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Phylogenetic relationships and biogeographical history of the genus *Rhinoclemmys* Fitzinger, 1835 and the monophyly of the turtle family Geoemydidae (Testudines: Testudinoidea)

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Rhinoclemmys is an interesting genus of turtles biogeographically and ecologically, being the only genus of the family Geoemydidae that occurs in the New World and inhabiting a wide range of habitats from aquatic to highly terrestrial. Here we present a molecular phylogeny of *Rhinoclemmys* using both mitochondrial and nuclear genes. Our results strongly support the monophyletic and subfamilial status of *Rhinoclemmys* within the monophyletic family Geoemydidae. Within *Rhinoclemmys*, two clades are strongly supported, i.e. *R. annulata* + *R. pulcherrima* and *R. areolata* + *R. punctularia* + *R. diademata* + *R. funerea* + *R. melanosterna*, but the positions of *R. nasuta* and *R. rubida* are still weakly supported. In terms of the biogeographical history, the results of this study, coupled with palaeontological evidence, corroborate the hypothesis that this group migrated from Asia to the Americas across the Bering Strait during the early Eocene. The radiation of *Rhinoclemmys* in Central and South America corresponds well with vicariance events, including the emergence of the Sierra Madres of Mexico and the Nuclear Highland, and dispersals across the Panama land bridge. Interestingly, our resulting phylogeny suggests this group invaded South America at least four times and that dispersal of *R. nasuta* to South America probably took place in the early Miocene before the emergence of the Isthmus of Panama. We finally discuss our phylogenetic results with regard to the monophyly of the family Geoemydidae and in the context of previous morphological analyses.

ADDITIONAL KEYWORDS: 12S – 16S – biogeography – c-mos – cyt-*b* – Geoemydidae – phylogenetics – Rag-1 – *Rhinoclemmys*.

INTRODUCTION

The turtle family Geoemydidae (Theobald, 1868), previously known as Bataguridae Gray, 1870, is the most diverse living turtle group, consisting of more than 70 described species and 23 genera, more than one-fifth of the world's turtle species. The genus *Rhinoclemmys*, with nine species, is the only group in the family that occurs in Central and South America. Most other living members of this family are distributed in Asia, with three species of the genus *Mauremys* living in the western Palearctic, including Europe and North Africa (Fig. 1). The ecology of *Rhinoclemmys* is particularly interesting because members of the genus vary from highly aquatic (*R. nasuta*) to terrestrial (*R. rubida*).

Although several studies have examined the phylogeny of *Rhinoclemmys* using morphological and molecular, including non-DNA sequence, characters

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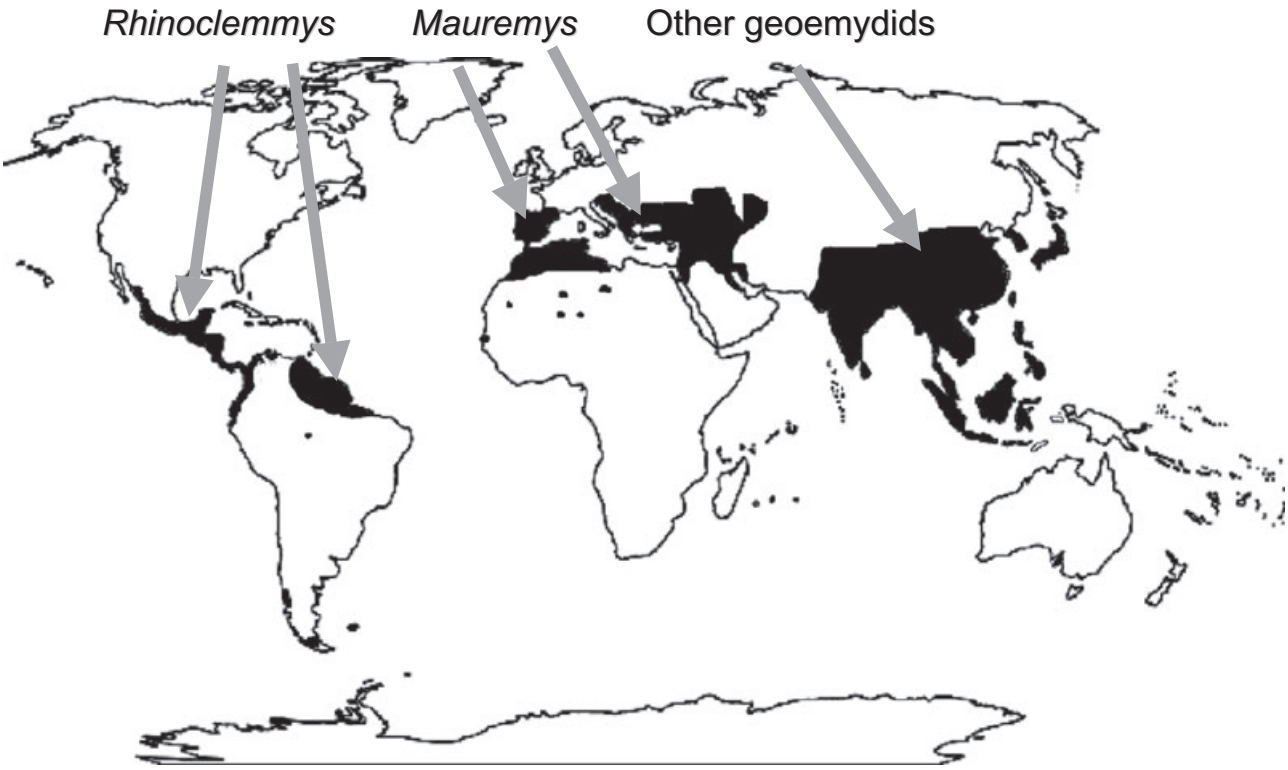

Figure 1. Distribution of the family Geoemydidae (data compiled from Iverson, 1992).

(i.e. Ernst, 1978; Sites, Greenbaum & Bickham, 1981; Hirayama, 1984; Sites *et al.*, 1984; Carr, 1991; Yasukawa, Hirayama & Hikida, 2001; Spinks *et al.*, 2004; Sasaki *et al.*, 2006), the inter- and intrageneric phylogenetic relationships have not been well resolved (Fig. 2). Specifically, there are discrepancies among these studies over the monophyly of *Rhinoclemmys*, its relationship to other members of the family Geoemydidae, and its interspecific relationships. In their morphological analyses, Hirayama (1984) and Yasukawa *et al.* (2001) argued that *Rhinoclemmys* is paraphyletic, because this genus does not have its own synapomorphic characters (Fig. 2). By contrast, Carr (1991) using both morphological and non-DNA sequence, including karyotypic and biochemical, characters and Claude & Tong (2004) based on morphological data proposed the monophyly of this group. The recent molecular study by Spinks *et al.* (2004) also strongly supports this monophyletic relationship, but an analysis of short interspersed nuclear element (SINE) insertion in this group did not recover this monophyly (Sasaki *et al.*, 2006). Nevertheless, thus far no molecular study has included all species in the analysis.

The position of *Rhinoclemmys* among other geoemydids is also unclear. Previous morphological studies (McDowell, 1964; Hirayama, 1984; Carr, 1991; Yasukawa *et al.*, 2001) all concurred that the genus falls within the *Geoemyda* complex, which consists of all narrow-jawed geoemydid species. This hypothesis is also largely supported by Sasaki *et al.*'s (2006) study of SINE insertion. Other molecular studies, however, placed the genus outside the rest of the geoemydids, but its position remains ambiguous (McCord *et al.*, 2000; Spinks *et al.*, 2004; Diesmos *et al.*, 2005). Specifically, although Spinks *et al.*'s (2004) maximum-likelihood cladogram based on cyt-*b* alone weakly supported the monophyly of the family inclusive of this genus, their cladogram based on combined data indicated a sister relationship of *Rhinoclemmys* with testudinids, thus rendering the family Geoemydidae paraphyletic. Diesmos *et al.* (2005) reanalysed data from Spinks *et al.* (2004) and added one species, *Siebenrockiella leytensis*, and also found that the monophyly of the family, including *Rhinoclemmys*, was weakly supported in their maximum-parsimony analysis.

