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Phylogeny of the Eurasian freshwater turtles of the genus *Mauremys* Gray 1869 (Testudines), with special reference to a close affinity of *Mauremys japonica* with *Chinemys reevesii*

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Abstract

Phylogenetic relationships of the freshwater turtles of the genus *Mauremys* and representatives of several other batagurid genera were inferred from variations in 863 base positions of mitochondrial 12S and 16S rRNA genes. Results strongly suggested the non-monophyly of *Mauremys* by indicating the closest affinity of *Mauremys japonica* with *Chinemys reevesii*, the type species of the genus *Chinemys*. Recent morphological analyses of the batagurid genera suggested that *Mauremys* is a basal stock of the subfamily Geoemydinae, whereas *Chinemys* is a member of the subfamily Batagurinae as supported by several putative synapomorphs with other batagurine genera. It is thus probable that the morphological character states used to define *Mauremys* actually represent symplesiomorphy, and that morphological character states shared between *Chinemys* and other batagurine genera have resulted from convergence. Also, our results did not support a sister-group relationship between *Mauremys annamensis* and *Mauremys mutica*, which has been implicitly or explicitly assumed by a number of previous authors on the basis of morphological data. Instead, *M. annamensis* was indicated to be closest to *Mauremys iversoni*, a species assumed to be most divergent among the East Asian *Mauremys* by previous authors.

Key words: Phylogeny – Biogeography – Bataguridae – *Mauremys* – *Chinemys*

Introduction

Mauremys is a batagurid geoemydine genus characterized by a maxillo-vomer separation and an almost consistent exclusion of the foramen praepalatium from the vomer (Hirayama 1984). For this genus, the following seven species of freshwater turtles are generally recognized – *Mauremys leprosa* from south-western Europe and northern Africa, *Mauremys caspica* from the eastern Mediterranean to Iran, *Mauremys annamensis* from central Vietnam, *Mauremys iversoni* from eastern China, *Mauremys japonica* from the main-islands of Japan, *Mauremys mutica* from Vietnam, southern and eastern China (including Hainan Island and Taiwan) and the southern Ryukyus, and *Mauremys pritchardi* from Myanmar (Pritchard and McCord 1991; Iverson 1992; Iverson and McCord 1994; Yasukawa et al. 1996; McCord 1997) (Fig. 1). Of these, *M. leprosa* was formerly regarded as a subspecies of *M. caspica* (Busack and Ernst 1980), and *M. annamensis* as constituting a distinct monotypic genus *Annamemys* (Ernst and Barbour 1989; Iverson 1992; David 1994).

On the basis of skull characters and some other morphological characters, McDowell (1964), revised the generic classification of the family Emydidae at that date (i.e. Emydidae plus Bataguridae in the currently prevailing classification of Testudines: Gaffney and Meylan 1988). He demonstrated a distinct morphological divergence of the Eurasian species recognized as being of the emydine genus *Clemmys* then, i.e. *Clemmys caspica*, *Clemmys japonica*, *Clemmys mutica*, and *Clemmys bealei*, from the North American congeners, reassigning the first three and the fourth species to the resurrected batagurine genera *Mauremys* and *Sacalia*, respectively (McDowell 1964).

Since then, most efforts on the systematics of the genus *Mauremys* have been devoted to its specific and subspecific classification (Busack and Ernst 1980; Iverson and McCord 1989; Pritchard and McCord 1991; Yasukawa et al. 1996; McCord 1997), or to its relationships with other genera (Hirayama 1984; Sites et al. 1984; Wu et al. 1999). Only Iverson and McCord (1994) addressed problems relevant to the infrageneric phylogeny of *Mauremys*, and, on the basis of phenetic analysis of morphological characters of the East Asian species, hypothesized the relationships within the genus as: (Mediterranean–West Asian taxa (*M. iversoni* (*M. japonica* (*M. annamensis*, *M. mutica*)))). However, it is argued that such an approach seriously suffers as for the inference of interspecific phylogeny (e.g. Honda et al. 1999a). Furthermore, no attempts have been made to examine the monophyly of *Mauremys* in the current taxonomic arrangement (see above).

Analysis of sequence variations in mitochondrial 12S and 16S ribosomal RNA (rRNA) genes is known to be effective for the estimation of phylogenetic relationships among reptile species and genera (Honda et al. 2000a, b). We thus partially sequenced these genes for five species of the genus *Mauremys* and representatives of several other batagurid genera, and analysed the resultant data phylogenetically. Our purposes were: (1) to examine the monophyly of *Mauremys*, and (2) to test the phylogenetic hypothesis derived from the morphological analysis.

Materials and methods

Tissues were obtained from the four East Asian (*M. annamensis*, *M. iversoni*, *M. japonica*, and *M. mutica*) and one Mediterranean–West Asian species of *Mauremys* (*M. caspica*). We could not examine the other two recognized species of the genus, *M. leprosa* and *M. pritchardi*, but their absence from materials analysed may not have brought any substantial noise to the results of the analyses, because the sister-group relationships of *M. leprosa* with *M. caspica* (see Busack and Ernst 1980; Hirayama 1984), and of *M. pritchardi* with *M. mutica* seem to deserve little doubt (McCord 1997; Yasukawa, unpublished observation).

