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Deep genealogical lineages in the widely distributed African helmeted terrapin: Evidence from mitochondrial and nuclear DNA (Testudines: Pelomedusidae: *Pelomedusa subrufa*)

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ABSTRACT

We investigated the phylogeographic differentiation of the widely distributed African helmeted terrapin *Pelomedusa subrufa* based on 1503 base pairs of mitochondrial DNA (partial cyt *b* and ND4 genes with adjacent tRNAs) and 1937 bp of nuclear DNA (partial Rag1, Rag2, R35 genes). Congruent among different analyses, nine strongly divergent mitochondrial clades were found, representing three major geographical groupings: (1) A northern group which includes clades I from Cameroon, II from Ghana and Ivory Coast, III from Benin, Burkina Faso and Niger, IV from the Central African Republic, and V from Kenya, (2) a northeastern group consisting of clades VI from Somalia, and VII from Saudi Arabia and Yemen, and (3) a southern group comprising clade VIII from Botswana, the Democratic Republic of Congo, Madagascar and Malawi, and clade IX from South Africa. Malagasy and continental African populations were not clearly differentiated, indicating very recent arrival or introduction of *Pelomedusa* in Madagascar. The southern group was in some phylogenetic analyses sister to *Pelusios*, rendering *Pelomedusa* paraphyletic with respect to that genus. However, using partitioned Bayesian analyses and sequence data of the three nuclear genes, *Pelomedusa* was monophyletic, suggesting that its mitochondrial paraphyly is due to either ancient introgressive hybridization or phylogenetic noise. Otherwise, nuclear sequence data recovered a lower level of divergence, but corroborated the general differentiation pattern of *Pelomedusa* as revealed by mtDNA. This, and the depth of the divergences between clades, indicates ancient differentiation. The divergences observed fall within, and in part exceed considerably, the differentiation typically occurring among chelonian species. To test whether *Pelomedusa* is best considered a single species composed of deep genealogical lineages, or a complex of up to nine distinct species, we suggest a future taxonomic revision that should (1) extend the geographical sampling of molecular data, specifically focusing on contact zones and the possible sympatric occurrence of lineages without admixture, and (2) evaluate the morphology of the various genealogical lineages using the type specimens or topotypical material of the numerous junior synonyms of *P. subrufa*.

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1. Introduction

The evaluation of inter- and intraspecific patterns of genetic diversity is essential for understanding and preserving regional and global biodiversity. In the past years, phylogeographic analyses have revealed that many previously recognized species represent multiple genealogical lineages which often led to taxonomic revisions and a reconsideration of conservation strategies (e.g., Fouquet et al., 2007; Fritz et al., 2008, 2010; Praschag et al., 2007a; Rissler et al., 2006; Vieites et al., 2009; Whittaker et al., 2005). The workhorse of phylogeography has traditionally been the analysis of mitochondrial DNA (mtDNA; Avise, 2000, 2004). However, in recent years evolutionary conclusions based exclusively on mtDNA are viewed with concern, and the inclusion of nuclear genomic markers in phylogeographic studies is recommended. Nevertheless, mtDNA remains in many cases an ideal tool for deciphering differentiation processes, especially in recently diverged lineages (see reviews in Brito and Edwards, 2009; Zink and Barrowclough, 2008).

The present study aims at analyzing the geographical differentiation of the most widely distributed African chelonian, the helmeted terrapin *Pelomedusa subrufa*, using both marker systems to provide an assessment of its diversity. Although chelonians (tortoises, turtles, and terrapins) are prominent animals and their species diversity is limited with approximately 315 extant species (Fritz and Havaš, 2007), their intraspecific genetic differentiation is often unknown. This situation is due to the fact that chelonians are rarely collected during field work. Turtles and terrapins are difficult to catch and the large size of most chelonian species challenges fixation and transport. In addition, tissue samples often originate from animals from the food or pet trade, and their uncertain provenance renders them useless for phylogeographic purposes (Shaffer et al., 2007). Consequently, phylogeographic data are completely missing even for some major chelonian clades. One of these is *P. subrufa*, the sole extant representative of its genus. Together with its sister taxon *Pelusios*, comprising about 20 species, it forms the Afrotropical family Pelomedusidae (de Broin, 1988; Fritz and Havaš, 2007). Pelomedusids are most closely related to the South American–Malagasy river turtle family Podocnemididae (Fujita et al., 2004; Gaffney and Meylan, 1988; Krenz et al., 2005), indicating an ancient Gondwana origin of both (Noonan and Chippindale, 2006; Vargas-Ramírez et al., 2008).

