

MATERIALS AND METHODS

SAMPLES

Twenty-eight specimens of *Mauremys* were collected from 20 localities (Table 1). Of these localities, 24 contained *M. rivulata*, two contained *M. caspica* and two contained *M. leprosa*. Two published sequences of other Batagurids were used in the analysis database: *Heosemys spinosa* (GenBank U81362, Shaffer *et al.*, 1997) and *Cuora aurocapitata* (GenBank AF043262, Wu *et al.*, unpublished).

Homologous sequences of the emydid turtle *Emys orbicularis* (Linnaeus, 1758) and the tortoise *Testudo marginata* (Schoepff, 1795) were included in the study as outgroups.

TABLE 1. Museum number, exact locality and accession number of each specimen, used in the analysis (code is used in Figs. 1, 3, 4, 5 and Table 2)

Species	Museum number	Locality	Code	Accession number
Mauremys rivulata	NHMC 80.3.15.41	Greece, Crete, Almyros River	1	AF487640
Mauremys rivulata	NHMC 80.3.15.26	Greece, Crete, Almyros River	2	AF487638
Mauremys rivulata	NHMC 80.3.15.16	Greece, Crete, Almyros River	3	AF487639
Mauremys rivulata	NHMC 80.3.15.125	Greece, Crete, Preveli	4	AF487633
Mauremys rivulata	NHMC 80.3.15.71	Greece, Crete, Gavdos	5	AF487637
Mauremys rivulata	NHMC 80.3.15.66	Greece, Crete, Gavdos	6	AF487641
Mauremys rivulata	NHMC 80.3.15.93	Greece, Crete, Krya Vrysi	7	AF487632
Mauremys rivulata	NHMC 80.3.15.45	Greece, Crete, Zakros	8	AF487627
Mauremys rivulata	NHMC 80.3.15.79	Greece, Crete, Georgioupoli	9	AF487629
Mauremys rivulata	NHMC 80.3.15.77	Greece, Crete, Georgioupoli	10	AF487628
Mauremys rivulata	NHMC 80.3.15.75	Greece, Crete, Akrotiri	11	AF487625
Mauremys rivulata	NHMC 80.3.15.128	Greece, Chios isl.	12	AF487630
Mauremys rivulata	NHMC 80.3.15.127	Greece, Chios isl.	13	AF487631
Mauremys rivulata	NHMC 80.3.15.98	Greece, Lesvos isl.	14	AF487636
Mauremys rivulata	NHMC 80.3.15.53	Greece, Kyklades, Naxos isl.	15	AF487634
Mauremys rivulata	NHMC 80.3.15.83	Greece, Dodekanisa, Rodos isl.	16	AF487635
Mauremys rivulata	NHMC 80.3.15.100	Greece, Dodekanisa, Ikaria isl.	17	AF487619
Mauremys rivulata	NHMC 80.3.15.103	Greece, Samos isl.	18	AF487622
Mauremys rivulata	NHMC 80.3.15.104	Greece, Samos isl.	19	AF487620
Mauremys rivulata	NHMC 80.3.15.132	Greece, Dodekanisa, Kos isl.	20	AF487621
Mauremys rivulata	NHMC 80.3.15.135	Greece, Thessalia, Larisa	21	AF487623
Mauremys rivulata	NHMC 80.3.15.136	Greece, Thessalia, Larisa	22	AF487626
Mauremys rivulata	NHMC 80.3.15.137	Greece, Peloponnisos, Kakkavas	23	AF487624
Mauremys rivulata	NHMC 80.3.15.56	Jordan, 25 km south of Jarash	24	AF487642
Mauremys caspica	NHMC 80.3.112.1	Syria, 5 km after As Suwar towards Marqadah	25	AF487644
Mauremys caspica	NHMC 80.3.112.62	Syria, lake Al Asad	26	AF487643
Mauremys leprosa	NHMC 80.3.113.119	Morocco, Qued Tensift	27	AF487645
Mauremys leprosa	NHMC 80.3.113.120	Morocco, Qued Tensift	28	AF487646
Emys orbicularis	NHMC 80.3.16.6	Greece, Thessalia, Larisa	29	AF487648
Emys orbicularis	NHMC 80.3.16.7	Greece, Thessalia, Larisa	30	AF487649
Testudo marginata	NHMC 80.3.22.6	Greece, Kythira isl.	31	AF487647