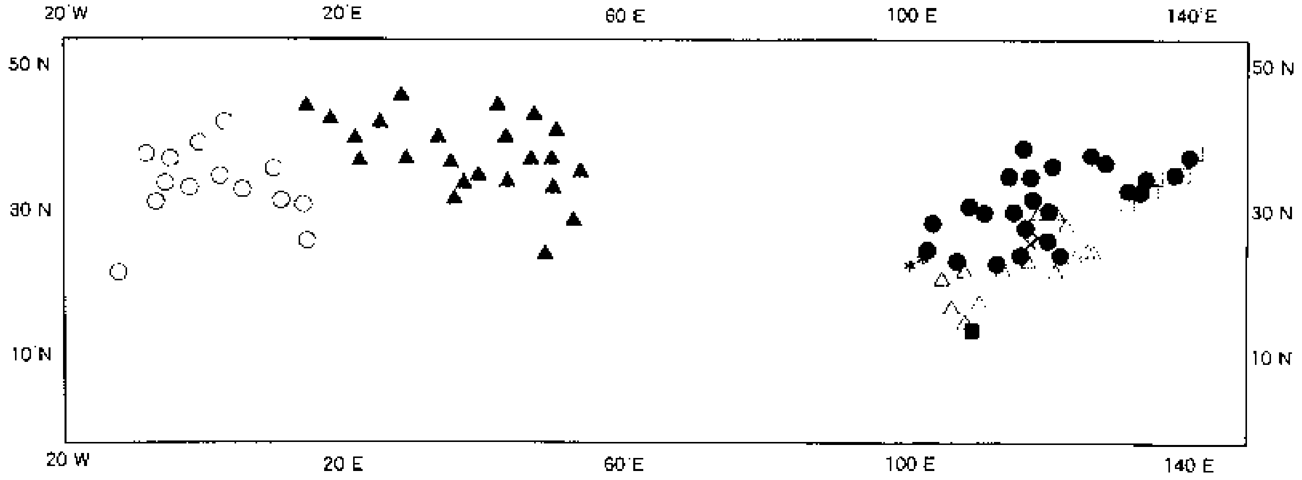

Fig. 1. A map showing natural distributions of species of the genus *Mauremys* (closed rectangles, *M. annamensis*; closed triangles, *M. caspica*; crosses, *M. iversoni*; open rectangles, *M. japonica*; open circles, *M. leprosa*; open triangles, *M. mutica*; asterisks, *M. pritchardi*;) and *Chinemys reevesii* (closed circles) on the basis of Iverson (1992), Joger and Lambert (1996) Yasukawa et al. (1996), McCord (1997) and Yasukawa's unpublished data

We also incorporated representatives of two other geoemydine genera, *Sacalia bealei* and *Cyclemys* sp., and two batagurine genera, *Chinemys reevesii* and *Orlitia borneensis* (see Appendix for further details). Of these batagurines, *Chinemys* was selected because a recent preliminary study on the molecular phylogeny of Testudines suggested its relatively close affinity with a few geoemydine genera including *Mauremys* (Wu et al. 1999). *Orlitia* was expected to function as an outgroup to root dendrograms resulting from phylogenetic analyses, because recent morphological analyses suggested this genus to be one of the most primitive members of Batagurinae (Hirayama 1984; Gaffney and Meylan 1988).

Methods for extraction, amplification and sequencing of DNA have been described in detail elsewhere (Honda et al. 1999a, b). A part of the mitochondrial 12S and 16S rRNA genes consisting of approximately 860 base pairs (bp) were amplified using the polymerase chain reaction (PCR: Saiki et al. 1988) with primers L1091, H1478, L2606, and H3056 (Kocher et al. 1989; Hedges et al. 1993).

Alignments for DNA sequences were determined, based on maximum nucleotide similarity using CLUSTAL X 1.8 (Thompson et al. 1994) with default gap penalties. Using CLUSTAL X 1.8, the neighbour-joining (NJ) clustering (Saitou and Nei 1987) was also performed to infer relationships among the taxa on the basis of a pairwise matrix of distance (not shown) from Kimura's (1980) two-parameter model. Maximum-likelihood (ML) and maximum-parsimony (MP) analyses were also conducted using the empirical frequencies and one category of substitution rate options of PHYLIP 3.54c (Felsenstein 1993) and the branch-and-bound search option of PAUP* 4.0b (Swofford 1998), respectively. In all these analyses, gap sites were excluded and confidences in the topology of resultant trees were assessed by 1000 bootstrap resamplings (Felsenstein 1985). As different weighting algorithms for transition and transversion (1 : 1–1 : 10) yielded identical branching topology in both ML and MP analyses, we provide here results of these analyses with weighting of 1 : 5 only, according to the ratio observed for the ingroup taxa (transition : transversion = 1 : 4.55).

The interpretation of bootstrap proportions (BPs) is still in a state of uncertainty (see Felsenstein and Kishino 1993; Hillis and Bull 1993). We tentatively followed Shaffer et al. (1997), and considered BPs ≥ 90% as highly significant, 70 ≤ BPs < 90% as marginally significant, and 50% ≤ BPs < 70% as constituting limited evidence of monophyly.

