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## Molecular phylogeny of Central and South American slider turtles: implications for biogeography and systematics (Testudines: Emydidae: *Trachemys*)

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### Abstract

We analyse phylogeny, systematics and biogeography of slider turtles (*Trachemys* spp.) using sequence data of four mitochondrial genes (3242 bp) and five nuclear loci (3396 bp) of most South American and southern Central American taxa and representatives of northern Central American, West Indian and North American slider species (16 species and subspecies) and allied North American species (genera *Chrysemys*, *Deirochelys*, *Graptemys*, *Malaclemys*, *Pseudemys*). By applying maximum likelihood, relaxed molecular clock and ancestral range analyses, we provide evidence for two successive colonizations of South America by slider turtles. In addition, we show that the current species delineation of Central and South American slider turtles is incorrect. Our data suggest that *Trachemys grayi* is a distinct polytypic species that embraces, besides the nominotypical subspecies, *T. g. emolli* and *T. g. panamensis*. *Trachemys ornata* is also polytypic with the subspecies *T. o. ornata*, *T. o. callirostris*, *T. o. cataspila*, *T. o. chichiriviche* and *T. o. venusta*. Moreover, *T. aditrix* should be regarded as a subspecies of *T. dorbigni*. All studied *Trachemys* species are inferred to have originated in the Late Miocene to Early Pliocene. The ancestor of the two subspecies of *T. dorbigni* colonized South America most probably prior to the establishment of the land bridge connecting Central and South America, whereas the two South American subspecies of *T. ornata* represent a younger independent immigration wave from Central America.

**Key words:** Ancestral range analysis – phylogeography – molecular clock – numt – species delineation – *Chrysemys* – *Deirochelys* – *Graptemys* – *Malaclemys* – *Pseudemys*

### Introduction

The speciose slider turtle genus *Trachemys* Agassiz, 1857 represents one of the most widely distributed American reptile groups. Several parapatric species range over large parts of North and Central America to northern South America (Colombia and Venezuela); two further, widely disjunct South American species occur in northern Brazil (state of Maranhão) and in the region of the Rio de la Plata (Brazil, Argentina, Uruguay). Four additional species live in the West Indies (Seidel 1988, 2002; Fritz and Havaš 2007; Rhodin et al. 2010; Fig. 1; Table 1). All *Trachemys* species are highly aquatic, medium- to large-sized turtles that leave the water only for basking, short overland movements and nesting (Gibbons 1990; Gibbons et al. 1990; Ernst et al. 2000; Ernst and Lovich 2009). The best-known taxon is the North American red-eared slider, *Trachemys scripta elegans*, which was traded for decades in enormous numbers as a pet. As a consequence, it has been naturalized worldwide in many regions outside its native range (Ernst et al. 2000; Fritz and Havaš 2007). *Trachemys* belongs to the mainly Nearctic family Emydidae and is its only genus that colonized Central and South America and the West Indies. Since the land connection of the two Americas did not form before the Pliocene (approximately 3 million years ago; Marshall 1979, 1985, 1988; Lessios 2008; Molnar 2008; Woodburne 2010), *Trachemys* has been postulated to be a recent invader of Central and South America (Savage 1966; Pritchard and Trebbau 1984; Vanzolini and Heyer 1985;

Legler 1990; Moll and Moll 1990; Seidel and Jackson 1990; Vanzolini 1995; de la Fuente et al. 2002).

Slider turtles generally do not occur in closed forest or rainforest, probably because of the difficulty in finding suitable, open nesting sites (Moll and Legler 1971; Pritchard and Trebbau 1984; Moll and Moll 1990). Accordingly, the South American species are confined to regions north and south of the Amazon Basin. This conspicuous distribution pattern suggests that the two disjunct southernmost species, *T. aditrix* and *T. dorbigni*, might have crossed the Amazon Basin during a phase of rainforest fragmentation (Pritchard and Trebbau 1984; Moll and Moll 1990) and that the northern South American *T. callirostris* is their closest relative (Williams 1956). If this scenario should be correct, it would strongly support the contentious 'forest refugia hypothesis'. According to it, increasing aridity and cooling during the Pleistocene glaciations resulted in isolated rainforest patches being separated by savannahs and deserts (e.g. Haffer 1969, 1997; Prance 1973; Potts and Behrensmeyer 1992; Hooghiemstra and van der Hammen 1998). This hypothesis was widely accepted until a few years ago, but has been severely criticized recently (e.g. Kastner and Goñi 2003; Pennington et al. 2004) because pollen cores show no evidence of reduced rainforest cover in the Amazon Basin (e.g. Colinvaux et al. 2001; Mayle et al. 2004), dynamic vegetation model simulations reject the hypothesis of widespread grasslands in Amazonia during the last glacial maximum (e.g. Cowling et al. 2001) and genetic data suggest that diversification in tropical rainforest animals generally predates the Pleistocene (Moritz et al. 2000; Glor et al. 2001). Yet, the genetic structure of two open-habitat reptiles argues in favour of former forest fragmentation (*Chelonoidis carbonaria*: Vargas-Ramírez et al. 2010a; *Crotalus durissus*: Wüster et al. 2005; Quijada-Mascareñas et al. 2007). At least in the case of *Ch. carbonaria*, the onset of the vicariant event corresponding to forest fragmentation seems to predate

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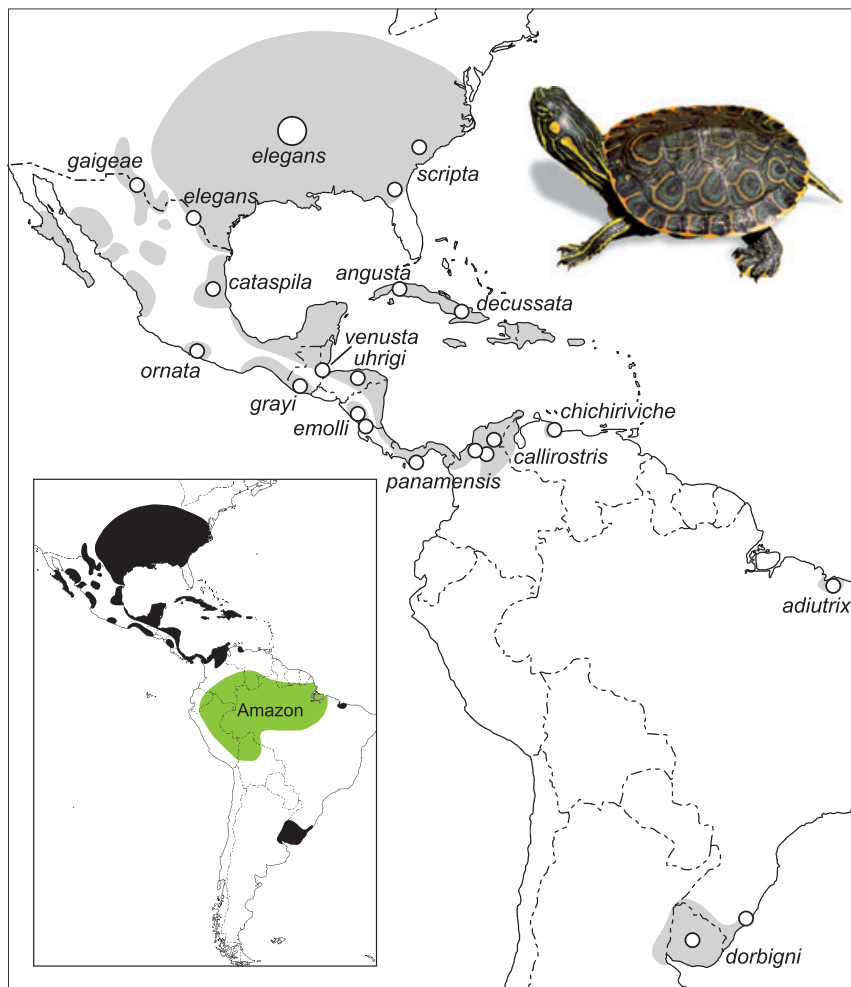