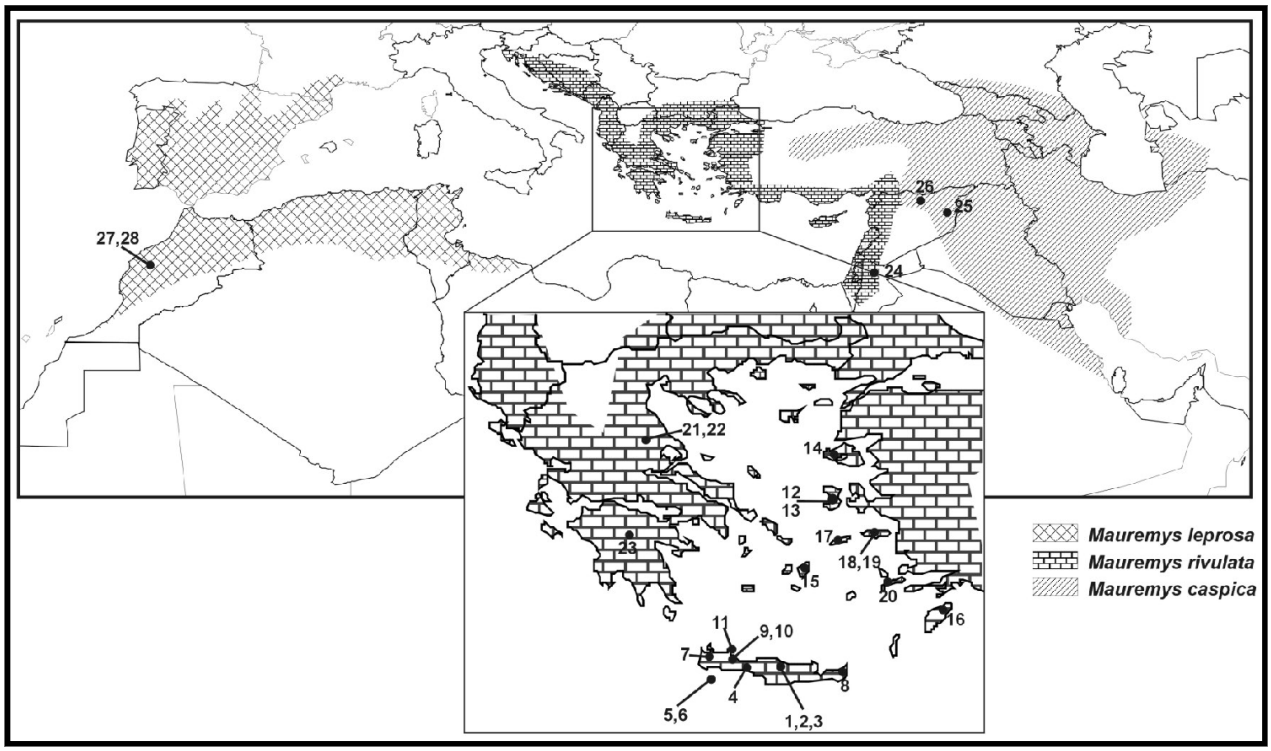

FIG. 1. Present distribution of the three Mediterranean *Mauremys* species and geographic origin of the studied specimens (numbers correspond to the code listed in Table 1).

This material was collected from 1999 to 2001 during several field trips. The geographical origin of the studied specimens is shown in Fig. 1.

Blood samples were obtained from every animal, except from animals with numbers NHMC 80.3.112.62, NHMC 80.3.15.98 and NHMC 80.3.22.6, which died and from which tissue sample was isolated and kept in ethanol (95%). The blood was collected by coccygeal vein puncture as described by Haskell & Porkas (1994), preserved in ethanol and stored at 4°C. After blood sampling the animals were released. Museum numbers were given to each animal that was captured and although they were released the numbers still correspond to the tissue samples taken from each one (Table1).

DNA Extraction, Amplification and Sequencing

Blood samples were first centrifuged at 13 000 rpm for 4 min. Ethanol was removed and samples were left at 37° C for 1 hour. Total genomic DNA was extracted following standard proteinase-k protocol, with standard salt extraction method (Sambrook *et al.*, 1989).

PCR amplification, targeting a segment of the cyt-*b* gene of the mitochondrial genome (mtDNA) was done on all extractions. The universal primers L14724 and H15175 (Palumbi, 1996) were used to amplify a 451 bp fragment of the mitochondrial cyt-*b* gene.

Amplifications were performed in a 10μl total reaction volume, where 1μl of template DNA was mixed with 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 4 pmol of each primer and 0.5 units of Taq Polymerase (GibcoBRL). Thermocycling was then performed in a PTC-100 (MJ-Research) thermocycler. The cycle programme comprised of an initial denaturation step of 2 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, extension for 1 min at 72°C and a final extension at 72°C for 10 min.

Sequencing of double-stranded DNA was performed in both directions in a PE-ABI377 sequencer (using dye-terminator chemistry). The primers in the sequencing reactions were the same as in the amplification procedure.

Sequences Alignment and Phylogenetic Analysis

Multiple sequence alignment was performed using a ClustalX program package (version 1.8: Thompson *et al.*, 1997), using the default parameters, alternative gap opening and gap extension penalties, with minor modifications made by eye.

Pairwise sequence comparisons were made for the cyt-*b* data set using MEGA (v.2, Kumar *et al*., 2001) in order to determine the number, nature, distance and distribution of base substitutions. Genetic distance was estimated using the Kimura two-parameter model (Kimura, 1980).

Evolutionary relationships, which result from DNA sequence data, are reliable only if sites are not saturated by multiple substitutions (Swofford *et al*., 1996). To assess potential saturation of substitutions of the cyt-*b* sequences, the numbers of transitions (Ts) and transversions (Tv) were plotted against the corresponding uncorrected *P*-distances for all pairwise comparisons.

Phylogenetic relationships among specimens were inferred via neighbour-joining (NJ, Saitou & Nei 1987), maximum parsimony (MP, Swofford *et al.*, 1996), and maximum likelihood methods (ML, Felsenstein 1981). NJ trees were implemented by MEGA (v. 2.0, Kumar *et al*., 2001) using Kimura's (1980) two-parameter distance estimate, even though the distance metric used in NJ had no effect on topology. MP and ML trees were constructed using PAUP (Windows Version 4.0b8a, Swofford, 2002).

