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Complex phylogeography in *Rhinoclemmys melanosterna*: conflicting mitochondrial and nuclear evidence suggests past hybridization (Testudines: Geoemydidae)

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Abstract

We examined differentiation within the Colombian wood turtle *Rhinoclemmys melanosterna,* and among *R. melanosterna* and the closely allied species *R. diademata*, *R. funerea* and *R. punctularia*, based on 1060 base pairs of the mitochondrial cyt *b* gene. We also assessed the phylogenetic relationships among these species using 2050 bp of mtDNA (partial cyt *b*, 12S and 16S genes) and 3620 bp of nuclear DNA (partial Rag 1, Rag 2, C-mos, R35 and ODC genes). There is considerable phylogeographic structuring within *R. melanosterna*, with seven distinct clades distributed across the species' range. These clades correlate to some extent with previously described differences in the dorsal pattern of head coloration. Individual and combined analyses of mitochondrial and nuclear DNA indicated contradictory relationships among *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia*. Mitochondrial DNA sequences revealed *R. melanosterna* to be non-monophyletic with respect to *R. diademata*, *R. funerea* and *R. punctularia*. In contrast, *R. melanosterna* constituted a well-supported monophyletic clade using nuclear DNA. This conflict between mitochondrial and nuclear data suggests past gene flow among the allopatrically and parapatrically distributed species *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia*. Compared to the other *Rhinoclemmys* species, the taxa under study are weakly differentiated. To assess their taxonomic status, further research is warranted using additional nuclear markers and additional samples of *R. diademata*, *R. funerea* and *R. punctularia*. For the time being, a continued classification of *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia* as distinct species is justified owing to their allopatric and parapatric distributions, and to conserve the established usage of names that is based on morphological and karyotypic differentiation.

Key words: Molecular phylogeny, mtDNA, nDNA, phylogeography, *Rhinoclemmys diademata*, *Rhinoclemmys funerea*, *Rhinoclemmys punctularia*, South America

Introduction

The advent of molecular genetic methods has contributed significantly to an enhanced understanding of the diversity and phylogenetic relationships of turtles and tortoises (Fritz & Havaš 2007). However, although only approximately 320 extant species are currently recognized (Fritz & Havaš 2007; Fritz 2011), their phylogeographic differentiation is often completely unknown. This is especially true for species occurring in the Neotropics (Central and South America), where relatively few phylogeographic studies have been performed so far (for a review see Vargas-Ramírez *et al.* 2012a). Such studies are crucial for unravelling evolutionarily significant units (Moritz 1994) that may correspond to unrecognized taxa or cryptic species (for chelonians, e.g., Fritz *et al*. 2005, 2008, 2011, 2012a; Praschag *et al*. 2007, 2011; Vargas-Ramírez *et al*. 2010; Stuckas & Fritz 2011) and have relevance for conservation. Moreover, such investigations elucidate underlying historical events and processes that may have shaped the current genetic diversity (e.g., Vargas-Ramírez *et al*. 2010, 2012b; Fritz *et al*. 2012b, c).

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In the present paper, we focus on the phylogeography of the Colombian wood turtle *Rhinoclemmys melanosterna* (Gray, 1861). Together with eight other species, it belongs to the genus *Rhinoclemmys* Fitzinger, 1835, the only Central and South American representatives of the otherwise purely Old World family Geoemydidae (McDowell 1964; Ernst & Barbour 1989; Ernst *et al*. 2000; Fritz & Havaš 2007; van Dijk *et al*. 2011). The ancestor of *Rhinoclemmys* is thought to have invaded the Neotropics from Asia via North America (McDowell 1964; Savage 1966; Carr 1991; Hutchison 2006). Extant *Rhinoclemmys* species are distributed from northwestern Mexico to northern South America and occur in a wide range of habitats, from aquatic to terrestrial (Ernst 1978; Smith & Smith 1980; Ernst & Barbour 1989; Ernst *et al*. 2000; Rueda-Almonacid *et al*. 2007). *Rhinoclemmys melanosterna* is semi-aquatic and prefers lentic water bodies within forest (Ernst & Barbour 1989; Ernst *et al*. 2000; Rueda-Almonacid *et al*. 2007). Its distribution range comprises the Caribbean versant of eastern Panama; in addition, it occurs throughout the Caribbean coast of Colombia including the northern and central portion of the Magdalena river basin, and in the Pacific drainages of western Panama and Colombia to northwestern Ecuador (Castaño-Mora & Medem 2002; Rueda-Almonacid *et al*. 2007; Fig.1). Pronounced geographical variation occurs with respect to coloration and shape of the conspicuous head stripes of *R. melanosterna* (Medem 1962; Carr 1991; Castaño-Mora & Medem 2002; Rueda-Almonacid *et al*. 2007). However, it remains unknown whether this variation corresponds to genetically distinct populations.

According to three previous phylogenetic analyses (Carr 1991; Spinks *et al*. 2004; Le & McCord 2008), *R. melanosterna* is most closely related to *R. funerea*, *R. diademata* and *R. punctularia*, three parapatrically or allopatrically distributed species with a similar natural history (Fig. 1; Rueda-Almonacid *et al*. 2007). Previously, many authors considered *R. melanosterna*, *R. diademata* and *R. punctularia* conspecific (e.g., Boulenger 1889; Mertens 1954; Ernst 1978, 1981), and only Pritchard (1979) elevated the taxa to full species level due to the "absence of any indication of intergradation" and their mutually exclusive distribution ranges. However, Ernst & Barbour (1989) and Ernst *et al*. (2000) continued to treat *R. diademata* as a subspecies of *R. punctularia*, while they recognized *R. melanosterna* as a distinct species. Carr (1991) endorsed the classification of all four taxa as distinct species, based on their morphological and karyotypic differences.

Spinks *et al*. (2004) used the mitochondrial cytochrome *b* (cyt *b*) and 12S rRNA genes and an intron of the nuclear RNA fingerprint protein 35 gene (R35) to examine the phylogenetic relationships of *Rhinoclemmys* and other geoemydids. Le & McCord (2008) relied on sequence data of three mitochondrial genes (cyt *b*, 12S, 16S) and two nuclear loci (C-mos, Rag 1) for studying the phylogeny and biogeography of *Rhinoclemmys*. Both of these studies agreed that *R. melanosterna* is the sister species of *R. funerea*, and that these two species together constitute the sister group of a clade containing *R. diademata* and *R. punctularia*, a conclusion previously reached by Carr (1991) on the basis of a combined analysis of morphology and genetic markers (isozymes). However, Spinks *et al*. (2004) and Le & McCord (2008) used only one individual per *Rhinoclemmys* species or subspecies, and Carr (1991) had genetic samples of *R. melanosterna* from Ecuador only. Hence, a comprehensive study assessing phylogeographic differentiation within *R. melanosterna*, and with respect to the closely allied species *R. funerea*, *R. diademata* and *R. punctularia*, has never been performed.

The present study aims to fill this gap by using a range-wide sampling and cyt *b* sequences to study the phylogeography of *R. melanosterna*. The observed genetic pattern will be compared to the geographical variation of head stripes in this species. In addition, evolutionary relationships among *R. melanosterna* populations and the closely allied species *R. diademata*, *R. funerea* and *R. punctularia* will be assessed using mitochondrial and nuclear DNA sequences.

Materials and methods

Sampling and laboratory procedures. Blood or tissue samples of 74 *Rhinoclemmys melanosterna* from throughout its range were collected (Fig. 1; Appendix I), and sequences of the nearly complete mitochondrial cyt *b* gene were generated. As far as possible, the head pattern of the sampled turtles was photographed.

For assessing phylogenetic relationships among *R. melanosterna*, *R. funerea*, *R. diademata* and *R. punctularia*, samples of *R. melanosterna* were selected that represented all clades revealed by phylogeographic analyses. Using these samples, two further mitochondrial genes, the partial 12S and 16S rRNA genes, and five nuclear loci were sequenced. The nuclear loci were the partial recombination activating genes 1 and 2 (Rag 1, Rag 2), the gene for

the oocyte maturation factor Mos (C-mos), the intron 1 of the RNA fingerprint protein 35 gene (R35), and the partial ornithine decarboxylase (ODC) gene. The ODC sequences included coding and non-coding regions. If these data were not available for the other species from GenBank, the corresponding sequences were generated using samples from the tissue collection of the Museum of Zoology, Senckenberg Dresden (see Appendix I).