Results

Aligned sequences from 12S and 16S rRNA genes are presented in Fig. 2. The 12S rRNA fragment consisted of 402 total aligned sites, of which 80 were variable. For the 16S rRNA fragment, there were 461 total aligned sites, of which 87 were variable. Inter-generic nucleotide replacements varied from 26 bp (*Ch. reevesii* versus *M. japonica* or *M. caspica*) to 75 bp (*Orlitia borneensis* versus *M. japonica*). Nucleotide replacements between congeneric species of *Mauremys* ranged from 5 bp (*M. annamensis* versus *M. iversoni*) to 42 bp (*M. japonica* versus *M. mutica*).

The NJ dendrogram derived from the distance matrix from aligned sequences is shown in Fig. 3(a). Five nodes (nodes 1–5) were supported with significant BPs (≥ 70%). In this dendrogram, *Cyclemys* sp. and *Sacalia bealei* first diverged from the remaining ingroup taxa, constituting a marginally supported cluster (BP = 71%: node 1), whereas *Ch. reevesii* was located within the cluster otherwise consisting of the five *Mauremys* species. Monophyly of the latter assemblage was supported with a relatively high BP value (87%: node 2). Furthermore, a sister-group relationship of *Ch. reevesii* with *M. japonica* was even more strongly supported (BP = 93%: node 5), and this cluster was then rather weakly connected with *M. caspica* (BP = 60%). The remaining three species of *Mauremys* constituted another marginally significantly supported cluster (BP = 70%: node 3), in which the closest affinity between *M. annamensis* and *M. iversoni* was supported in all bootstrap iterations (i.e. BP = 100%: node 4).

Both ML and MP dendrograms exhibited branching topology identical with that of the NJ dendrogram except for the position of *M. mutica*, which was located outside the cluster consisting of the remaining *Mauremys* species and *Ch. reevesii* in these trees (Fig. 3b,c). Even so, however, BP values supporting the 'non-mutica' *Mauremys*–*Chinemys* cluster in the ML and MP trees were relatively low, being 54 and 61%, respectively. Monophyly of the *M. caspica* – node 5 assemblage, only weekly supported in the NJ tree, received higher bootstrap supports in the ML (BP = 76%) and MP dendrograms (BP = 78%). In the MP dendrogram, node 1 was not significantly supported by bootstrap iterations (BP = 64%).

Discussion

Results of the phylogenetic analyses using the three different procedures, although showing a discrepancy to some extent