The paraphyly of this family is also supported by an earlier molecular study (Lamb & Lydeard, 1994) and the SINE insertion analysis (Sasaki *et al.*, 2006). The paraphyletic relationship of the Geoemydidae with the Testudinidae has also been hypothesized in previous morphological studies (McDowell, 1964; Hirayama, 1984), but these studies did not employ

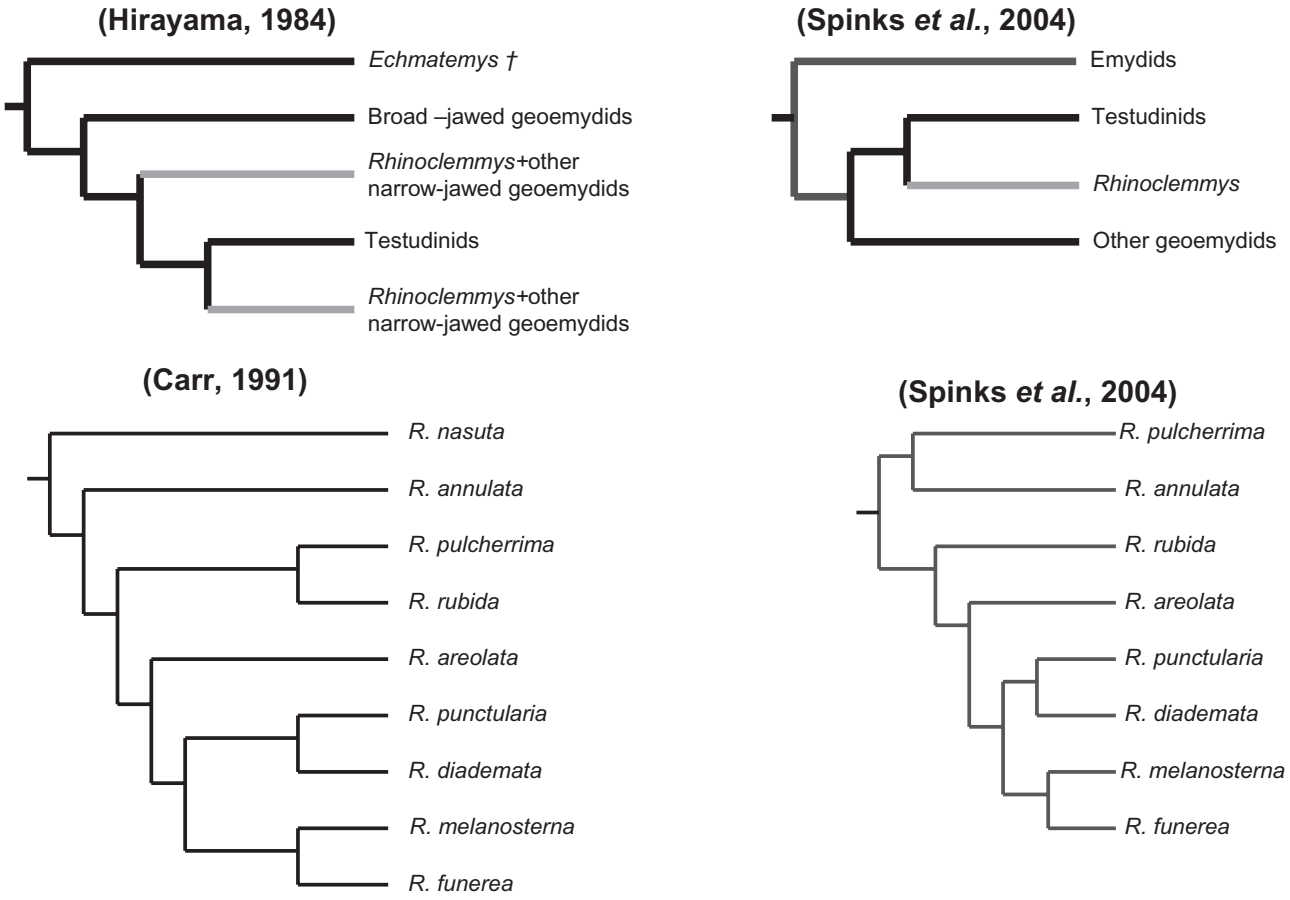

Figure 2. Previous hypotheses regarding the position of *Rhinoclemmys* among geoemydids (upper cladograms) and the relationships among the species of the genus (lower cladograms). †Fossil taxon.

any phylogenetic methods. A recent morphological study (Claude & Tong, 2004) proposed the monophyly of this group, but again the data were not formally analysed. So far, it is unclear if the family has any synapomorphies as several characters proposed by McDowell (1964) and Hirayama (1984) have been considered variable between the ingroup and the outgroup or more appropriately regarded as pleisiomorphies, given that they occur widely among other cryptodires (Waagen, 1972; Ehrenfeld & Ehrenfeld, 1973; Gaffney & Meylan, 1988; Shaffer, Meylan & McKnight, 1997; Weldon & Gaffney, 1998; Jamniczky & Russell, 2004; Joyce & Bell, 2004). In the present study, we assess the monophyly of this problematic family in order to clarify the position of *Rhinoclemmys* within it.

Regarding the biogeographical history of *Rhinoclemmys*, it is still unclear how members of the genus migrated to Central and South America. Ernst (1978) and Hirayama (1984) hypothesized that this group migrated from Asia to North America across the Beringean region, but they did not provide any phylogenetic evidence to support this claim. Another possibility that has not been explored in previous studies is that *Rhinoclemmys* reached North America across the Atlantic Ocean. Other groups have been shown to have invaded North America via the Thulean and De Geer Bridges after colonizing Europe (McKenna, 1983; Tiffney, 1985; Sanmartin, Enghoff & Ronquist, 2001). The Thulean Bridge was particularly important for cross-Atlantic invasion (Sanmartin *et al.*, 2001). Alternatively, the genus might have dispersed over the ocean from Africa to South America using equatorial currents as suggested for the tortoise genus *Chelonoidis* and other groups of animals, such as platyrrhine monkeys, caviomorph rodents and *Mabuya* skinks (Houle, 1999; Huchon & Douzery, 2001; Mouchaty *et al.*, 2001; Carranza & Arnold, 2003; Le *et al.*, 2006).

The radiation of *Rhinoclemmys* in Central and South America has been addressed by Carr (1991) and Savage (2002). Savage (2002) suggested that the genus belongs to the northern herpetofauna, which invaded Central America in the Eocene (corresponding to dis-

persal event D2 in Savage, 2002). An interesting geological event, which might have had a significant influence on the biogeographical pattern of the genus, is the emergence of the Isthmus of Panama. Currently, there are conflicting hypotheses regarding the time of diversification of *Rhinoclemmys* through this Isthmus. While Carr (1991) hypothesized that some species might have dispersed to South America prior to the its closure, other authors proposed that they migrated subsequent to the closure of this land bridge (Duellman, 1979; Vanzolini & Heyer, 1985). In the present study, we use our best phylogenetic estimate and molecular calibration of radiation times of this group in the region to test these hypotheses.

MATERIAL AND METHODS

Taxonomic sampling and choice of outgroups

As our primary goal was to examine the phylogenetic relationships of the genus *Rhinoclemmys*, we included all nine recognized species. We were able to obtain tissue from three of the subspecies of *R. pulcherrima*, but were unable to obtain tissue for *R. rubida perixantha*, *R. punctularia flammigerra* or *R. pulcherrima pulcherrima*, because we could not locate their specimens. In addition, we selected 12 other geoemydid species, representing all major lineages of this group (after Spinks *et al.*, 2004). To test the hypothesis that *Rhinoclemmys* reached the Americas through Europe or Africa we included all three western Palearctic species of *Mauremys*. Because all species of *Mauremys* have been shown to form a monophyletic group with strong statistical support in previous studies (i.e. Barth *et al.*, 2004; Spinks *et al.*, 2004), we did not include Asian *Mauremys* in this study. In addition, in order to examine the monophyly of geoemydids with regard to testudinids, we sampled five species of tortoises. These taxa represent high genetic diversity within the family Testudinidae (after Le *et al.*, 2006). Two members of the family Emydidae were selected as outgroups as its sister position to testudinids and geoemydids is supported by both morphological and molecular studies (Gaffney & Meylan, 1988; Krenz *et al.*, 2005; Near, Meylan & Shaffer, 2005) (see supplementary Appendix S1).