Fig. 1. Distribution of *Trachemys*. Inset shows the Amazon rainforest separating the widely disjunct South American species *Trachemys adiutrix* and *T. dorbigni*. Circles indicate the approximate collection sites of samples used in the present study

the Pleistocene, however (Vargas-Ramírez et al. 2010a), and fossil land mammals argue for a Pliocene, and not Pleistocene, trans-Amazonian savannah corridor (Marshall 1979, 1985). Due to their confinement to open habitats, slider turtles are an attractive additional model for assessing former Amazonian forest fluctuations and their age. Unfortunately, the systematics and phylogeny of *Trachemys* are badly understood. For a long time, most authors placed all or nearly all continental American *Trachemys* populations in a single polytypic species (*T. scripta* sensu lato; Moll and Legler 1971; Ernst 1990; Legler 1990; Ernst et al. 2000). By contrast, at present, most authors recognize up to 11 in part polytypic continental American slider species (plus four additional West Indian species; Seidel 2002; Fritz and Havaš 2007; Jackson et al. 2008; Rhodin et al. 2010; Table 1; but see Wiens et al. 2010, who still adhere to a more inclusive concept of *T. scripta*). However, neither assessment has been sufficiently tested by molecular means. Jackson et al. (2008) used mtDNA sequences (768 bp; partial ND4 gene plus flanking tRNA genes) of 12 species and additional six subspecies of *Trachemys* to reconstruct their phylogeny, but these authors did not study some crucial Central and South American species (*T. ornata*, *T. adiutrix*) or representatives of the allied North American genera *Graptemys*, *Malaclemys* and *Pseudemys*. Jackson et al. (2008) achieved only a weak resolution for the phylogeny of Central and South American *Trachemys*. Two other, more general papers (Spinks et al. 2009; Wiens et al. 2010) used more genes,

but distinctly fewer taxa of *Trachemys* (four or five, respectively) and obtained in part contradictory branching patterns (see Discussion).

Here, we aim at clarifying the phylogeny of *Trachemys* using the sequence variation of four mitochondrial genes (3242 bp) and five nuclear loci (3396 bp) of most South American and southern Central American taxa and representatives of northern Central American, West Indian and North American slider species (16 species and subspecies) and allied North American taxa (Tables 1, S1). Based on the obtained phylogeny, we try to elucidate the historic biogeography of *Trachemys*. In particular, we examine whether the separation of the South American species *T. adiutrix* and *T. dorbigni* can be used for assessing the age of Amazon rainforest fragmentation. To gauge former range shifts and their timing, we apply a relaxed molecular clock calibrated with fossil evidence and an ancestral range analysis.

## Materials and Methods

### Gene selection and taxon sampling

Three mitochondrial genes and five nuclear loci that had been shown to reveal phylogenetic relationships and differences among terminal chelonian taxa (e.g. Le et al. 2007; Fritz and Bininda-Emonds 2007; Fritz et al. 2008a, 2010a; Vargas-Ramírez et al. 2008, 2010b; Wiens et al. 2010) were chosen. The mitochondrial genes were the partial 12S ribosomal RNA (12S rRNA) gene, the cytochrome *b* (*cytb*) gene and

Table 1. Species and subspecies of *Trachemys* according to Seidel (2002), Fritz and Havaš (2007) and Rhodin et al. (2010). Terminal taxa included in the present study are asterisked

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\**Trachemys adiutrix* Vanzolini, 1995  
*Trachemys callirostris* (Gray, 1856)  
 \**T. c. callirostris* (Gray, 1856)  
 \**T. c. chichiriviche* (Pritchard and Trebbau, 1984)  
*Trachemys decorata* (Barbour and Carr, 1940)  
*Trachemys decussata* (Gray, 1831)  
 \**T. d. decussata* (Gray, 1831)  
 \**T. d. angusta* (Barbour and Carr, 1940)  
 \**Trachemys dorbigni* (Duméril and Bibron, 1835)  
 \**Trachemys emolli* (Legler, 1990)  
*Trachemys gaigeae* (Hartweg, 1939)  
 \**T. g. gaigeae* (Hartweg, 1939)  
*T. g. hartwegi* (Legler, 1990)  
*Trachemys nebulosa* (Van Denburgh, 1895)  
*T. n. nebulosa* (Van Denburgh, 1895)  
*T. n. hiltoni* (Carr, 1942)  
 \**Trachemys ornata* (Gray, 1831)  
*Trachemys scripta* (Schoepff, 1792)  
 \**T. s. scripta* (Schoepff, 1792)  
 \**T. s. elegans* (Wied, 1839)  
*T. s. troostii* (Holbrook, 1836)  
*Trachemys taylori* (Legler, 1960)  
*Trachemys venusta* (Gray, 1856)  
 \**T. v. venusta* (Gray, 1856)  
 \**T. v. cataspila* (Günther, 1885)  
 \**T. v. grayi* (Bocourt, 1868)  
*T. v. iversoni* McCord, Joseph-Ouni, Hagen and Blanck, 2010  
 \**T. v. panamensis* McCord, Joseph-Ouni, Hagen and Blanck, 2010  
 \**T. v. uhrigi* McCord, Joseph-Ouni, Hagen and Blanck, 2010  
*Trachemys stejnegeri* (Schmidt, 1928)  
*T. s. stejnegeri* (Schmidt, 1928)  
*T. s. vicina* (Barbour and Carr, 1940)  
*T. s. malonei* (Barbour and Carr, 1938)  
*Trachemys terrapen* (Lacépède, 1788)  
*Trachemys yaquia* (Legler and Webb, 1970)

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the NADH dehydrogenase subunit 4 (ND4) gene. In addition, sequences of the mitochondrial NADH ubiquinone oxidoreductase chain 4L (ND4L) gene were generated. The nuclear genomic loci were the protein-coding oocyte maturation factor Mos (C-mos) gene, the ornithine decarboxylase (ODC) gene, the intron 1 of the RNA fingerprint protein 35 (R35) gene and the partial recombination activating genes 1 and 2 (Rag1, Rag2). Ethanol-preserved blood, saliva or tissue samples of 59 individuals representing 16 species and subspecies of *Trachemys* were studied, plus two samples of *Chrysemys picta dorsalis* and one sample each of *Deirochelys reticularia*, *Graptemys barbouri*, *G. gibbonsi*, *G. nigrinoda delticola*, *G. pseudogeographica*, *Malaclemys terrapin centrata*, *M. t. tequesta*, *Pseudemys concinna*, *P. floridana*, *P. nelsoni* and *P. rubriventris* (Table S1). The latter taxa are allied to *Trachemys* and belong to the same subfamily (Deirochelyinae; Gaffney and Meylan 1988; Wiens et al. 2010). Mitochondrial sequences were generated for all samples, and nuclear sequences for at least one representative sample of each mitochondrial clade. Remaining samples and DNA are stored at  $-80^{\circ}\text{C}$  in the tissue collection of the Museum of Zoology, Dresden.

### Laboratory procedures

Total genomic DNA was extracted from tissue or blood samples using the DTAB method (Gustincich et al. 1991) or the innuPREP DNA Mini Kit for tissue and saliva samples and the innuPREP Blood DNA Mini Kit (both Analytik, Jena, Germany) for blood samples.

The 12S, C-mos, Rag1 and Rag2 fragments were amplified and sequenced using the same primers as in Le et al. (2007) and Fritz et al. (2010a). The nuclear loci ODC and R35 were amplified and sequenced using the primers of Friesen et al. (1999) and Fujita et al. (2004), respectively.

Initially, the universal turtle primers cytbG, mt-E-rev2, mt-c-for2 and mt-f-na (Spinks et al. 2004; Fritz et al. 2006a; Prasczag et al. 2007; Table S2) were used for amplifying and sequencing the cytb gene in two fragments overlapping by approximately 300 bp. However, this procedure sometimes yielded mismatching sequences from the same sample, suggestive of the involvement of numts. The presence of such nuclear copies of mitochondrial genes has previously been shown for some turtle and tortoise species (Stuart and Parham 2004; Spinks and Shaffer 2007; Fritz et al. 2010b), and specifically designed PCR primers turned out to be a powerful tool for amplifying authentic mtDNA (Fritz et al. 2010b). Accordingly, the universal PCR primers were abandoned, and the new specific primer pair Trach\_1.for/Trach\_2.rev (Table S2) was designed using a complete mitochondrial genome of *Trachemys scripta* from GenBank (accession number FJ392294) and the software OLIGOEXPLORER 1.2 (<http://www.genelink.com/tools/gl-oe.asp>). The new primer pair was used then for the amplification of the complete cytb gene in one PCR product.

Based on this approach, specific primers were also developed for amplifying and sequencing the complete ND4L and ND4 genes in three partially overlapping fragments (Table S2).