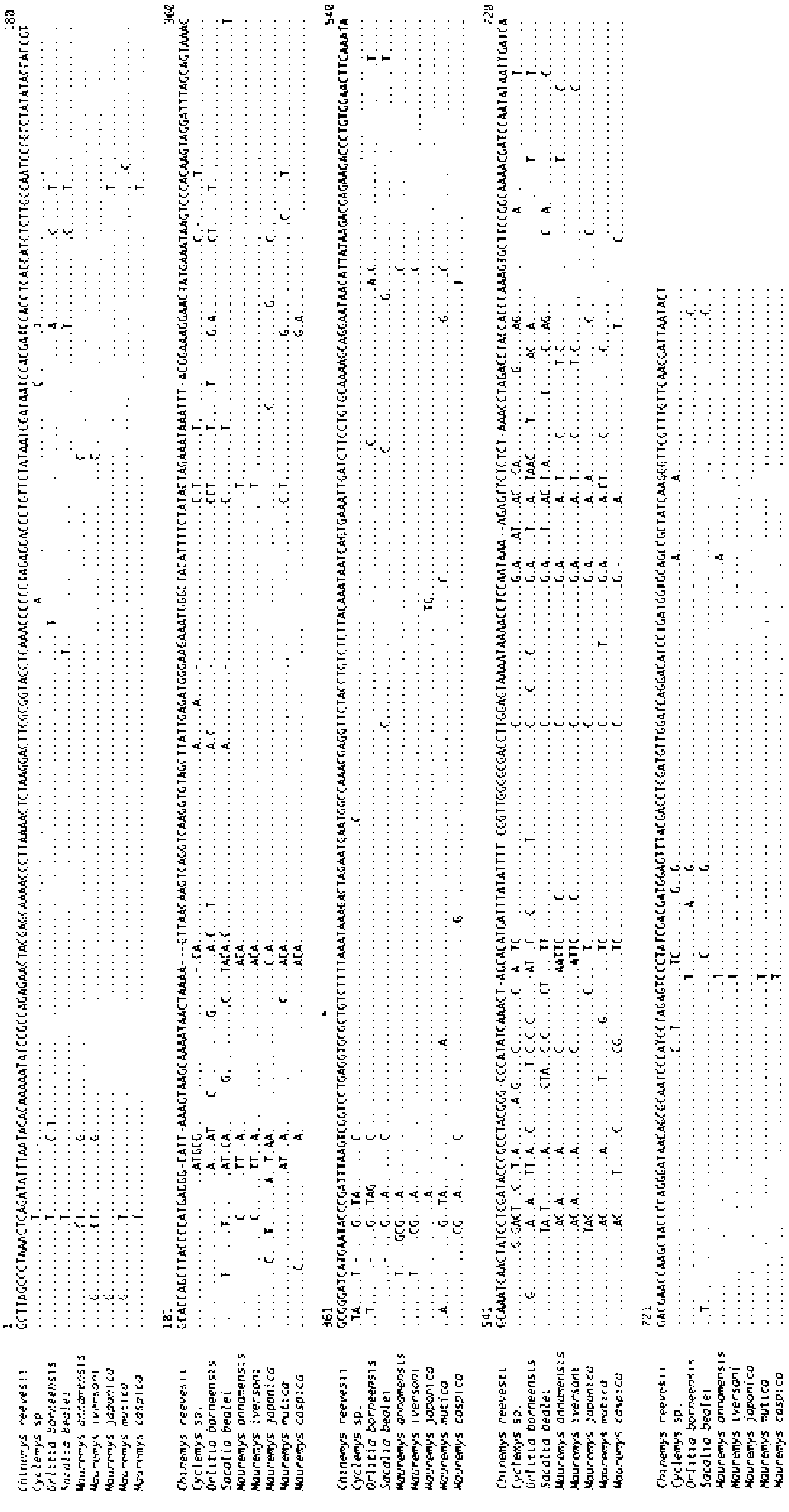

Fig. 2. Aligned sequences of a 863 bp segment of the 12S and 16S rRNA genes. The initial 401 bp in each row correspond to the 12S rRNA gene domain. The 16S rRNA gene domain begins at the asterisk. The dot indicates an identity with the first sequence; dash denotes a gap

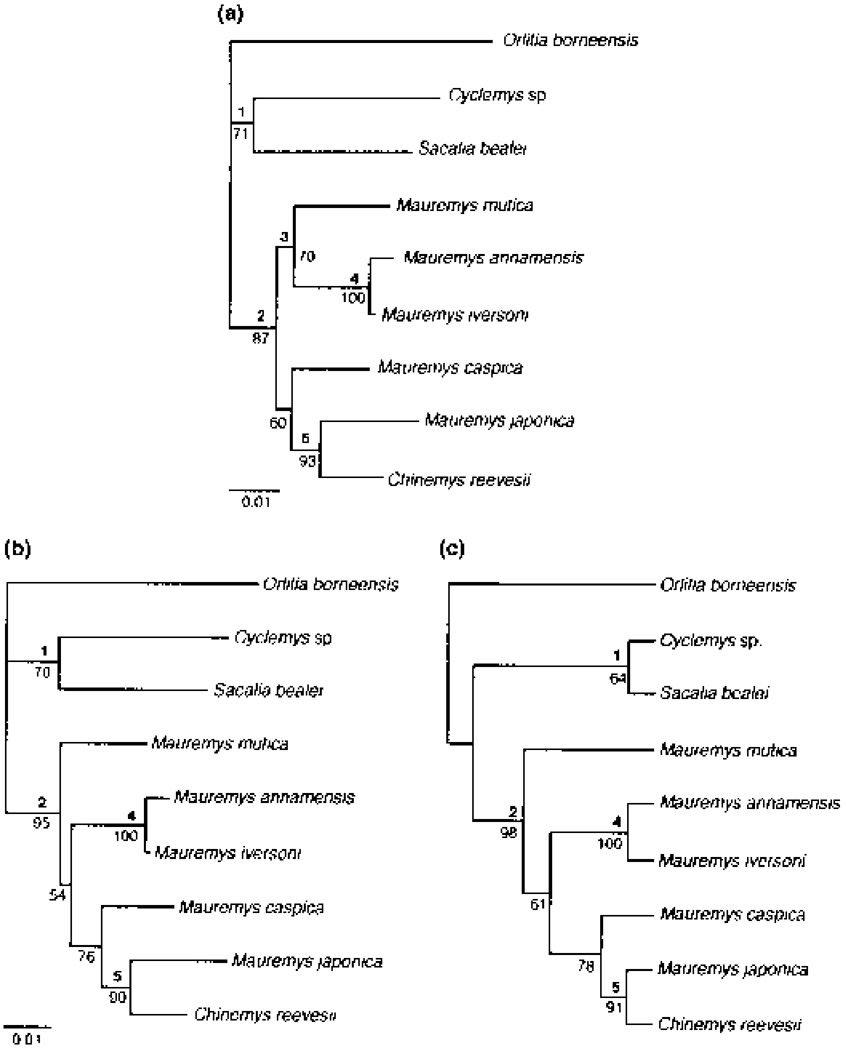

Fig. 3. (a) Neighbour-joining (NJ) dendrogram derived from analysis of distance matrix from 12S and 16S rRNA sequence data. Numbers beneath branches are bootstrap proportions (BPs) in the 1000 bootstrap pseudoreplications. Values less than 50% are omitted. Nodes with bold numbers are identical with those in the ML and MP dendrograms below. Bar equals 0.1 unit of Kimura's (1980) two-parameter distance. (b) Maximum-likelihood (ML) dendrogram (ln likelihood = –2256.0). All branches were supported as significantly positive ($p < 0.01$). Branches without BP values were not supported in ≥ 50% of the replicates. Bar equals 0.1 unit. (c) Maximum-parsimony (MP) cladogram using the branch-and-bound search option (347 steps, 65 bp informative under the condition of parsimony, consistency index = 0.87, homoplasy index = 0.13, and retention index = 0.72). Branches without BP values were not supported in ≥ 50% of the replicates

with respect to the position of *M. mutica*, are consistent with each other in topology of the remaining taxa (Fig. 3a–c). We thus hypothesize the relationships among the genera and species examined here as: (*O. borneensis* (*S. bealei*, *Cy.* sp.) (*M. mutica* (*M. annamensis*, *M. iversoni*) (*M. caspica* (*M. japonica*, *Ch. reevesii*)))).