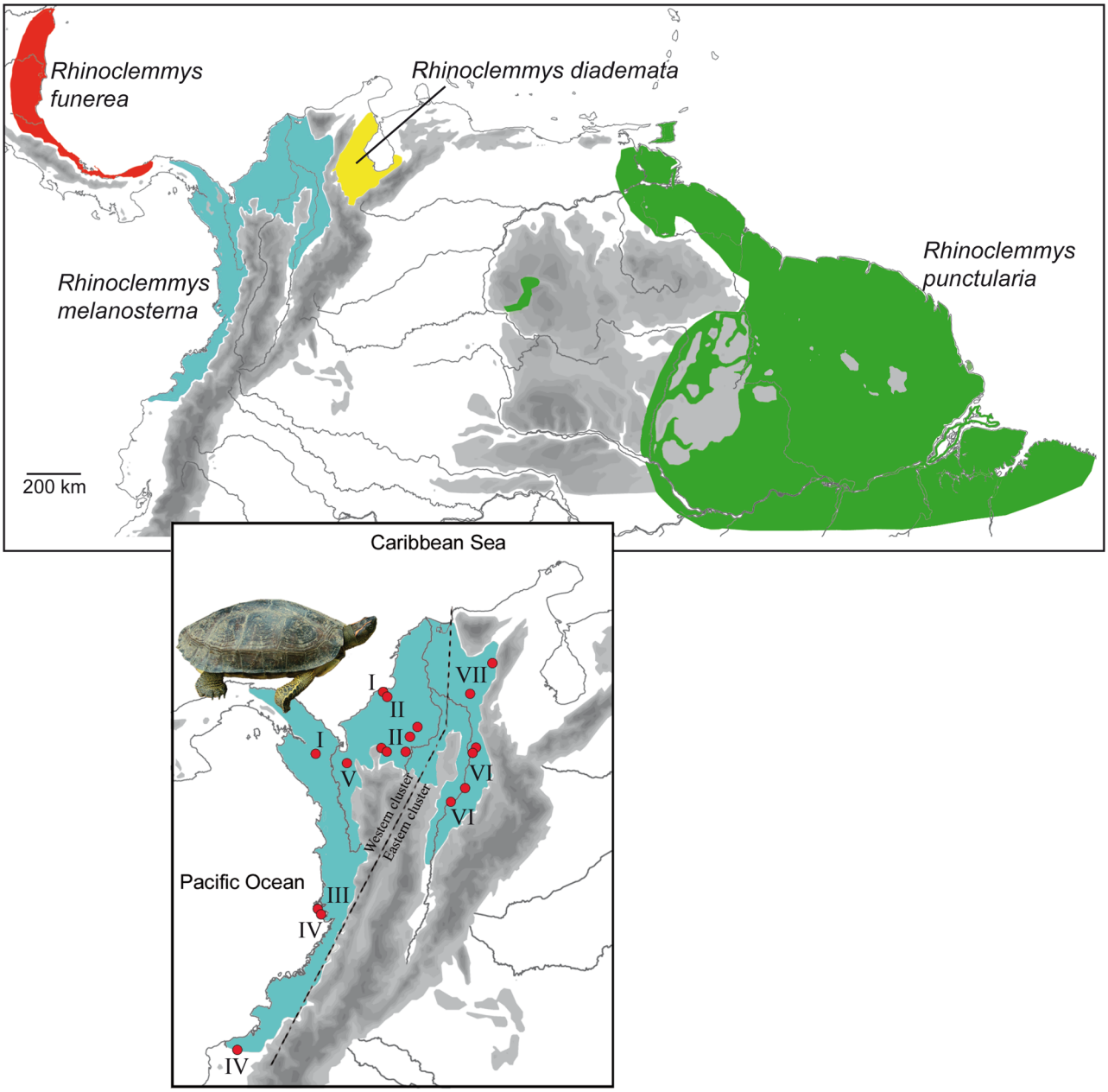

FIGURE 1. Approximate ranges of *Rhinoclemmys melanosterna* and the allied species *R. funerea*, *R. diademata* and *R. punctularia* (top; based on Rueda-Almonacid *et al*. 2007) and sampling sites for *R. melanosterna* (bottom; red dots). Stippled line separates the distribution of the two clusters of mitochondrial haplotypes of *R. melanosterna*; Roman numerals indicate haplotypes. Inset: Female *R. melanosterna* from Cangrejo, Córdoba (Colombia).

Total genomic DNA was extracted using a Qiagen DNA blood extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The cyt *b* gene was amplified and sequenced in two fragments overlapping by approximately 300 bp using the primer pairs mt-a-neu3 + mt-E-Rev2 and mt-c-For2 + mt-f-na (Fritz *et al*. 2006; Praschag *et al*. 2007), the 12S rRNA gene with the primers L1091 and H1478 (Kocher *et al*. 1989), and the 16S rRNA gene using the primers 16Sa and 16Sb (Palumbi *et al*. 2002). For the nuclear loci the following primer pairs were used: for Rag 1, Rag1878 and Rag2547 (Le *et al*. 2007); for Rag 2, F2-1 and R2-1 (Le *et al*. 2006); for C-mos, G136 and G137 (Georges *et al*. 1998); for R35, R35Ex1 and R35Ex2 (Fujita *et al*. 2004);

and for ODC, the chicken primers of Friesen *et al*. (1999). PCR was carried out in a total volume of 50 µl containing 1 unit *Taq* polymerase (Bioron, Ludwigshafen, Germany), 1 x buffer (as recommended by the supplier), a final concentration of 0.4 µM of the respective primer, and 0.2 mM of each dNTP (Fermentas, St. Leon-Rot, Germany). PCR products were purified using the ExoSAP-IT enzymatic cleanup (USB Europe GmbH, Staufen, Germany; modified protocol: 30 min at 37°C, 15 min at 80°C) and sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Thirty-one additional sequences were downloaded from GenBank and included in the alignments. Sequences were edited and aligned using CHROMAS 1.51 (http://technelysium.com.au/?page_id=13) and BIOEDIT 7.0.5.2 (Hall 1999). The nuclear sequences contained no heterozygous sites. GenBank accession numbers of new and downloaded sequences are listed in Appendix I.

In several species of turtles and tortoises, insertions of mtDNA in the nuclear genome (numts) are known to occur, specifically numts derived from the cyt *b* gene (Fritz *et al*. 2010, 2012c; Wiens *et al*. 2010; Kindler *et al*. 2012). However, base frequencies of our sequences as well as their ratios of synonymous and non-synonymous substitutions corresponded to the expectations for mtDNA. In addition, no internal stop codons were detected, and the two DNA fragments resulting from different PCR primers yielded consistent sequences. Therefore, we conclude that we amplified and sequenced authentic mtDNA.

Phylogeographic and phylogenetic analyses. Phylogeographic differentiation within *R. melanosterna*, and among this species and the closely allied species *R. diademata*, *R. funerea*, and *R. punctularia*, was assessed using a 1060-bp-long fragment corresponding to the nearly complete mitochondrial cyt *b* gene. Moreover, phylogenetic relationships among *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia* were inferred using three data sets: (1) a 2050-bp-long mitochondrial data set containing 1060 bp of the cyt *b* gene plus 17 bp of the adjacent DNA coding for tRNA-Thr, the partial 12S (407 bp) and 16S rRNA genes (566 bp); (2) a 3620-bp-long nuclear data set, corresponding to the concatenated Rag 1 (642 bp), Rag 2 (689 bp), C-mos (495 bp), R35 (880 bp) and ODC sequences (914 bp); and (3) a 5670-bp-long supermatrix of the merged mitochondrial and nuclear data sets. In addition, exploratory analyses were run for each gene alone.

Moreover, for cyt *b* sequences of *R. melanosterna* and the closely allied species *R. diademata*, *R. funerea* and *R. punctularia*, parsimony networks were calculated using TCS 1.21 (Clement *et al*. 2000) and uncorrected *p* distances were obtained using MEGA 4.0.2 (Tamura *et al*. 2007).

For phylogenetic analyses, identical cyt *b* sequences representing the same haplotype were removed. For computing trees based on cyt *b* sequences, GenBank data and newly generated sequences of the remaining eight *Rhinoclemmys* species (*R. annulata*, *R. areolata*, *R. diademata*, *R. funerea*, *R. nasuta*, *R. pulcherrima*, *R. punctularia*, *R. rubida*) were added. *Mauremys caspica* was used as a distantly related outgroup for tree rooting (for GenBank accession numbers, see Appendix I). For the three other data sets of 2050 bp (mtDNA), 3620 bp (nDNA) and 5670 bp length (mtDNA + nDNA = supermatrix), sequences of *R. melanosterna* were aligned with corresponding data for *R. diademata*, *R. funerea*, *R. punctularia* and *R. nasuta*. *Mauremys caspica* was again used for tree rooting. The mitochondrial and nuclear data sets and the supermatrix were examined using the following partition schemes: (i) unpartitioned, (ii) partitioned by gene, i.e., each gene corresponds to a distinct partition, and (iii) maximum partitioning, i.e., using each codon of each protein-coding gene and the R35 and ODC sequences as a distinct partition. To find the best partitioning scheme and substitution models for phylogenetic analyses, the software PARTITIONFINDER (Lanfear *et al.* 2012) was used, resulting in the selection of the maximum partitioning scheme for the mitochondrial data set and the supermatrix, whereas no partitioning was favoured for the nuclear DNA sequences.

For each set of concatenated sequences, ML analyses were then performed in RAxML 7.2.6 (Stamatakis 2006) using raxmlGUI 0.93 (Silvestro & Michalak 2012) and the best partitioning scheme. The default GTR+G model was applied across all partitions. To explore the robustness of the branching patterns, five independent ML searches were run using the fast bootstrap algorithm. Subsequently, 1000 thorough bootstrap replicates were calculated and plotted against the tree with the best likelihood value. In addition, using the same partitioning scheme for each data set and individual best-fit models for nucleotide substitution (Appendix II), Bayesian analyses (BA) were run in MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). The substitution models were incorporated into a single tree search (mixed model partition approach; Nylander *et al*. 2004) and two parallel runs were carried out using four Markov chains, each starting from a random tree. The Markov chains were run for ten

million generations, sampling every 100 generations. The burn-in was set to sample only the plateau of the most likely trees, which were used for generating a 50% majority rule consensus. The posterior probabilities in this tree are a measure for clade frequency and, thus, credibility.

Further, for the mitochondrial data set and the supermatrix, monophyly of *R. melanosterna* was examined using the Shimodaira-Hasegawa test as implemented in PAUP* 4.0b10 (Swofford 2002).

Results

Phylogeography

Twenty-one distinct haplotypes were found among the 74 cyt *b* sequences of *Rhinoclemmys melanosterna*. These haplotypes were assigned to seven weakly to well-supported clades by ML analysis and to six weakly to well-supported clades by BA (Fig. 2; clades consecutively numbered with Roman numerals I–VII in Figs 1–2). Under BA, the monophyly of clade I was not supported. The haplotypes of each clade are parapatrically distributed (Fig. 1).