P. subrufa is a semi-aquatic, medium-sized species, ranging throughout Africa, from Somalia and Ethiopia in the northeast to Senegal and Mali in the northwest, and southwards through central and eastern Africa to the Cape Peninsula. In addition, it occurs in the southwestern Arabian Peninsula and in Madagascar (Boycott and Bourquin, 2008; Branch, 2008; Ernst et al., 2000; Gasperetti et al., 1993). This range (Fig. 1) includes numerous different habitats and is transected by some important biogeographical barriers, such as the Mozambique Channel that separates Madagascar from Africa, the Red Sea, and the African rift valley, suggesting that an assessment of the phylogeographic differentiation of this species could yield significant biogeographical insights. Of particular interest are the Malagasy and Arabian populations.

The fauna of Madagascar, often considered a veritable 'microcontinent', is characterized by extreme endemism that often extends to the level of genera and families (Goodman and Benstead, 2003). Only a few Malagasy amphibians and reptiles are assumed to be conspecific with African populations (Glaw and Vences, 2007), among them *P. subrufa*. For such taxa, different scenarios can be postulated for their origins and taxonomic status: (1) They might have been introduced by man who populated Madagascar not earlier than approximately 2300 years ago (Burney et al., 2004), in which case they are expected to have haplotypes identical to those found in Africa, and a comparatively low haplotype diversity; (2) they may be very recent natural colonizers that diverged only slightly from conspecifics in Africa; or (3) they may be recent natural colonizers that, however, have diverged enough to be considered separate species, as is possibly the case in the frog *Ptychadena mascareniensis* (Measey et al., 2007; Verneau et al., 2009).

For the Arabian helmeted terrapins, as for the Malagasy populations, an origin by either recent trans-oceanic dispersal or introduction by man can be hypothesized. Alternatively, the Arabian populations could be ancient relicts of a formerly continuous population that was at the latest separated from the extant African populations by the Early to Late Pliocene submersion of the Afar-Yemen land bridge (Bosworth et al., 2005; Redfield et al., 2003). Should the latter be true, the Arabian populations are expected to be clearly differentiated genetically.

Largely based on its characterization as a wide-ranging, generalized species, the helmeted terrapin is considered under no immediate threat in conservation assessments (Boycott and Bourquin, 2008) and, consequently, is not listed in the IUCN Red List of Threatened Species (IUCN, 2009). However, all previous analyses of geographical variation within *P. subrufa* were based on external morphology and only small sample sizes (review in Gasperetti et al., 1993). Although the helmeted terrapin was the first chelonian of which the complete mitochondrial genome was sequenced (Zardoya and Meyer, 1998a, b), molecular tools were never applied to elucidate its geographical variation. Here we use sequence data of two mitochondrial (1503 bp) and three nuclear genomic DNA fragments (1937 bp) of a nearly range-wide sample, to assess the phylogeography of *P. subrufa* and to place our findings in a biogeographical context, in order to provide a basis for future revisions of its taxonomy and conservational status.

2. Materials and methods

2.1. Sampling, laboratory procedures, and alignment of DNA sequences

Blood or tissue samples of 58 *P. subrufa* from throughout its distribution range were studied (Appendix 1; Fig. 1). For all samples, two mtDNA fragments were sequenced, a 664-bp-long part of the partial cytochrome *b* gene (cyt *b*) plus 23 bp of the adjacent tRNA threonine gene (tRNA-Thr); the second fragment comprised 667 bp of the nicotinamide adenine dinucleotide dehydrogenase subunit 4 gene (ND4) plus the adjacent tRNA genes (complete tRNA-His: 76 bp, complete tRNA-Ser: 58 bp, partial tRNA-Leu: 15 bp). This mitochondrial data set was complemented with partial sequences of three nuclear genes (nDNA). Probably due to partly degraded template DNA, attempts to sequence nuclear genes from several old samples were unsuccessful. Despite this problem, nDNA sequences from samples representing all mitochondrial clades and nearly all localities were successfully obtained. The nuclear genes comprised 700 bp of the intron 1 of the RNA fingerprint protein 35 gene (R35), 593 bp of the recombination-activating gene 1 (Rag1), and 644 bp of the recombination-activating gene 2 (Rag2) (Appendix 1).