Nucleotides were used as discrete, unordered characters. The shortest tree was looked for with the branch and bound search. When more than one minimal length tree was found, the strict consensus tree was presented. Confidence estimates were obtained via bootstrapping with 1000 replicates (Felsenstein, 1985).

For maximum likelihood (ML) analysis (Felsestein, 1981), the best fit model of DNA substitution and the parameter estimates used for tree construction were chosen by performing hierarchical likelihood ratio tests (Huelsenbeck & Crandall, 1997) using Modeltest 3.06 (Posada & Crandall, 1998). Heuristic ML searches were performed with 10 replicates of random sequence addition and tree bisection-reconnection (TBR) branch swapping. ML bootstraps employed 1000 iterations. The model parameters (substitution rate matrix, gamma distribution approximation with four rate classes, and empirical nucleotide frequencies) were estimated initially from the starting trees generated by the approach described above (Huelsenbeck & Crandall, 1997). These estimates were used in a ML analysis to produce a tree from which the parameters were then reestimated. In an iterative fashion, these steps were repeated until the ML score converged to its maximum value (Swofford *et al.* 1996).

A minimum spanning network was constructed among *M. rivulata* haplotypes, by using Arlequin v.2000 (Schneider *et al.*, 2000).

Tajima's relative rate test (Tajima, 1993; Nei & Kumar, 2000) was carried out using MEGA (v.2.0) in order to assess differences in rates among separate lineages. Statistical estimation of the validity of the molecular clock hypothesis was performed using the χ^2 test proposed by Fitch (1976). In addition, the maximum likelihood model was used to test the null hypothesis that the sequences were evolving at constant rates and therefore fit a molecular clock (Muse & Weir, 1992). This hypothesis may be tested once we have chosen one of the models of evolution, simply calculating the log likelihood score of the chosen model with the molecular clock enforced and comparing it with the log likelihood previously obtained without enforcing the molecular clock. In this case, the molecular clock is the null hypothesis. The number of degrees of freedom is the number of OTUs – 2. It should be mentioned that in this analysis we used not only the unique haplotypes, but all 33 sequences.

Clock assumptions must be treated cautiously since the differences in mtDNA evolution in higher vertebrate groups have not yet been fully identified, and many studies have shown considerable rate heterogeneity (Hillis *et al.*, 1996). Nevertheless, the use of clock assumptions for closely related taxa is generally considered to be more reliable than for distantly related taxa (Caccone *et al*., 1997), which stems from the premise that rates of evolution of a particular gene are likely to be stable in closely related taxonomic groups, with similar life histories, metabolic rates, and generation times. In this respect, the estimation of ''local'' rates for closely related taxa might be preferable over a ''universal'' rate (Hillis *et al*., 1996). In the present study we use an evolutionary rate suggested for turtle mtDNA (Avise *et al*., 1992; Lamb *et al*., 1994; Lenk *et al.*, 1999), instead of the universal rate.

The published sequence used in the relative rate test as outgroup (one specimen), was that of *Staurotypus triporcatus* (GenBank U81349, Shaffer *et al.*, 1997).

The sequence data from this study were deposited in the GenBank Data library under the accession numbers AF487619–AF487649.

RESULTS

Base Composition

A total of 11 unique haplotypes from the 28 specimens of *Mauremys* were obtained in this study (Table 2), the lengths of which ranged from 365 to 427 bp. Within the cyt-*b* gene of the presented sequences, no insertions, no deletions and no premature stop codons were encountered.

A total of 435 base pairs were aligned, of which 42 sites (9.65%) were variable among the *Mauremys* species (26.66% including outgroups) and 25 (5.75%) were parsimony informative (16.78% including outgroups). Nine (21.43%) of the 42 variable positions represent changes in the first codon position, 8 (19.05%) in the second and 25 (59.52%) in the third.

Mean base composition of the fragment of cyt-*b* of the three codon positions is provided in Table 3. There is a strong bias in base composition (Bias C of Irwin *et al.*, 1991), a feature characteristic of cyt-*b* and other mitochondrial protein-coding genes in mammals and reptiles. This fact supports the authenticity of the mitochondrial sequences (Irwin *et al.*, 1991; McGuire & Heang, 2001; Lenk *et al.*, 2001; Surget-Groba *et al.*, 2001). As expected, the abundance of G's was low (12.9%), whereas the percentages of A, T and C were quite similar (26.9–31.0%). However, a significant compositional bias exists at the second and especially the third codon position. The frequency of guanine at the first position is 21.1%, while a marked under-presentation of guanine was observed at both second (16.2%) and third position (1.8%).

Genetic Divergence and Saturation

Summarized Kimura two-parameter distances between all pairs are given in Table 4. Sequence divergence ranged from 0.24% within *M. rivulata*

TABLE 2. Grouping of selected *Mauremys* samples into 11 unique haplotypes (numbers in parenthesis correspond to the code listed in Table 1)

	Haplotype	Samples	Frequency
M. rivulata			
	h1	Samos (19)	25%
		Akrotiri (11)	
		Kos (20)	
		Gavdos (5)	
		Gavdos (6)	
		Krya Vrysi (7)	
	h2	Ikaria (17)	29.16%
		Samos (18)	
		Larissa (21)	
		Larissa (22)	
		Peloponissos (23)	
		Jordan (24)	
		Preveli (4)	
	h3	Chios (13)	8.33%
		Lesvos (14)	
	h4	Zakros (8)	12.50%
		Georgioupoli (9)	
		Georgioupoli (10)	
	h5	Naxos (15)	8.33%
		Chios (12)	
	h6	Rodos (16)	4.16%
	h7	Almyros (1)	12.50%
		Almyros (2)	
		Almyros (3)	
M. caspica			
	h8	Syria (25)	50%
	h9	Syria (26)	50%
M. leprosa			
	h10	Morocco (27)	50%
	h11	Morocco (28)	50%

TABLE 3. Percentage base composition at first, second and third codon position for all 33 specimens. Compositional bias index (CBI) is calculated as $C=(2/3)\Sigma|c_i - 0.25|$ where C is the compositional bias index and c_i the frequency of *i*th base (Irwin *et al.*, 1991).