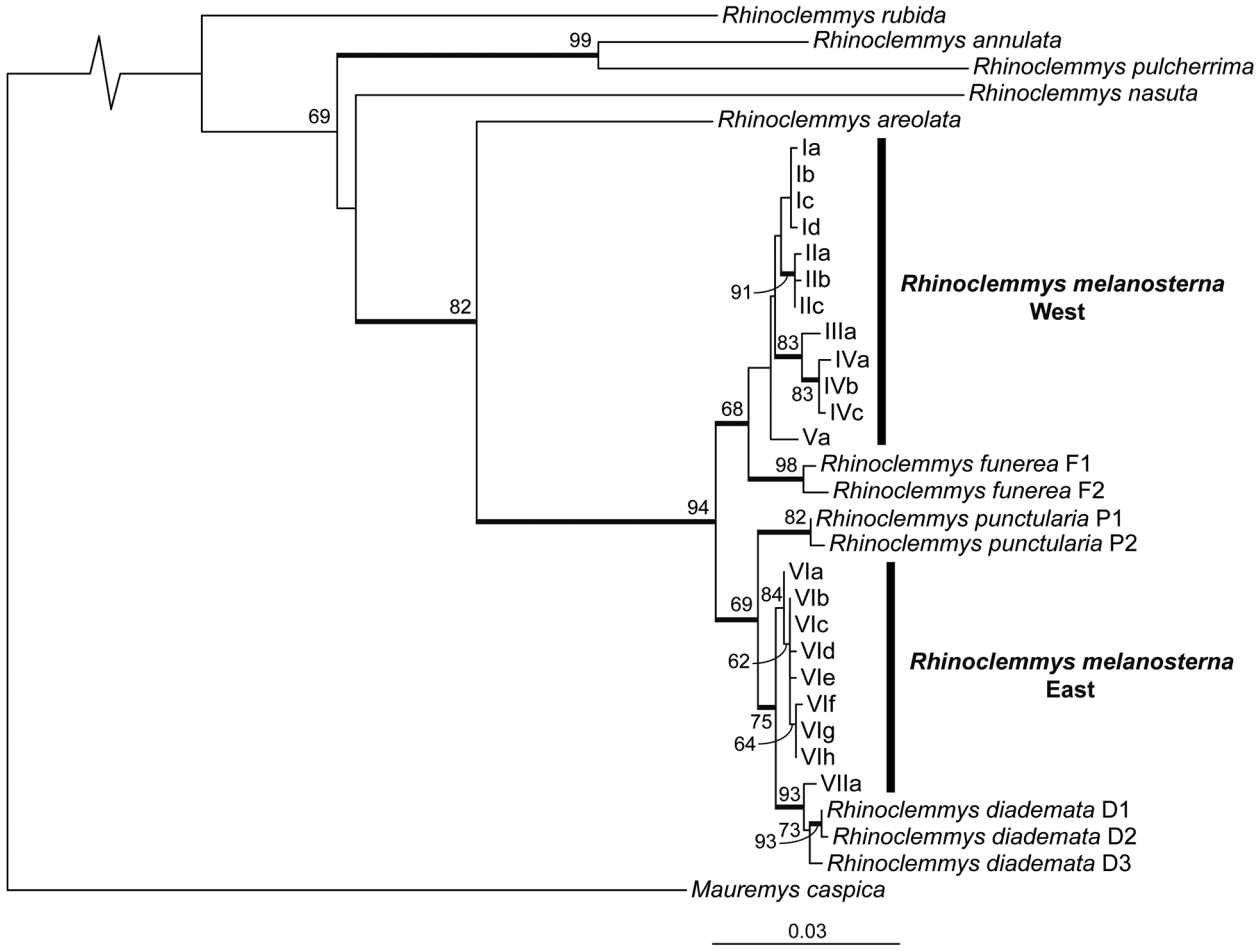

FIGURE 2. Maximum Likelihood tree for cyt *b* haplotypes (1060 bp) of *Rhinoclemmys melanosterna*, including sequences of the other eight *Rhinoclemmys* species. Haplotype codes correspond to Figure 3 and Appendix I (see there for GenBank accession numbers). Support values along branches are thorough bootstrap values > 50. Bold branches are supported by posterior probabilities > 0.95 in Bayesian analyses. Root length shortened by 75%. Note the polyphyly of *R. melanosterna.*

Both ML and BA returned polyphyletic topologies for haplotypes of *R. melanosterna* (Fig. 2). Haplotypes of *R. melanosterna* constituted together with sequences of *R. funerea*, *R. punctularia* and *R. diademata* a well-supported clade, and within this clade, the six to seven clades of *R. melanosterna* corresponded to two more inclusive clades distributed in the west and east of the species' range. Haplotypes of the western clade of *R. melanosterna* clustered with *R. funerea*, albeit only with weak or moderate support (ML bootstrap support of 68, Bayesian posterior probability of 0.97). The monophyly of the western clade of *R. melanosterna* was only very weakly supported under both methods. Haplotypes of the eastern clade of *R. melanosterna* were paraphyletic with respect to *R. diademata* with high or moderate support (ML: 75, BA: 0.96) and *R. punctularia* constituted the successive sister of this paraphyletic group with weak or moderate support (ML: 69, BA: 0.97). The monophyly of haplotype VIIa of *R. melanosterna* + *R. diademata* received high bootstrap and Bayesian support values of 93 and 1.0. The western haplotypes of *R. melanosterna* plus *R. funerea* and the eastern haplotypes of *R. melanosterna* plus *R. diademata* and *R. punctularia* together represented a well-supported major clade of which *R. areolata*, *R. nasuta*, *R. annulata* + *R. pulcherrima* and *R. rubida* were the successive sister taxa. This relationship among cyt *b* haplotypes of *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia* was also reflected in parsimony networks (Fig. 3). Under the 95% criterion, three unconnected networks were obtained (not shown; connection limit: 14 steps). One network corresponded to the two haplotypes of *R. funerea* and the two others to the eastern and western cluster of *R. melanosterna*, respectively. Haplotypes of *R. diademata* and *R. punctularia* were connected with the eastern cluster. If a connection among these three networks was enforced (Fig. 3), the haplotypes of *R. funerea* differed by a minimum of 16 steps from the most similar haplotype of *R. melanosterna* (western cluster), while the minimal difference between haplotypes of the western and eastern clusters of *R. melanosterna* was 22 steps. Haplotypes of *R. diademata* differed from haplotypes of the eastern cluster of *R. melanosterna* only by a minimum of five steps, and haplotypes of *R. punctularia* differed from haplotypes of the eastern cluster of *R. melanosterna* by a minimum of 12 steps. Within the western cluster of *R. melanosterna* (exclusive of *R. funerea*), haplotypes differed by a maximum of 13 steps. Among haplotypes of the eastern cluster of *R. melanosterna* (exclusive of *R. diademata* and *R. punctularia*), the maximum difference was 11 steps.

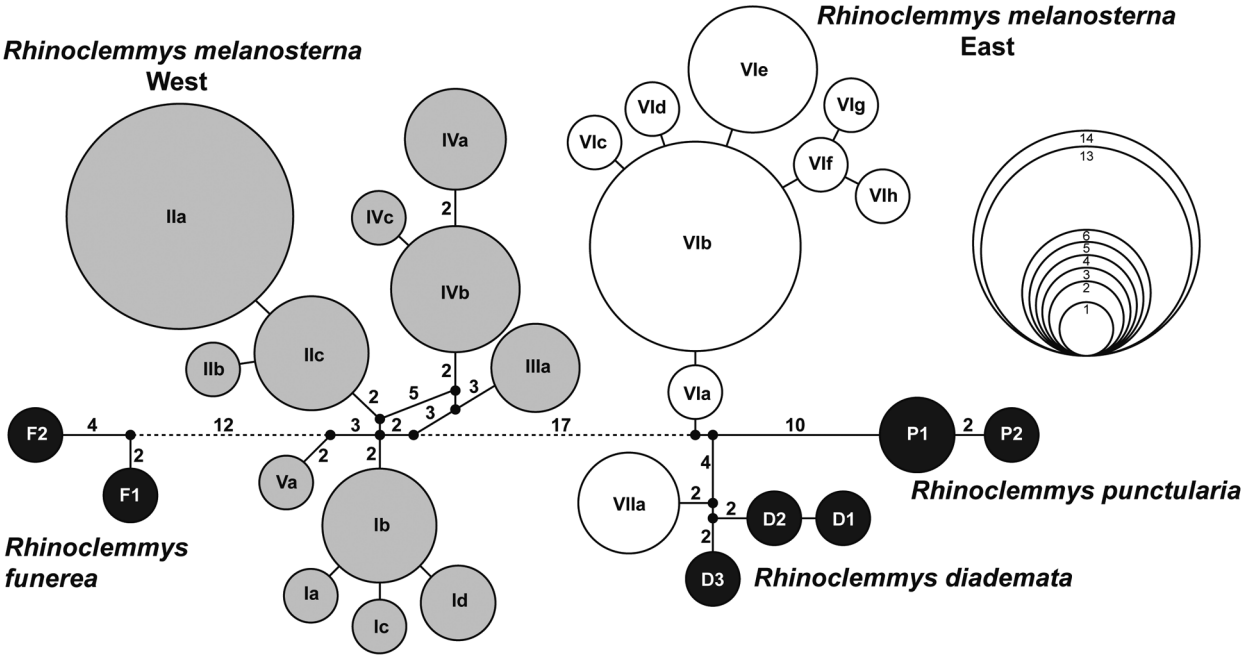

FIGURE 3. Parsimony network of cyt *b* haplotypes of *Rhinoclemmys melanosterna*, including sequences of *R. diademata, R. funerea* and *R. punctularia*, based on an alignment of 1060 bp length. Circle size indicates haplotype frequency. Missing node haplotypes are shown as small black circles. Each line connecting haplotypes corresponds to one mutational step, if not otherwise indicated by bold numbers. Stippled connections were not established under the 95% criterion. Haplotype codes refer to Appendix I.

TABLE 1. Mean uncorrected *p* distances between and within the seven mitochondrial clades of *Rhinoclemmys melanosterna* (I-VII) and the remaining *Rhinoclemmys* species, based on 1060 bp of the cyt *b* gene. Below diagonal, average percentages; above diagonal, standard error (500 bootstrap replicates). On diagonal, within-clade divergences in bold (mean ± SE); *n* = number of sequences.

	n	I	II	III	IV	V	VI	VII	*ann*	*are*	*dia*	*fun*	*nas*	*pul*	*pun*	*rub*
I	9	**0.06 ± 0.04**	0.21	0.28	0.30	0.23	0.45	0.48	1.09	0.81	0.49	0.39	1.15	1.21	0.50	1.14
II	21	0.48	**0.06 ± 0.05**	0.32	0.30	0.28	0.47	0.49	1.09	0.83	0.50	0.40	1.17	1.17	0.52	1.16
III	3	0.89	1.12	**0**	0.24	0.34	0.48	0.52	1.10	0.85	0.52	0.42	1.19	1.25	0.52	1.16
IV	11	0.92	1.01	0.65	**0.01 ± 0.07**	0.34	0.50	0.51	1.10	0.82	0.52	0.44	1.17	1.22	0.53	1.14
V	1	0.60	0.83	1.24	1.32	—	0.56	0.49	1.14	0.82	0.49	0.36	1.17	1.21	0.51	1.17
VI	25	2.22	2.46	2.48	2.53	2.38	**0.08 ± 0.04**	0.28	1.11	0.81	0.28	0.49	1.23	1.19	0.34	1.12
VII	4	2.64	2.87	2.91	2.99	2.81	0.91	**0**	1.10	0.84	0.18	0.52	1.25	1.21	0.37	1.14
R. annulata	1	10.15	10.18	10.11	10.04	10.80	9.77	10.00	—	1.07	1.11	1.15	1.13	0.88	1.10	1.14
R. areolata	1	6.44	6.67	6.94	6.66	6.61	6.63	7.04	10.43	—	0.84	0.82	1.04	1.14	0.81	1.05
R. diademata	3	2.71	2.93	2.97	3.01	2.87	1.01	0.51	10.15	6.97	**0.32 ± 0.13**	0.52	1.25	1.20	0.38	1.16
R. funerea	2	1.86	1.90	2.21	2.22	1.63	2.74	3.20	10.89	6.81	3.20	**0.57± 0.23**	1.20	1.20	0.54	1.18
R. nasuta	1	12.46	12.72	12.76	12.45	12.64	12.75	12.89	11.61	10.57	13.07	12.95	—	1.22	1.25	1.24
R. pulcherrima	1	12.15	12.17	12.45	12.15	12.57	12.01	12.22	7.71	11.48	12.32	12.60	14.03	—	1.19	1.26
R. punctularia	3	2.52	2.74	2.78	2.81	2.68	1.34	1.60	10.05	6.34	1.69	3.01	12.85	12.04	**0.13 ± 0.08**	1.18
R. rubida	1	12.81	13.08	13.24	13.29	13.00	12.79	12.77	12.76	11.47	13.05	13.23	13.97	14.33	12.71	—

The same pattern was echoed by uncorrected *p* distances (Table 1). Values ranged from 0.48% between clades I and II of *R. melanosterna* to 2.99% between clades IV and VII. Values among the clades of the western cluster were 0.48–1.32%, and 0.91% between clades VI and VII constituting the eastern cluster. Sequence divergences within clades of *R. melanosterna* ranged from 0% to 0.08%. The western and eastern clusters differed by 2.22–2.99%. When *R. diademata*, *R. funerea* and *R. punctularia* were compared to *R. melanosterna*, most values fell within the range observed among *R. melanosterna* clades. Only the pairwise differences between clade IV and *R. diademata* (3.01%) and between clade VII and *R. funerea* (3.20%) slightly exceeded the maximum value (2.99%) within *R. melanosterna*. By contrast, among the remaining five *Rhinoclemmys* species, and when these were compared to *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia*, significantly higher sequence divergence values of 6.34–14.33% were observed.