Molecular data

Most previous molecular studies (e.g. Wu, Zhou & Yang, 1998, 1999; Honda *et al.*, 2002; Honda, Yasukawa & Ota, 2002; Barth *et al.*, 2004) used only mtDNA. Spinks *et al.* (2004) also used one nuclear intron, but the authors sequenced only a small number of the ingroup taxa and three testudinids for this gene. Because many basal nodes in previous molecular studies were not well supported, we used a combination of two nuclear, Rag-1 and c-mos, and three mitochondrial genes, 12S, 16S and cyt-*b*. A similar approach has been employed successfully in recent studies of this and other turtle groups (Georges *et al.*, 1999; Engstrom, Shaffer & McCord, 2004; Le *et al.*, 2006; Le, McCord & Iverson, 2007). We sequenced the complete cyt-*b* sequences, and fragments of the 12S, 16S, c-mos and Rag-1 genes, and downloaded available cyt-*b*, 12S, 16S and c-mos sequences from GenBank (see supplementary Appendix S1). All primers used for the present study are shown in Table 1. DNA was extracted from tissues and blood samples, and sequenced using the same methods specified in Le *et al.* (2006) and Le *et al.* (2007).

Phylogenetic analysis

We aligned molecular data using ClustalX v1.83 (Thompson *et al.*, 1997) using default settings. All the data were then combined in a single matrix and analysed using maximum parsimony (MP) and maximum likelihood (ML) via PAUP*4.0b10 (Swofford, 2001), and Bayesian analyses were conducted using MrBayes v3.1 (Huelsenbeck & Ronquist, 2001). For parsimony analysis, we ran a heuristic analysis with 100 random taxon addition replicates using the tree-bisection and reconnection (TBR) branch swapping algorithm in PAUP. Bootstrap support values (BP) (Felsenstein, 1985a) were evaluated using 1000 pseudoreplicates and 100 random taxon addition replicates. Decay or Bremer indices (BI) (Bremer, 1994) were measured using Tree Rot 2c (Sorenson, 1999). All characters were equally weighted and unordered. Gaps in sequence alignments were treated as a fifth character state (Giribet & Wheeler, 1999). The congruence of the five molecular datasets was assessed by the incongruence length difference (ILD) test (Farris *et al.*, 1994).

For ML analysis the optimal model for nucleotide evolution was determined using Modeltest v3.7 (Posada & Crandall, 1998). Analyses used a randomly selected starting tree, and heuristic searches with simple taxon addition and the TBR branch swapping algorithm. Support for the likelihood hypothesis was evaluated by bootstrap analysis with 100 replications and simple taxon addition.

For Bayesian analyses we used the optimal model determined using Modeltest with parameters estimated by MrBayes v3.1. Analyses were conducted with a random starting tree and run for 5×10^6 generations. Four Markov chains, one cold and three heated (utilizing default heating values), were sampled every 1000 generations. Log-likelihood scores of sample points were plotted against generation time to detect stationarity of the Markov chains. Trees generated prior to stationarity were removed

Table 1. Primers used in this study

Primer	Position	Sequence	Reference
L1091 (12S)	491	5′-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3′	Kocher *et al.* (1989)
H1478 (12S)	947	5′-TGACTGCAGAGGGTGACGGGCGGTGTGT-3′	Kocher *et al.* (1989)
AR (16S)	1 959	5′-CGCCTGTTTATCAAAAACAT-3′	Palumbi *et al.* (1991)
BR (16S)	2 561	5′-CCGGTCTGAACTCAGATCACGT-3′	Palumbi *et al.* (1991)
CytbG (cytb)	14 368	5′-AACCATCGTTGTWATCAACTAC-3′	Spinks *et al.* (2004)
GLUDGE (cytb)	14 358	5′-TGATCTTGAARAACCAYCGTTG-3′	Palumbi *et al.* (1991)
CytbJSi (cytb)	15 011	5′-GGATCAAACAACCCAACAGG-3′	Spinks *et al.* (2004)
CytbJSr	15 030	5′-CCTGTTGGGTTGTTTGATCC-3′	Spinks *et al.* (2004)
THR (cytb)	15 593	5′-TCATCTTCGGTTTACAAGAC-3′	Spinks *et al.* (2004)
THR-8 (cytb)	15 585	5′-GGTTTACAAGACCAATGCTT-3′	Spinks *et al.* (2004)
CM1 (Cmos)	163	5′-GCCTGGTGCTCCATCGACTGGGA-3′	Barker *et al.* (2002)
CM2 (Cmos)	820	5′-GGGTGATGGCAAAGGAGTAGATGTC-3′	Barker *et al.* (2002)
Cmos1 (Cmos)	163	5′-GCCTGGTGCTCCATCGACTGGGATCA-3′	Le *et al.* (2006)
Cmos3 (Cmos)	812	5′-GTAGATGTCTGCTTTGGGGGTGA-3′	Le *et al.* (2006)
Rag1878	1 717	5′-GAAGACATCTTGGAAGGCATGA-3′	Le *et al.* (2007)
Rag2547	2 406	5′-TGCATTGCCAATGTCACAGTG –3′	Le *et al.* (2007)

Cmos and Rag1 primer positions correspond to the positions in the complete Cmos and Rag1 sequences of chicken with GenBank numbers M19412 and M58530, respectively; primer positions for mitochondrial genes correspond to the positions in the complete mitochondrial genome of *Chrysemys picta* (Mindell *et al.*, 1999).

from the final analyses using the burn-in function. Two independent analyses were started simultaneously. The posterior probability (PP) values for all clades in the final majority rule consensus tree are reported. We ran analyses on both combined and partitioned datasets to examine the robustness of the tree topology (Nylander *et al.*, 2004; Brandley, Schmitz & Reeder, 2005). In the partitioned analyses, we divided the data into 11 separate partitions, including 12S and 16S, and the other nine based on gene codon positions (first, second, and third) in cyt-*b*, c-mos and Rag-1. Optimal models of molecular evolution for each partition were selected using Modeltest and then assigned to these partitions in MrBayes v3.1 using the command APPLYTO. Model parameters were estimated independently for each data partition using the UNLINK command.

Biogeographical analysis

To test alternative hypotheses of relationships, corresponding tree topologies were compared using the Wilcoxon signed-ranks and Shimodaira–Hasegawa (SH) tests (using RELL optimization for the latter test) (Templeton, 1983; Felsenstein, 1985b; Shimodaira & Hasegawa, 1999), to determine if tree length difference could have resulted from chance alone (Larson, 1998). Alternative tree topologies were constructed in MacClade (Maddison & Maddison, 2001) and then used as constraint trees by importing to PAUP. Specifically, the two alternative hypotheses regarding the migration of geoemydids to the Americas, i.e. the shortest trees supporting *Rhinoclemmys* as sister to Asian vs. European lineages, were tested.

Point locality data for *Rhinoclemmys* were obtained from Iverson (1992). These points were plotted on the Global 30-Arc-Second Digital Elevation Model (DEM GTOPO30) produced by the US Geological Survey using the software ArcView 3.2 (ESRI, 1999) to facilitate the assignment of distribution to each area of endemism. To examine the patterns of biogeographical diversification of *Rhinoclemmys*, we constructed an area cladogram from our best phylogenetic hypothesis. We used the areas of endemism of herpetofauna in Central America as described in Savage (2002) for units of analysis because these areas are well corroborated among different groups of reptiles and amphibians. The area cladogram was then compared with that of other reptile and amphibian groups (Savage, 2002).

To estimate the divergence times of the phylogeny, we first tested the molecular clock hypothesis of combined data by running an ML analysis with clock constraint in PAUP. After the clock-like hypothesis was rejected [$\delta = 2 \times (\ln L_{NO\ CLOCK} - \ln L_{CLOCK}) = 2 \times (1812.36 - 18781.70) = 61.32$; d.f. = 30; $P < 0.001$], divergence times were calculated using a relaxed clock model (Drummond *et al.*, 2006) as implemented in the computer program BEAST v.1.4.5 (Drummond & Rambaut, 2006). The program BEAUti v.1.4.5 was used to set criteria for the analysis. All geoemydid species were considered monophyletic, and this node

Table 2. Data partitions subject to phylogenetic analyses with maximum parsimony

Data	Total no. of aligned sites	Parsimony-informative characters	Variable characters	MP tree length	RI	CI	No. of equally parsimonious trees
12S	409	123	168	436	0.70	0.51	6
16S	580	141	193	572	0.66	0.49	3
cyt-*b*	1140	444	539	2089	0.54	0.37	1
Cmos	602	54	95	123	0.83	0.83	29
Rag1	642	36	63	75	0.92	0.82	75
All mtDNA	2129	708	900	3127	0.58	0.40	1
All Nuclear	1244	90	158	203	0.85	0.83	96
All data	3373	798	1058	3337	0.59	0.43	3

was constrained to 54 Myr with 95% confidence interval from 50 to 55 (see Discussion). A GTR model using gamma + invariant sites with four gamma categories was used along with the assumption of a relaxed molecular clock. As for the priors, we used all default settings, except for the Tree Prior category being set to Yule Process as suggested by the program manual. In addition, the UPGMA tree was employed as a starting tree. For this analysis, the length chain was set to 5×10^6, and the Markov chain was sampled every 1000 generations. After the dataset with the above settings was analysed in BEAST, the resulting likelihood profile was then examined by the program Tracer v1.1 to determine the burn-in cutoff point. The final tree with calibration estimates was computed using the program TreeAnnotator v1.4.5 as recommended by the manual of the program BEAST.