Nucleotide	Position			Mean
	First	Second	Third	
A	30.0	21.4	40.8	31.0
T	26.8	37.5	16.6	26.9
C	22.1	24.9	40.8	29.2
G	21.1	16.2	1.8	12.9
CBI	0.091	0.167	0.421	

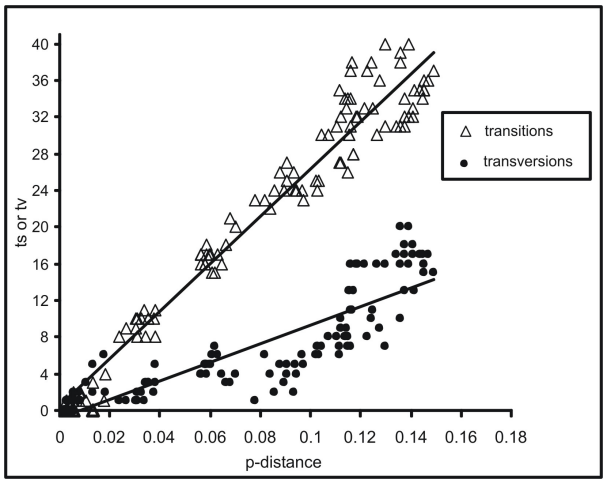

FIG. 2. Relationships between genetic distance, transitions and transversions.

haplotypes to 16.97% between *E. orbicularis* and *M. leprosa*. If we consider only *Mauremys* species, sequence divergence ranged from 0.24% (within *M. rivulata*) to 7.45% between *M. leprosa* and *M. caspica*.

The results of saturation analysis are presented in Fig 2. Both transitions and transversions show an approximately linear relationship with distances, which indicates that saturation has not occurred.

PHYLOGENETIC RELATIONSHIPS

For the phylogenetic analyses, a data set of 16 cyt-*b* sequences (11 unique haplotypes of *Mauremys* spp.,

TABLE 4. Genetic distances (Kimura two-parameter) between the different taxa. In-group sequence divergence is given in diagonal. The range of genetic distances is given in the parentheses.

	M. rivulata	*M. caspica*	*M. leprosa*	*C. aurocapitata*	*H. spinosa*	*T. marginata*	*E. orbicularis*
M. rivulata	0.7						
	(0.24-1.78)						
M. caspica	3.36	1.83					
	(2.45-3.95)						
M. leprosa	6.16	7.14	0.28				
	(5.9-6.66)	(6.83-7.45)					
C. aurocapitata	9.56	8.94	10.49	n/c			
	(8.41-10.26)	(8.81-9.07)	(10.34-10.63)				
H. spinosa	11.49	11.03	12.53	12.91	n/c		
	(10.23-12.37)	(10.56-11.5)	(12.36-12.71)				
T. marginata	12.88	13.06	14.51	15.42	13.91	n/c	
	(12.41-13.89)	(12.36-13.76)	(14.34-14.68)				
E. orbicularis	14.64	14.54	16.23	16.44	12.87	15.47	0.25
	(12.72-16.31)	(12.70-16.63)	(15.49-16.97)				

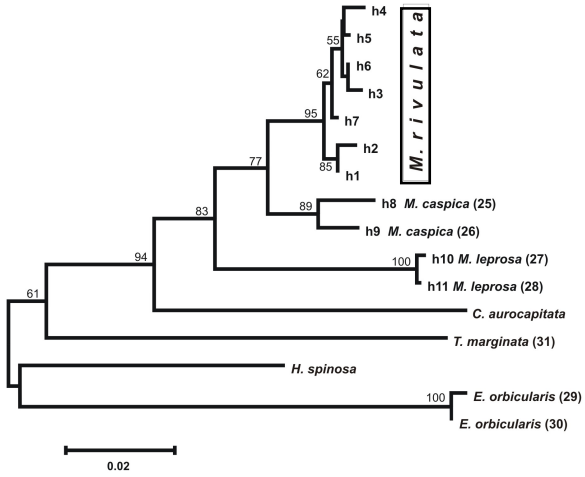

FIG. 3. Phylogenetic analysis of the mitochondrial cyt-*b* gene of *Mauremys*. Tree inferred by the neighbour-joining (NJ) method, based on 1000 replicates.

two of *Emys orbicularis* and one of *Testudo marginata* from this study, two of other Batagurid species from the literature) was used. Tree length distribution, determined from random sampling of 10^6 unweighted trees, was significantly skewed to the left (g1=-0.471), suggesting strong phylogenetic signal in the data (P<0.01; Hillis & Huelsenbeck, 1992).