Head pattern and correlation with mitochondrial clades. In all *R. melanosterna* studied, the dorsal head coloration was dark brown to black, with a light-coloured dorsolateral stripe running on each side of the head from the rostrum to the back of the head. These stripes were red, orange, or greenish-yellow to yellow coloured. Table 2 and Figure 4 summarize the geographical variation in stripe form and colouration and its correspondence with the mitochondrial cyt *b* clades. Figure 4 also presents diagrams of the dorsal head pattern of the closely related species *R. diademata*, *R. funerea* and *R. punctularia*.

TABLE 2. Relationship between mitochondrial clades of *Rhinoclemmys melanosterna* and different types of dorsolateral head stripes. The column *n* refers to the number of individuals for which the head pattern was recorded.

Clade	Geographical distribution	*n*	Head stripes
I	Northern Córdoba, Colombia	8	Complete, narrow, orange, reaching nostrils (Fig. 4a, b)
II	Southern Córdoba and northern Antioquia, Colombia	17	Complete or interrupted, wide, bright red, reaching orbits (Fig. 4c, d)
III	Chocó, Colombia	9	Complete, narrow, light greenish yellow, reaching nostrils (Fig. 4e)
IV	Valle del Cauca, Colombia; Esmeraldas, Ecuador	9	Complete or interrupted, narrow, light greenish yellow, reaching nostrils (Fig. 4e)
V	Urabá, Antioquia, Colombia	–	Unknown
VI	Eastern Antioquia, Santander and southern Cesar, Colombia	25	Interrupted, narrow, orange, not reaching orbits (Fig. 4f, g)
VII	Northern Cesar, Colombia	4	Interrupted, narrow, orange, not reaching orbits (Fig. 4f, g)

Phylogenetic analyses of additional mitochondrial and nuclear DNA sequences. For most mitochondrial clades of *R. melanosterna*, five nuclear loci (Rag 1, Rag 2, C-mos, R35, ODC) and two additional mitochondrial genes (12S, 16S) were sequenced. However, these sequences could not be obtained for the only sample of cluster V due to poor DNA quality. Homologous sequence data were generated for *R. diademata*, *R. funerea* and *R. punctularia*, if such sequences were not available from GenBank (for GenBank accession numbers of newly generated and downloaded sequences, see Appendix I). Phylogenetic information for each gene was explored by computing ML and BA trees. In addition, mitochondrial and nuclear sequences were concatenated and the resulting data sets (mtDNA: 2050 bp, nDNA: 3620 bp, supermatrix of mtDNA and nDNA: 5670 bp) analyzed using ML and Bayesian Inference.

In the individual analyses of 12S and 16S sequences (trees not shown), *R. melanosterna* was not monophyletic with respect to *R. diademata*, *R. funerea* and *R. punctularia*, but the trees were weakly resolved compared to those based on cyt *b* data. The trees based on the concatenated mitochondrial gene sequences (Fig. 5) agreed well with the trees based on cyt *b* alone (Fig. 2), even though support values for the monophyly of the western clade of *R. melanosterna* plus *R. funerea* and of the eastern clade plus *R. diademata* and *R. punctularia* decreased or did not improve significantly compared to the cyt *b*-only trees. Like in the trees based on cyt *b* alone, *R. melanosterna* was polyphyletic (Fig. 5). A Shimodaira-Hasegawa test comparing the polyphyletic *R. melanosterna* with a constrained tree having *R. melanosterna* monophyletic found both topologies significantly different ($p = 0.001$) and supported the polyphyletic topology.

Individual analyses of the nuclear genes resulted in weakly resolved trees (not shown). The trees based on ODC, Rag 1 and Rag 2 placed the sequences of *R. melanosterna* together with *R. diademata*, *R. funerea* and *R. punctularia* in polytomies. In all trees based on C-mos and R35 sequences, *R. melanosterna* was monophyletic, albeit with very weak support. However, in the trees based on the concatenated five nuclear loci (Fig. 6), *R. melanosterna* was monophyletic with high support, with *R. diademata* + *R. punctularia* and *R. funerea* as consecutive sister taxa. While the clade comprising *R. diademata* and *R. punctularia* received high support under ML, it was only weakly supported under BA. The monophyly of the more inclusive clade with *R. funerea* was well-supported under both methods.

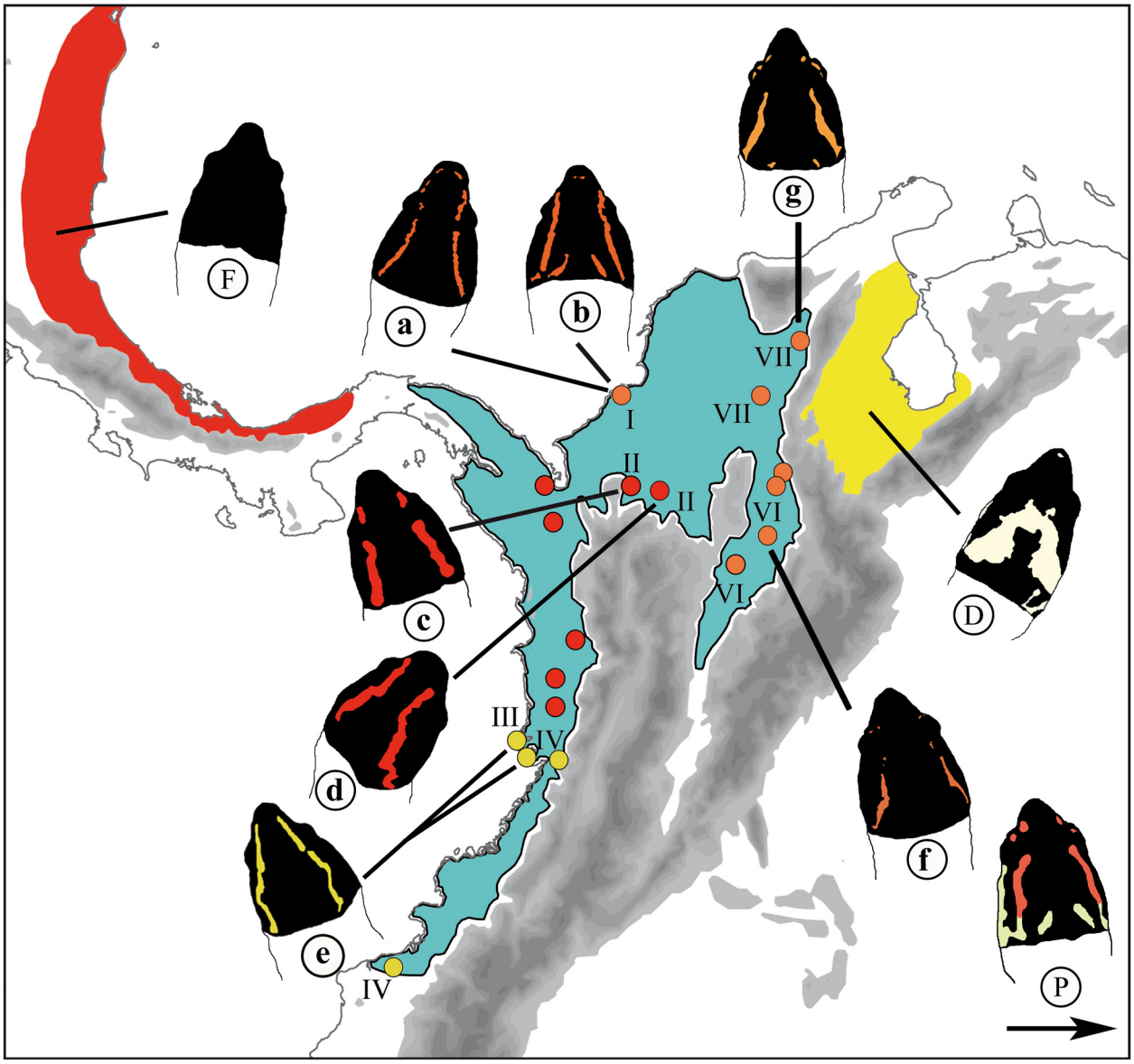

FIGURE 4. Geographical variation of head pattern in *Rhinoclemmys melanosterna*. Figured turtles are: (a) MTD T 4885, Cazuela, Lorica, Córdoba, Colombia; (b) MTD T 4888, Sicara, Lorica, Córdoba, Colombia; (c) MTD T 4726, Montelibano, Córdoba, Colombia; (d) MTD T 4569, Qda. Carmelo, Caucasia, Antioquia, Colombia; (e) MTD T 9167, Ladrilleros, Valle del Cauca, Colombia; (f) MTD T 9171, Ciénaga de Barbacoas, Antioquia, Colombia; (g) MTD T 4565, Caño Grande, Cesar, Colombia; F = *R. funerea*; D = *R. diademata*; P = *R. punctularia*. Colour of ranges of *R. funerea*, *R. melanosterna* and *R. diademata* correspond to Figure 1. Roman numerals indicate mitochondrial clades of *R. melanosterna*, coloured circles symbolize different colours of head stripes. Symbols without Roman numerals refer to specimens described by Medem (1962); vouchers are in the collection of the Instituto de Ciencias Naturales, Bogotá, Colombia (see text).