Although these specific primers worked excellent for all ingroup species, contradictory results were still obtained in *Deirochelys*. Therefore, a long-range PCR approach (Thalmann et al. 2004) was chosen to overcome this problem. Primers were designed to amplify an approximately 5.6-kb-long mtDNA fragment embracing several genes (tRNA-Arg, ND4L, ND4, tRNA-His, tRNA-Ser, tRNA-Leu, ND5, ND6, tRNA-Glu, cytb, tRNA-Thr; Table S2). To confirm earlier obtained sequences, this approach was used not only for *Deirochelys*, but also for representative ingroup samples.

Individual gene fragments were amplified in a total volume of 20  $\mu\text{l}$  containing 1 unit *Taq* polymerase (Bioron, Ludwigshafen, Germany), 1 $\times$  buffer (as recommended by the supplier), 0.5  $\mu\text{M}$  of each primer and 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany). PCR protocols are summarized in the Supporting Information (Table S3).

Long-range PCR was performed using 2.5 units TaKaRa LA *Taq*<sup>TM</sup> (TaKaRa, Shiga, Japan) in a final volume of 75  $\mu\text{l}$  containing 1 $\times$  buffer (as recommended by the supplier), 2.5 mM  $\text{MgCl}_2$ , 67 nM of each primer and 1.5 mM of each dNTP. The cycling protocol was basically divided into two steps: after an initial denaturation at  $94^{\circ}\text{C}$  for 3 min, there was a touch-down PCR step over seven cycles, with denaturation at  $94^{\circ}\text{C}$  for 60 s, annealing at temperatures ranging from 62 to  $59^{\circ}\text{C}$  (lowering  $0.5^{\circ}\text{C}$  per cycle) for 90 s and 8 min at  $70^{\circ}\text{C}$  for extension. Subsequently, 35 PCR cycles were performed, with initial denaturation at  $94^{\circ}\text{C}$  for 60 s, annealing at  $58^{\circ}\text{C}$  for 90 s and extension at  $70^{\circ}\text{C}$  for 8 min. This cycling protocol was finalized by an extension step at  $72^{\circ}\text{C}$  for 10 min.

PCR products were purified using the ExoSAP-IT enzymatic cleanup (USB Europe GmbH, Staufien, Germany; modified protocol: 30 min at  $37^{\circ}\text{C}$ , 15 min at  $80^{\circ}\text{C}$ ). Subsequent sequencing reactions were performed using the primers indicated in Table S2, and nucleotide sequences were resolved on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Cycle sequencing products were purified by precipitation under the following conditions: 1 volume sequencing reaction (10  $\mu\text{l}$ ), 0.1 volume 3 M NaAc (1  $\mu\text{l}$ , pH 4.6) and 2.5 volumes EtOH (100%; 27.5  $\mu\text{l}$ ) or by using Sephadex (GE Healthcare, München, Germany).

### Alignment, partitioning and phylogenetic analyses

GenBank accession numbers of sequences produced in this study are given in the Supporting Information (Table S1). All sequences aligned perfectly in BIOEDIT 7.0.5.2 (Hall 1999). Sequences were further inspected in MEGA 5.05 (Tamura et al. 2011), and uncorrected *p* distances were calculated using this software. From concatenating the mtDNA fragments, an alignment of 3242 bp (including gaps) was obtained. The partial 12S rRNA gene contributed 401 bp; the nearly complete cytb plus the adjacent tRNA-Thr gene, 1140 bp plus 29 bp, respectively; and the overlapping ND4L and ND4 genes, 1672 bp. Concatenating the five nuclear fragments yielded an alignment of 3396 bp of which 563 bp corresponded to C-mos, 620 bp to ODC,

962 bp to R35, 614 bp to Rag1, and 637 bp to Rag2. The mtDNA and nDNA data sets used for calculations contained only sequences from turtles of which none or, in two cases of the nDNA data set, one of the DNA fragments was missing (Table S1). For combined analyses of nDNA and mtDNA, mitochondrial sequences of the same samples were added to the nDNA data set, yielding an alignment of 6638 bp in total.

Maximum likelihood (ML) trees were computed with RAxML 7.0.3 (Stamatakis 2006) and the implemented evolutionary model GTR + G for each data set, i.e., mtDNA, nDNA and mtDNA combined with nDNA (mtDNA + nDNA). To explore the influence of the partition scheme on tree topology, three ML trees were calculated for each of these data sets using the fast parametric bootstrap algorithm and (1) unpartitioned alignments, (2) alignments partitioned by gene and (3) alignments partitioned by gene and codon, that is, using each non-protein-coding DNA fragment (tRNA-Thr, introns) and additionally each codon of protein-coding genes as a distinct partition. The resulting trees were compared and no conflicting topologies observed. However, the highest bootstrap support was obtained for partition scheme (3), which is why an in-depth analysis was performed using this scheme. For doing so, five independent ML searches were conducted using different starting conditions and the fast bootstrap algorithm to examine the robustness of the branching patterns by comparing the best-scored trees. Subsequently, 1000 non-parametric thorough bootstrap replicates were calculated and plotted against the best-scoring tree with the highest likelihood value. All trees were rooted with *Deirochelys reticularia* according to the assumption that this species constitutes the sister taxon of all other Deirochelyinae (Wiens et al. 2010).

### Molecular dating

Based on the concatenated mtDNA + nDNA data set, split ages of taxa were estimated by an uncorrelated relaxed clock as implemented in BEAST 1.4.8 (Drummond and Rambaut 2007). Due to computational limitation, calculations were based on a reduced partitioned data set that included only one representative of each taxon, or when the same taxon appeared in different clades, representatives of each of these clades. Evolutionary models for each partition were selected using MRMODELTEST (Nylander 2004); other settings were random starting tree (tree prior speciation: yule process) and 30 million generations. The 'auto optimize' option was activated to adjust automatically the tuning parameters. Input sequence data were manually partitioned according to the different gene fragments in the XML file generated with BEAUTI. The following substitution models were a priori assigned to each partition according to the estimates with MRMODELTEST: 12S rRNA – GTR+G, ND4L – HKY+I, ND4 – HKY+G, *cytb* – GTR+G+I, tRNA-Thr – K80, C-mos – GTR, ODC – HKY+I, R35 – HKY+I, Rag1 – GTR+I and Rag2 – GTR+G. Linearized consensus trees including posterior probabilities and 95% highest posterior density (HPD) intervals for tMRCA estimates were inferred from the tree output files using TREEANNOTATOR (as implemented in the BEAST package) with the burn-in parameter set to 9000.

For tree calibration, two fossil *Trachemys* species were used. *Trachemys inflata* is known from the Late Miocene of Florida (approximately 7 million years = Ma; Seidel and Jackson 1990) and somewhat younger Hemphillian sites (approximately 5 Ma) of Nebraska and Tennessee (Parmalee et al. 2002; Holman and Parmley 2005). Although the exact relationships of *T. inflata* are somewhat contentious (Parmalee et al. 2002), it is clear from its strongly serrated peripheral shell bones that it is allied to the extant North American and West Indian slider turtles (*T. scripta* sensu stricto, *T. decorata*, *T. decussata*, *T. stejnegeri*, *T. terrapen*). Only these species share this character with *T. inflata*, but not any Central and South American *Trachemys* species. Therefore, *T. inflata* was used for calibrating the divergence between the most inclusive clade containing North American and West Indian sliders and its sister group by setting the time for their most recent common ancestor (tMRCA) to a minimum age of 7.0 Ma. The first true representative of the lineage of *T. scripta* sensu stricto is the widespread Pliocene species *T. idahoensis* (approximately 4 Ma; Seidel and Jackson 1990), which was used to

constrain the split between *T. scripta* sensu stricto and its sister taxon by setting tMRCA to a minimum age of 4.0 Ma. Molecular dating was performed in two independent runs with priors for the most recent common ancestors (tMRCA) having a lognormal or uniform distribution, respectively.