In the phylogenetic analysis carried out by the neighbour joining method, the resulting tree (Fig. 3), rooted by *E. orbicularis*, showed *Mauremys* species as a monophyletic group (83% bootstrap support, b.s.). Three lineages are evident in the tree: an early offshoot of *M. leprosa* (100% b.s.), followed by *M. caspica* (89% b.s.) and *M. rivulata* (95% b.s.).

The parsimony analysis of the 16 cyt-*b* sequences, using *E. orbicularis* as outgroup, resulted in 176 equally parsimonious trees of 170 steps (consistency index CI=0.788 and homoplasy index HI=0.212), the strict consensus of which is shown (with bootstrap values) in Fig 4. The topology of this tree is similar to the NJ tree, regarding the main clades.

For the maximum likelihood analysis, likelihood ratio tests indicated that the Tamura-Nei model with general time reversible option was the most appropriate for subsequent ML analyses (Table 5). The phylogeny recovered by the ML analysis was similar to that recovered by the MP and NJ analysis and is illustrated in Fig. 5. One ML tree was identified (-ln$L$=1383.01; final parameters estimates: base frequencies A=0.30, C=0.30, G=0.14, T=0.26, a=0.3159, P_{inv}=0.000, and A/G=3.59, C/T=11.04).

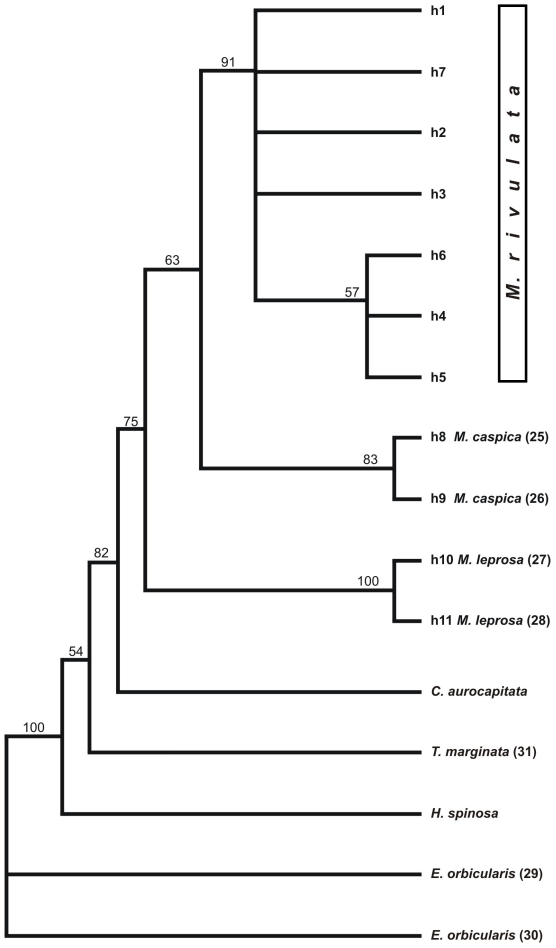

FIG. 4. A Maximum Parsimony (MP) tree derived from cyt-*b* sequences of *Mauremys* species. The strict consensus of the 176 equally most parsimonious trees is presented. Probability percentages of bootstrap replicates (1000) supporting each branching pattern are given beside the corresponding nodes (numbers correspond to the code listed in Table 1).

Geographic distribution of *M. rivulata* haplotypes is shown in Fig. 6. The minimum spanning network among *M. rivulata* haplotypes is presented in Fig.7.

TABLE 5. Test of hypotheses relating to the model of evolution appropriate for phylogeny reconstruction (Huelsenbeck & Crandall, 1997). *P*- values were obtained with Modeltest (Posada & Crandall, 1998).

Null hypothesis	Models compared	-lnL_0	-lnL_1	df	*P*
Equal nucletide frequencies	H_0:JC69, H_1:F81	1513.1719	1481.9972	3	0.0000
Equal Ti and Tv rates	H_0:81, H_1:HKY85	1481.9972	1425.8398	1	0.0000
Equal Ti rates	H_0:HKY85, H_1:TrN	1425.8398	1417.3188	1	0.0000
Equal Tv rates	H_0:TrN, H_1:TIM	1417.3188	1416.5848	1	0.2257
Equal rates among sites	H_0:TrN, H_1:TrN+G	1417.3189	1387.3484	1	0.0000
Proportion of invariable sites	H_0:TrN+G, H_1:TrN+I+G	1387.3484	1387.2443	1	0.3241