RESULTS

Phylogenetic analyses

We were able to attain sequences for all five genes for all taxa. The final matrix consists of 3373 aligned characters from 32 species in which Rag-1 contains 642 characters; c-mos, 602; cyt-*b*, 1140; 12S, 409; and 16S, 580. We found no indels among the nuclear and cyt-*b* sequences, but indels were present in 12S and 16S. The ILD test indicated no significant incongruence between mitochondrial genes, between nuclear genes, and between nuclear and mitochondrial partitions. Overall, we conducted five tests: 12S vs 16S ($P = 0.5$), 12S vs cyt-*b* ($P = 0.31$), cyt-*b* vs 16S ($P = 0.2$), c-mos vs Rag-1 ($P = 0.14$) and nuclear DNA vs mtDNA ($P = 0.94$).

The combined mitochondrial data were three times more variable than the combined nuclear data (42% of sites variable compared with 14%). Although the nuclear genes were less variable, the MP analysis showed high consistency indices compared with those from mitochondrial genes (Table 2). The analysis of combined nuclear data showed strong support for the monophyly of geoemydids (BP = 76%) (Fig. 3B). The monophylies of testudinids and geoemydids + testudinids were also strongly supported (BP = 88 and 100%, respectively). However, even though many groups received strong support, the relationship among major clades was unresolved. In addition, only 70% of nodes received strong support (BP $\geq$ 70%) and the monophyly of *Rhinoclemmys* was weakly supported (BP = 60%).

In the analysis of combined mitochodrial data, about 79% of nodes received strong support. However, there were some discrepancies between the combined nuclear and mitochondrial trees. For example, the position of *Geoemyda japonica* was markedly different from that in the nuclear cladogram despite both hypotheses receiving weak support. The positions of *R. rubida* and *R. areolata* supported by nuclear data were also distinct from those shown in the cladogram derived from mitochondrial markers (Fig. 3A). In addition, even though MP analyses of some partitions, i.e. 12S, 16S and cyt-*b*, produced trees with some resolution, these trees either showed topologies inconsistent with the consensus topologies of combined data obtained using all three methods or had lower support values at the deep nodes. Based on this and the results of the IDL tests, we consider the combined approach is the best representation of our data.

In the MP analysis of combined data, three most parsimonious trees were found and the strict consensus tree is shown in Figure 4. The tree is well resolved with approximately 90% of the nodes receiving strong support (BP > 70%) (Hillis & Bull, 1993), 4% receiving reasonable support (BP > 65%) and the rest with low support (BP < 60%). The four with low support are the most basal node of the sister clade to *Rhinoclemmys*, the nodes representing the sister

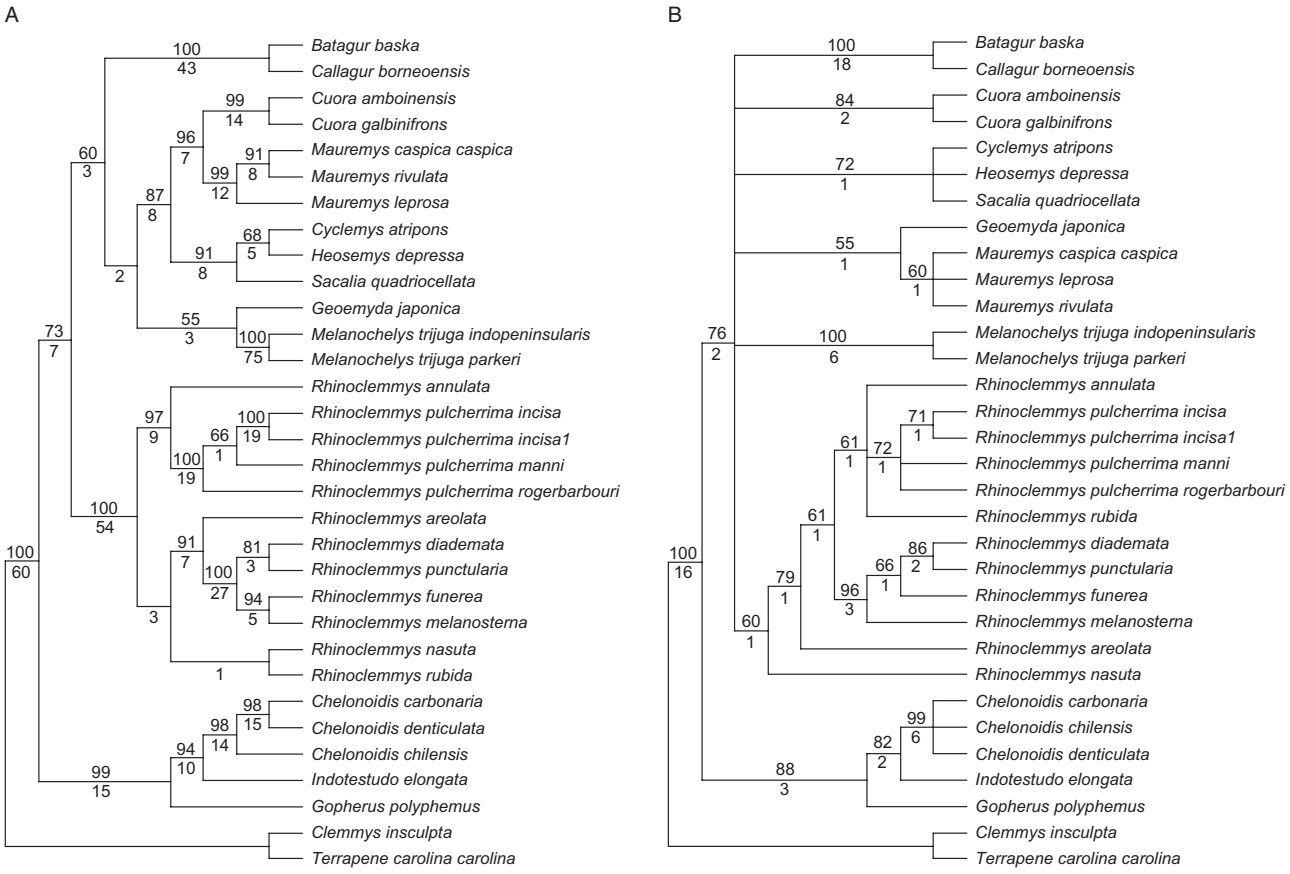

Figure 3. A, the single most parsimonious tree derived from 2129 aligned characters of mitochondrial genes (12S, 16S, cyt-*b*) (CI = 0.40; TL = 31; RI = 0.58) using maximum parsimony. Of these, 1229 characters are constant and 708 characters are parsimony-informative. Numbers above branches are bootstrap values and below are Bremer values. B, strict consensus of 96 trees generated from 1244 aligned characters of nuclear genes (Rag1 and Cmos) (CI = 0.82; TL = 205; RI = 0.84) using maximum parsimony. Of these, 1086 characters are constant and 90 are parsimony-informative. Numbers above branches are bootstrap values and below are Bremer values.

relationship between *Geoemyda* and *Melanochelys*, and the positions of *R. nasuta* and *R. rubida*.

The results of the ML and combined Bayesian analyses are shown in Figure 5. The ML analysis produced a single tree with topology identical to those generated by both the combined and the partitioned Bayesian analyses. The tree is totally resolved with 90% of nodes receiving strong support (BP > 70%) and the rest with low support (BP < 60%). Three nodes with low support include the positions of *R. nasuta* and *R. rubida* and the sister relationship between *R. p. manni* and *R. p. incisa*.