As fossils are thought to represent minimum ages, fossil calibrations are often provided with an additional soft maximum constraint (Benton et al. 2009). In our first dating approach, the probability density (lognormal distribution range) was codified by manually adjusting lognormal means and standard deviations to such maximum constraints to match the distribution in real space. For the lognormal tMRCA prior distribution of the younger node, the age of the next older fossil was chosen as maximum constraint (mean = 0.5, standard deviation = 0.3). For the older node, the beginning of the Tortonian (12.0 Ma; Walker and Geissman 2009) was used as soft maximum (mean = 0.75, standard deviation = 0.4). In the second calibration, the uniform prior range was the same for the younger node (lower bound = 4.0 Ma, upper bound = 7.0 Ma). However, a broadened uniform prior range was assigned to the older node by setting the upper bound to '100.0' (acknowledging that for this node only a minimum fossil constraint is available and that there is no true upper bound; prior goes to 'infinity').

### Biogeography

Ancestral distribution ranges of clades were inferred using the ancestral state reconstruction package of MESQUITE 2.5 (Maddison and Maddison 2008). As phylogenetic backbone, the RAxML tree (partitioned by gene and codon) was loaded in a MESQUITE file and slightly adjusted in the tree window. Only one lineage per taxon was retained in the stored tree, and all bifurcations with bootstrap support below 75% were collapsed (with one exception RAxML bootstrap support >75% corresponded to node support from Bayesian posterior probabilities with BEAST >0.97; only the node uniting West Indian sliders with their North American relatives received weaker support from Bayesian analysis, but it was nevertheless retained in MESQUITE analysis). According to the distribution ranges of slider turtles, regions were coded in a character matrix by successive numbers: 0 = North America, 1 = Mexico, 2 = Central America exclusive of Mexico, 3 = West Indies, 4 = northern South America (Colombia, Venezuela) and 5 = South America (Brazil, Uruguay). Since taxa were always restricted to one region, no polymorphic character states occurred.

Ancestral ranges were inferred by parsimony reconstruction under a user-defined step-matrix model (Table S4). Parsimony reconstructions find the ancestral states that minimize the number of steps of character change. The step-matrix model is based on the premise that faunal interchange among neighbouring bioregions is common and likely to occur with a minimum cost assumed, whereas interchange between two regions becomes less likely with increasing distance and the number of interspersed regions, as reflected by higher costs. Accordingly, the cost of dispersal to a neighbouring region was set to '1' and costs were assumed to be doubled with each region to be crossed, i.e., the maximum costs in the step-matrix model corresponded to dispersal from North America (region 0) across Mexico, Central America and northern South America to region 5 in South America (Brazil, Uruguay: cost =  $1 \times 2 \times 2 \times 2 = 8$ ).

In addition, the likelihood reconstruction method of MESQUITE was applied for comparative purposes. The likelihood reconstruction searches for each node for the ancestral states that maximize the probability that the observed distributions would evolve under a stochastic model of evolution (Schluter et al. 1997; Pagel 1999). The likelihood reconstruction finds, for each node, the state assignment that maximizes the probability of arriving at the observed states in the terminal taxa, given the model of evolution, and allowing the states at all other nodes to vary (Maddison and Maddison 2008). In our reconstruction, the Markov k-state 1 parameter model (Mk1 model) as implemented in MESQUITE was applied, with rate of character state change as the single parameter constrained to be equal for all state changes (which might be a slight disadvantage compared with the step-matrix model).

## Results

### Phylogeny

The trees based on mtDNA sequences and concatenated mtDNA + nDNA sequences were largely congruent and well resolved, with most clades having high bootstrap support (Figs 2 and 3). By contrast, the clearly different topology of the tree based on the five nuclear loci (C-mos, ODC, R35, Rag1, Rag2; 3396 bp) contained many multifurcations and weakly supported clades (Fig. S1). Among the non-trivial clades with high support in the latter tree, two are worthwhile being mentioned. The first clade embraces the sequences of the two *Malaclemys* samples that constitute with high bootstrap support of 98 the sister group of *Graptemys*. The second clade, having a support value of 92, contains all sequences of Central and South American *Trachemys*, while the North American and Antillean *Trachemys* are excluded from this group.

The tree (Fig. 2) based on 3242 bp of mtDNA (12S, cytb, ND4, ND4L) revealed a well-supported sister group relationship of *Chrysemys* + *Pseudemys*; all other ingroup taxa constituted the well-supported sister group of this *Chrysemys* + *Pseudemys* clade. *Graptemys*, *Malaclemys* and *Trachemys* were each found to be monophyletic, all with high support, and *Graptemys* and *Malaclemys* were the successive sister groups of *Trachemys*. Within *Graptemys*, the small-headed species *G. nigrinoda* and *G. pseudogeographica* constituted a well-supported clade, and the large-headed *G. barbouri* and *G. gibbonsi* constituted another one. Within *Trachemys*, a number of deeply divergent, well-supported clades occurred that agreed only in part with the currently accepted taxonomy. Well-supported clades or highly distinct terminals corresponded to sequences of, in the tree from top to bottom, (1) *T. decussata*, (2) *T. gaigeae*, (3) *T. scripta*, (4) *T. adiutrix*, (5) *T. dorbigni*, (6) *T. emolli* + *T. v. grayi* + *T. v. panamensis* and (7) a major clade comprising sequences of *T. c. callirostris*,

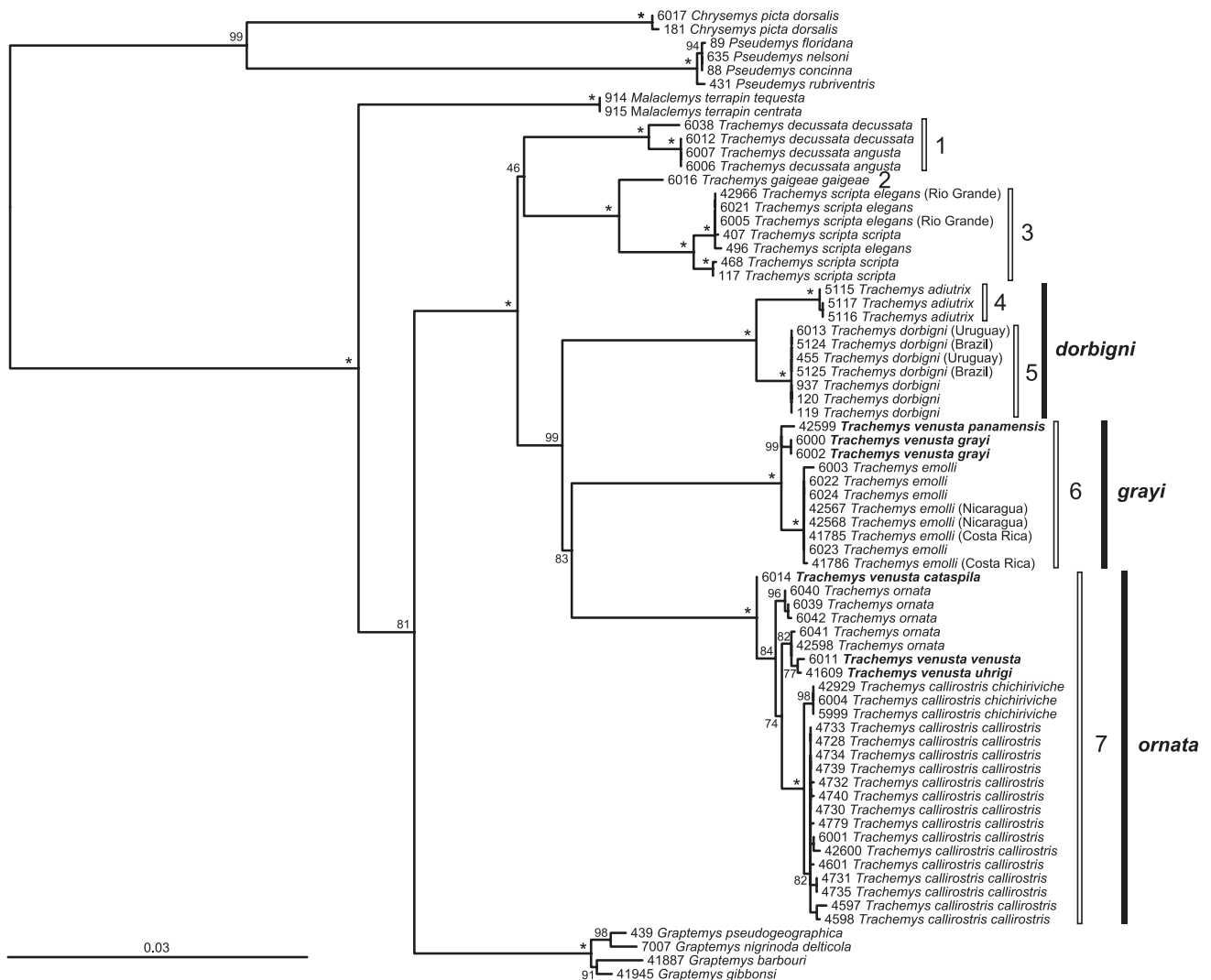


Fig. 2. Phylogeny of *Trachemys* and allied emydids (RAxML tree, partitioned analysis by gene and codon) based on sequence information of four mitochondrial genes (12S, cytb, ND4, ND4L; 3242 bp). Note the conflicting placement of sequences of *T. venusta* (in bold). Sample codes preceding taxon names are MTD D or MTD T numbers (Table S1). Except for most terminal clades with short branches, bootstrap values are shown at nodes; asterisks indicate maximum support. Outgroup (*Deirochelys reticularia*) removed for clarity. Clade numbers on the right refer to the text. Black bars indicate revised species delineation (see Discussion)

