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Molecular phylogeny and divergence times of ancient South American and Malagasy river turtles (Testudines: Pleurodira: Podocnemididae)

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Abstract

The eight extant podocnemidid species are the last survivors of a speciose ancient group of turtles known to have existed since the Cretaceous. One species, representing the monotypic genus *Erymnochelys*, occurs on Madagascar; the remaining seven species are confined to South America (*Peltocephalus*: one species; *Podocnemis*: six species). Phylogenetic relationships of all extant species were reconstructed from six mitochondrial (3385 bp) and six nuclear DNA fragments (4115 bp) in separate and combined analyses (Bayesian inference, Maximum Likelihood, Maximum Parsimony). In a total evidence approach for all concatenated genes, all methods yielded the same well-supported phylogenetic hypothesis for the three basal lineages. The Malagasy genus *Erymnochelys* is sister to the South American *Podocnemis*, and *Peltocephalus* constitutes the sister taxon to *Erymnochelys* + *Podocnemis*. Within *Podocnemis*, *P. unifilis* + (*P. erythrocephala* + *P. lewyana*) constitute a well-supported crown clade; *P. sextuberculata*, *P. vogli*, and *P. expansa* were revealed as successive sister taxa. According to Bayesian relaxed molecular clock calculations calibrated with fossil evidence, *Peltocephalus* originated during a period of the Late Cretaceous (~86 mya), when a contiguous Gondwana landmass exclusive of Africa is likely to have still existed. The Late Cretaceous split between *Erymnochelys* and *Podocnemis* (~78 mya) coincides with the supposed submergence of the land bridge between Madagascar and Antarctica + South America, suggesting that the origin of those genera is linked to this vicariant event. The extant *Podocnemis* species evolved from the Late Eocene (~37 mya) to the Middle Miocene (~15 mya), during a phase characterized by dramatic global cooling, aridification, and massive Andean uplift.
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Keywords: *Erymnochelys*; *Peltocephalus*; *Podocnemis*; Zoogeography; Phylogeny

Introduction

The chelonian family Podocnemididae, known to have existed since the Cretaceous, comprises many extinct and eight extant large freshwater turtle species from South America and Madagascar (Pritchard and Trebbau 1984; Wood 1984, 1997; de Lapparent de Broin 2000, 2001; Carvalho et al. 2002; Danilov 2005). Podocnemididae and the Afrotropical family Pelomedusidae represent the last survivors of a highly diverse radiation of turtles, the Pelomedusoides.

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Pelomedusoides together with the South American and Australian family Chelidae constitute the clade Pleurodira (side-necked turtles; Gaffney et al. 2006), traditionally treated as one of the two extant chelonian suborders (Fritz and Havaš 2007). During the Cretaceous and Paleogene, pelomedusoid turtles were geographically widespread and occurred in littoral and freshwater habitats. Records exist from all landmasses except Antarctica and Central Asia (Gaffney et al. 2006).

Two of the three extant podocnemidid genera occur in South America. The sole species of the genus *Peltocephalus* is *P. dumerilianus*; *Podocnemis* contains six extant species (*P. erythrocephala*, *P. expansa*, *P. lewyana*, *P. sextuberculata*, *P. unifilis*, and *P. vogli*). The Malagasy genus *Erymnochelys* is also monotypic, the only included species being *E. madagascariensis* (Frair et al. 1978; Ernst et al. 2000; Fritz and Havas 2007). While the disjunct 'Gondwana distribution' of podocnemidids (Wood 1984) inspired two recent zoogeographic investigations using molecular tools and one representative of each genus (Noonan 2000; Noonan and Chippindale 2006), a complete molecular evaluation of the phylogeny and zoogeography of podocnemidids was never attempted before, and the phylogenetic relationships of the six *Podocnemis* species remain unknown. Using six mitochondrial (3385 bp) and six nuclear DNA fragments (4115 bp), the present study aims at (i) providing the first complete molecular phylogeny for all extant podocnemidids, and (ii) estimating their diversification times based on a Bayesian relaxed molecular clock approach.

Material and methods

Sampling

Blood samples of all podocnemidid species except *Erymnochelys madagascariensis* and *Podocnemis erythrocephala* were obtained by MVR during field work in Colombia. Blood samples of *E. madagascariensis* were donated by the Zoo Landau, Germany; for *P. erythrocephala*, a sample from the collection of the Museum of Zoology Dresden was used (Río Casiquiare, Venezuela; MTD T 403). Samples were preserved in an EDTA buffer (0.1 M Tris, pH 7.4, 10% EDTA, 1% NaF, 0.1% thymol) or in ethanol. Frozen blood samples of Colombian specimens are permanently housed in the Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá; samples of *E. madagascariensis* and DNA of the Colombian turtles are stored at −80 °C in the tissue sample collection of the Museum of Zoology, Dresden (MTD T 4232, 4498–4503).