Based on the cladistic analysis of morphological characters, Hirayama (1984) surmised a close affinity of *M. japonica* with the two Mediterranean–West Asian congeners (*M. caspica* and *M. leprosa*) rather than with *M. mutica*. However, in the work focusing on the phylogenetic relationships among the East Asian *Mauremys*, Iverson and McCord (1994) took an *a priori* assumption that the initial dichotomy of the genus occurred between the Mediterranean–West Asian lineage and the East Asian assemblage including *M. japonica* (see their Fig. 7). The present results support Hirayama's (1984) view and negate the adequacy of the presumption adopted by Iverson and McCord (1994). This further points to a risky aspect of delimitation of subjects for phylogenetic analysis solely on the basis of their geographic distributions (e.g. Tu et al. 2000).

Wu et al. (1999), on the basis of sequence variations in a 400 bp fragment of the mitochondrial 12S rRNA gene, provided an NJ dendrogram for representatives of a number of Chelonian genera and families. In that dendrogram, the genus *Mauremys* (as represented by *M. mutica*) is shown to be closely located to *Chinemys* (as represented by *Ch. reevesii*) and *Ocadia* (as represented by *Ocadia sinensis*), genera currently classified to the subfamily Batagurinae on the basis of several morphological features, rather than to most other geoemydine genera. Even so, bootstrap supports to such relationships were not sufficiently high (i.e. < 90%), and most other clusters were not supported with significant BPs (i.e. ≥ 70%), most likely due to the insufficiency in the number of base pairs determined. The present results, although confirming the close affinity of *Mauremys* with *Chinemys* as predicted by Wu et al. (1999), go even further and indicate a sister relationship of the latter with *M. japonica*, rendering *Mauremys* in the current delimitation to a non-monophyletic assemblage.

Based on the phylogenetic analyses of morphological characters, Yasukawa (1997) claimed the paucity of unequivocally derived character states shared between *Mauremys* and other geoemydine turtles. He thus argued that *Mauremys* may constitute a basal stock to other genera of the subfamily. Likewise, although there are a number of morphological characters diagnostic of *Mauremys*, very few of them are in unequivocally derived states (Yasukawa 1997). On the other hand, *Chinemys* is currently classified to the Batagurinae because of the presence of several putative synapomorphies with other genera of this subfamily (Hirayama 1984). Our results, demonstrating an exclusively close affinity of *M. japonica* and *Chinemys*, thus suggest that the morphological character states used to define *Mauremys* (e.g. separation or only weak contact of maxilla and vomer, and almost consistent allocation of foramen praepalatinum within premaxilla: Hirayama 1984; Yasukawa 1997) actually represent symplesiomorphy, and that the character states exclusively shared between *Chinemys* and other batagurine genera (e.g. consistently broad fissura ethmoidalis, and formation of the secondary palate: Hirayama 1984) have resulted from convergence.

Biogeographically, the relationships (*M. caspica* (*M. japonica*, *Chinemys*)), suggest that the initial divergence in this clade occurred between the Mediterranean–West Asian region and East Asia. The broad geographical gap between these two regions, mostly occupied by arid lands, implies that this divergence was enhanced by the increase of arid area in central Asia since the Tertiary period with the rising of the Himalayas (Borkin 1999). Such a geological event may be responsible for similar phylogeographical patterns exhibited by a few other semi-aquatic vertebrates, such as *Bombina*, the Eurasian *Hyla*, and *Salamandrella* (Borkin 1999). Of the East Asian species, *M. japonica* is endemic to the main islands of Japan, whereas *Ch. reevesii* has a broader range that includes the main islands of Japan, Taiwan, Korea, and eastern continental China (Iverson 1992). Considering the greater morphological variation in the continental populations in *Ch. reevesii* [see Lovich et al. (1985)] and the endemic occurrence of the other species of the genus, *Chinemys nigricans*, in south-eastern continental China (Iverson 1992), it is likely that the initial divergence in the *M. japonica*–*Chinemys* clade took place between the continent and the main islands of Japan, and that the current sympatry of *M. japonica* and *Ch. reevesii* in the main islands of Japan is a consequence of more recent dispersals of the latter species from the continent.

The closest affinity of *M. annamensis* with *M. iversoni*, which is firmly indicated in the present analyses (invariably 100% BP), contradicts the currently prevailing view, in which *M. annamensis* is regarded as closest to *M. mutica*, and *M. iversoni* as phylogenetically the most divergent among the East Asian *Mauremys* (Iverson and McCord 1994). The present results suggest that the morphometric similarity between *M. annamensis* and *M. mutica* actually reflects remnants of primitive states, and that a distinct phenetic divergence between *M. iversoni* and *M. annamensis* [see Iverson and McCord 1994] only recently initiated and has progressed rapidly.