In the combined Bayesian analysis, –lnL scores reached equilibrium after 17 000 generations in both runs and 86% of nodes received strong support (PP > 95%). Of four nodes with weak support in the Bayesian analysis, three are identical to those with weak support in the ML analysis and the other represents the sister relationship between *Melanochelys* and a clade including *Sacalia*, *Cyclemys*, *Cuora*, *Mauremys* and *Heosemys*. However, this node is strongly supported in the ML analysis (BP = 84%). In the partitioned Bayesian analysis, –lnL scores stabilized after 15 000 generations in both runs. Compared with the combined Bayesian analysis, only one node representing the sister relationship between *R. p. rogerbarbouri* and *R. p. incisa* has significantly higher support (PP = 97% vs. 56%). Other nodes have roughly similar PP values: the basal node of geoemydids (99% vs. 98%), the *R. rubida* position (86% vs. 84%), the *R. nasuta* position (87% vs. 79%), and the sister relationship between *Melanochelys* and *Sacalia* + other taxa (83% vs. 92%).

Three differences were observed between the MP and Bayesian and ML tree topologies. First, the positions of *R. nasuta* and *R. rubida* are unresolved in the MP analysis, but they are shown to be sister to the clade containing *R. areolata* and others in the

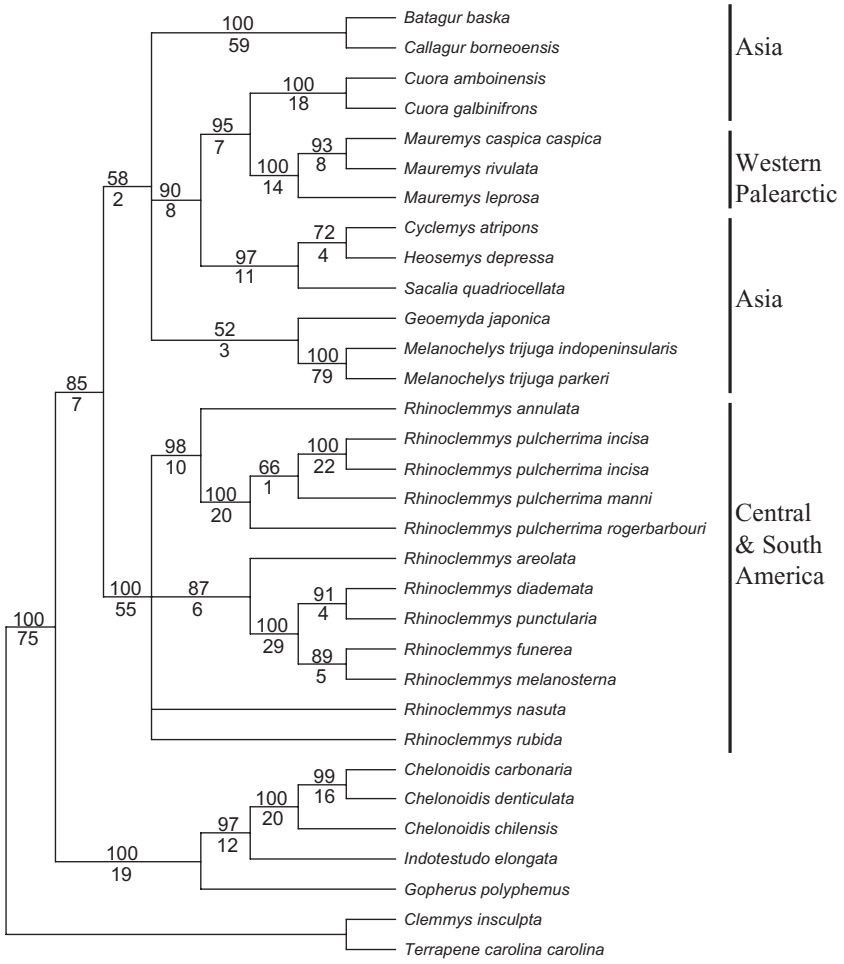

Figure 4. Strict consensus of three most parsimonious trees produced from 3373 aligned characters (TL = 3337; CI = 0.43; RI = 0.59) using maximum parsimony. Of these, 2315 are constant characters and 798 are potentially parsimony-informative. Numbers above and below branches are bootstrap (> 50%) and Bremer values, respectively.

Bayesian and ML analyses with weak support values. The MP tree is also unresolved at the deepest node of the large clade consisting of all other geoemydids excluding *Rhinoclemmys*, while the Bayesian and ML tree demonstrates that *Batagur*, *Callagur* and *Geoemyda* are basal to other taxa. Finally, *Geoemyda japonica* is sister to *Melanochelys* with weak support (BP = 52%) in the MP analysis, but sister to *Batagur* + *Callagur* in the Bayesian and ML analyses with strong support (BP = 85%, PP = 100%).

Overall, all of our analyses recovered two major well-supported clades within *Rhinoclemmys*, *R. annulata* + *R. pulcherrima* and *R. areolata* + *R. funerea* + *R. melanosterna* + *R. diademata* + *R. punctularia*. Within the latter clade, the sister relationships between *R. funerea* and *R. melanosterna* and between *R. punctularia* and *R. diademata* are also well supported in all of our analyses. In addition, our results strongly supported the monophyly of geoemydids, with *Rhinoclemmys* being sister to all other species within the family. The monophyly of the family Testudinidae is also strongly supported by all of the analyses.

BIOGEOGRAPHICAL ANALYSES

The comparison between the best-supported trees (Figs 4, 5) and the best tree constrained to place *Rhinoclemmys* as sister to *Mauremys* was significantly different in both Wilcoxon and SH tests ($P = 0.0001$). In the Wilcoxon test, the most parsimonious tree representing the latter hypothesis was 33 steps longer than the shortest tree. This result thus strongly supports the migration of *Rhinoclemmys* to the Americas across the Bering Strait. The cladogram in Figure 7 shows that nearly all major clades within *Rhinoclemmys* have members with distribution north of Panama, including *R. annulata*, *R. pulcherrima*,