*T. c. chichiriviche*, *T. ornata*, *T. v. venusta*, *T. v. cataspila* and *T. v. uhrigi*. This latter major clade (7) with Central and South American taxa was, with moderate support, sister to the Central American clade (6). The South American species *T. adiutrix* (clade 4) and *T. dorbigni* (clade 5) were well-supported sister taxa, and the clade containing *T. adiutrix* and *T. dorbigni* occurred with high support in a more inclusive clade embracing also clades (6) + (7). The basal branching pattern of the remaining *Trachemys* clades (1, 2, 3), corresponding to the species *T. decussata*, *T. gaigeae* and *T. scripta*, was not well resolved. However, *T. gaigeae* (clade 2) and *T. scripta* (clade 3) were well-supported sister species.

Furthermore, the following observations should be highlighted: the Central American species *T. venusta* was polyphyletic, with some sequences occurring in clade (6) and others in clade (7), suggesting that current species delineation is incorrect. The South American species *T. adiutrix* and *T. dorbigni* were sister taxa. However, their successive sister was not *T. callirostris* from northern South America. Rather, the latter species was more closely allied to Central American sliders, in particular *T. ornata*, *T. v. venusta*, *T. v. cataspila* and *T. v. uhrigi* (clade 7). Sequences of *T. ornata* were paraphyletic with respect to *T. v. venusta*, *T. v. uhrigi* and *T. callirostris*, and *T. v. cataspila* was sister to this paraphyletic assemblage. Sequences of *T. v. panamensis* and *T. v. grayi* occurred along

with *T. emolli* in the deeply divergent clade (6). Within *T. decussata* and *T. scripta*, the studied subspecies yielded distinct haplotypes but shared haplotypes among the subspecies occurred as well. The haplotypes of the two morphologically distinctive specimens of *T. s. elegans* from the Rio Grande were not differentiated from other *T. s. elegans*.

The tree (Fig. 3) based on the concatenated mitochondrial and nuclear DNA sequences (6638 bp) basically mirrored the topology of the mtDNA tree, except that *Malaclemys* + *Graptemys* and *T. decussata* + (*T. gaigeae* + *T. scripta*), respectively, were now returned as weakly to moderately supported clades. *Graptemys* and *Malaclemys* were not sister taxa using the mtDNA data set but constituted the successive sister groups of *Trachemys*, and support for the monophyly of *T. decussata* + (*T. gaigeae* + *T. scripta*) was very low (Fig. 2).

### Molecular dating

The results from both independent molecular dating approaches (lognormal and uniform tMRCa prior distributions) yielded similar results (Fig. 4; Table 2). The basal splits among major lineages of *Trachemys* were consistently dated to the Late Miocene and Early Pliocene with mean estimates ranging from 11.50 to 5.14 Ma (nodes 16–20 in Fig. 4), suggesting that *T. decussata*, *T. gaigeae*, *T. scripta* and the more inclusive

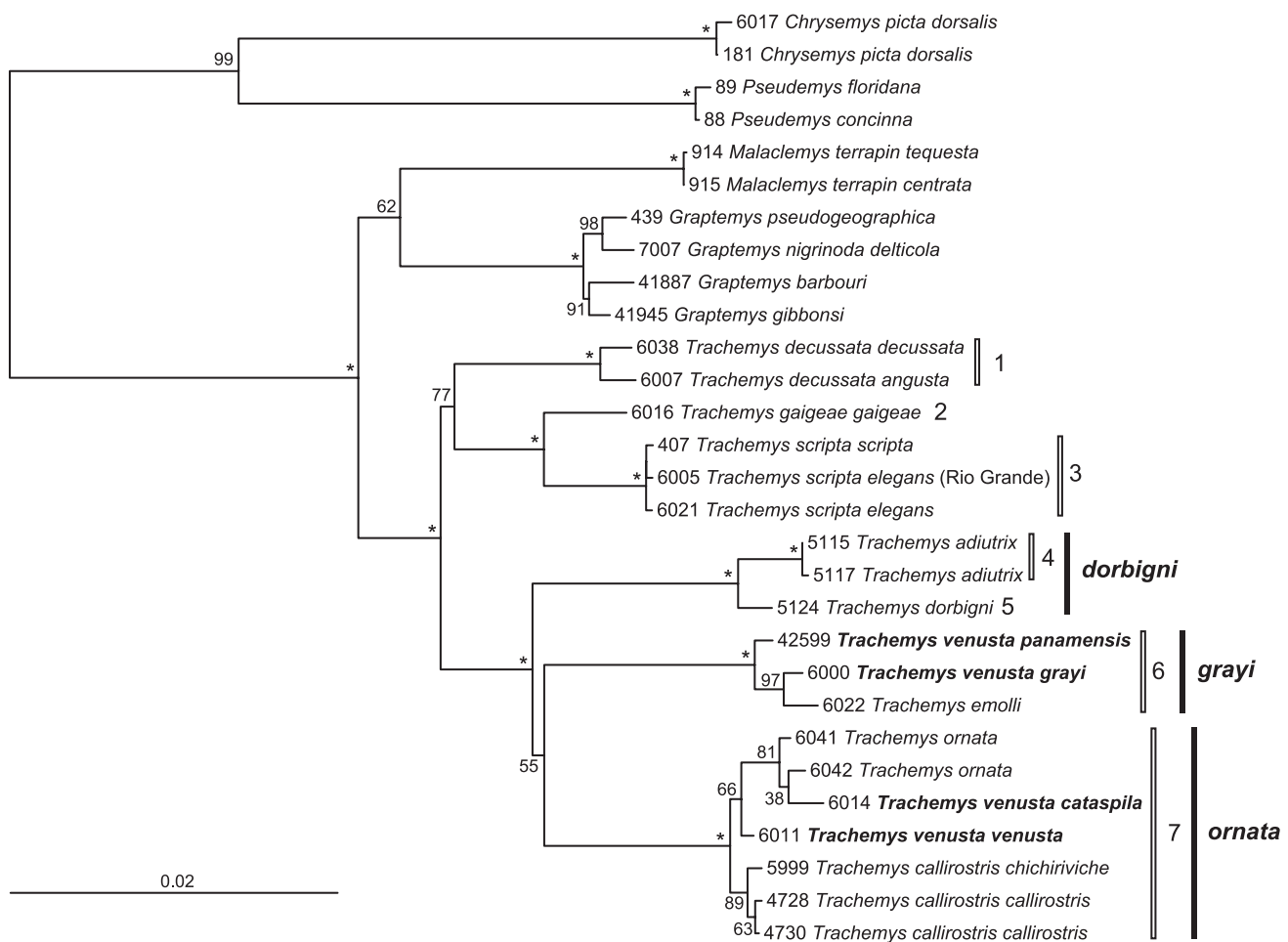


Fig. 3. Phylogeny of *Trachemys* and allied emydids (RAxML tree, partitioned analysis by gene and codon) based on sequence information of four mitochondrial genes and five nuclear loci combined (6638 bp). Outgroup (*Deirochelys reticularia*) removed for clarity. For further information, see Fig. 2