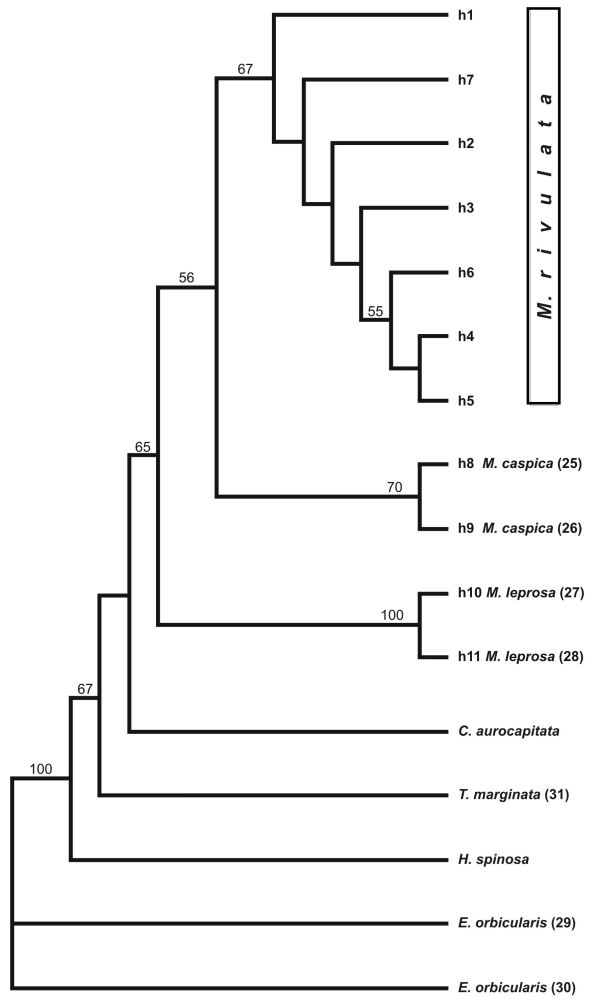

FIG. 5. A Maximum Likelihood (ML) tree derived from cyt-*b* sequences of *Mauremys* species. Probability percentages of bootstrap replicates supporting each branching pattern are given beside the corresponding nodes (numbers correspond to the code listed in Table 1). Bootstrap values >50% are shown.

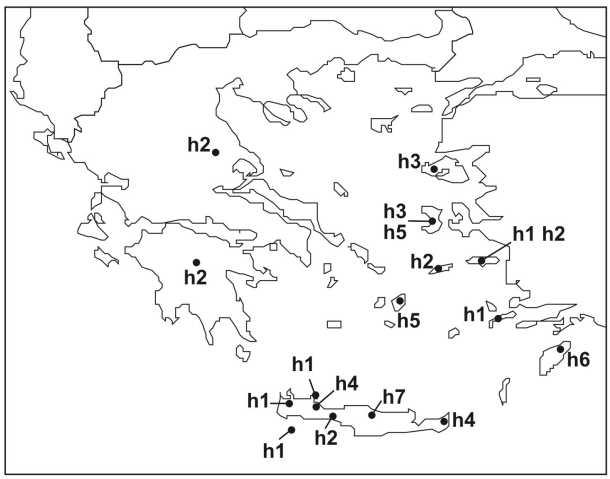

FIG. 6. Geographic distribution of *M. rivulata* haplotypes. Note the absence of geographic patterns.

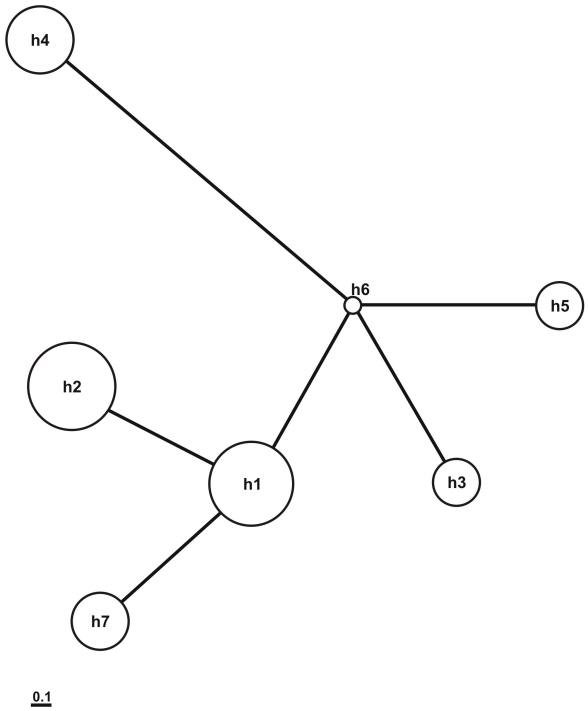

FIG. 7. Minimum spanning network for the 7 *M. rivulata* haplotypes. Size of each circle is proportional to the frequency of the haplotypes.

TABLE 6. Estimated splitting time between clades. The splitting time is estimated using a divergence rate of 0.3%–0.4% per Myr. A: *M. rivulata* clade, B: *M. caspica* clade, C: *M. leprosa* clade.

Clades	Da	Splitting Time	
		Rate: 0.3% per Myr	Rate: 0.4% per Myr
(A&B) vs. C	5.88%	19.6 Myr ago	14.7 Myr ago
A vs. B	2.1%	7 Myr ago	5.3 Myr ago

Tajima's relative rate test was carried out for many different pair-combinations of the examined clades and resulted that all these clades evolve with the same rate (χ^2 <3.84, *P*>0,05). The likelihood ratio test was employed to investigate the rate of homogeneity for the analysed species (Huelsenbeck & Crandall, 1997). Because the simpler (clocklike) tree cannot be rejected at a significance level of 5% (LRT=17.66, df=31, $\chi_{critical}$=19.28), we do not reject the application of a molecular clock to the species used in the analysis.

Since our results are compatible with the molecular clock hypothesis, we can use the suggested evolving rate for mtDNA of Emydidae (0.3% -0.4% per Myr) (Lenk *et al.*, 1999). The resulting estimated splitting times between clades are summarized in Table 6.