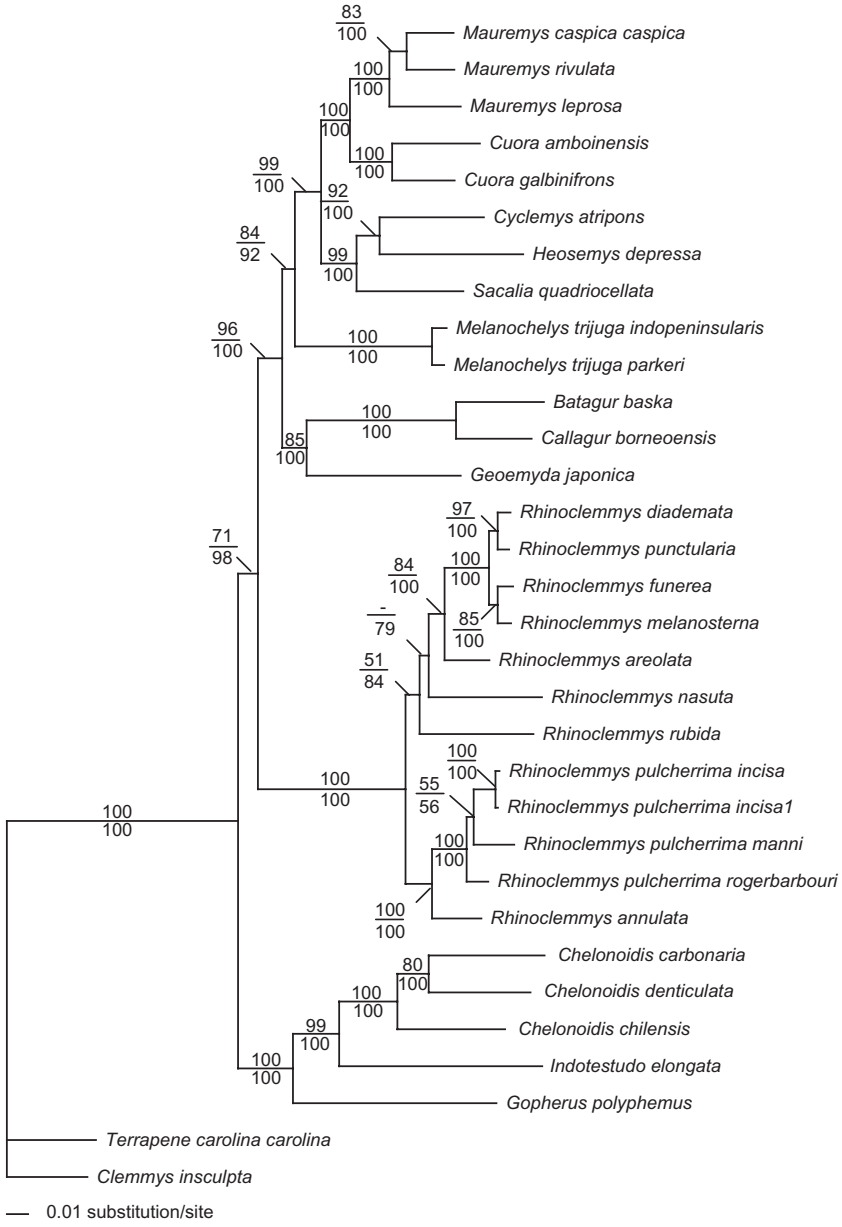

Figure 5. Phylogram generated from ML and Bayesian analysis using the GTR+G+I model of molecular evolution with the following parameters: K = 10; Base frequencies: freqA = 0.2945, freqC = 0.2856, freqG = 0.1962, freqT = 0.2237; rate matrix: A–C = 3.2207, A–G = 12.8698, A–T = 2.6852, C–G = 0.7080, C–T = 37.6846, G–T = 1.0000; proportion of invariable sites (I) = 0.4881; gamma distribution shape parameter = 0.4998. Score of the best tree found in the ML analysis = 18781.70 and total number of rearrangements tried = 9661. Numbers above and below branches are bootstrap values (> 50%) from 100 replicates of the ML analysis and the posterior probability of the Bayesian analysis, respectively.

R. areolata and *R. rubida*. The exception is *R. nasuta*, which is endemic to the Choco Region, and the *R.* punctularia + *R.* diademata clade, distributed in the Maracaibo Basin and northern Amazon.

For time calibration analysis, after 500 trees were discarded in the burn-in, the tree generated by the program BEAST showed an identical topology to that supported by the ML and Bayesian analyses, except for the position of *R. p. rogerbarbouri* being interchanged with that of *R. p. manni* (Fig. 6). Age estimates and 95% confidence intervals for all nodes within *Rhinoclemmys* are shown in Table 3.

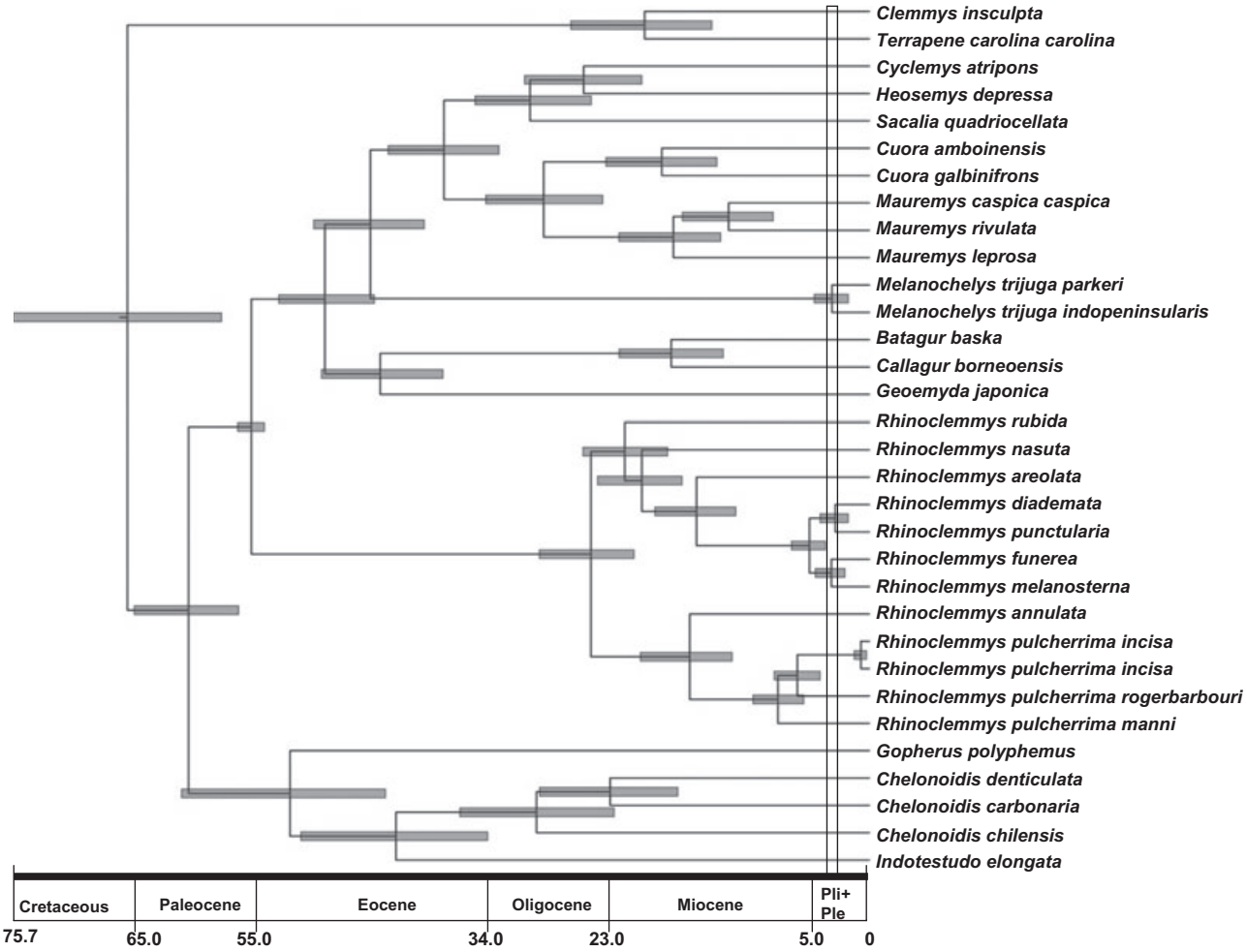

Figure 6. Time calibration using the program BEAST. The error bar on each node represents the 95% confidence interval calculated by the program. The column on the right shows the time slice of Isthmian closure (3.5–2.5 Mya; Coates & Obando, 1996). Pli + Ple: Pliocene + Pleistocene.

DISCUSSION

Phylogenetic relationships within *Rhinoclemmys*

Our results are apparently in conflict with the relationships proposed by several previous studies. Ernst (1978) first inferred the internal relationships within *Rhinoclemmys* phenetically from morphological characters and behaviour. He grouped *R. rubida* with *R. pulcherrima*, and placed *R. annulata*, *R. nasuta*, *R. punctularia* and *R. funerea* in the same cluster based on their external characters and coloration. *Rhinoclemmys areolata* was sister to all other species because it shares characters with both of the groups. In terms of habitat preference, terrestrial species include *R. annulata*, *R. pulcherrima manni*, and two subspecies of *R. rubida*; *R. areolata* is semi-terrestrial; *R. funerea* and *R. nasuta* are aquatic; and both subspecies of *R. punctularia*, *R. diademata*, *R. melanosterna* and subspecies of *R. pulcherrima* (except *manni*) are all semi-aquatic (Ernst & Barbour, 1989; WPM Pritchard & Trebbau, pers. observ., 1984). Habitat preferences, therefore,

Table 3. Time calibration for nodes within *Rhinoclemmys*. Node numbers are defined in Figure 7

Node	Age estimate (Mya)	95% CI (Mya)
1	24.6	20.8–29.1
2	15.8	12.1–20.2
3	21.6	17.8–25.3
4	20.1	16.5–24.0
5	15.3	11.8–18.9
6	5.3	3.8–6.9
7	3.0	1.8–4.3
8	3.3	2.2–4.8

do not seem to correspond well with the phylogenetic relationships.

Moreover, phylogenetic analysis of morphological characters also produced a different topology (Fig. 2). Hirayama (1984) and Yasukawa *et al.* (2001) proposed almost the same hypotheses as each other in which they placed *R.* annulata and rubida in one clade and areolata, pulcherrima, funerea and punctularia in the other. Their hypotheses also showed that *Rhinoclemmys* was paraphyletic, and embedded within other narrow-jawed genera including *Cuora*, *Pyxidea*, *Cyclemys*, *Heosemys*, *Mauremys* and *Sacalia*. The width of the jaw, therefore, is apparently attributable to morphological convergence within these groups. Morphological convergence seems common among testudinoids (Claude *et al.*, 2003, 2004; Claude, 2006), making phylogenetic analyses of morphological data alone potentially misleading (Claude, 2006). Remarkably, the study using SINE insertion by Sasaki *et al.* (2006) also hypothesized that *Rhinoclemmys* was placed within a group consisting of these narrow-jawed genera. Their results indicate that SINE insertion might also be subject to convergence.