Gene selection

We used mitochondrial and nuclear DNA sequences in order to determine the phylogenetic relationships between the three genera (*Erymnochelys*, *Peltocephalus*, and *Podocnemis*) and the eight species of Podocnemididae (*Erymnochelys madagascariensis*, *Peltocephalus dumerilianus*, *Podocnemis erythrocephala*, *P. expansa*, *P. lewyana*, *P. sextuberculata*, *P. unifilis*, and *P. vogli*). We sequenced the following mtDNA fragments: cytochrome *b* (cyt *b*) gene, nicotinamide–adenine dinucleotide deshydrogenase subunit 4 (ND4) plus the adjacent complete tRNA histidine gene (tRNA-His) and part of the tRNA serine gene (tRNA-ser), D-loop, cytochrome oxidase subunit I (COI), and 12S rRNA gene. With respect to nuclear genes, we produced partial sequences of the recombination-activating gene 2 (Rag2), the intron 1 of the RNA fingerprint protein 35 (R35), and the neurotrophin-3 (NT3) gene. Additional sequences were downloaded from GenBank (Table 1). The chosen markers have been used in previous studies to unravel relations on the terminal as well as deeper levels of chelonian phylogeny. The protein-coding mitochondrial cyt *b* gene is now routinely applied to resolve phylogeny and phylogeography of terminal taxa (e.g. Caccone et al. 1999; Weisrock and Janzen 2000; Fritz et al. 2006a, b, 2008a, b; Praschag et al. 2007), as are the fast-evolving markers ND4 and D-loop (Feldman and Parham 2004; Pearse et al. 2006; Rosenbaum et al. 2007; Amato et al. 2008). The 12S rRNA gene has been used widely in inter- and intrafamiliar studies (e.g. Seddon et al. 1997; Georges et al. 1998; Noonan 2000; Honda et al. 2002). The nuclear genes Rag2 and NT3 and the R35 intron 1 have performed well in resolving deeper nodes of chelonian phylogeny (Fujita et al. 2004; Spinks et al. 2004; Le et al. 2006; Noonan and Chippindale 2006; Fritz and Bininda-Emonds 2007).

Laboratory procedures

Total genomic DNA was extracted by overnight incubation at 37 °C in lysis buffer (10 mM Tris, pH 7.5, 25 mM EDTA, 75 mM NaCl, 1% SDS) including 1 mg of proteinase K (Merck, Whitehouse Station, NJ), followed by the standard phenol/chloroform protein extraction. DNA was precipitated from the supernatant with 0.8 volumes of cold isopropanol, centrifuged, washed, dried and resuspended in TE buffer. Using polymerase chain reaction (PCR), we amplified five mtDNA fragments (D-loop, ND4, 12S RNA, cyt *b*, and COI) and three regions of nuclear DNA (Rag2, R35, and NT3). PCR was performed in a 50 µl volume (Bioron PCR buffer or 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl, 0.5% Triton X-100, pH 8.5) containing 1 unit of Taq DNA polymerase

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Table 1. GenBank accession numbers of sequences used

Species	D-loop	ND4	12S rRNA	16S rRNA	cyt *b*	COI	Rag1	Rag2	R35	NT3	BDNF	POMC
Erymnochelys madagascariensis	–	FM165619	AM943824	AF113640	AM943834	AF113664	AY988099	AM943835	AM943849	AY988085	AY988078	AY988092
Peltocephalus dumerilianus	–	FM165622	AM943823	AF113643	AM943833	AF113667	AY988101	AM943837	AM943848	AY988087	AY988080	AY988093
Podocnemis erythrocephala	FM165612	FM165621	AM943822	–	AM943832	–	–	AM943841	AM943845	FM165607	–	–
Podocnemis expansa	FM165614	FM165620	AM943820	AF113642	AM943830	AF113666	AY988100	AM943839	AM943843	AY988086	AY988079	AY988097
Podocnemis lewyana	FM165610	FM165617	AM943817	–	AM943827	FM165625	–	AM943825	AM943826	FM165605	–	–
Podocnemis sextuberculata	FM165613	FM165616	AM943819	–	AM943831	–	–	AM943840	AM943844	FM165608	–	–
Podocnemis unifilis	FM165611	FM165623	AM943818	–	AM943829	–	–	AM943842	AM943847	FM165606	–	–
Podocnemis vogli	FM165615	FM165618	AM943821	–	AM943828	–	–	AM943838	AM943846	FM165609	–	–
Pelomedusa subrufa	–	FM165624	AF039066	AF113639	AF039066	AF113663	AY988102	AM943836	AY339639	AY988088	AY988081	AY988094
Staurotypus triporcatus	–	–	AB090018	AB090046	U81349	–	AY988105	–	AY339633	AY988091	AY988084	AY988096

(Bioron, Ludwigshafen, Germany), 10 pmol dNTPs (Fermentas, St. Leon-Roth, Germany), and 5 or 10 pmol of the respective primer. Primers and thermocycling conditions are summarized in Table 2. PCR products were purified by precipitation under the following conditions: 1 volume PCR product (30 µl), 1 volume 4 M NH_4Ac (30 µl), and 12 volumes EtOH (100%; 360 µl). DNA was pelleted by centrifugation (15 min at 13,000 rpm) and the pellet washed with 70% ethanol. The pellet was dissolved in 20 µl H_2O. PCR products were sequenced directly on both strands on an ABI 3130 sequencer (Applied Biosystems, Foster City, CA).