DISCUSSION

PHYLOGENETIC RELATIONSHIPS

Our results indicate that *M. rivulata*, *M. caspica* and *M. leprosa* are indeed genetically isolated taxa. These three taxa constitute a monophyletic group, which splits

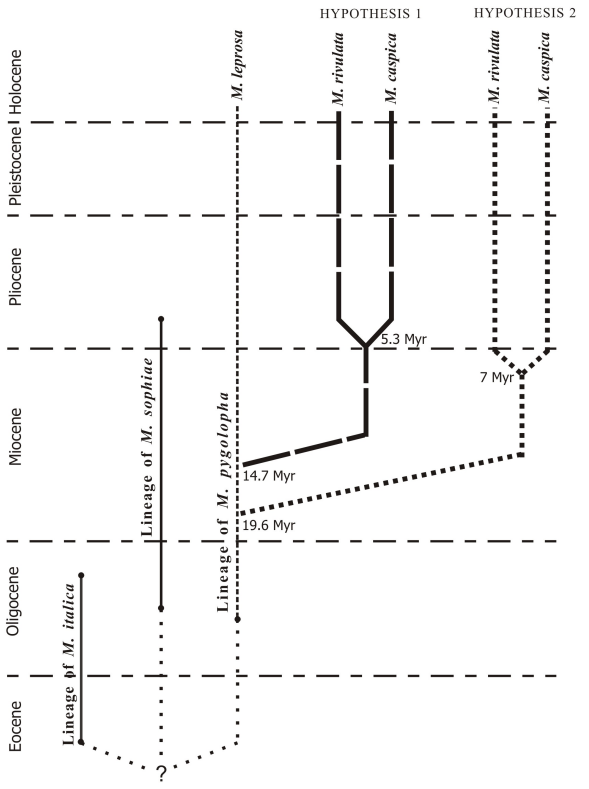

FIG. 8. Alternative hypothesis on the phylogenetic relationships of the three species in question. By applying mtDNA evolutionary rate of 0.4% per Myr or of 0.3% per Myr, we result in hypothesis 1 or hypothesis 2, respectively.

into two genealogical lineages. The first corresponds to the lineage leading to *M. leprosa*, whereas the second corresponds respectively to the lineage from which the *caspica-rivulata* group emerged. The very small within-group divergence of *M. rivulata* and the multifold between-group divergence (3.36% between *M. caspica* and *M. rivulata*) support the elevation of *M. rivulata* to the species level, as suggested by Fritz & Wischuf (1997), although further sampling in the area of contact is necessary to fully resolve this issue.

The elevation of *M. leprosa*, which was based on electrophoretic and morphometric data (Merkle, 1975; Busack & Ernst, 1980), is further supported from our results.

Specimens of *M. rivulata* (from the Middle East to Greece) cluster with a small intraspecific differentiation ranging from 0.24% to 1.78% (mean=0.7%). This divergence is not related to the geographic origin of the specimens (see Fig. 6, Fig. 7 and Appendix 1). The small intraspecific divergence observed in *M. rivulata*, is comparable to results reported by Lenk *et al.* (1999) for *Emys orbicularis*. The authors compared a great number of populations from Europe and N. Africa, which split to seven groups of haplotypes without being separated geographically.

In general, phylogeographic patterns are considered to be the result of a multifactorial process, which is somewhat arbitrary and variable among different species (Taberlet *et al.*, 1998). The combination of existing paleontological data with the results we present, permits a preliminary interpretation of the phylogeographic pattern of *M. rivulata* in the Eastern Mediterranean and especially in the area of the Aegean Sea.

According to Lenk *et al.* (1999), the climate change in Europe 3.2 Myr ago triggered a sudden radiation of *Emys orbicularis*. During the climatic oscillations of the Pleistocene, the range of *E. orbicularis* probably fragmented recurrently, with isolated populations along a slender belt throughout southern Europe. This belt has been shaped by cold climates to the north and by barriers of inappropriate habitat to the south. Thus, southern Italy and Greece served as refugia for the populations of *E. orbicularis*.

Kotsakis (1980) claimed that the lower Pleistocene glaciations, which provoked the southward shift of distribution of *E. orbicularis* in Italy, drove *Mauremys* populations to extinction in the peninsula.

Thereby, the main factor that influenced the present distribution of *Mauremys* in the Mediterranean region, and particularly the distribution of *M. rivulata* in the Balkans, is climatic change, which prevailed during the glacial periods of the Pleistocene. The cold periods resulted in the latitudinal shift of *Mauremys* populations in the Italian Peninsula towards the south, and ultimately led to their extinction. Something similar occurred in the Balkan Peninsula with *M. rivulata*, resulting in a latitudinal shift of the populations towards the south and east, but it did not result in extinction since they found refuges on the coast of Asia Minor and in the Aegean islands.

Consequently, the shifting and rearrangements of *M. rivulata* populations led to their mixing and the probable establishment of new populations on islands, where they did not exist before and where populations of *E. orbicularis* were not favoured due to ecological factors. According to Lenk *et al.* (1999), *E. orbicularis* is more favoured by continental climate conditions.

On the other hand, the genetic distances observed among *M.. rivulata* haplotypes, and their relationships based on minimum spanning network, are not related to respective geographic distances. This fact cannot be explained by the vicariance approach of the distributional pattern of *M. rivulata*, but can be better explained by a dispersal model. To propose a dispersal model we need to have evidence that *M. rivulata* is easily dispersed.