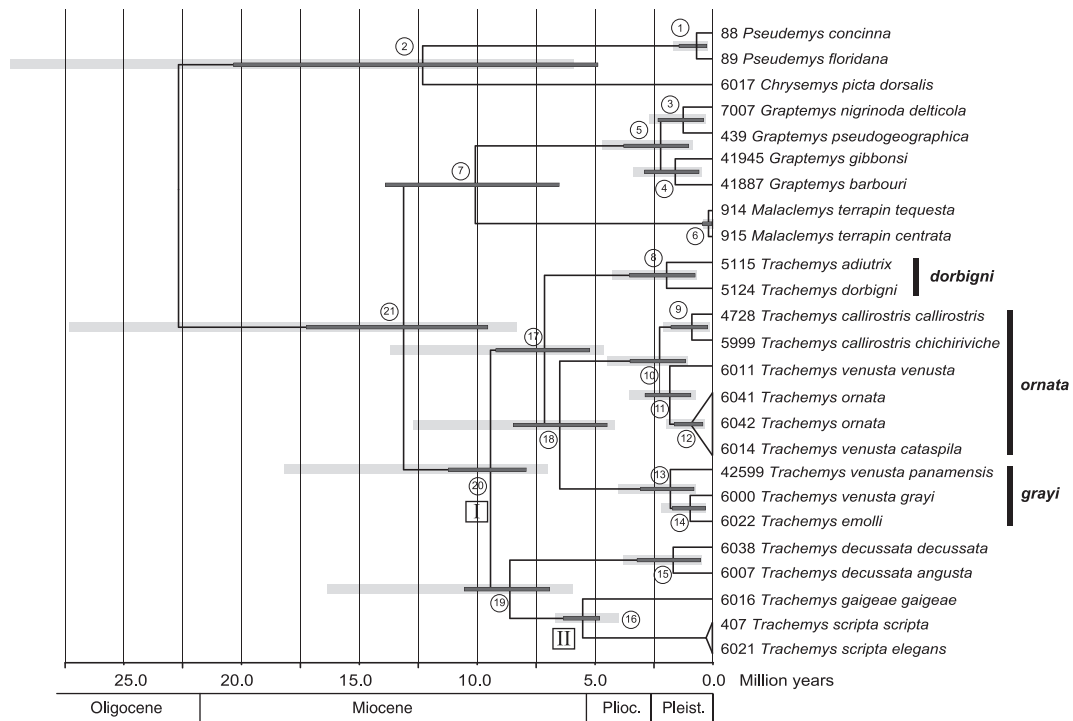


Fig. 4. Divergence times of slider turtles and allied emydids, estimated with prior distributions for the most recent common ancestors (tmrca) having a lognormal or uniform distribution. Branch lengths are in units of time and represent the means of the posterior distribution. Node bars correspond to the 95% highest posterior density (HPD) intervals of the posterior distribution; dark grey: lognormal distribution, light grey: uniform distribution. The backbone is the tree using the lognormal distribution. The *Graptemys* + *Malaclemys* node is not supported by the tree with a uniform distribution for the tmrca. Enclosed Roman numerals I and II correspond to the fossil calibration points, *Trachemys inflata* (7 Ma) and *T. idahoensis* (4 Ma). Encircled numbers denote nodes, see Table 2 for exact mean and 95% HPD values. The lower boundary of the Pleistocene is according to Walker and Geissman (2009). Black bars indicate revised species delineation (see Discussion)

Table 2. Divergence time estimates (means) and their 95% highest posterior density (HPD) intervals for the nodes in Fig. 4 (in million years)

Node	Lognormal distribution (95% HPD)	Uniform distribution (95% HPD)
1	0.73 (0.22–1.39)	0.85 (0.24–1.71)
2	12.27 (4.84–20.29)	16.70 (5.92–29.73)
3	1.22 (0.37–2.29)	1.42 (0.34–2.72)
4	1.56 (0.55–2.86)	1.78 (0.52–3.41)
5	2.18 (0.99–3.74)	2.49 (0.87–4.71)
6	0.15 (0–0.40)	0.16 (0–0.42)
7	10.04 (6.48–13.85)	–
8	1.92 (0.71–3.50)	2.28 (0.71–4.28)
9	0.85 (0.18–1.74)	0.98 (0.18–2.14)
10	2.22 (1.12–3.47)	2.52 (1.09–4.51)
11	1.79 (0.89–2.84)	2.04 (0.77–3.57)
12	0.95 (0.38–1.60)	1.11 (0.39–2.01)
13	1.76 (0.76–3.03)	2.12 (0.76–4.03)
14	0.92 (0.27–1.68)	1.11 (0.32–2.22)
15	1.64 (0.49–3.17)	1.95 (0.52–3.83)
16	5.48 (4.76–6.31)	5.14 (4.00–6.67)
17	7.10 (5.19–9.17)	8.60 (4.62–13.63)
18	6.45 (4.44–8.43)	7.88 (4.17–12.68)
19	8.57 (6.88–10.51)	10.21 (5.95–16.33)
20	9.39 (7.87–11.18)	11.50 (7.00–18.16)
21	13.08 (9.51–17.21)	15.13 (8.34–24.73)

clades embracing Central and South American slider turtles represent old lineages. The radiation of the crown groups of Central and South American *Trachemys* (nodes 8–14) was

generally dated to the Lower Pleistocene, like the split between the two Cuban lineages (*T. d. decussata* and *T. d. angusta*; node 15), with mean estimates ranging from 2.52 to 0.85 Ma.

The mean estimates for the divergence of *Trachemys* from its sister group were 13.08 and 15.13 Ma (node 21), albeit with very wide 95% HPD intervals. Within the studied *Graptemys* species divergence commenced only in the Lower Pleistocene (node 5, with mean estimates of 2.18 and 2.49 Ma). *Pseudemys concinna* and *P. floridana* seem to be even younger species (node 1, mean estimates of 0.73 and 0.85 Ma). According to our calculations, the mean estimates for the divergence of *Pseudemys* from its sister taxon *Chrysemys* were 12.27 and 16.70 Ma (node 2), but again with very wide 95% HPD intervals.

#### Historic biogeography

Ancestral ranges inferred by the parsimony and likelihood approaches were essentially identical (Fig. 5). A North American centre of origin was unambiguously suggested for the ancestor of the extant genera *Trachemys*, *Malaclemys*, *Graptemys*, *Pseudemys* and *Chrysemys*. The latter four genera and the ancestor of the West Indian and North American *Trachemys* taxa were inferred to have diverged only within North America, from where the West Indies have been reached. By contrast, the range of the last common ancestor of all Central and South American taxa was inferred to be Central America south of Mexico, suggesting a radiation following a southwards directed range shift. The extant South

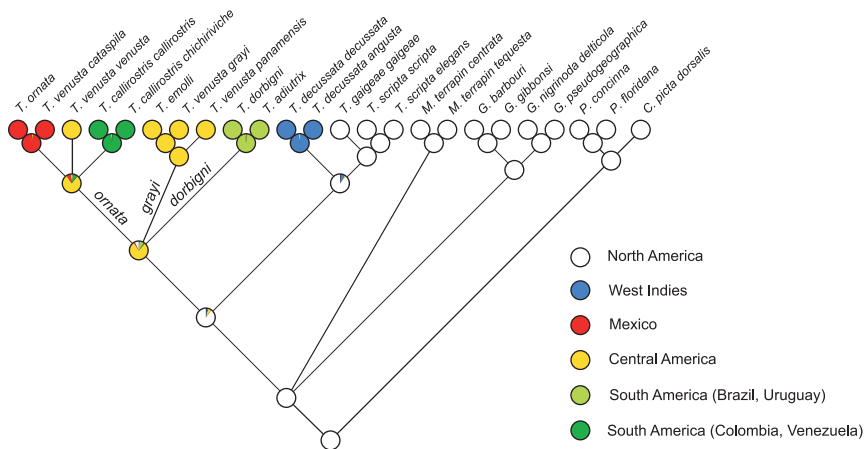


Fig. 5. Ancestral range analysis of slider turtles and allied emyids (likelihood analysis). Colour-coded circles at nodes and terminals indicate regions; slices, inferred proportional likelihoods of respective ancestral ranges. The parsimony reconstruction using the step-matrix resulted in essentially the same pattern; the inferred ancestral ranges correspond to the ones with the highest likelihoods. Species names along branches indicate revised species delineation (see Discussion)

American species were inferred to have colonized South America twice from southern Central America, from whence more northern Mexican regions (Guerrero: *T. ornata*; Tamaulipas: *T. v. cataspila*) were also reached.