Alignment

Sequences were aligned using BIOEDIT 7.0.5.2 (Hall 1999). For the ingroup taxa, all sequences for the protein-coding regions ND4, cyt *b*, COI, and Rag2 aligned perfectly, with no insertions or deletions. The D-loop fragment was highly variable with several insertions and deletions: *Podocnemis expansa* had a single deletion corresponding to position 225 and a 4 bp insertion (CCAA) corresponding to positions 253–256; *P. erythrocephala* had a single deletion corresponding to position 305 and a double insertion (GC) corresponding to positions 321–322; *P. sextuberculata* had a single insertion (C) corresponding to position 250 and a 3 bp deletion corresponding to positions 317–319; *P. unifilis* had a 7 bp insertion (ACCCCGG) corresponding to positions 250–256; and *P. vogli* had a 2 bp insertion (TG) corresponding to positions 83–84, a single insertion (A) corresponding to position 97, a 7 bp deletion corresponding to positions 200–206, and a single deletion at position 305. In the ND4-tRNA-His-tRNA-ser alignment of 797 bp, *Erymnochelys madagascariensis* had a 2 bp deletion corresponding to positions 754–755 (tRNA-ser); *Peltocephalus dumerilianus* had a single deletion corresponding to position 755. For the 12S rRNA gene, *P. dumerilianus* had a single insertion (A) at position 285 and one deletion at position 40; *Podocnemis expansa* had a single insertion (C) at position 143; *P. lewyana* a single insertion (T) at position 303. In the R35 intron, *E. madagascariensis* had a 3 bp deletion corresponding to positions 236–238, a 15 bp deletion corresponding to position 639–653, a 2 bp deletion corresponding to positions 687–688, and a 2 bp insertion (TT) corresponding to positions 403–404. *Peltocephalus dumerilianus* had a 2 bp deletion corresponding to positions 688–689 and a 2 bp deletion corresponding to positions 824–825; *Podocnemis expansa* had a 3 bp deletion corresponding to positions 236–238; *P. sextuberculata* had an 8 bp deletion corresponding to positions 723–730. In NT3, *E. madagascariensis* had a 3 bp deletion at position 206.

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Phylogenetic analyses

Phylogenetic relationships were inferred using probabilistic (Bayesian inference and Maximum Likelihood) and cladistic (Maximum Parsimony) methods. Bayesian analyses (BA) were run using MRBAYES 3.1 (Ronquist and Huelsenbeck 2003) for each individual marker, for partitioned and unpartitioned datasets of concatenated mitochondrial and nuclear genomic markers and of all markers together. For the partitioned analyses, data were divided into 12 partitions (Table 3). The best-fit model of nucleotide substitution was established for each partition and for concatenated sequences using MODELTEST 3.06 (Posada and Crandall 1998), and was incorporated into a single tree search (mixed model partition approach; Nylander et al. 2004). Four incrementally heated Markov chains were run for 10^7 generations with every 100th generation being saved; Bayesian posterior probabilities were obtained from the 50% majority rule consensus trees. For each independent run, the variation in likelihood scores was examined by plotting $-\ln L$ scores against the number of generations, and the burn-in was set to sample only the plateau of the most likely trees.

Maximum Likelihood (ML) and Maximum Parsimony (MP) trees were calculated with PAUP* 4.0b10 (Swofford 2002) for the same datasets, using heuristic searches with 100 random addition sequences of taxa and the tree bisection-reconnection branch swapping option. To examine the robustness of obtained trees, we ran 1000 bootstrap replicates for MP and 100 for ML under the same settings. *Pelomedusa subrufa* (Testudines: Pleurodira: Pelomedusidae) and *Staurotypus triporcatus* (Testudines: Cryptodira: Kinosternidae) served as outgroups (Table 1).

All datasets were tested for incongruence using the incongruence length difference (ILD) test (Farris et al. 1995) implemented in PAUP*, with 1000 replicates to generate the null distribution. No significant incongruence was revealed, neither between the nuclear genes ($p = 0.21$), nor between the mitochondrial genes ($p = 0.58$), nor between nuclear and mitochondrial partitions ($p = 0.94$).