Blood or tissue samples were preserved in an EDTA buffer (0.1 M Tris, pH 7.4, 10% EDTA, 1% NaF, 0.1% thymol) or in ethanol and kept at −20 °C until processing. Remaining tissue and blood samples are stored at −80 °C in the tissue sample collections of the Museum of Zoology, Senckenberg Dresden, Germany, and of the Museum of Vertebrate Zoology, Berkeley, CA, USA. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following manufacturer's protocols. PCR was performed in a 50 μL volume (Bioron PCR buffer or 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, 0.5% Triton X-100, pH 8.5) containing 1 U *Taq* DNA polymerase (Bioron, Ludwigshafen, Germany), 10 pmol dNTPs

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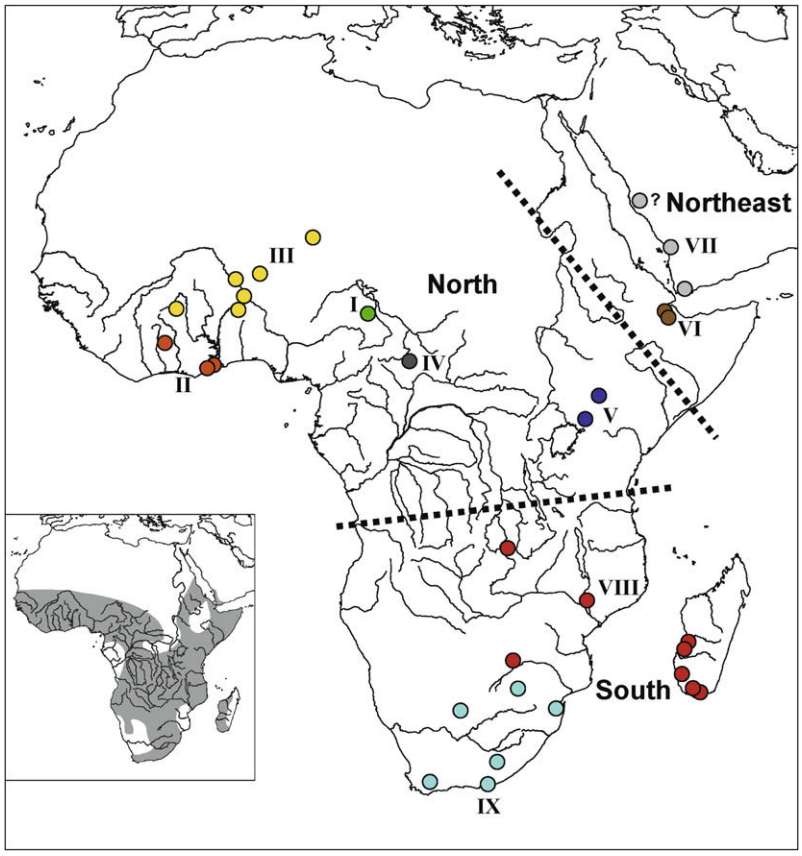

Fig. 1. Approximate range of *Pelomedusa subrufa* (inset; based on Boycott and Bourquin, 2008) and sampling sites. Colours and Roman numerals correspond to mitochondrial clades. Major inclusive clades (North, Northeast, South) indicated. Question mark denotes questionable locality. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fermentas, St. Leon-Roth, Germany) and 5 or 10 pmol of the respective primer. For the mtDNA fragment containing the partial cyt *b* gene, the primer pair Pel-for/Pel-rev (5′-CTACGGTTGCAACCG-GAA-3′/5′-GTCCCCCTAGAGAGAGTA-3′) was designed during this study; for other primers and thermocycling conditions see Table 1. PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and sequenced directly on both strands on an ABI 3130 XL sequencer (Applied Biosystems, Foster City, CA, USA).