## Discussion

### Phylogeny and taxonomy

In contrast to other recent studies (Spinks et al. 2009; Wiens et al. 2010) using mitochondrial and nuclear DNA sequences for reconstructing the phylogeny of emydid turtles, our mtDNA data suggest with high support the monophyly of *Trachemys* (Fig. 2). The two mentioned studies relied on universal turtle primers for producing their mtDNA sequences, and we suspect that their results have been biased by the inclusion of unrecognized numt sequences. This suspicion is further substantiated by the weak mitochondrial differentiation of some taxa (*Graptemys* spp.) in the data set of Wiens et al. (2010) compared with our findings (compare our Fig. 2 with Fig. 1 of Wiens et al. 2010). Using standard primers for PCR, we were regularly confronted with numt sequences and only specifically designed PCR primers and the application of long-range PCR enabled us to produce unambiguous mtDNA sequences (for details, see under Materials and Methods). This situation suggests that some recent considerations about conflicts between mitochondrial and nuclear DNA sequences (see review in Wiens et al. 2010) could be obsolete because they were not based on authentic mtDNA sequences but on numts.

On the alpha-taxonomic level, our data indicate that the currently accepted species delineation within *Trachemys* requires revision. Like Jackson et al. (2008), we found for some subspecies shared mitochondrial haplotypes or topologies that were not reciprocally monophyletic (Fig. 2: *T. d. decussata* and *T. d. angusta*; *T. s. scripta* and *T. s. elegans*). While this is in perfect agreement with gene flow among subspecies according to the Biological Species Concept (Mayr 1942), the allocation of sequences of *T. venusta* to two distinct major clades (clades 6 and 7 of Figs 2 and 3) is in clear conflict with current species delineation. Sequences of *T. v. venusta*, *T. v. cataspila* and *T. v. uhrigi* appear in clade (7) that comprises also the two subspecies of *T. callirostris* and *T. ornata*, whereas sequences of *T. v. grayi* and *T. v. panamensis* cluster with high support with *T. emolli* in clade (6).

Based on subtle morphological differences, Legler (1990) and McCord et al. (2010) suggested that Panamanian

populations of *T. venusta* are taxonomically distinct, and this is supported by our data. McCord et al. (2010) erected three new subspecies of *T. venusta*, and our samples from Guatemala and Honduras correspond within this framework to the subspecies *venusta* and *uhrigi*, respectively, and our sample from Panama to the subspecies *panamensis*. While the validity of the latter taxon is clearly supported by our data, the distinctiveness of *venusta* and *uhrigi* is not. The sequences of both nominal subspecies show only negligible differentiation (Fig. 2; Table 3), suggesting that the coloration and pattern differences described by McCord et al. (2010) represent only population-specific variation. However, the question remains of whether *panamensis* represents a distinct species or rather a subspecies of another species than *T. venusta*.

In recent years, several authors have used uncorrected *p* distances of the mitochondrial *cytb* gene for inferring or corroborating species borders of turtles and tortoises (see the review in Vargas-Ramírez et al. 2010b). In absence of direct evidence, this approach applies divergences of syntopically occurring species as a yardstick for the comparison of allopatric or parapatric taxa (Stuckas and Fritz 2011). With respect to *Trachemys*, the divergence between *T. gaigeae* and *T. scripta* may serve as a benchmark for closely related, but distinct species. Their species status is unambiguously supported by the observation of only sporadically occurring hybridization in syntopic populations (Seidel et al. 1999). By contrast, the broad-scale intergradation of *T. s. scripta* and *T. s. elegans* is well known (Ernst and Lovich 2009). Between these two *T. scripta* subspecies, a sequence divergence of 0.57% is observed in the *cytb* gene, while *T. gaigeae* differs from these two taxa by 1.67% and 1.47% (Table 3), suggesting that a threshold of 1.5% is reasonable for recognizing different species. Such a value is clearly lower than observed among many other turtle and tortoise species and underlines that rigid universal thresholds, as applied in the widely used barcoding approach (Hebert et al. 2003), might be misleading. Except for one *Cyclemys* species with introgressed mitochondrial genome (Fritz et al. 2008b), uncorrected *p* distances between other congeneric chelonian species range from 2.8% to 18.3% (Vargas-Ramírez et al. 2010b; Prashag et al. 2011; Stuckas and Fritz 2011).

When the values obtained for other *Trachemys* taxa (Table 3) are compared, it is obvious that the pairwise divergences of several taxa are below 1.5%, suggesting a revised taxonomy that agrees well with the placement of the respective taxa in phylogenetic analyses (Figs 2 and 3). Values

Table 3. Mean uncorrected *p* distances of the mitochondrial *cytb* gene of *Trachemys* taxa (percentages). Order of taxa as in Table 1. Below the diagonal, values between taxa are presented; on the diagonal, within-taxon divergences in bold. Introgressed *angusta* and *elegans* haplotypes were added to the respective maternal lineage

	<i>adiutrix</i>	<i>callirostris</i>	<i>chichiriviche</i>	<i>decussata</i>	<i>angusta</i>	<i>dorbigni</i>	<i>emolli</i>	<i>gaigeae</i>	<i>ornata</i>	<i>scripta</i>	<i>elegans</i>	<i>venusta</i> <sup>1</sup>	<i>cataspila</i>	<i>grayi</i>	<i>panamensis</i>	
	<i>n</i> = 3	<i>n</i> = 15	<i>n</i> = 3	<i>n</i> = 1	<i>n</i> = 3	<i>n</i> = 7	<i>n</i> = 8	<i>n</i> = 1	<i>n</i> = 5	<i>n</i> = 2	<i>n</i> = 5	<i>n</i> = 2	<i>n</i> = 1	<i>n</i> = 2	<i>n</i> = 1	
<i>adiutrix</i>	<b>0</b>															
<i>callirostris</i>	4.30	<b>0.09</b>														
<i>chichiriviche</i>	4.45	0.38	<b>0</b>													
<i>decussata</i>	4.79	4.05	4.19	–												
<i>angusta</i>	4.19	3.71	3.85	0.60	<b>0</b>											
<i>dorbigni</i>	1.11	3.88	4.02	4.11	3.51	<b>0</b>										
<i>emolli</i>	4.90	3.64	3.61	4.64	4.38	4.56	<b>0.04</b>									
<i>gaigeae</i>	4.53	3.62	3.76	2.57	2.31	4.02	3.44	–								
<i>ornata</i>	4.53	0.65	0.79	3.95	3.61	4.11	3.55	3.52	<b>0.24</b>							
<i>scripta</i>	5.18	4.35	4.49	3.46	3.29	4.83	4.77	1.67	4.25	<b>0.09</b>						
<i>elegans</i>	4.77	4.49	4.64	3.35	3.10	4.47	4.57	1.47	4.40	0.57	<b>0.03</b>					
<i>venusta</i> <sup>1</sup>	4.62	0.72	0.86	3.93	3.59	4.19	3.70	3.51	0.39	4.23	4.38	<b>0.17</b>				
<i>cataspila</i>	4.36	0.46	0.60	3.76	3.42	3.93	3.53	3.34	0.19	4.06	4.21	0.34	–			
<i>grayi</i>	4.70	3.45	3.42	4.45	4.19	4.36	0.36	3.25	3.46	4.75	4.55	3.51	3.34	<b>0</b>		
<i>panamensis</i>	4.70	3.62	3.59	4.62	4.36	4.36	0.53	3.42	3.63	4.92	4.72	3.68	3.51	0.17	–	

<sup>1</sup>Guatemala, Honduras (nominal subspecies *venusta* and *uhrigi* lumped).

below 1.5% sequence divergence occur between the two subspecies of *T. decussata*, but also between *T. adiutrix* and *T. dorbigni* and among the taxa of clades (6) and (7). This suggests that *T. adiutrix* should be treated as a subspecies of *T. dorbigni* (*T. d. adiutrix* Vanzolini, 1995) and that all terminal taxa of clades (6) and (7) are subspecies of two further species. The oldest available name for clade (6) is *Emys grayi* Bocourt, 1868, which has to be used as the valid species name of the included subspecies *T. g. grayi* (Bocourt, 1868), *T. g. emolli* (Legler, 1990) and *T. g. panamensis* McCord, Joseph-Ouni, Hagen and Blanck, 2010. The oldest available name for taxa within clade (7) is *Emys ornata* Gray, 1831. Consequently, all taxa of clade (7) are nomenclaturally to be regarded as subspecies of *T. ornata* (Gray, 1831), viz., *T. o. ornata* (Gray, 1831), *T. o. callirostris* (Gray, 1856), *T. o. cataspila* (Günther, 1885), *T. o. chichiriviche* (Pritchard and Trebbau, 1984) and *T. o. venusta* (Gray, 1856).