Molecular clock estimations

Divergence times for nodes were estimated using the Bayesian relaxed molecular clock as implemented in the MULTIDISTRIBUTE package (Thorne et al. 1998; Thorne and Kishino 2002). All calculations were performed on the topology obtained from the Bayesian analyses, using the total evidence dataset. In order to determine the appropriate nucleotide substitution model parameters, the total evidence dataset was analyzed using the program PAML 3.13 (Yang 1997). Subsequently, the program ESTBRANCHES was used for estimating branch lengths and their variance–covariance matrix (outgroup: *Staurotypus triporcatus*). Markov chains in MULTIDIVTIME were run for 10^6 generations, sampling every 100th generation for a total of 10^5 trees, with a burn-in of 10^4 trees before the first sampling of the Markov chain. The prior for the mean of the ingroup root age (rttm) was set to a mean of 100 million years ago (mya), corresponding to the split between Podocnemididae and Pelomedusidae (based on the mid-Cretaceous record of the turtles *Brasilemys* and *Araripemys*; Gaffney 1990). The mean of the rate of molecular evolution at the ingroup root node (rtrate) was 0.007555 substitutions per site and million years. Settings for the Brownian motion constant (brownmean) and bigtime were 0.1 and 210 mya (split of the chelonian suborders Cryptodira and Pleurodira; Gaffney 1990), respectively. The node of the clade *Erymnochelys* + *Podocnemis* was calibrated with 65 mya, based on the early Paleocene record of an *Erymnochelys*-like fossil (Gaffney and Forster 2003). Calibration points were open-ended minimum ages, assuming that the first appearance of the group in the fossil record represents its minimum age (Noonan and Chippindale 2006).

Results

Single-gene analyses

All trees based on single genes were weakly resolved to entirely unresolved. As expected, 12S rRNA, cyt *b*, ND4, NT3, and R35 returned Podocnemididae as a well-supported monophylum, and all sequences except the D-loop confirmed with high support the monophyly of the South American genus *Podocnemis*. In part, the branching patterns differed also between tree-building methods (Fig. 1). For the D-loop (not shown), ML and MP produced entirely unresolved trees; in the otherwise unresolved BA tree, (*P. expansa* + *P. vogli*) and (*P. unifilis* + *P. sextuberculata*) clustered together with weak support (posterior probabilities of 0.72 and 0.62, respectively).

Mitochondrial and nuclear datasets

The analyses resulted in very similar tree topologies (Fig. 2). Both datasets suggested with moderate to high support a paraphyly of the South American genera *Peltocephalus* and *Podocnemis* with respect to the Malagasy *Erymnochelys*, and a sister group relationship of *Erymnochelys* + *Podocnemis*. Within *Podocnemis*, *P. expansa* was revealed as the basal species by BA and ML (mtDNA) and by all tree-building methods using the nDNA dataset. All methods and both

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Table 2. Primers and thermocycling conditions used

Gene	Primer	References	Thermocycling conditions					
			ID	C	D	A	PE	FE
D-loop	Pro, CSB	Pearse et al. (2006)	3 min, 94 °C	40	30 s, 94 °C	30 s, 55 °C	60 s, 72 °C	5 min, 72 °C
ND4, tRNA-his, tRNA-ser	L-ND4, H-Leu	Stuart and Parham (2004)	5 min, 94 °C	40	45 s, 94 °C	30 s, 50 °C	60 s, 72 °C	10 min, 72 °C
12S rRNA	L1091, H1478	Kocher et al. (1989)	3 min, 94 °C	35	30 s, 94 °C	45 s, 50 °C	45 s, 72 °C	10 min, 72 °C
cyt *b*	mt-c-For2, mt-E-Rev2	Fritz et al. (2006a)	5 min, 94 °C	35–40	45 s, 94 °C	52 s, 50–60 °C	80 s, 72 °C	10 min, 92 °C
	mt-a-neu3	Praschag et al. (2007)	4 min, 94 °C	30	45 s, 94 °C	60 s, 52 °C	120 s, 72 °C	10 min, 92 °C
COI	M72, M73	Georges et al. (1998)	3 min, 94 °C	35	45 s, 94 °C	45 s, 48 °C	60 s, 72 °C	10 min, 72 °C
Rag2	F2-1, R2-1	Le et al. (2006)	5 min, 95 °C	39	30 s, 95 °C	45 s, 52 °C	60 s, 72 °C	10 min, 72 °C
R35	R35Ex1, R35Ex2	Fujita et al. (2004)	5 min, 94 °C	35–40	30 s, 94 °C	90 s, 50–60 °C	120 s, 72 °C	10 min, 72 °C
NT3	NT3-F3, NT3-R4	Noonan and Chippindale (2006)	5 min, 95 °C	35	45 s, 95 °C	45 s, 55 °C	80 s, 72 °C	10 min, 72 °C

Abbreviations: ID = initial denaturing, C = number of cycles, D = denaturing, A = annealing conditions, PE = primer extension, FE = final elongation.