The results of our combined analysis show two clades of *Rhinoclemmys* are well supported, i.e. *R. annulata* + *R. pulcherrima* and *R. areolata* + *R. funerea* + *R. melanosterna* + *R.diademata* + *R. punctularia*. Nevertheless, the positions of *R. nasuta* and *R. rubida* are still unresolved in the MP tree and their positions are weakly supported in the ML and Bayesian analyses. Carr's (1991) study also supported the monophyly and an identical topology of the well-supported group including *R. areolata* and other species. However, his analysis proposed the sister relationship between *R. pulcherrima* and *R. rubida* and the basal position of *R. nasuta* (Fig. 2). Our ML and Bayesian topology, excluding *R. nasuta*, resembles the tree proposed by Spinks *et al.* (2004) based on combined data, but our MP topology without *R. nasuta* is identical to that in Diesmos *et al.* (2005) and Spinks *et al.* (2004) based on cyt-*b* alone.

Our intraspecific data also suggest that *R. pulcherrima rogerbarbouri* is the most divergent subspecies of its species. The cyt-*b* data, the most variable gene in this study (Table 2), indicate that *R. pulcherrima incisa* is about 3% divergent from *R. pulcherrima rogerbarbouri* and is about 4.5% divergent from *R. pulcherrima manni*. In addition, *R. pulcherrima manni* is about 5% divergent from *R. pulcherrima rogerbarbouri*. Sites *et al.* (1981) also found high genetic divergence and little or no gene flow between *R. p. manni* and *R. p. incisa*. Ernst & Barbour (1989) distinguished these apparently allopatric subspecies based on coloration of the carapace, plastron and bridge. They also differ in the degree of doming of the shell. *Rhinoclemmys p. rogerbarbouri* is distributed from southern Sonora to Colima, Mexico. *Rhinoclemmys p. pulcherrima* only occurs in Guerrero, Mexico, *R. p. incisa* from Oaxaca, Mexico, to northern Nicaragua, and *R. p. manni*, the most terrestrial of this complex, from southern Nicaragua to Costa Rica. These results show that a range-wide phylogeographical study of *R.* pulcherrima is likely to discover high genetic divergence within this species complex.

Biogeography of *Rhinoclemmys*

Our biogeographical results do not support the hypothesis that *Rhinoclemmys* migrated to the New World from Europe or Africa. In addition, as the sister clade of *Rhinoclemmys* within the Geoemydidae has all basal lineages restricted to Asia and the fossils of the family putative ancestors, the Lindholmemydidae, are all Asiatic (Sukhanov, 2000; Claude & Tong, 2004), the origin of geoemydids can safely be placed in Asia. Other palaeontological evidence corroborates the hypothesis that the ancestors of the group migrated over the Bering Land Bridge during the warmer period in the early Eocene as did the mammals (Sukhanov, 2000; Beard, 2002; Bowen *et al.*, 2002).

The Bering Strait separating Asia and northwestern America was formed about 100 Mya and remained open occasionally until the Pleistocene, but due to its northern latitude it is clear that animals only migrated over the Bridge during warm periods (Sanmartin *et al.*, 2001). According to Bowen *et al.* (2002) and Beard (2002), a short global warming period facilitated the dispersal of at least three mammal groups (uintatheres, rodents and hyaenodontids) through this route in the late Tiffanian (57 Mya), early Clarkforkian (56 Mya) and early Wasatchian (55 Mya).

The abrupt occurrence of geoemydid fossils (genus *Echmatemys*) in North America (Wyoming and South Dakota) around 55 Mya (in the earliest Wasatchian, early Eocene) (Hutchison, 1996) is congruent with this hypothesis. The monophyly of *Rhinoclemmys* suggests that this group only colonized the Americas once, and this colonization probably corresponds with the third wave of mammal invasion of the Americas. Other fossil records of *Bridgeremys*, a genus related to *Rhinoclemmys*, were also found in Wyoming in the middle Eocene between 46 and 49 Mya (Hutchison, 2006).

The fossil record also indicates that the diversification of geoemydids occurred very early in their history with fossils found in the early Eocene in North America and Europe (Godinot & de Broin, 2003; Claude & Tong, 2004). This implies that the family had very widespread distribution and that the current distribution may just be relict. Thus far, palaeontological evidence supports two separate

migration routes from Asia. According to Godinot & de Broin (2003), fossil forms of Europe and North America in the early Eocene are completely different from each other even though they both seem to be related to the Asian forms (but see Hutchison, 1996). The same hypothesis has been proposed for the migrations of tortoises, family Testudinidae, to Europe and North America (Le *et al.*, 2006). This pattern shows that these two families had already diversified in Asia well before they migrated to Europe and the Americas, and that there was no exchange of turtle fauna between Europe and North America.

In the Americas, the ancestors of *Rhinoclemmys* may have dispersed to tropical regions in Central America during the cooling period of the Eocene, as did other groups of reptiles and amphibians, due in part to the formation of uplands in western North America and Mexico (Savage, 2002). Nevertheless, living lineages of this genus only started to diversify in the late Oligocene (Fig. 6). Thus, the emergence of the Sierra Madres of Mexico, the Nuclear Highlands – a combination of the highlands of Chiapas, Guatemala and Honduras – and the Panama land bridge in the Oligocene, Miocene and Pliocene, respectively, substantially influenced the biogeographical patterns of the local herpetofauna (Savage, 1982, 2002). Mountain uplift in northern Mexico, i.e. Sierra Madre Oriental, might have isolated the *R. areolata* group (Lowland Atlantic) from *R. rubida* (Lowland Pacific) during the early Miocene. This vicariance event also had important impacts on other reptiles and amphibians, resulting in similar divergence of frogs in the *Hyla microcephala* complex and lizard species in the *Enyaliosaurus* group of the genus *Ctenosaura* (Savage, 1982, 2002).

The emergence of the Nuclear Highlands in the middle Miocene may have caused the divergences of *R. pulcherrima* and *R. annulata*, and also isolated *R. areolata*, largely distributed in the Yucatán Peninsula, from the *R. punctularia* + *R. funerea* group. Interestingly, our estimate indicates that these two events took place almost simultaneously (Fig. 6, Table 3). The species of the *R*. areolata group show a clear progression rule, where younger species are found further south, from the Lowland Atlantic to Amazon South (Fig. 7). As shown by the phylogenetic

