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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.


***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