Table 3. Data partitions analysed with Maximum Parsimony, Maximum Likelihood, and Bayesian inference of phylogeny

Partition	Fragment length (aligned)	Variable characters	Parsimony informative characters	MP tree length	RI	CI	ML $-\ln L$	BA $-\ln L$	Source	Selected AIC model
D-loop	341	165	67	263	0.39	0.81	1511.04	1955.31	This study	HKY + G
ND4	797	393	206	446	0.35	0.90	4145.9	3973.76	This study	GTR + G
12S rRNA	407	154	68	251	0.50	0.77	1737.44	1684.53	This study	GTR + G
16S rRNA	420	140	43	222	0.51	0.90	1506.27	–	Noonan and Chippindale (2006)	GTR + G
cyt *b*	1044	502	311	1124	0.37	0.63	6166.24	5715.52	This study	GTR + I + G
COI	376	113	36	165	0.53	0.89	1230.55	–	Georges et al. (1998); this study	GTR + G
All mtDNA	3385	1467	731	2970	0.38	0.71	16612.28	15494.49	This study	TVM + G
Rag1	769	105	11	114	0.72	0.97	1674.42	–	Noonan and Chippindale (2006)	HKY
Rag2	636	84	19	88	0.93	0.97	1372.08	1392.84	This study	HKY
BDNF	714	44	12	49	0.58	0.89	1267.94	–	Noonan and Chippindale (2006)	K80 + G
POMC	440	95	13	112	0.38	0.92	1138.95	–	Noonan and Chippindale (2006)	GTR + I
R35	976	267	91	320	0.85	0.92	2919.22	2927.74	This study	HKY
NT3	580	52	14	58	0.90	0.94	1149.56	1158.61	This study	TIM + I
All nDNA	4115	647	160	747	0.81	0.93	9709.63	8998.57	This study	HKY + G
Total evidence	7500	2123	891	3538	0.45	0.75	27531.55	24499.91	This study	TVM + I + G

Under MP, a single most parsimonious tree was obtained for each partition.

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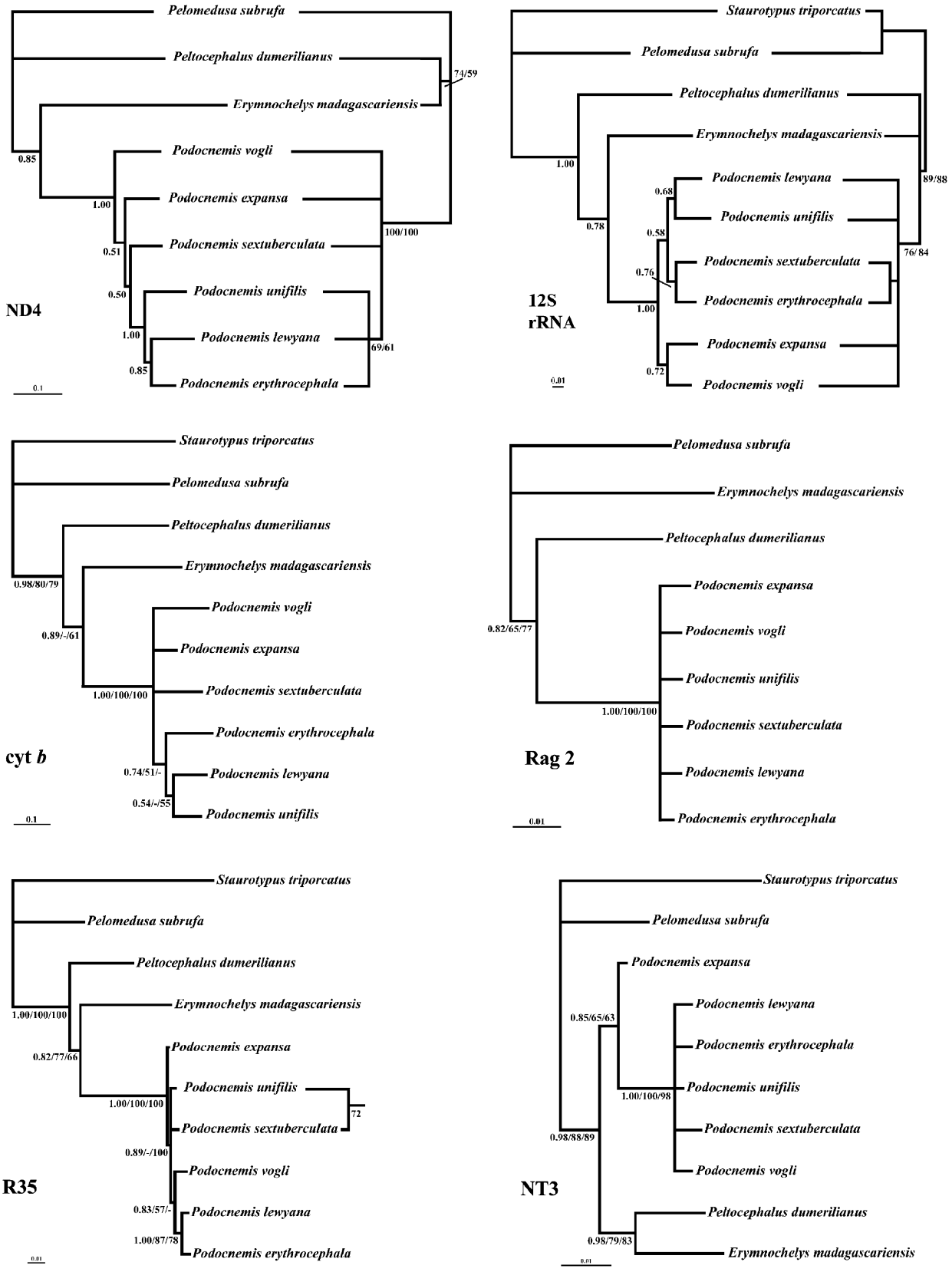

Fig. 1. Bayesian topologies obtained for ND4, 12S rRNA, cyt *b*, Rag2, R35, and NT3. Bayesian posterior probabilities and bootstrap support under Maximum Likelihood and Maximum Parsimony greater than 50% indicated. For ND4 and12S rRNA, the different branching pattern of ML and MP shown on the right; for R35, alternative branching pattern of ML on the right. Dashes indicate support values below 50%.