According to Lenk *et al.* (1999), marine straits represent no absolute barriers for *E. orbicularis* and coastal corridors could have promoted genetic exchange. This is probably also the case for *M. rivulata* since this species inhabits also brackish waters (Gasith & Sidis, 1983; Sidis & Gasith, 1985; Engelmann *et al.*, 1993). Consequently, the easily accessible marine straits, which appeared in the Aegean area repeatedly during Pliocene and Pleistocene, and the great dispersal capacity of *M. rivulata* through coastal corridors, are supporting the dispersal model. The extant populations of *M. rivulata* are probably still under the influence of this dispersal procedure.

PHYLOGENETIC RELATIONS INFERRED BY PALAEONTOLOGICAL DATA

Bergounioux (1955) suggested that there are three parallel evolutionary lines in the Western Palearctic turtles of the genus *Mauremys* (Fig. 8): (1) the lineage of *Mauremys italica*, which starts in the Eocene with the species *M. italica* and *M. vidali* and ends in the Oligocene with *M. chainei*; (2) the lineage of *Mauremys sophiae*, which consists of *M. batalleri* from the Oligocene, goes on in the Miocene with *M. batalleri* and *M. sophiae* and ends in the Pliocene with *M. sophiae*; (3) the lineage of *M. pygolopha*, which starts in the Oligocene, with *M. subpyrenaica*, continues in the Miocene with *M. rotundiformis* and *M. pygolopha* and in the Pliocene with *M. romani* and *M. gaudryi.* Broin (1977) suggests that *M. romani* and *M. gaudryi* are synonymous. According to Melentis (1966), *M. steinheimensis* from the Miocene belongs to this lineage as well. Among these we can also place *M. sarmatica* from the Miocene, which is supposed to be the direct ancestor of *M. gaudryi* from the Pliocene (Kotsakis, 1980). The fossils considered most akin to the extant *Mauremys* species belong to this third lineage (Melentis, 1966; Kotsakis, 1980).

Combining our results with the available paleontological data allows us to suggest a hypothesis on the phylogenetic relationships of the three species in question. The three species share a common ancestor, which is situated in the lineage of *M. pygolopha*. The first to separate from this lineage is the branch that led to the extant *M. leprosa* with the intermediate form of *M. gaudri* (Broin, 1977; Kotsakis, 1980). When applying the above mentioned evolutionary rate (0.3%-0.4% per Myr) to our data, the common ancestor of the two main genealogical lines dates to the Lower Miocene/Middle Miocene (from 19.8 Myr ago to 14.9 Myr ago; Fig. 8). This contradicts Busack & Ernst's (1980) suggestion that this isolation started during the Pliocene. The information on the second branch, which led to *M. caspica* and *M. rivulata*, is very restricted. The only hypothesis we may initially support is that the two species have shared a long common history. The common ancestor of the genealogical lines, which led to *M. rivulata* and *M. caspica*, is dated in the Upper Miocene/Lower Pliocene (from 7.2 Myr ago to 5.4 Myr ago). Because the lack of relevant paleontological data and geological events inhibit making a more accurate dating, further study is necessary, which will have to include more specimens of the three Mediterranean *Mauremys* species.

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APPENDIX 1

Genetic distances (Kimura two-parameter) between the *Mauremys* haplotypes and other taxa used in the analysis. *M.r.*, *M. rivulata*; *M.c.*, *M. caspica*; *M.l.*, *M. leprosa*; *C.a., C. aurocapitata; H.s., H. spinosa*; *T.m., T. marginata*; *E.o., E. orbicularis.*

	h1 (*M.r.*)	h2 (*M.r.*)	h3 (*M.r.*)	h4 (*M.r.*)	h5 (*M.r.*)	h6 (*M.r.*)	h7 (*M.r.*)	h8 (*M.c.*)	h9 (*M.c.*)	h10 (*M.l.*)	h11 (*M.c.*)	*C.a.*	*H.s.*	*T.m.*	*E.o.* (30)
h1 (*M.r.*)															
h2 (*M.r.*)	0.24														
h3 (*M.r.*)	1.31	1.78													
h4 (*M.r.*)	0.84	1.35	0.79												
h5 (*M.r.*)	0.55	1.07	0.53	0.53											
h6 (*M.r.*)	0.28	0.80	0.26	0.53	0.26										
h7 (*M.r.*)	0.27	0.79	0.52	0.80	0.53	0.53									
h8 (*M.c.*)	3.54	3.54	3.28	3.37	3.07	3.06	3.83								
h9 (*M.c.*)	2.39	2.66	3.79	3.73	3.40	3.08	3.02	1.81							
h10 *(M.l.)*	6.07	6.16	6.04	5.77	5.79	5.79	6.32	7.00	6.45						
h11 (*M.l.*)	5.62	5.98	5.87	5.61	5.61	5.61	5.87	6.80	6.60	0.28					
C. a.	8.76	9.04	9.03	9.33	8.94	8.58	7.77	8.39	8.19	9.72	9.46				
H. s.	10.73	11.02	11.21	10.33	10.26	10.23	9.39	9.68	10.45	11.46	11.15	11.58			
T. m.	11.62	11.64	12.40	11.48	11.41	11.38	11.17	12.26	11.20	12.75	12.95	13.6	12.46		
E. o. (30)	11.58	11.82	13.56	14.08	13.73	13.41	12.64	14.33	11.53	13.74	14.53	14.53	11.68	13.58	
E. o. (29)	11.88	12.13	13.90	14.45	14.08	13.76	12.98	14.68	11.53	14.12	14.90	14.53	11.68	13.90	0.25