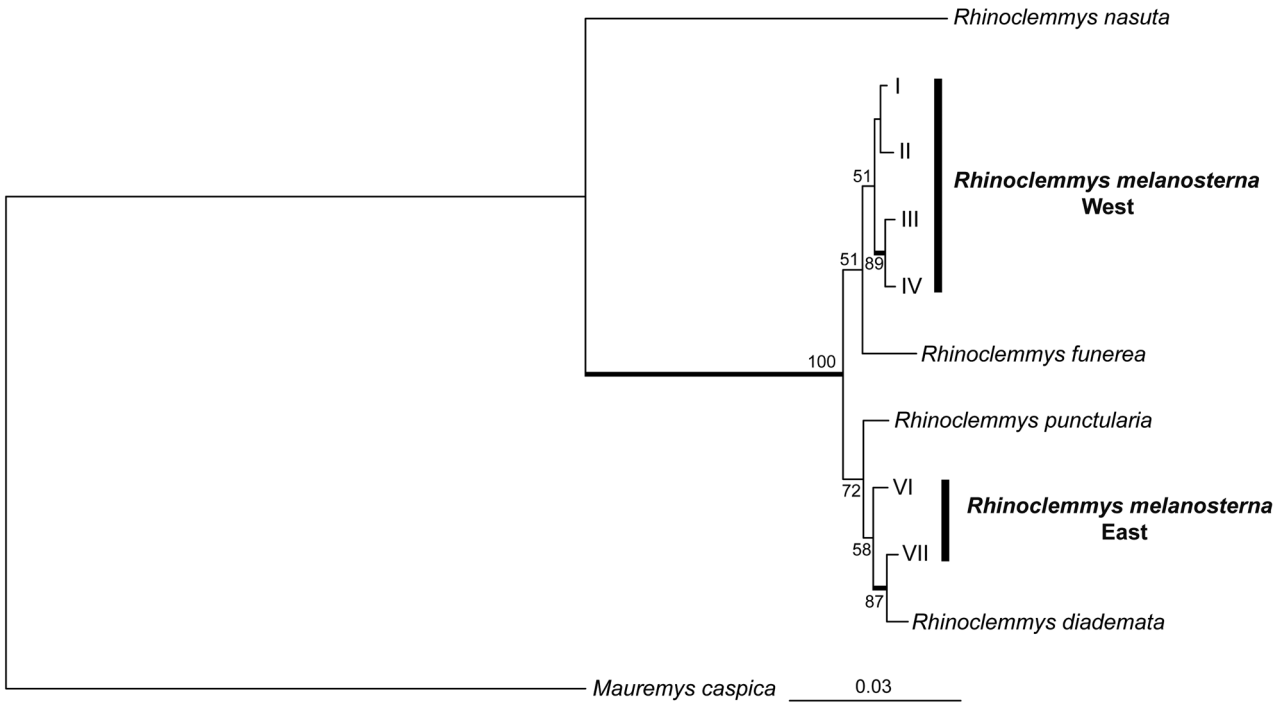

FIGURE 5. Maximum Likelihood tree for *Rhinoclemmys melanosterna*, *R. diademata*, *R. funerea*, *R. punctularia* and *R. nasuta*, based on 2050 bp of mitochondrial DNA (partial cyt *b*, 12S and 16S genes). Support values along branches are thorough bootstrap values > 50. Bold branches are supported by posterior probabilities of 1.0 in Bayesian analyses (no other branches had support values equal to or greater than 0.95). Note the polyphyly of *R. melanosterna.*

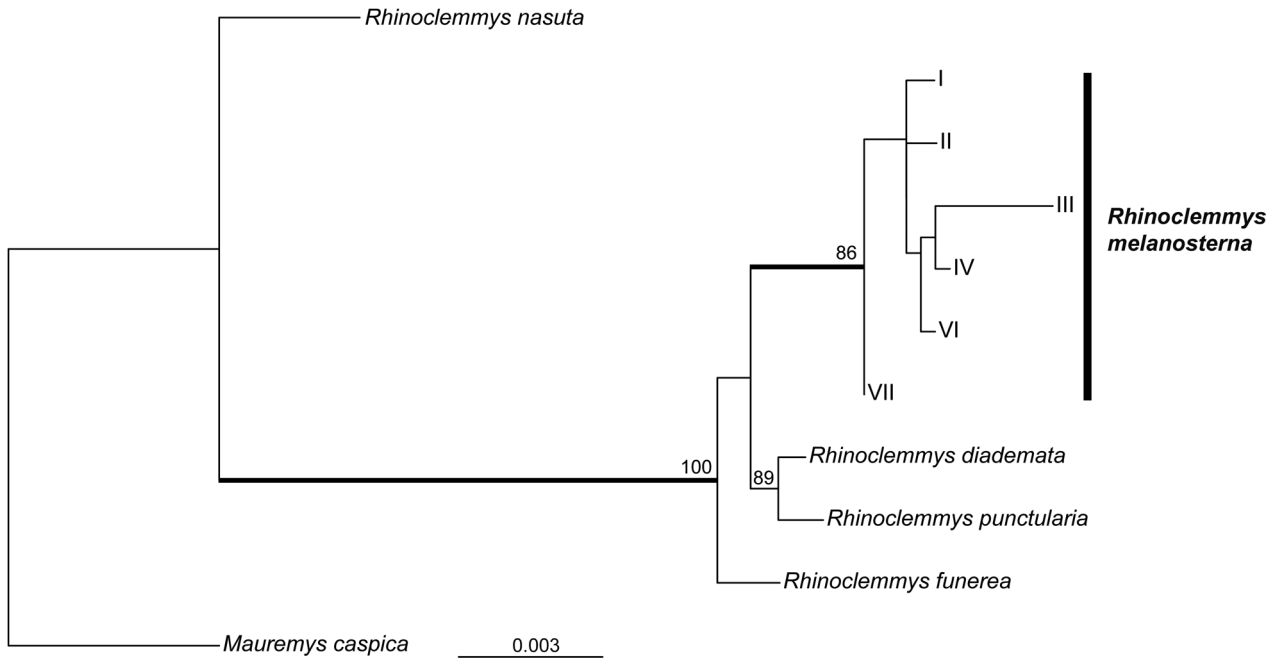

FIGURE 6. Maximum Likelihood tree for *Rhinoclemmys melanosterna*, *R. diademata*, *R. funerea*, *R. punctularia* and *R. nasuta*, based on 3620 bp of nuclear DNA (partial Rag 1, Rag 2, C-mos, R35 and ODC genes). Support values along branches are thorough bootstrap values > 50. Bold branches are supported by posterior probabilities > 0.95 in Bayesian analyses. Note the monophyly of *R. melanosterna*; lineages I–IV are distributed in the western part, lineages VI and VII in the eastern part of the range.

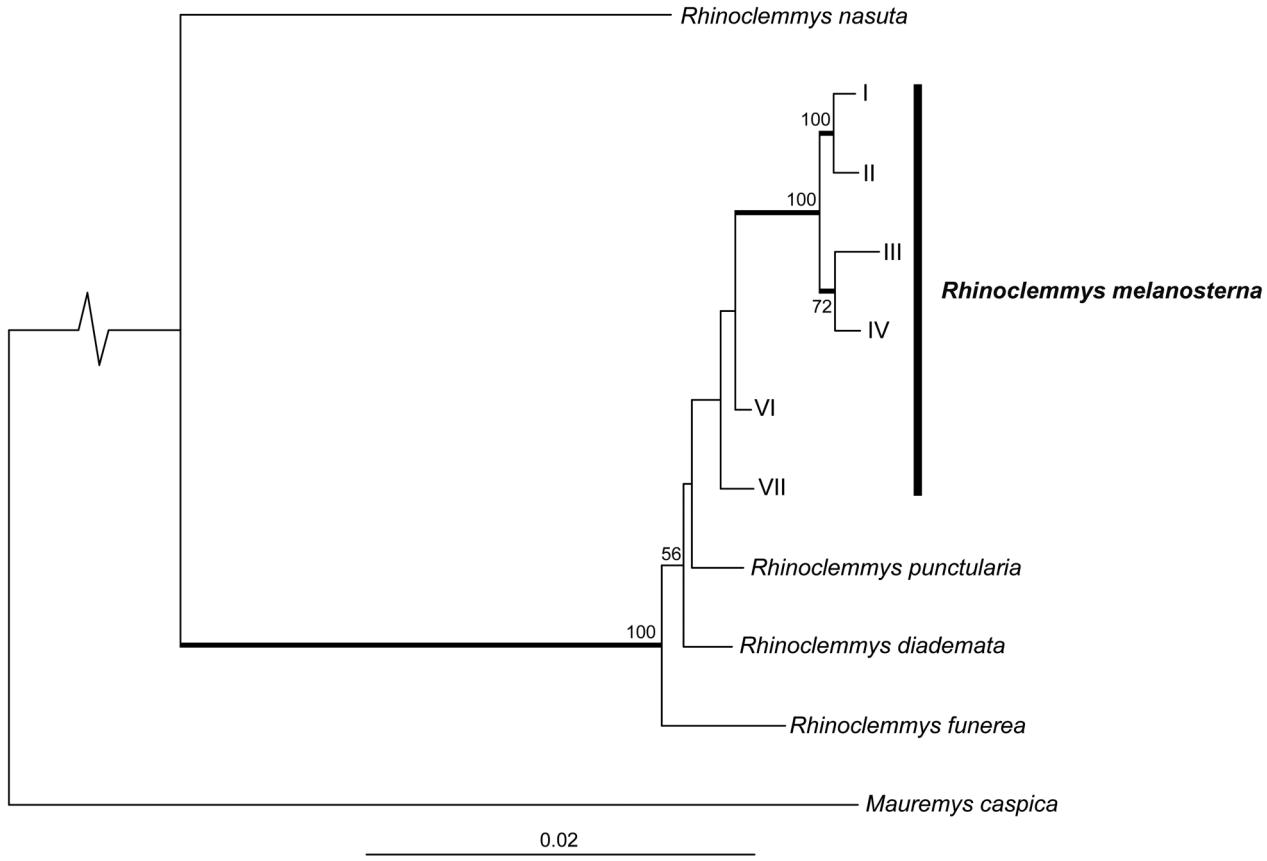

FIGURE 7. Maximum Likelihood tree for *Rhinoclemmys melanosterna*, *R. diademata*, *R. funerea*, *R. punctularia* and *R. nasuta*, based on the supermatrix of 2050 bp of mitochondrial DNA (partial cyt *b*, 12S and 16S genes) concatenated with 3620 bp of nuclear DNA (partial Rag 1, Rag 2, C-mos, R35 and ODC genes). Support values along branches are thorough bootstrap values > 50. Bold branches are supported by posterior probabilities of 1.0 in Bayesian analyses (no other branches had support values equal to or greater than 0.95; for further explanation see text). Root length shortened by 80%. Note the weak support for the monophyly of *R. melanosterna* and most other clades.