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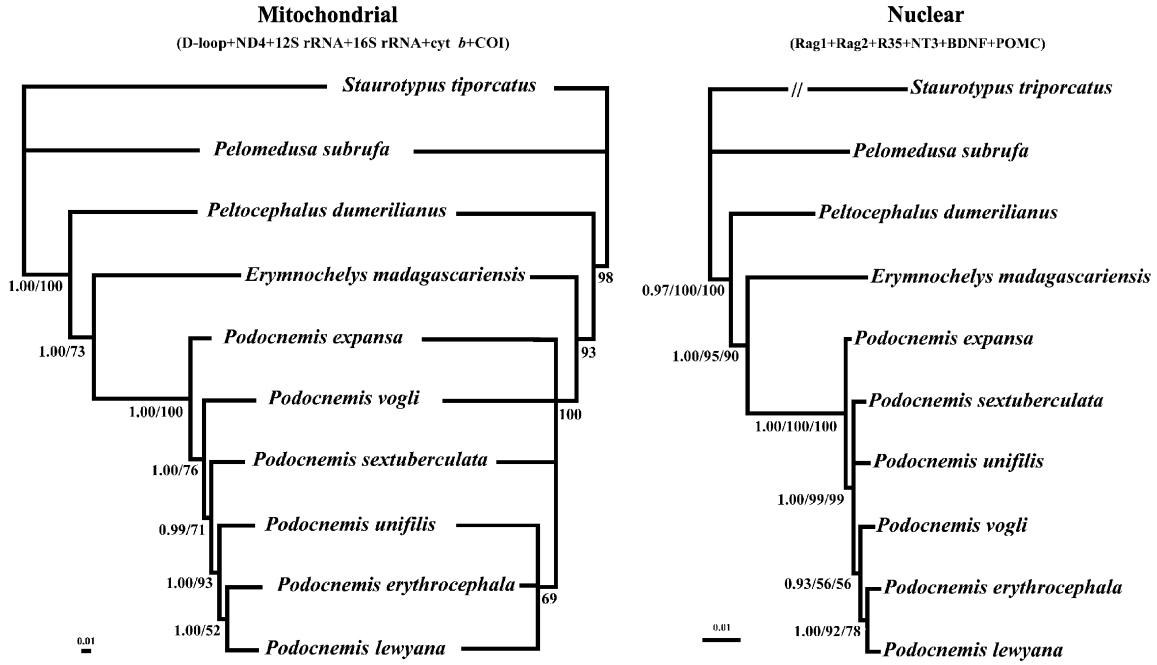

Fig. 2. Bayesian topologies obtained for mitochondrial and nuclear partitions. Bayesian posterior probabilities and bootstrap support under Maximum Likelihood and Maximum Parsimony greater than 50% indicated (MP topology for mtDNA on the right).

datasets, except MP for mtDNA, agreed further in placing *P. erythrocephala* and *P. lewyana* as sister species. From mtDNA, the branching pattern for the *Podocnemis* species was weakly resolved under MP, while the two other methods agreed with moderate to high support values that *P. sextuberculata*, *P. vogli*, and *P. expansa* are the successive sister taxa of the crown clade formed by *P. unifilis* + (*P. erythrocephala* + *P. lewyana*). Using the nDNA dataset, *P. vogli* was suggested with weak support as the sister taxon of *P. erythrocephala* + *P. lewyana*; these three species occurred together with *P. sextuberculata* and *P. unifilis* in a well-supported multifurcation.

Total evidence analyses

The branching pattern for the partitioned and unpartitioned analyses of the total evidence dataset consisting of 7500 characters matches exactly the BA and ML topology of the concatenated mtDNA dataset. The basal splits between the three podocnemidid genera are well-supported under all methods (99–100%). For some nodes within *Podocnemis* weaker support values were achieved under ML and particularly under MP (Fig. 3); all nodes received 100% support in the unpartitioned BA (not shown).