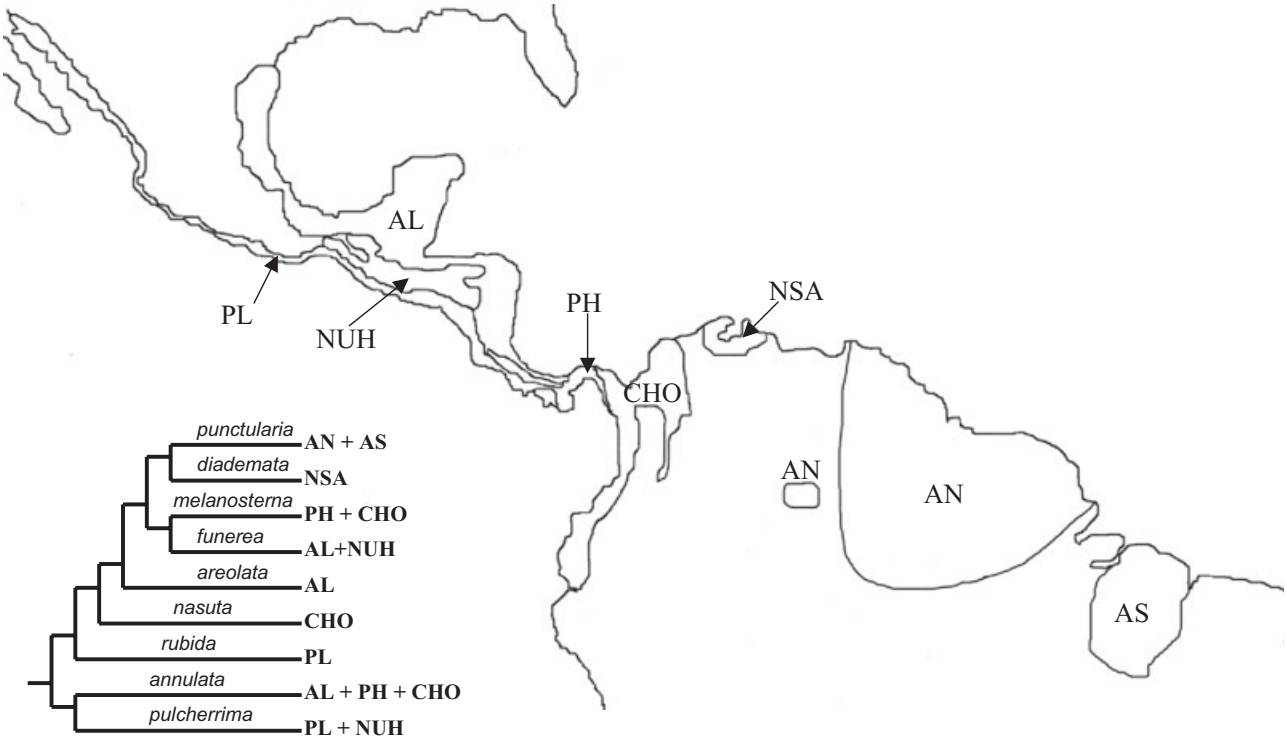

Figure 7. Areas of endemism and the area cladogram illustrating the relationship among species of *Rhinoclemmys*. Sources of these areas of endemism are from Savage (2002) (Central America), Cracraft (1985), and Haffer (1985) as assigned to zoographical regions of Stotz, Fitzpatrick & Moskovits (1996) (South America): AL, Atlantic Lowland; AN, Amazon North; AS, Amazon South; CHO, Choco; NSA, Northern South America; NUH, Nuclear Highland; PH, Panamanian Herpetofauna; PL, Pacific Lowland.

results and by the distribution of *R. punctularia* + *R. funerea*, *R. annulata* and *R.* nasuta, *Rhinoclemmys* invaded South America at least four times. Three of these invasions are likely to have taken place after the emergence of the Isthmus (see Fig. 6).

Due to the fact that *R. nasuta* is endemic to the Choco, there are two explanations for its current distribution. *Rhinoclemmys nasuta* invaded the Choco through the Panamanian Land Bridge as did other species in the Pliocene and its populations in the north subsequently went extinct (Carr, 1991). However, this hypothesis conflicts with the fact that all other species representing other major clades within this genus, *R. pulcherrima*, *R. areolata* and *R. rubida*, still occur in the north. Alternatively, *R. nasuta* migrated to South America before the emergence of the Isthmus of Panama. Due to the general limited dispersal ability and limited tolerance to seawater in turtles, it might have used different means to reach South America rather than island hopping and waif dispersal as shown in many groups of mammals (Marshall, 1979; Webb, 1985). It is very likely that this species inhabited the Choco in the early Miocene when this region was still a series of volcanic islands close to Central America (Savage, 2002). The subsequent movement of the Choco block led to its collision with northern South America in the late Miocene (Duque-Caro, 1990; Savage, 2002), probably bringing with it part of the Central American fauna. The close relationship between the Choco and Central America has also been reported in different bird groups (Cracraft & Prum, 1988; Brumfield & Capparella, 1996; Bates, Hackett & Cracraft, 1998).

The diversification within the *R. punctularia* and funerea groups is likely to have been influenced by dispersals across the Isthmus of Panama, although the split between these two groups might have occurred before the closure of the land bridge (Figs 6, 7). In addition, the Pleistocene effect in South America might have had impacts on the distribution of *R. diademata* and *R. punctularia*. In particular, their distribution seems to fit well with the refugia hypothesis (Haffer, 1969) with the former being restricted to the Maracaibo Basin and the later distributed in the lower Amazon Basin. The increased aridity in the lowlands during the Pleistocene may explain the gap between the distributions of these two species. Because both of them are semi-aquatic, this phenomenon can have a significant impact on constraining their ranges. In fact, *Rhinoclemmys* presumably had a much wider range because fossils have been found in Brazil hundreds of kilometres south of its current range, and on the Santa Elena Peninsula (Carr, 1991).

Relationship between *Rhinoclemmys* and other genera and the monophyly of the family Geoemydidae

The results from the present study strongly support the monophyly of the family Geoemydidae (BP = 85% in MP, BP = 71% in ML; PP = 98%) inclusive of *Rhinoclemmys*. This is the first broad-sampling molecular analysis that strongly supports the monophyly of the family with regard to testudinids, and also supports the subfamilial status of *Rhinoclemmys*. We herein propose to raise this genus to subfamily rank with the name of Rhinoclemminae. Morphologically, all species of the genus share at least two synapomorphies, the absence of lateral keels throughout their life (Claude & Tong, 2004) and the shape of the upper triturating surface (our pers. observ.). Examination of 60 specimens of all species in this genus and 63 specimens of other species (see supplementary Appendix S2) belonging to other major clades of the family reveals that the upper triturating surface in this group is different from that of other geoemydid species in that it is narrower in the anterior portion and expanded in the posterior potion. In addition, the upper triturating surface has a minimal lingual ridge on the inner rim. Carr (1991) proposed other synapomorphies for this group, but these characters either vary among other geoemydids or could not be checked because they are either karyotypic or biochemical characters.

Although the other major clade of geoemydids, exclusive of *Rhinoclemmys*, is not strongly supported by the MP analysis, it is consistently recovered in all of our analyses and received strong support from the Bayesian and ML analyses. Biogeographically, it is a distinct clade containing mostly Asian taxa. Within this clade, our MP results show the same topology as the one recovered by Diesmos *et al.* (2005). However, several basal nodes have significantly higher BP values, such as the clade consisting of *Cuora* + *Mauremys* + *Cyclemys* + *Heosemys* + *Sacalia* (BP = 90% vs. 57%) and the clade of *Cyclemys* + *Heosemys* + *Sacalia* (BP = 97% vs. 74%). The topology resulting from our Bayesian and ML analyses for this major clade is the same as one proposed by Spinks *et al.* (2004), but the support values in all nodes are generally higher. The most significant discrepancy between our MP and the Bayesian and ML analyses is the position of *Geoemyda*. It is likely, however, that this problem could be eliminated by increasing taxon sampling. We are currently investigating this problem using more taxa and more molecular data.

In terms of the synapomorphy of this family, we agree with Hirayama (1984) and Yasukawa *et al.* (2001) that the presence of inguinal and axillary musk duct foramina is the character uniting all geoemydids. This hypothesis has been criticised as musk

duct foramina are also present in other groups of turtles (Waagen, 1972; Ehrenfeld & Ehrenfeld, 1973; Gaffney & Meylan, 1988; Weldon & Gaffney, 1998; Joyce & Bell, 2004). Nevertheless, the musk duct positions and formation can be used to differentiate geoemydids from emydids and testudinids. Within testudinoids, emydids tend to have only one pair of musk duct foramina in the axillary region and testudinids do not possess these foramina. Geoemydids commonly have two pairs, in the axillary and inguinal buttresses. The genus *Morenia* used to be considered the only geoemydid genus that does not possess musk duct foramina (Waagen, 1972; Yasukawa *et al.*, 2001; Joyce & Bell, 2004), but our observation of a *Morenia ocellata* specimen (see supplementary Appendix S2) revealed that they do have two pairs of small musk duct foramina as do other geoemydids. Yasukawa *et al.* (2001) also proposed that expanded iliac blades distinguish the family Geoemydidae from all other families, but we were unable to check this character due to the rarity of complete iliac blades in examined specimens.

CONCLUSIONS

Analyses of combined mitochondrial and nuclear markers along with morphological examination of 124 specimens strongly support the monophyly of the family Geoemydidae, inclusive of the genus *Rhinoclemmys*. Importantly, this result helps settle controversies over the past 40 years regarding the paraphyletic relationship of this family, thus resolving a major problem in cryptodire systematics. Moreover, the monophyly of this family coupled with palaeontological evidence shows that the genus *Rhinoclemmys* might have dispersed to the Americas across the Bering Strait during the early Eocene and subsequently invaded Central and South America. Despite the results of this study, some areas of the family's phylogeny are in need of further investigation, including the monophyly of the other clade of geoemydids, exclusive of *Rhinoclemmys*, and the positions of *Geoemyda*, *R. nasuta* and *R. rubida*. Future research should strive to include more data as well as more complete sampling. In addition, phylogeographical patterns of the species or species complex within *Rhinoclemmys*, such as *R. pulcherrima*, punctularia and rubida, should be further studied to uncover cryptic diversity in this group.

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SUPPLEMENTARY MATERIAL

The following material is available for this article online:

Appendix S1. GenBank accession numbers and associated samples were used in this study.
Appendix S2. Specimens examined for morphological characters in this study.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1096-3642.2008.00413.x
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