Sixteen additional sequences were downloaded from GenBank and included in the alignments where appropriate (Appendix 1); sequences were aligned using BIOEDIT 7.0.5.2 (Hall, 1999) and sequence statistics were calculated using MEGA 4.0.2 (Tamura et al., 2007). For GenBank accession numbers of new and downloaded sequences, see Appendix 1. Alignments and trees not shown in this study were submitted to TreeBase (study accession number S2648; matrix accession numbers M5085-M5087). To assess the levels of substitution saturation for the cyt *b* and ND4 fragments, pairwise differences were plotted for transitions and transversions against corrected pairwise divergences (F84 distances; Xia and Xie, 2001). Data sets of both mitochondrial DNA fragments were partitioned by codon positions to obtain separate plots for each partition. All *Pelomedusa* sequences aligned perfectly for R35 and the protein-coding regions of cyt *b*, Rag1 and Rag2, with no insertions or deletions. No heterozygous sites were detected in the sequenced nuclear DNA fragments. Some indels occurred in the other alignments; for details, see online Supplementary Information.

The base frequencies of the ND4 and cyt *b* fragments as well as their ratios of synonymous and non-synonymous substitutions corresponded to expectations for mitochondrial sequences. In addition, in the protein-coding parts no stop codons were found

Table 1
Primers and thermocycling conditions.

Fragment	Primers	Reference	Thermocycling conditions					
			ID	*C*	D	A	PE	FE
mtDNA (cyt *b* + tRNA-Thr)	Pel-for/Pel-rev	This study	5 min, 94 °C	35–40	45 s, 94 °C	52 s, 50–60 °C	80 s, 72 °C	10 min, 92 °C
mtDNA (ND4 + tRNA-His + tRNA-Ser + tRNA-Leu)	L-ND4/H-Leu	Stuart and Parham (2004)	5 min, 94 °C	40	45 s, 94 °C	30 s, 50–53 °C	60 s, 72 °C	10 min, 72 °C
nDNA (intron 1 of R35)	R35Ex1/R35Ex2	Fujita et al. (2004)	5 min, 94 °C	35–40	30 s, 94 °C	90 s, 50–60 °C	120 s, 72 °C	10 min, 72 °C
nDNA (Rag1)	Rag1878/Rag2547	Le et al. (2007)	5 min, 95 °C	39–42	40 s, 95 °C	45 s, 52–55 °C	60 s, 72 °C	10 min, 72 °C
nDNA (Rag2)	F2-1/R2-1	Le et al. (2006)	5 min, 95 °C	39	30 s, 95 °C	45 s, 52 °C	60 s, 72 °C	10 min, 72 °C

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

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and nucleotides successfully translated into amino acids. Therefore, we conclude to have sequenced authentic mitochondrial DNA fragments and not nuclear copies of mtDNA.

2.2. Sequence analyses

Phylogenetic relationships were inferred using three different data sets: (1) A 1508 bp mitochondrial data set, containing concatenated sequences of the two mtDNA fragments containing the partial cyt *b* and ND4 genes and several tRNA genes (see above), (2) a 1950 bp nuclear data set, comprising concatenated Rag1, Rag2 and R35 sequences of samples representing all mitochondrial clades, and (3) a total evidence data set of 3458 bp, consisting of the mtDNA and nDNA sequences of samples for which nuclear data were available (Table 2). Concatenated mitochondrial and nuclear sequences of *P. subrufa* were collapsed into haplotypes using TCS 1.21 (Clement et al., 2000), resulting in 35 mitochondrial and 12 nuclear haplotypes (Appendix 1). Identical sequences were generally removed for phylogenetic analyses. For the mitochondrial data set, the *P. subrufa* sequences were aligned with corresponding data of *Pelusios sinuatus*; *Erymnochelys madagascariensis* and *Podocnemis expansa* were used as distantly related outgroups. In the nuclear and total evidence partitions, sequences of *P. sinuatus*, *P. expansa*, *Peltocephalus dumerilianus*, and *E. madagascariensis* were included. In all cases, *Erymnochelys* was defined as the outgroup for tree rooting in BA. *Pelusios* is the sister genus of *Pelomedusa*, whereas *Erymnochelys*, *Peltocephalus* and *Podocnemis* are representatives of the family Podocnemididae that constitutes the sister group of Pelomedusidae (Fujita et al., 2004; Gaffney and Meylan, 1988; Krenz et al., 2005). The combinability of all sequence partitions was tested using the incongruence length difference test (ILD test; Farris et al., 1995) as implemented in PAUP* 4.0b10 (Swofford, 2002), with 1000 replicates to generate the null distribution. No significant incongruence was revealed for any of the tested settings (mitochondrial fragments: $p = 0.08$; nuclear sequences: $p = 0.49$; nuclear and mitochondrial data sets combined: $p = 0.25$).