Divergence times

The results of the relaxed molecular clock calculations suggested, in agreement with the underlying fossil

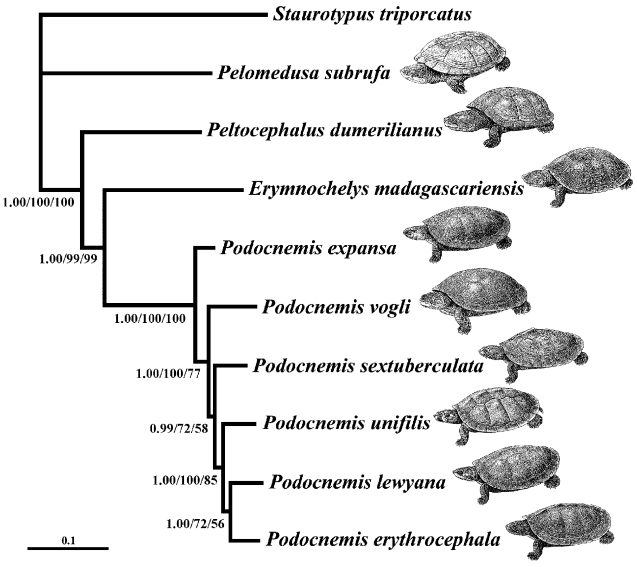

Fig. 3. Phylogenetic hypothesis for Podocnemididae based on total evidence (Bayesian tree; partitioned analysis). Numbers along nodes are BA, ML, and MP support values. Species icons from http://www.flmnh.ufl.edu.

evidence, that the podocnemidid–pelomedusid split occurred during the Early Cretaceous (Albian; 109.25 mya; SD = 0.68; Fig. 4). The branching-off of *Peltocephalus* was dated to 85.74 mya (SD = 0.67), the separation of *Erymnochelys* and *Podocnemis* to 78.46 mya (SD = 0.67), both corresponding to the Late Cretaceous. Within *Podocnemis*, the basal species *P. expansa* diverged during the Late Eocene (36.86 mya,

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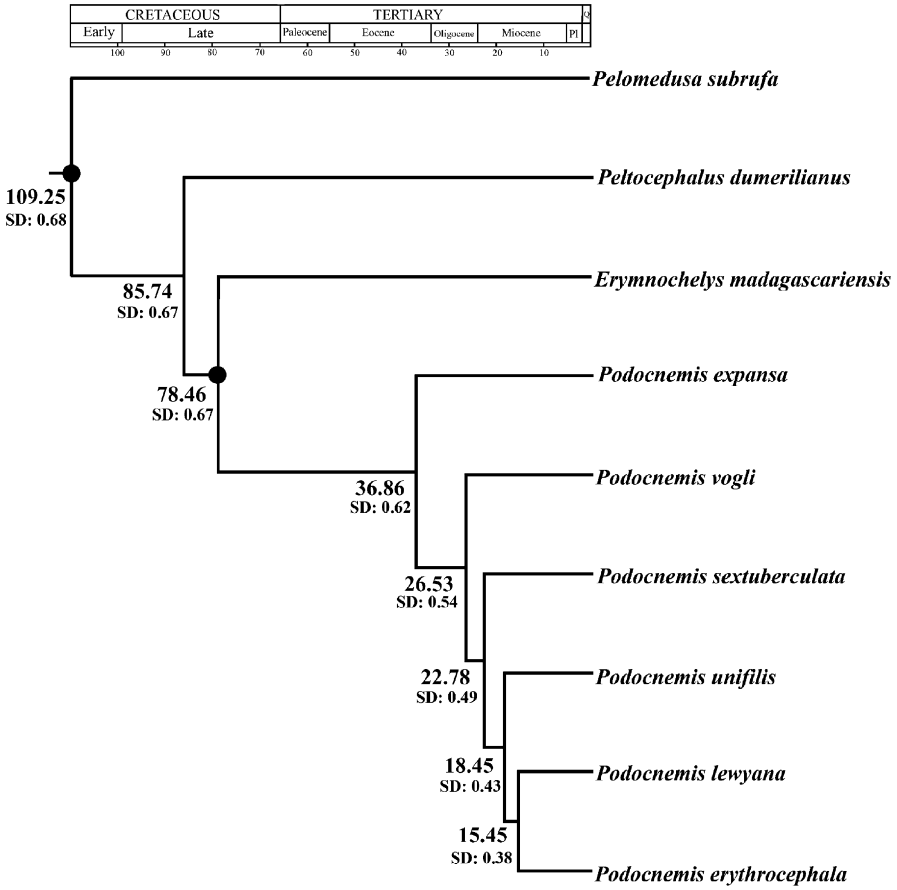

Fig. 4. Relaxed molecular clock estimates for ages of extant Podocnemididae (Bayesian tree). Black circles correspond to fossil calibration points: 100 mya for the podocnemidid–pelomedusid split (Gaffney 1990), 65 mya for the *Erymnochelys–Podocnemis* split (Gaffney and Forster 2003). Inferred ages of podocnemidid divergences with standard deviations indicated. Abbreviations: Pl = Pliocene, Q = Quaternary.

SD = 0.62), *P. vogli* in the Late Oligocene (26.53 mya, SD = 0.54), *P. sextuberculata* and *P. unifilis* in the Early Miocene (22.78 mya, SD = 0.49 and 18.45 mya, SD = 0.43, respectively), and *P. erythrocephala* and *P. lewyana* split during the Middle Miocene at 15.45 mya (SD = 0.38).