Further analyses by incorporating samples of additional species, especially of *M. leprosa*, *M. pritchardi*, and *Ch. nigricans*, as well as representatives of *Ocadia* (see above), are desirable in order to verify the phylogenetic hypothesis and relevant evolutionary scenarios discussed above. Then, the subfamilial and generic classifications of the Bataguridae, as well as diagnoses for each of the taxa recognized, should be revised accordingly.

Acknowledgements

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Zusammenfassung

Phylogenie der eurasischen Süßwasserschildkröten der Gattung Mauremys *Gray 1869 (Testudines), mit besonderem Hinweis auf eine enge Ähnlichkeit von Mauremys japonica mit Chinemys reevesii.*

Die phylogenetischen Beziehungen zwischen den Süßwasserschildkröten der Gattung Mauremys und Vertretern einiger anderer bataguriner Gattungen wurde aus den Unterschieden an 863 Positionen der 12S- und der 16S-rRNA-Gene des Mitochondriengenoms ermittelt. Die Ergebnisse weisen sehr stark darauf hin, daß die Gattung Mauremys wegen der starken Ähnlichkeit zwischen *M. japonica* und *Chinemys reevesii* nicht monophyletisch sein kann. Neue morphologische Analysen der batagiurinen Gattungen ließen vermuten, daß Mauremys einen basalen Ast der Unterfamilie Geomydinae darstellt, während Chinemys zur Unterfamile Batagurinae gehört, was aus den anzunehmenden Synapomorphien mit anderen batagurinen Gattungen gestützt wird. Es ist daher auch wahrscheinlich, daß die zur Beschreibung von Mauremys verwendeten morphologischen Merkmalszustände eher Plesiomorphien darstellen und daß die morphologischen Merkmalsausprägungen, die Chinemys und anderen batagurinen Gattungen gemeinsam sind, auf Konvergenz beruhen. Unsere Ergebnisse unterstützen auch nicht eine Schwestergruppen-Beziehung zwischen *M. anamensis* und *M. mutica*, was direkt oder indirekt von einer Zahl von früheren Untersuchern auf Grund morphologischer Daten angenommen wurde. Statt dessen erwies sich *M. anamensis* am engsten verwandt mit *M. iversoni*, einer Art, die von früheren Autoren als am stärksten divergent zu den ostasiatischen Mauremys-Arten angesehen wurde.

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Appendix

Catalogue numbers and sampling localities of specimens examined in this study

The list includes one unidentified juvenile specimen of *Cyclemys*, in which infrageneric taxonomy is remarkably controversial (Fritz et al. 1997; Iverson and McCord 1997). We thus refer to this specimen as *Cyclemys* sp. here.

Chinemys reevesii: Ibo River, Hyogo Prefecture, Japan, Herpetological Collection of the Department of Zoology, Kyoto University (KUZ) 36609

Cyclemys sp. locality unknown (imported by a pet dealer), KUZ 36654

Mauremys annamensis: locality unknown (imported by a pet dealer), KUZ36700

Mauremys iversoni: locality unknown (imported by a pet dealer)

Mauremys japonica: Oharano, Kyoto City, Japan, KUZ 36599

Mauremys mutica kami: Yonagunijima Island, the southern Ryukyus, Japan, KUZ 47020

Mauremys caspica rivulata: locality unknown (imported by a pet dealer), KUZ 36680

Sacalia bealei: locality unknown, a living animal kept in Zoo, Okinawa

Orlitia borneensis: locality unknown (imported by a pet dealer), a living animal kept by Y. Yasukawa.