MP and ML analyses were performed in PAUP* 4.0b10 using heuristic searches with 100 random addition sequences of taxa using the tree bisection-reconnection branch swapping option; bootstrap support values were calculated for MP with 1000 and for ML with 100 replicates. For ML analyses, the appropriate model of sequence evolution was estimated using the Akaike Information Criterion (AIC) as implemented in MODELTEST 3.06 (Posada and Crandall, 1998; see Table 2).

Bayesian analyses (BA) were run in MrBAYES 3.1 (Ronquist and Huelsenbeck, 2003) using four incrementally heated Markov chains; posterior probabilities were obtained from the 50% majority rule consensus tree. For each independent run, the variation in likelihood scores was examined by plotting $-\ln L$ scores against the number of generations, and the burn-in was set to sample only the plateau of the most likely trees. In a conservative approach, 40% of all sampled trees were discarded, although the plateau of likelihood values had been reached before. For all three data sets (mitochondrial, nuclear, and combined), we used a Bayes factor analysis to determine the most appropriate partition scheme for BA. Each gene (tRNAs merged in one partition), and within each gene the individual codon positions, were examined for the appropriate model of sequence evolution using AIC in MrMODELTEST (Nylander, 2002); for the selected models, see Supplementary Information. Subsequently, for each data set (mitochondrial, nuclear, and mitochondrial and nuclear sequences combined), the following three partition schemes were applied: (1) unpartitioned, (2) by-gene, i.e., each gene corresponding to a distinct partition with its own evolutionary model and all tRNAs merged in one additional partition, and (3) maximum partitions, i.e., a scheme using each non-coding gene as distinct partition (again with the tRNA genes merged in a single partition) and additionally each codon of each protein-coding gene as distinct partition. For each scheme, analyses were run for five million generations with every 100th tree sampled; the trees corresponding to the first two million generations were discarded. According to Brandley et al. (2005), the harmonic means of the likelihoods were then used to calculate Bayes factors. Bayes factors suggested the maximum partition scheme as the best for the mitochondrial and nuclear data sets (see Supplementary Information) and the by-gene partition scheme for the total evidence data set. Then, BA were run for 50 million generations under the respectively selected partition schemes, sampling every 1000th tree. The trees corresponding to the first 20 million generations were excluded as burn-in.

In addition to these phylogenetic analyses, genealogical relationships of mitochondrial and nuclear haplotypes within *P. subrufa* were examined by calculating parsimony networks for concatenated mitochondrial sequences and nuclear haplotypes (Rag1, Rag2, R35) using TCS 1.21. Fixed differences between haplotype clusters were computed in DnaSP 5.00.07 (Librado and Rozas, 2009). Moreover, the influence of geographical distances on genetic variation was examined with a Mantel test in the software IBD (Isolation-by-Distance; Bohonak, 2002) using uncorrected *p* distances of mitochondrial sequences and geographical distances (km). Furthermore, a categorical matrix coding the impact of ancestry was used to assess the influence of long-term historical processes vs. isolation-by-distance. In this matrix, haplotypes of different clades were coded with 0 (corresponding to no impact of ancestry) and haplotypes of the same clade with 1 (impact of ancestry). This matrix was used in a second Mantel test to determine whether the phylogenetic association co-varied with genetic distances. In addition, in order to evaluate the effect of historical divergence and geography simultaneously, partial Mantel tests (Legendre and Legendre, 1998) were run in IBD.