Also the ML tree based on the supermatrix (mitochondrial and nuclear genes concatenated) returned *R. melanosterna* as monophyletic, however, with only weak support, as for most of the other branches (Fig. 7). Only the monophyly of the major clade embracing *R. funerea*, *R. diademata*, *R. punctularia* and *R. melanosterna* received maximum support, as well as the western cluster of *R. melanosterna* with its two component haplotype clades I + II and III + IV. In contrast to the trees based on the five nuclear loci (Fig. 6), *R. punctularia* and *R. diademata* were not monophyletic, but rather the consecutive sister taxa of *R. melanosterna*, but this branching pattern was only very weakly supported. In the BA tree based on the supermatrix (not shown), *R. melanosterna* was not monophyletic. The topology of the BA tree closely resembled the tree based on the three concatenated mitochondrial genes (Fig. 5), but most branches were only weakly supported (for well-supported branches, see Fig. 7). A Shimodaira-Hasegawa test comparing the polyphyletic BA topology of the supermatrix with a tree having *R. melanosterna* monophyletic was inconclusive in that both competing topologies were not significantly different ($p = 0.391$).

Discussion

While our phylogenetic trees for *Rhinoclemmys* were in agreement with those of previous studies for some species (Carr 1991; Spinks *et al*. 2004; Le & McCord 2008), they were in clear conflict regarding the placement of *R. melanosterna* and the allied species *R. diademata*, *R. funerea* and *R. punctularia*. Spinks *et al*. (2004) and Le & McCord (2008) used mitochondrial and nuclear DNA sequences (cyt *b*, 12S, R35 and cyt *b*, 12S, 16S, C-mos, Rag

1, respectively) for their phylogeny reconstructions, whereas Carr (1991) used a combination of morphological and genetic (karyotype and isozyme) characters. All three studies recovered a sister group relationship between *R. melanosterna* and *R. funerea*, and these two species together were sister to a clade containing *R. diademata* and *R. punctularia*. However, using nuclear data alone, Le & McCord (2008) found a different topology with a *R. diademata* and *R. punctularia* clade with high support, and with *R. melanosterna* and *R. funerea* as the successive sister taxa with weak support. Although the study by Carr (1991) included multiple specimens of *R. melanosterna* and the other species, the genetic data for *R. melanosterna* only came from Ecuador; the studies by Spinks *et al*. (2004) and Le & McCord (2008) were based on only one individual per *Rhinoclemmys* taxon.

Using a comprehensive range-wide sampling of *R. melanosterna* and the same mitochondrial genes as Le & McCord (2008), we found in individual and combined analyses of mitochondrial sequences that *R. melanosterna* was consistently non-monophyletic. Sequences of *R. melanosterna* were always either paraphyletic or polyphyletic with respect to *R. diademata*, *R. funerea* and *R. punctularia*, and *R. melanosterna* exhibited considerable phylogeographic variation (Figs 2 and 5). This argues for caution when only one individual per taxon is used for phylogeny reconstructions, especially in closely related species.

In contrast to our mitochondrial results, combined analyses of five nuclear DNA loci (Rag 1, Rag 2, C-mos, R35, ODC) returned *R. melanosterna* as a well-supported monophyletic clade. Under ML, *R. diademata* was with high support sister to *R. punctularia*, while the phylogenetic relationship of this clade to *R. melanosterna* and *R. funerea* was only weakly resolved (Fig. 6). However, the sister group relationship of *R. diademata* and *R. punctularia* was only very weakly supported under BA (posterior probability of 0.65). Combined analyses of mitochondrial and nuclear sequences resulted only in weakly resolved and very weakly supported topologies and suggested either a monophyletic (ML, Fig. 7) or polyphyletic *R. melanosterna* (BA), and a Shimodaira-Hasegawa test found both topologies not significantly different.

This situation warrants an in-depth discussion. To examine the distinct gene partitions, we calculated ML and BA trees for each partition and for mitochondrial and nuclear genes concatenated, i.e., for a mitochondrial data set of 2050 bp length, a nuclear data set of 3620 bp length and a supermatrix of 5670 bp length, in which the mitochondrial and nuclear sequences were merged. When trees based on the individual nuclear gene partitions were compared to the trees based on the concatenated nuclear data, both resolution and support values increased distinctly using the concatenated sequences. This is suggestive of positive synergy caused by hidden branch support and hidden synapomorphies. In contrast, based on the three concatenated mitochondrial genes resolution and support of many nodes did not improve compared to the cyt *b* trees (Figs 2 and 5), and when the mitochondrial and nuclear data sets were combined in the supermatrix, resolution and support clearly decreased for most nodes, a situation that indicates data conflict (Thompson *et al*. 2012).

It could be argued that the conflict between the topologies of the nuclear and mitochondrial trees results from incomplete mitochondrial lineage sorting among closely related taxa (*cf*. Maddison 1997), and this hypothesis seems to be corroborated by some weakly supported branches. Based on mitochondrial data, the monophyly of the western clade of *R. melanosterna* and *R. funerea* is only weakly supported, as is the monophyly of *R. punctularia* plus the eastern clade of *R. melanosterna*, including *R. diademata* (Figs 2 and 5). Likewise, the monophyly of *R. diademata* and *R. punctularia* is not unambiguously supported in the BA analysis of the nuclear data set (see above). However, in the ML analysis of nuclear sequences *R. diademata* appeared well-supported as sister of *R. punctularia*, and the monophyly of *R. melanosterna* is unequivocally well-supported under ML and BA using the five nuclear loci (Fig. 6). In contrast, *R. diademata* and haplotype VIIa of *R. melanosterna* form a well-supported clade using mitochondrial data (Figs 2 and 5), and this clade renders the eastern clade of *R. melanosterna* paraphyletic with respect to *R. diademata*.

Therefore, we conclude that incomplete sorting cannot completely explain the contradictory patterns. In fact, the smaller effective population size of mitochondrial DNA should lead first on the mitochondrial level, and not the nuclear genomic level, to reciprocally monophyletic gene trees. It is well-known that mitochondrial DNA introgresses more easily than nuclear DNA (Ballard & Whitlock 2004; Currat *et al*. 2008; Toews & Brelsford 2012). Hence, we believe that gene flow among the four species in question caused or at least contributed to the conflict between the mitochondrial and nuclear data. If so, the non-monophyly of mitochondrial haplotypes of *R. melanosterna* were the result of substantial mitochondrial introgression. Since the four involved taxa are parapatrically or allopatrically distributed (Fig. 1), the current pattern must then mirror past gene flow.

It is obvious that each of the two haplotype clusters of *R. melanosterna* harbours haplotypes resembling its

geographically neighbouring species, suggestive of former secondary contact zones. Haplotypes of the western cluster of *R. melanosterna* resemble those of *R. funerea*, from which the putative distribution range of the western cluster is separated by a gap of only approximately 100 km (Fig. 1). Haplotypes of the eastern clade of *R. melanosterna* resemble haplotypes of *R. diademata* and *R. punctularia*. The ranges of *R. diademata* and of the eastern clade of *R. melanosterna* are only separated by the Serranía de Perijá, a narrow Andean mountain chain, and *R. punctularia* is widely disjunct farther eastwards (Fig. 1).

Mitochondrial haplotypes of *R. diademata* are much more similar to *R. melanosterna* than haplotypes of *R. funerea* and *R. punctularia* (Fig. 3). Their nearly abutting ranges together with the genetic similarity suggest that the mitochondrial divergence between *R. melanosterna* and *R. diademata* is much younger than the divergences among *R. melanosterna* and the other taxa. We cannot exclude the possibility that gene flow still continues through valleys across the Serranía de Perijá, and that a denser sampling of *R. diademata* could reveal shared mitochondrial haplotypes with the eastern clade of *R. melanosterna*.

Several recent studies have used uncorrected *p* distances of the mitochondrial cyt *b* gene as a tool for species delimitation in chelonians (Vargas-Ramírez *et al*. 2010; Praschag *et al*. 2011; Stuckas & Fritz 2011; Fritz *et al*. 2012b, c; Kindler *et al*. 2012; Liebing *et al*. 2012), similar to the barcoding approach (e.g., Hebert *et al*. 2003). When this method is applied to *Rhinoclemmys*, we see that *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia* differ by sequence divergences of 0.51% to 3.20%. These figures closely resemble the pairwise divergences among the seven haplotype clades within *R. melanosterna* (0.48–2.99%; Table 1). In contrast, among the remaining *Rhinoclemmys* species, and when these species are compared to *R. melanosterna* and its allied species, significantly higher values of 6.34% to 14.33% are observed. These values highlight the fact that differentiation within *R. melanosterna*, and among this species and *R. diademata*, *R. funerea* and *R. punctularia*, reflects a much lower level of divergence compared to the remaining *Rhinoclemmys* species. Moreover, the close genetic relationships among *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia* render their classification as distinct species a matter of convention. Under the Biological Species Concept (Mayr 1942; Coyne & Orr 2004), they would qualify equally well as subspecies. Carr (1991) applied the Evolutionary Species Concept (Wiley 1981; Frost & Hillis 1990) to *Rhinoclemmys* and recognized all four taxa as species based on their morphological and karyotypic differences (*cf*. Ernst 1978; Pritchard 1979; Pritchard & Trebbau 1984; Carr & Bickham 1986; Carr 1991). We propose to conserve this classification of *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia* as distinct species, which is justifiable under various species concepts owing to their allopatric and parapatric distribution ranges, as well as their morphological and karyotypic differentiation.

Within *R. melanosterna*, the distinct cyt *b* clades correspond to some extent to previously described morphological differences with respect to the colour pattern of the head (Medem 1962; Carr 1991; Castaño-Mora & Medem 2002; Rueda-Almonacid *et al*. 2007). In accordance with our findings (Fig. 4; Table 2), Medem (1962) reported that 17 turtles from the Chocó department (Colombia) had heads with red stripes. In addition, Medem (1962) and Carr (1991) found that individuals from Pacific drainages beginning at the mouth of the Río San Juan in Colombia and south into Ecuador had green, greenish-yellow, or yellow head stripes. The latter colour pattern matches Gray's (1861) description of *Geoclemmys melanosterna* and the head pattern its two putative syntypes in the collection of the Natural History Museum London (Carr 1991; Echeverri-García *et al*. 2012; Carr pers. obs.).