The very close relationship of the subspecies of *T. ornata* sensu lato is also illustrated by their weak mitochondrial differentiation (Fig. 2) leading to a paraphyly of *T. o. ornata* with respect to other subspecies, suggestive of incomplete lineage sorting.

Parenthetically, it may be noted that we found no genetic differentiation of the morphologically distinctive Rio Grande population of *T. s. elegans* compared with two pet trade specimens of the same subspecies that are thought to represent the gene pool occurring in the Mississippi Basin. This underlines that population-specific morphological differences may be misleading for morphological taxon delineation, in particular when coloration and pattern characters are concerned, like in the Rio Grande population of *T. s. elegans* or the Honduran population of *T. o. venusta*.

### Biogeography

An unexpected outcome of our study was that the South American slider turtles represent two distinct evolutionary lineages that are not sister groups. *Trachemys ornata callirostris* and *T. o. chichiriviche* from northern South America are

allied to the Central American *T. ornata* subspecies, but not to the other South American slider species *T. dorbigni* as thought before (Williams 1956). According to our results, the latter species can be regarded as polytypic, with the subspecies *T. d. dorbigni* (Duméril and Bibron, 1835) from the Rio de la Plata Region of Argentina, Brazil and Uruguay, and the widely disjunct *T. d. adiutrix* Vanzolini, 1995 from Maranhão, Brazil. Consequently, the phylogenetic placement of the South American sliders rejects our original hypothesis that their split age can be used for dating Amazon rainforest fragmentation, although it seems still likely that *T. dorbigni* crossed South America during a phase of fragmented rainforest that may predate the Pleistocene.

Our results suggest that South America was reached by two independent and successive colonization waves of slider turtles from Central America. According to their extant distribution, it seems likely that the wave giving rise to *T. d. dorbigni* and *T. d. adiutrix* reached South America first. According to the mean divergence time estimates from our relaxed molecular clock calculations, the radiation of all basal *Trachemys* lineages took place from the Late Miocene to Early Pliocene (Fig. 4; Table 2), in a period ranging from approximately 11.5 to 5.1 Ma ago. The ancestor of the extant subspecies of *T. dorbigni* was estimated to have branched off about 8.6 or 7.1 Ma ago (mean age estimates of uniform and lognormal distributions, respectively). Even when the 95% HPD intervals are considered, its origin predates by far the Pleistocene.

Our ancestral range analyses (Fig. 5) indicate that the last common ancestor of all Central and South American sliders has lived in southern Central America and that it descended from North American stock. This ancestral turtle most probably colonized Central America during the Miocene (cf. Fig. 4; Table 2). This contradicts previous hypotheses suggesting that *Trachemys* is a recent invader of Latin America, which used the emerging land bridge for a southward range expansion only during the Pliocene or Pleistocene (Savage 1966; Pritchard and Trebbau 1984; Vanzolini and Heyer 1985; Moll and Moll 1990; Seidel and Jackson 1990; Vanzolini 1995; de la Fuente et al. 2002). Legler (1990) even believes that

Mexico and Central America were colonized not before the 'Pleistocene or later'. However, North America, Mexico and northern Central America were contiguous, exposed land masses since at least the Middle Eocene (Heinicke et al. 2007). Moreover, before the formation of the Central American isthmus, the Central American peninsula reached far southward. In the Middle Miocene, southern Central America was connected with North America and islands existed between the southern Central American land mass and South America, with only the about 100-km-wide Atrato Seaway separating the island chain from South America (Kirby and MacFadden 2005; Kirby et al. 2008; Woodburne 2010).

Previously, the closure of the Central American landbridge in the Pliocene (Marshall 1979, 1985, 1988; Lessios 2008; Molnar 2008), allowing the 'Great American Interchange' (Stehli and Webb 1985), was thought to be responsible for the colonization of South America by slider turtles. However, when the ability of freshwater turtles to cross sea straits (Fritz et al. 2006b, 2007, 2008a; Suzuki and Hikida 2011) is taken into account, it seems likely that the ancestors of *T. dorbigni* arrived in South America prior to the establishment of a solid landbridge via island hopping or transoceanic dispersal. Indeed, the emergence of the so-called Panama Island Archipelago between southernmost Central America and South America (Woodburne 2010; fully established at approx. 6 Ma) coincides well with our mean divergence estimates for the ancestor of *T. dorbigni*, supporting this hypothesis. The same dispersal mode is nowadays also recognized for the first immigration wave of North American mammals into South America (Woodburne 2010). By contrast, the second slider species present in South America, *T. ornata*, seems to be a recent invader indeed. The mean estimates of 2.2 and 2.5 Ma (Table 2: node 10) for the divergence between the ancestor of the two South American subspecies *T. o. callirostris* and *T. o. chichiriviche* and their Central American conspecifics are in line with the idea of a Pleistocene colonization.

Turning to Central America, the deep divergence between the two species *T. grayi* and *T. ornata*, both distributed there, argues for their long-lasting isolation. Our molecular clock calculations indicate that this split occurred in the Late Miocene (mean estimates of 6.5 and 7.9 Ma; Table 2: node 18). It might be speculated that the Central American Cordillera acted as a barrier between the involved east coast taxa (*T. o. cataspila*, *T. o. venusta*) and west coast taxa (*T. g. grayi*, *T. g. emolli*, *T. g. panamensis*). However, *T. o. ornata* from the west coast is closely related to its east coast conspecifics and not to *T. grayi*, suggesting that the Cordillera was circumvented at least once. For a better understanding of the biogeography of Central American *Trachemys*, a denser sampling of northern Mexican taxa is a future challenge. The detected differentiation and the patchy distribution of slider turtles there (Fig. 1) imply a complex pattern of dispersal and vicariance events.

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## Resumen

*Filogenia molecular de las tortugas Icoeteas Centro y Suramericanas: Implicaciones para su biogeografía y sistemática (Testudines: Emydidae: Trachemys)*

Analizamos la filogenia, sistemática y biogeografía de las tortugas Icoeteas (*Trachemys* spp.) usando información de secuencias de cuatro genes mitocondriales (3242 bp) y cinco loci nucleares (3396 bp) de la mayoría de taxa Suramericanos y de la parte sur de Centroamérica, especies representantes de la parte norte de Centroamérica, Antillas y Norteamérica (16 especies y subespecies) y especies Norteamericanas relacionadas (géneros *Chrysemys*, *Deirochelys*, *Graptemys*, *Malaclemys*, *Pseudemys*). Mediante la aplicación de máxima verosimilitud, reloj molecular relajado y análisis de rango ancestral, presentamos evidencia de dos colonizaciones sucesivas de las Icoeteas a Suramérica. Además, demostramos que la delimitación actual de especies de Centro y Suramérica no es correcta. Nuestros datos sugieren que *Trachemys grayi* es una especie politípica distintiva y agrupa, aparte de la subespecie nominotípica, *T. g. emolli* and *T. g. panamensis*. *Trachemys ornata* también es politípica con las subespecies *T. o. ornata*, *T. o. callirostris*, *T. o. cataspila*, *T. o. chichiriviche* y *T. o. venusta*. Adicionalmente, *T. adiutrix* debe ser considerada como subespecie de *T. dorbigni*. Se infirió que todas las especies estudiadas de *Trachemys* se originaron en el Mioceno tardío a Plioceno temprano. El ancestro de las dos subespecies de *T. dorbigni* colonizo Suramérica más probablemente antes del establecimiento de la conexión terrestre entre Centro y Suramérica, mientras las dos subespecies Suramericanas de *T. ornata* representan un evento de migración independiente más reciente desde Centroamérica.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Phylogeny of *Trachemys* and allied emydid (RAxML tree, partitioned analysis by gene and codon) based on sequence information of five nuclear loci (C-mos, ODC, R35, Rag1, Rag2; 3396 bp).

**Table S1.** Samples used in the present study.

**Table S2.** Primers used for amplification and sequencing of ND4L, ND4 and *cytb*.

**Table S3.** PCR protocols for mitochondrial and nuclear genes.

**Table S4.** Step-matrix model applied in the parsimony reconstruction of ancestral states with MESQUITE.

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