3. Results

3.1. Phylogeny and geographical distribution of mitochondrial haplotypes

The concatenated sequences of the two mtDNA fragments containing the partial cyt *b* and ND4 genes represented 35, in

Table 2
Data partitions (including outgroups) analysed with Maximum Parsimony, Maximum Likelihood, and Bayesian inference of phylogeny, their sequence statistics, and evolutionary models. See Supplementary Information for the substitution models in Bayesian partitioned analysis.

Partition	Fragment length (aligned)	Variable characters	Parsimony informative characters	MP tree length	RI	CI	ML $-\ln L$	BA $-\ln L$	AIC model
mtDNA	1508	659	458	1378	0.85	0.61	10345.42	9079.03	GTR + G
nDNA	1950	297	112	339	0.88	0.90	4416.44	4409.41	HKY + 2
Total evidence	3458	1036	609	2023	0.60	0.65	16417.79	16444.46	GTR + 1 + G

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part highly distinct, haplotypes. All tree-building methods revealed for these mitochondrial data a strong phylogenetic structure with nine well-supported clades within *P. subrufa*, each corresponding to a well-delimited geographical region (clades consecutively numbered with Roman numerals I–IX; Figs. 1 and 2). However, a major difference was observed between the maximally partitioned BA analysis on one hand and all other analyses on the other, with respect to the relationships of the *Pelomedusa* clades and *Pelusios sinuatus*. The analysis based on the maximum number of partitions (suggested as most appropriate by the Bayes factor analysis) revealed, as expected, a monophyletic *Pelomedusa*, albeit with weak support. By contrast, the ML and MP analyses as well as BA based on the unpartitioned and by-gene partition schemes suggested a paraphyly of *Pelomedusa* with respect to *P. sinuatus* (Fig. 2). Yet, all analyses agreed in that the nine *Pelomedusa* clades clustered in three weakly to well-supported, more inclusive clades from the northern, northeastern, and southern parts of the species' range. The major clade from the north, only weakly to moderately supported, included haplotype Ia (Cameroon), haplotype IVa (Central African Republic), and the well-supported subclades II (Ghana, Ivory Coast), III (Benin, Burkina Faso, Niger), and V (Kenya). The well-supported major clade from the northeast included the two well-supported subclades VI (Somalia) and VII (Saudi Arabia, Yemen). The weakly supported major clade from the south, placed by unpartitioned BA and ML and MP analyses with moderate support as sister to *P. sinuatus* (Fig. 2: inset), contained the remaining *Pelomedusa* haplotypes, corresponding to two well-supported subclades. In one of these, subclade VIII, the haplotypes from Madagascar (VIIIa, VIIIb, VIIId) occurred together with haplotypes from Botswana (VIIIc), Malawi (VIIIe) and the Democratic Republic of Congo (VIIIf); this subclade was sister to the well-supported South African subclade IX.

3.2. Phylogeny of nuclear DNA sequences and combined phylogenetic analyses

Nuclear sequences could not be generated for all samples, and for some samples only one or two of the three nuclear DNA fragments could be sequenced (see Section 2; Appendix 1). Nonetheless, it was obvious that distinctly less variation occurred compared to mtDNA. For at least one specimen representative of each mitochondrial clade of *Pelomedusa*, a complete set of all three nuclear sequences could be compiled, and sequences of samples for which not all nuclear sequences were available represented no additional haplotypes (see also below under Section 3.3.), suggesting that the available nuclear sequences are a good proxy for the total nDNA variation. In the phylogenetic analyses of nuclear data, at least one representative of each mitochondrial clade was included and these sequences were concatenated with the corresponding mitochondrial data from the same samples for a total evidence analysis.