Conclusions

Rhinoclemmys melanosterna is a species with clear phylogeographic structure and the clades identified correspond to some extent to morphological differentiation in the dorsal head pattern. This suggests that each mitochondrial clade constitutes an evolutionarily significant unit (Moritz 1994) and, hence, is of substantial conservation relevance. Compared to previous studies (Carr 1991; Spinks *et al*. 2004; Le & McCord 2008), we found much more complicated phylogenetic relationships among *R. melanosterna*, *R. diademata*, *R. funerea* and *R. punctularia*. With respect to the prior molecular studies (Spinks *et al*. 2004; Le & McCord 2008), this discrepancy highlights the point that phylogenetic investigations of closely related taxa may be misleading when just one individual per species is used, and when those specimens are of unknown geographical provenance. Compared to the remaining *Rhinoclemmys* species, the four taxa in question constitute a closely related species complex for which we found evidence for extensive past hybridization. With respect to the molecular data alone, their classification as distinct

species to maintain the current nomenclatural usage is merely a matter of convention, but justifiable owing to their allo- and parapatric distribution ranges and differences in other character sets. To gain a better understanding of past gene flow, future research should include a more extensive sampling of *R. diademata*, *R. funerea* and *R. punctularia*, and use additional nuclear genes and quickly evolving markers like microsatellites. Le & McCord (2008) suggested that two ancestral species, one giving rise to *R. melanosterna* + *R. funerea* and the other to *R. diademata* + *R. punctularia*, reached South America after the closure of the Central American land bridge and diverged in South America into the extant species. Our novel genetic results do not necessarily conflict with this view. However, the close relationship among the four taxa makes it possible that just one species reached South America and diversified there, as suggested by Carr (1991).

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Online Appendices

Appendix I. GenBank accession numbers of DNA sequences used in the present study. MTD T numbers refer to samples in the tissue collection of the Museum of Zoology (Museum für Tierkunde), Senckenberg Dresden; h = haplotype, HP = head pattern: Oc = orange complete, Oi = orange incomplete, Y = yellow, Yc = yellow complete, Yi = yellow incomplete, R = red.

Appendix II. Models selected by the Bayesian Information Criterion in PARTITIONFINDER (Lanfear *et al.* 2012) for the optimal partitioning schemes.

Both Appendices are available from the Dryad Repository using the link http://dx.doi.org/10.5061/dryad.q9s30

References

Ballard, J.W.O. & Whitlock, M.C. (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, 13, 729–744. http://dx.doi.org/10.1046/j.1365-294X.2003.02063.x

Boulenger, G.A. (1889) *Catalogue of the Chelonians, Rhynchocephalians, and Crocodiles in the British Museum (Natural History)*. British Museum (Natural History), London, x + 311 pp., 6 plates.

Carr, J.L. (1991) *Phylogenetic analysis of the Neotropical turtle genus* Rhinoclemmys *Fitzinger (Testudines: Emydidae)*. Ph.D. Dissertation, Southern Illinois University, Carbondale, IL, xx + 290 pp.

Carr, J.L. & Bickham, J.W. (1986) Phylogenetic implications of karyotypic variation in the Batagurinae (Testudines: Emydidae). *Genetica*, 70, 89–106. http://dx.doi.org/10.1007/BF00123580

Castaño-Mora, O.V. & Medem, F. (2002) *Rhinoclemmys melanosterna. In:* Castaño-Mora, O.V. (Ed.), *Libro rojo de reptiles de Colombia.* Libros rojos de especies amenazadas de Colombia. Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Ministerio del Medio Ambiente, Conservación Internacional Colombia, Bogotá, pp. 116–117.

Clement, M., Posada, D. & Crandall, K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657–1660. http://dx.doi.org/10.1046/j.1365-294x.2000.01020.x

Coyne, J.A. & Orr, H.A. (2004) *Speciation*. Sinauer Associates, Sunderland, MA, xiii + 545 pp.

Currat, M., Ruedi, M., Petit, R.J. & Excoffier, L. (2008) The hidden side of invasions: massive introgression by local genes. *Evolution*, 62, 1908–1920. http://dx.doi.org/10.1111/j.1558-5646.2008.00413.x

Echeverri-García, L.P., Carr, J.L., Garcés-Restrepo, M.F., Galvis-Rizo, C.A. & Giraldo, A. (2012) *Rhinoclemmys melanosterna*

(Gray, 1861). *In:* Páez, V., Morales-Betancourt, M.A., Lasso, C.A., Castaño-Mora, O.V. & Bock, B. (Eds.), *Biología y Conservación de las Tortugas Continentales de Colombia*. *Serie Editorial: Recursos Hidrobiológicos y Pesqueros Continentales de Colombia*. Instituto de Investigación de los Recursos Biológicos Alexander von Humboldt (IAvH), Bogotá, pp. 310–316.

Ernst, C.H. (1978) A revision of the Neotropical turtle genus *Callopsis* (Testudines: Emydidae: Batagurinae). *Herpetologica*, 34, 113–134.

Ernst, C.H. (1981) *Rhinoclemmys punctularia* (Daudin), spotted-legged terrapin. *Catalogue of American Amphibians and Reptiles*, 276, 1–2.

Ernst, C.H. & Barbour, R.W. (1989) *Turtles of the World*. Smithsonian Institution Press, Washington, D.C., xii + 313 pp., 16 plates.

Ernst, C.H., Altenburg, R.G.M. & Barbour, R.W. (2000) *Turtles of the World.* World Biodiversity Database, version 1.2. Biodiversity Center of ETI, Amsterdam, CD-ROM.

Friesen, V.L., Congdon, B.C., Kidd, M.G. & Birt, T.P. (1999) Polymerase chain reaction (PCR) primers for the amplification of five nuclear introns in vertebrates. *Molecular Ecology*, 8, 2147–2149.
http://dx.doi.org/10.1046/j.1365-294x.1999.00802-4.x

Fritz, U. (2011) Order Testudines Batsch, 1788. *In:* Zhang, Z.-Q. (Ed.), Animal biodiversity: an outline of higher-level classification and survey of taxonomic richness. *Zootaxa*, 3148, 61–62.

Fritz, U. & Havaš, P. (2007) Checklist of chelonians of the world. *Vertebrate Zoology,* 57, 149–368.

Fritz, U., Fattizzo, T., Guicking, D., Tripepi, S., Pennisi, M.G., Lenk, P., Joger, U. & Wink, M. (2005) A new cryptic species of pond turtle from southern Italy, the hottest spot in the range of the genus *Emys*. *Zoologica Scripta*, 34, 351–371.
http://dx.doi.org/10.1111/j.1463-6409.2005.00188.x

Fritz, U., Auer, M., Bertolero, A., Cheylan, M., Fattizzo, T., Hundsdörfer, A.K., Martín Sampayo, M., Pretus, J.L., Široký, P. & Wink, M. (2006) A rangewide phylogeography of Hermann's tortoise, *Testudo hermanni* (Reptilia: Testudines: Testudinidae): implications for taxonomy. *Zoologica Scripta*, 35, 531–543.
http://dx.doi.org/10.1111/j.1463-6409.2006.00242.x

Fritz, U., Guicking, D., Auer, M., Sommer, R.S., Wink, M. & Hundsdörfer, A.K. (2008) Diversity of the Southeast Asian leaf turtle genus *Cyclemys*: how many leaves on its tree of life? *Zoologica Scripta*, 37, 367–390.
http://dx.doi.org/10.1111/j.1463-6409.2008.00332.x

Fritz, U., Daniels, S.R., Hofmeyr, M.D., González, J., Barrio-Amorós, C.L., Široký, P., Hundsdörfer, A.K. & Stuckas, H. (2010) Mitochondrial phylogeography and subspecies of the wide-ranging sub-Saharan leopard tortoise *Stigmochelys pardalis* (Testudines: Testudinidae) – a case study for the pitfalls of pseudogenes and GenBank sequences. *Journal of Zoological Systematics and Evolutionary Research*, 48, 348–359.
http://dx.doi.org/10.1111/j.1439-0469.2010.00565.x

Fritz, U., Branch, W.R., Hofmeyr, M.D., Maran, J., Prokop, H., Schleicher, A., Široký, P., Stuckas, H., Vargas-Ramírez, M., Vences, M. & Hundsdörfer, A.K. (2011) Molecular phylogeny of African hinged and helmeted terrapins (Testudines: Pelomedusidae: *Pelusios* and *Pelomedusa*). *Zoologica Scripta*, 40, 115–125.
http://dx.doi.org/10.1111/j.1463-6409.2010.00464.x

Fritz, U., Branch, W.R., Gehring, P.-S., Harvey, J., Kindler, C., Meyer, L., Du Preez, L., Široký, P., Vieites, D.R. & Vences, M. (2012a) Weak divergence among African, Malagasy and Seychellois hinged terrapins (*Pelusios castanoides*, *P. subniger*) and evidence for human-mediated oversea dispersal. *Organisms Diversity & Evolution*, published online ahead of print.
http://dx.doi.org/10.1007/s13127-012-0113-3

Fritz, U., Alcalde, L., Vargas-Ramírez, M., Goode, E.V., Fabius-Turoblin, D.U. & Praschag, P. (2012b) Northern genetic richness and southern purity, but just one species in the *Chelonoidis chilensis* complex. *Zoologica Scripta*, 41, 220–232.
http://dx.doi.org/10.1111/j.1463-6409.2012.00533.x

Fritz, U., Stuckas, H., Vargas-Ramírez, M., Hundsdörfer, A.K., Maran, J. & Päckert, M. (2012c) Molecular phylogeny of Central and South American slider turtles: implications for biogeography and systematics (Testudines: Emydidae: *Trachemys*). *Journal of Zoological Systematics and Evolutionary Research*, 50, 125–136.
http://dx.doi.org/10.1111/j.1439-0469.2011.00647.x

Frost, D.R. & Hillis, D.M. (1990) Species in concept and practice: herpetological applications. *Herpetologica*, 46, 86–104.

Fujita, M.K., Engstrom, T.N., Starkey, D.E. & Shaffer, H.B. (2004) Turtle phylogeny: insights from a novel nuclear intron. *Molecular Phylogenetics and Evolution*, 31, 1031–1040.
http://dx.doi.org/10.1016/j.ympev.2003.09.016

Georges, A., Birrel, J., Saint, K.M., McCord, W.P. & Donnellan, S.C. (1998) A phylogeny for side-necked turtles (Chelonia: Pleurodira) based on mitochondrial and nuclear gene sequence variation. *Biological Journal of the Linnean Society*, 67, 213–246.
http://dx.doi.org/10.1111/j.1095-8312.1999.tb01862.x

Gray, J.E. (1861) On a new species of water-tortoise (*Geoclemmys melanosterna*) from Darien. *Proceedings of the Zoological Society of London*, 1861, 204–205.