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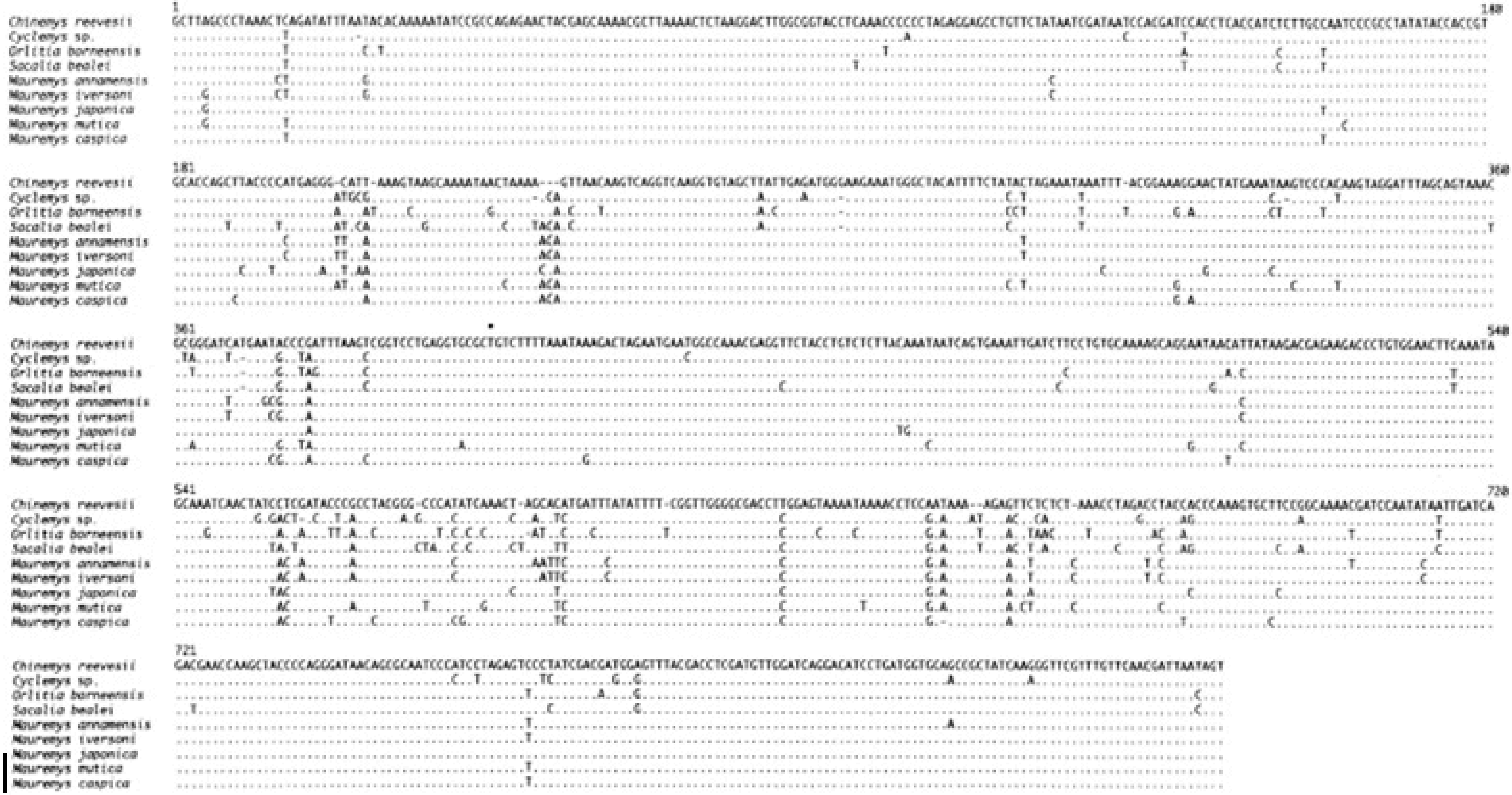
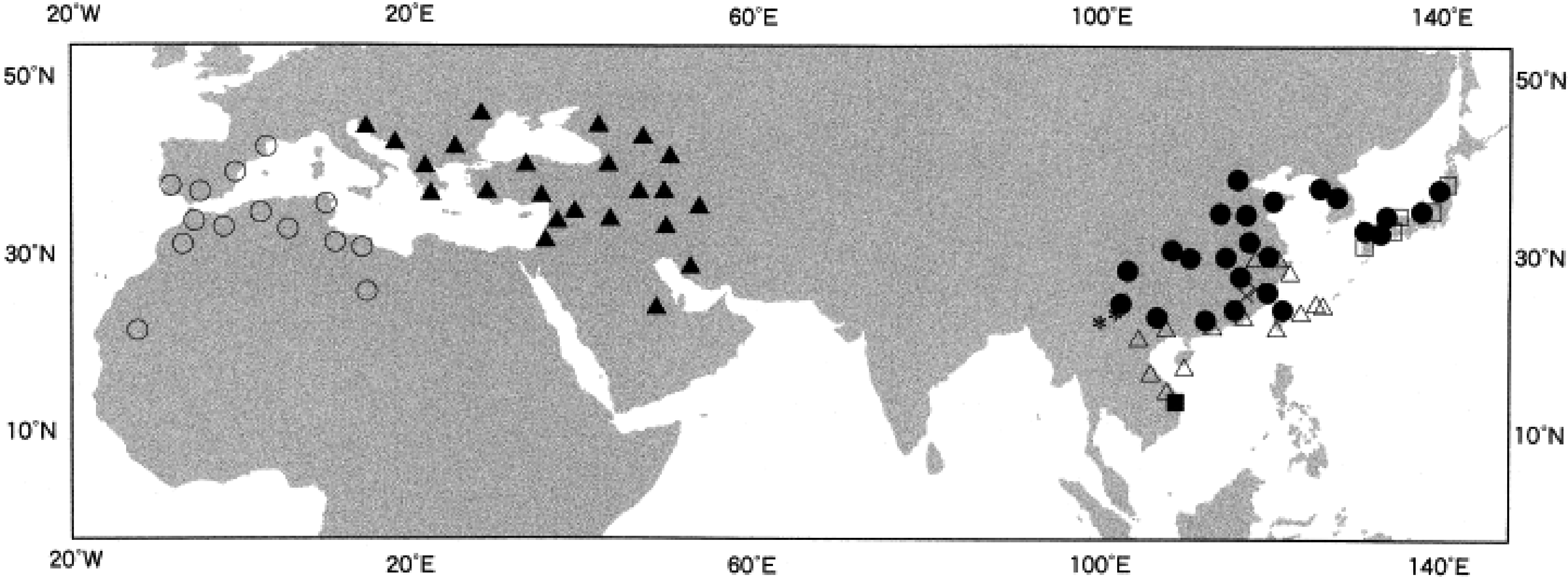