All phylogenetic analyses of the nuclear data set consistently revealed *P. subrufa* as well-supported monophyletic group (Fig. 3A), including BA using the favoured partition scheme (maximum number of partitions) as well as using the unpartitioned and by-gene partition schemes. Nuclear haplotypes clustered only in two major clades, one corresponding to the two major mitochondrial clades from the north and northeast, and the other perfectly matching

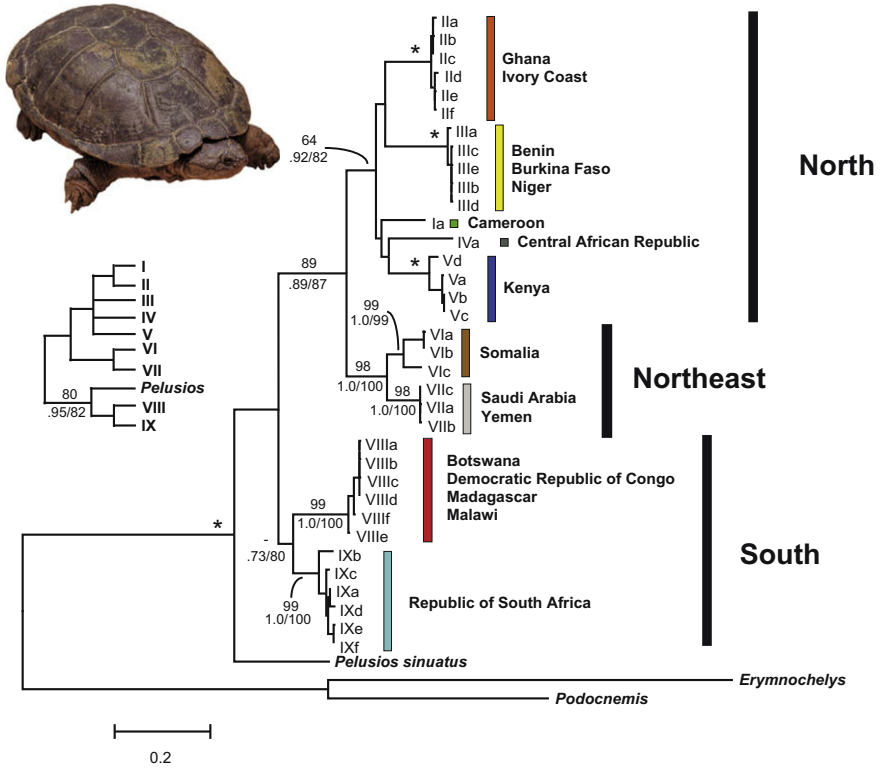

Fig. 2. Bayesian inference tree for mitochondrial haplotypes of *Pelomedusa subrufa*, including sequences of *Pelusios sinuatus*, *Podocnemis expansa* and *Erymnochelys madagascariensis* (partitioned analysis based on the maximum partition scheme with seven partitions). Each individual haplotype is indicated by a different letter following the Roman numeral of the respective clade. Geographical origins on the right. Inset: Simplified gross topology as obtained under ML, MP and BA based on unpartitioned and by-gene partition schemes (note the paraphyly of *Pelomedusa*). Support values are ML bootstrap values (top) and Bayesian posterior probabilities/MP bootstrap values (bottom). Values not shown for nodes with <0.95 posterior probabilities and <70% bootstrap support, and for nodes within clades I–IX. Branches with maximum support values asterisked. Colour code as in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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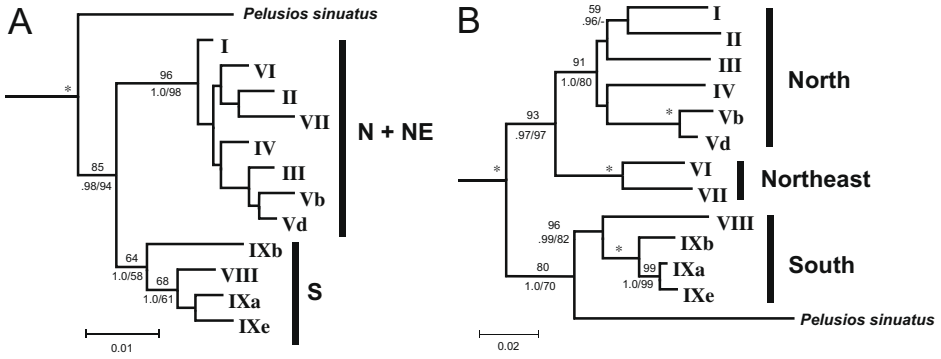

Fig. 3. Bayesian inference topologies obtained for (A) nuclear and (B) total evidence data sets of *Pelomedusa subrufa* haplotypes and *Pelusios sinuatus*. Outgroups (*Erymnochelys madagascariensis*, *Peltocephalus dumerilianus*, *Podocnemis expansa*) removed for clarity. Analyses based on the favoured partition schemes (maximum partitions for the nuclear data sets, by-gene partitions for the total evidence data set). Mitochondrial haplotypes and their geographical origins indicated on the right. The data sets used for analysis had no missing sequences. Support values are ML bootstrap values (top) and Bayesian posterior probabilities/MP bootstrap values (bottom). Branches with maximum support values asterisked.

the third major mitochondrial clade from the southern part of the range.