Hall, T.A. (1999) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.

Hebert, P.D.N., Ratnasingham, S. & de Waard, J.R. (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences

among closely related species. *Proceedings of the Royal Society London B*, 270, S96–S99.
http://dx.doi.org/10.1098/rsbl.2003.0025

Hutchison, J.H. (2006) *Bridgeremys* (Geoemydidae, Testudines), a new genus from the Middle Eocene of North America. *Russian Journal of Herpetology*, 13 (Supplement: Fossil Turtle Research, Vol. 1.), 63–83.

Kindler, C., Branch, W.R., Hofmeyr, M.D., Maran, J., Široký, P., Vences, M., Harvey, J., Hauswaldt, J.S., Schleicher, A., Stuckas, H. & Fritz, U. (2012) Molecular phylogeny of African hinge-back tortoises (*Kinixys*): implications for phylogeography and taxonomy (Testudines: Testudinidae). *Journal of Zoological Systematics and Evolutionary Research*, 50, 192–201.
http://dx.doi.org/10.1111/j.1439-0469.2012.00660.x

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X. & Wilson, A.C. (1989) Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA*, 86, 6196–6200.
http://dx.doi.org/10.1073/pnas.86.16.6196

Lanfear, R., Calcott, B., Ho, S.Y.W. & Guindon, S. (2012) PARTITIONFINDER: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, 29, 1695–1701.
http://dx.doi.org/10.1093/molbev/mss020

Le, M. & McCord, W.P. (2008) Phylogenetic relationships and biogeographical history of the genus *Rhinoclemmys* Fitzinger, 1835 and the monophyly of the turtle family Geoemydidae (Testudines: Testudinoidea). *Zoological Journal of the Linnean Society*, 153, 751–767.
http://dx.doi.org/10.1111/j.1096-3642.2008.00413.x

Le, M., Raxworthy, C.J., McCord, W.P. & Mertz, L. (2006) A molecular phylogeny of tortoises (Testudines: Testudinidae) based on mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution*, 40, 517–531.
http://dx.doi.org/10.1016/j.ympev.2006.03.003

Le, M., McCord, W.P. & Iverson, J.B. (2007) On the paraphyly of the genus *Kachuga* (Testudines: Geoemydidae). *Molecular Phylogenetics and Evolution*, 45, 398–404.
http://dx.doi.org/10.1016/j.ympev.2007.05.002

Liebing, N., Praschag, P., Gosh, R., Vasudevan, K., Rashid, S.M.A., Rao, D.-Q., Stuckas, H. & Fritz, U. (2012) Molecular phylogeny of the softshell turtle genus *Nilssonia* revisited, with first records of *N. formosa* for China and wild-living *N. nigricans* for Bangladesh. *Vertebrate Zoology*, 62, 261–272.

Maddison, W.P. (1997) Gene trees in species trees. *Systematic Biology*, 46, 523–536.
http://dx.doi.org/10.1093/sysbio/46.3.523

Mayr, E. (1942) *Systematics and the Origin of Species from the Viewpoint of a Zoologist*. Columbia University Press, New York, NY, xxxv + 334 pp.

McDowell, S.B. (1964) Partition of the genus *Clemmys* and related problems in the taxonomy of the aquatic Testudinidae. *Proceedings of the Zoological Society of London*, 143, 239–279.
http://dx.doi.org/10.1111/j.1469-7998.1964.tb03860.x

Medem, F. (1962) La distribución geográfica y ecología de los Crocodylia y Testudinata en el Departamento del Chocó. *Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales*, 11, 279–303.

Mertens, R. (1954) Zur Kenntnis der Schildkrötenfauna Venezuelas. *Senckenbergiana biologica*, 35, 3–7.

Moritz, C. (1994) Defining 'Evolutionarily Significant Units' for conservation. *Trends in Evolution & Ecology*, 9, 373–375.
http://dx.doi.org/10.1016/0169-5347(94)90057-4

Nylander, J.A.A., Ronquist, F., Huelsenbeck, J.P. & Nieves-Aldrey, J.L. (2004) Bayesian phylogenetic analysis of combined data. *Systematic Biology*, 53, 47–67.
http://dx.doi.org/10.1080/10635150490264699

Palumbi, S., Martin, A., Romano, S., McMillan, W.O., Stice, L. & Grabowski, G. (2002) *The Simple Fool's Guide to PCR, Version 2*. Department of Zoology and Kewalo Marine Laboratory, University of Hawaii, Honolulu, HI, 45 pp.

Praschag, P., Hundsdörfer, A.K. & Fritz, U. (2007) Phylogeny and taxonomy of endangered South and South-east Asian freshwater turtles elucidated by mtDNA sequence variation (Testudines: Geoemydidae: *Batagur*, *Callagur*, *Hardella*, *Kachuga*, *Pangshura*). *Zoologica Scripta*, 36, 429–442.
http://dx.doi.org/10.1111/j.1463-6409.2007.00293.x

Praschag, P., Stuckas, H., Päckert, M., Maran, J. & Fritz, U. (2011) Mitochondrial DNA sequences suggest a revised taxonomy of Asian flapshell turtles (*Lissemys* Smith, 1931) and the validity of previously unrecognized taxa (Testudines: Trionychidae). *Vertebrate Zoology*, 61, 147–160.

Pritchard, P.C.H. (1979) *Encyclopedia of Turtles*. T.F.H. Publications, Hong Kong, 895 pp.

Pritchard, P.C.H. & Trebbau, P. (1984) *The Turtles of Venezuela*. SSAR, Oxford, OH, VIII + 403 pp., 47 plates, 16 maps.

Ronquist, F. & Huelsenbeck, J.P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572–1574.
http://dx.doi.org/10.1093/bioinformatics/btg180

Rueda-Almonacid, J.V., Carr, J.L., Mittermeier, R.A., Rodríguez-Mahecha, J.V., Mast, R.B., Vogt, R.C., Rhodin, A.G.J., de la Ossa-Velásquez, J., Rueda, J.N. & Goettsch-Mittermeier, C. (2007) *Las tortugas y los cocodrilianos de los países andinos del trópico*. Conservación Internacional (Serie de guías tropicales de campo), Bogotá, 538 pp.

Savage, J.M. (1966) The origins and history of the Central American herpetofauna. *Copeia*, 1966, 719–766.
http://dx.doi.org/10.2307/1441404

Silvestro, D. & Michalak, I. (2012) raxmlGUI: a graphical frontend for RAxML. *Organisms, Diversity & Evolution*, 12, 335–337.
http://dx.doi.org/10.1007/s13127-011-0056-0

Smith, H.M. & Smith, R.B. (1980) *Synopsis of the Herpetofauna of Mexico, Guide to Mexican Turtles, Bibliographic Addendum III*. John Johnson, North Bennington, VT, xviii + 1044 pp.

Spinks, P.Q., Shaffer, H.B., Iverson, J.B. & McCord, W.P. (2004) Phylogenetic hypotheses for the turtle family Geoemydidae. *Molecular Phylogenetics and Evolution*, 32, 164–182.
http://dx.doi.org/10.1016/j.ympev.2003.12.015

Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, 22, 2688–2690.
http://dx.doi.org/10.1093/bioinformatics/btl446

Stuckas, H. & Fritz, U. (2011) Identity of *Pelodiscus sinensis* revealed by DNA sequences of an approximately 180-year-old type specimen and a taxonomic reappraisal of *Pelodiscus* species (Testudines: Trionychidae). *Journal of Zoological Systematics and Evolutionary Research*, 49, 335–339.
http://dx.doi.org/10.1111/j.1439-0469.2011.00632.x

Swofford, D.L. (2002) *PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods)*. Sinauer, Sunderland, MA.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596–1599.
http://dx.doi.org/10.1093/molbev/msm092

Thompson, R.S., Bärmann, E.V. & Asher, R.J. (2012) The interpretation of hidden support in combined data phylogenetics. *Journal of Zoological Systematics and Evolutionary Research*, 50, 251–263.
http://dx.doi.org/10.1111/j.1439-0469.2012.00670.x

Toews, D.P.L. & Brelsford, A. (2012) The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology*, 21, 3907–3930.
http://dx.doi.org/10.1111/j.1365-294X.2012.05664.x

van Dijk, P.P., Iverson, J.B., Shaffer, H.B., Bour, R. & Rhodin, A.G.J. (2011) Turtles of the world, 2011 update: annotated checklist of taxonomy, synonymy, distribution, and conservation status. *Chelonian Research Monographs*, 5, 000.165–000.242.

Vargas-Ramírez, M., Vences, M., Branch, W.R., Daniels, S.R., Glaw, F., Hofmeyr, M.D., Kuchling, G., Maran, J., Papenfuss, T.J., Široký, P., Vieites, D.R. & Fritz, U. (2010) Deep genealogical lineages in the widely distributed African helmeted terrapin: evidence from mitochondrial and nuclear DNA (Testudines: Pelomedusidae: *Pelomedusa subrufa*). *Molecular Phylogenetics and Evolution*, 56, 428–440.
http://dx.doi.org/10.1016/j.ympev.2010.03.019

Vargas-Ramírez, M., Michels, J., Castaño-Mora, O.V., Cárdenas-Arevalo, G., Gallego-García, N. & Fritz, U. (2012a) Weak genetic divergence between the two South American toad-headed turtles *Mesoclemmys dahli* and *M. zuliae* (Testudines: Pleurodira: Chelidae). *Amphibia-Reptilia*, 33, 373–385.
http://dx.doi.org/10.1163/15685381-00002840

Vargas-Ramírez, M., Stuckas, H., Castaño-Mora, O.V. & Fritz, U. (2012b) Extremely low genetic diversity and weak population differentiation in the endangered Colombian river turtle *Podocnemis lewyana* (Testudines: Podocnemididae). *Conservation Genetics*, 13, 65–77.
http://dx.doi.org/10.1007/s10592-011-0263-4

Wiens, J.J., Kuczynski, C.A. & Stephens, P.R. (2010) Discordant mitochondrial and nuclear gene phylogenies in emydid turtles: implications for speciation and conservation. *Biological Journal of the Linnean Society*, 99, 445–461.
http://dx.doi.org/10.1111/j.1095-8312.2009.01342.x

Wiley, E.O. (1981) *Phylogenetics: The Theory and Practice of Phylogenetic Systematics*. John Wiley and Sons, New York, NY, 456 pp.