Discussion

Prior to the present work, the phylogeny of extant podocnemidids was only incompletely assessed using DNA sequence data, and all previous studies focused on the relationships of the three genera *Erymnochelys*, *Peltocephalus*, and *Podocnemis*, but never tackled the phylogeny of the six *Podocnemis* species. Traditionally, all extant podocnemidids were placed in the same genus (*Podocnemis*; e.g. Wermuth and Mertens 1961, 1977). Based on serological evidence, Frair et al. (1978) removed one Amazonian species and the Malagasy species from *Podocnemis* and transferred them to two monotypic genera, *Peltocephalus* and *Erymnochelys*, respectively. Using morphological characters, Gaffney (1988) and Gaffney and Meylan (1988) proposed that the Malagasy *Erymnochelys* constitutes the sister taxon of a purely South American clade, *Peltocephalus* + *Podocnemis*. Georges et al. (1998) and Noonan (2000) analyzed the phylogenetic relationships of *Erymnochelys madagascariensis*, *Peltocephalus dumerilianus*, and *Podocnemis expansa*, using 1382 bp of the mitochondrial COI, 12S and 16S rRNA genes and the nuclear genomic c-mos gene or 921 bp of the 12S and 16S rRNA genes, respectively. Both studies contradicted Gaffney (1988) and Gaffney and Meylan (1988) and suggested that Neotropical podocnemidids are paraphyletic with respect to *Erymnochelys*, the latter being sister to *Podocnemis*. In a subsequent study, Noonan and Chippindale (2006) analyzed the divergence times of the three genera, using portions of four nuclear genes (Rag1, NT3, BDNF, and POMC) and two mitochondrial loci (12S rRNA and 16S rRNA) for *Erymnochelys madagascariensis*, *Peltocephalus dumerilianus*, and *Podocnemis expansa*. Phylogenetic analyses of these genes

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resulted in the same branching pattern as in the two preceding studies by Georges et al. (1998) and Noonan (2000). Based on morphological evidence some authors still believe that South American podocnemidids are monophyletic, however (de Lapparent de Broin 2000; de la Fuente 2003; Romano and Azevedo 2006).

Our total evidence analyses of nuclear and mitochondrial DNA sequences have yielded a completely resolved phylogeny of extant podocnemidids and confirm the findings of Georges et al. (1998), Noonan (2000), and Noonan and Chippindale (2006) regarding the generic relationships. Furthermore, we present for the first time a phylogenetic hypothesis for the six extant *Podocnemis* species. Within *Podocnemis*, the close relations of *P. lewyana* with *P. erythrocephala* and *P. unifilis*, revealed by all applied tree-building methods, was unexpected considering the morphological similarity and distribution of *P. lewyana* and *P. vogli*. The latter two are the only extant *Podocnemis* species occurring outside the Amazon Basin, with restricted neighbouring ranges (*P. lewyana* west of the eastern Cordillera in the Magdalena and Sinu river basins of Colombia; *P. vogli* west of the eastern Cordillera and northwest of the Guyana Shield; Pritchard and Trebbau 1984; Ernst et al. 2000).

Our inferred age for the branching-off of *Peltocephalus* (~86 mya) falls in a period of the Late Cretaceous when a contiguous Gondwana landmass exclusive of Africa is likely to have still existed (Krause et al. 1997; Sampson et al. 1998; Noonan and Chippindale 2006). This suggests that *Peltocephalus* originated in what later became South America, and remained stationary there. Our age of divergence of *Erymnochelys* and *Podocnemis* (~78 mya), being in line with the estimate obtained by Noonan and Chippindale (2006), coincides with the supposed submergence of the land bridge between Madagascar and Antarctica + South America, and corroborates the hypothesis of Noonan and Chippindale (2006) and Romano and Azevedo (2006) that this vicariant event led to the origin of *Erymnochelys*. The novel time frame for the diversification of *Podocnemis* presented here, from the Late Eocene (~37 mya) to the Middle Miocene (~15 mya), corresponds with major environmental changes in South America as well as with the diversification ages of South American marsupials (Steiner et al. 2005) and xenarthrans (Delsuc et al. 2004), suggesting that these radiations were triggered by the same environmental forces. The Late Eocene origin of *P. expansa* agrees well with the first phase of Andean uplift, which incited a change from the Paleocene–Eocene warm woodland habitats to more arid and cooler conditions (Flynn and Wyss 1998; Zachos et al. 2001). The radiation of the other *Podocnemis* species, from approximately 27 to 15 mya, took place during a phase of environmental instability after the Eocene–Oligocene boundary that was characterized by further global cooling and aridification. Permanent Antarctic ice sheets developed then, resulting in a dramatic drop of the sea level and changes in oceanic and atmospheric circulation, and inducing significant alterations of terrestrial habitats (Haq et al. 1997; Zachos et al. 2001). A second phase of Andean uplift was associated in the Miocene with a regression of tropical forests, the spread of savannahs (Steiner et al. 2005), and changing river drainage patterns (Hoorn et al. 1995), all factors suggestive of vicariant events responsible for the diversification of the river-dwelling *Podocnemis* species.

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