1
180
181
360
361
540
541
720
721
Chinemys reevesii
Cyclemys sp.
Orlitia borneensis
Sacalia bealei
Mauremys annamensis
Mauremys iversoni
Mauremys japonica
Mauremys mutica
Mauremys caspica


20°W
20°E
60°E
100°E
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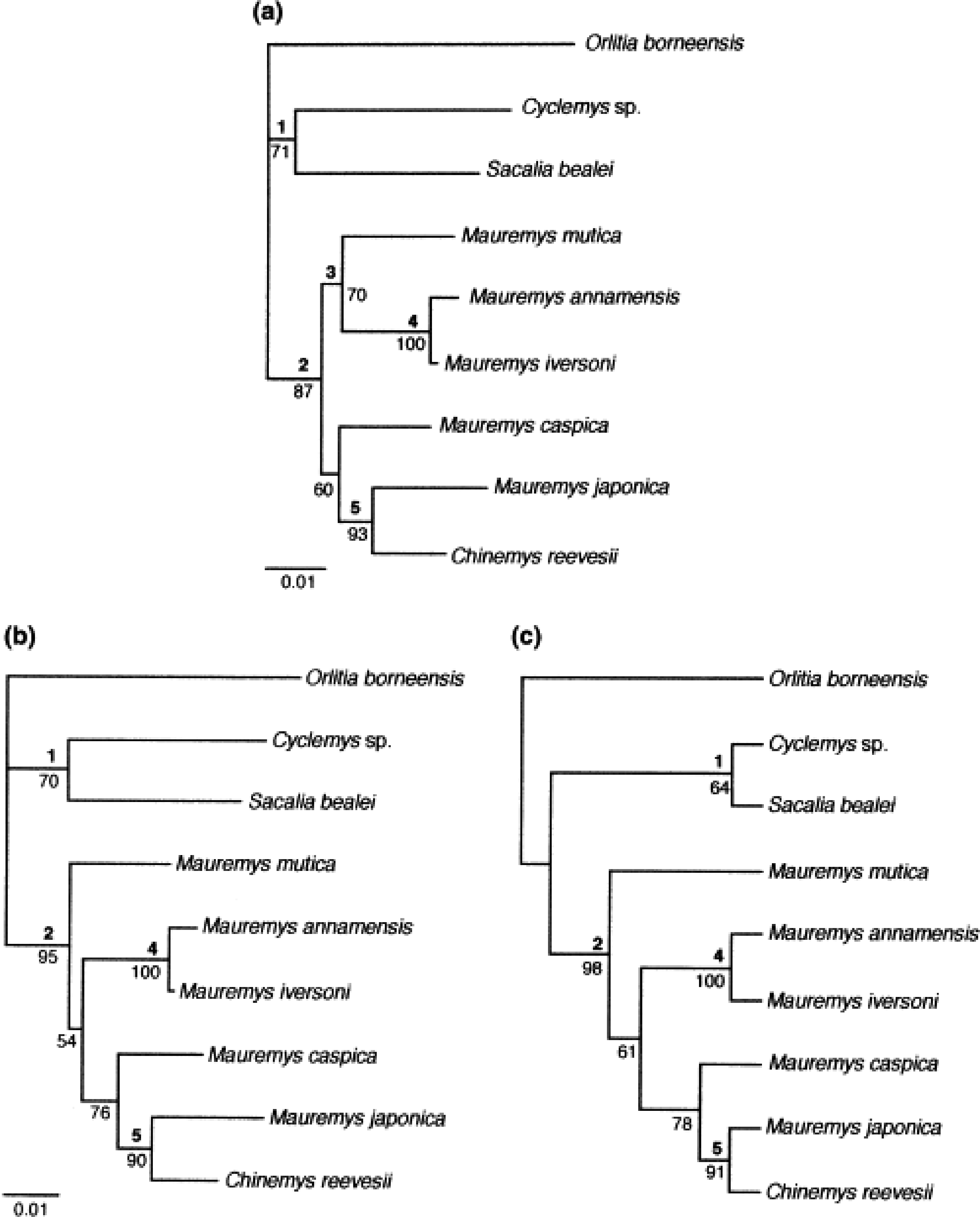
10°N
20°W
20°E
60°E
100°E
140°E


(a)
Orlitia borneensis
Cyclemys sp.
Sacalia bealei
Mauremys mutica
Mauremys annamensis
Mauremys iversoni
Mauremys caspica
Mauremys japonica
Chinemys reevesii
1
71
2
87
3
70
4
100
60
5
93
0.01
(b)
Orlitia borneensis
Cyclemys sp.
Sacalia bealei
Mauremys mutica
Mauremys annamensis
Mauremys iversoni
Mauremys caspica
Mauremys japonica
Chinemys reevesii
1
70
2
95
4
100
54
76
5
90
0.01
(c)
Orlitia borneensis
Cyclemys sp.
Sacalia bealei
Mauremys mutica
Mauremys annamensis
Mauremys iversoni
Mauremys caspica
Mauremys japonica
Chinemys reevesii
1
64
2
98
4
100
61
78
5
91