The total evidence approach resulted in a branching pattern resembling the mitochondrial tree, with the major exception that *P. subrufa* was paraphyletic with respect to *Pelusios* (Fig. 3B) in all analyses, including BA with the favoured partition scheme (by-gene). However, all of the three major inclusive clades of *Pelomedusa* received now high support values.

3.3. Network analyses of mitochondrial and nuclear sequences

In the parsimony network analyses of the 35 mitochondrial haplotypes, all clades revealed by phylogenetic analyses corresponded to unconnected haplotype clusters, either when the 90–95% thresholds were applied or when the connection limit was set to 50 steps (Fig. 4). In addition, haplotype VIc remained unconnected to haplotypes VIa and VIb (all three haplotypes

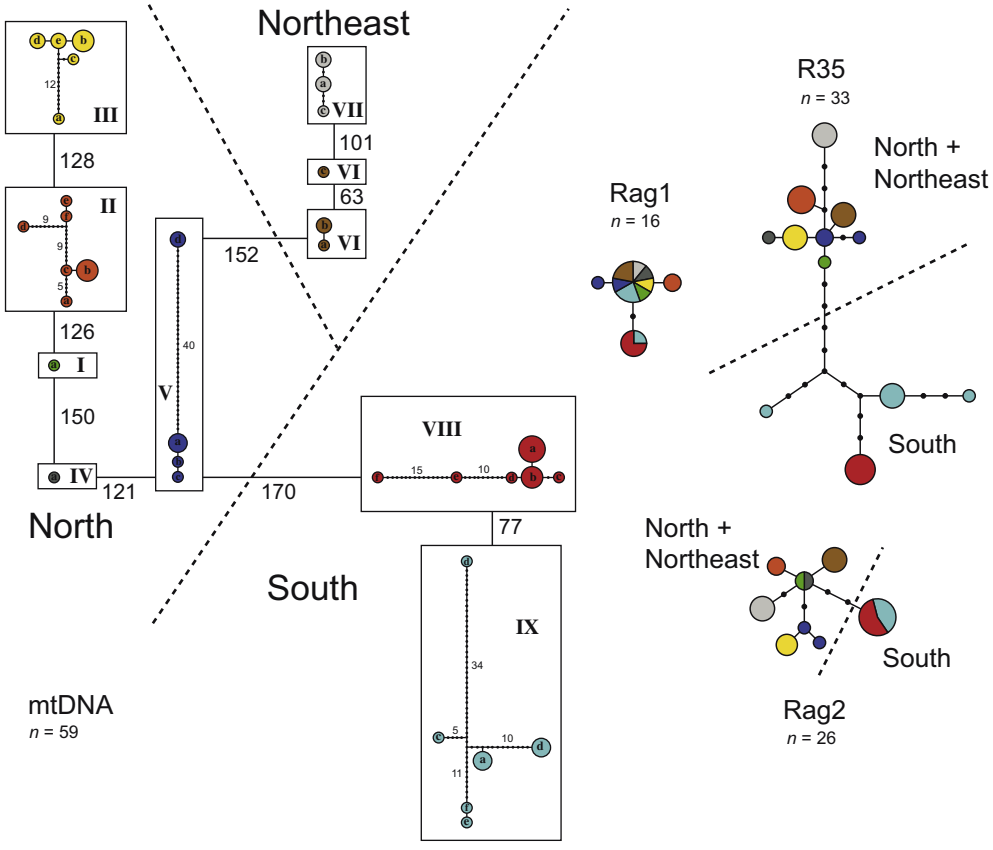

Fig. 4. Parsimony networks for mitochondrial (left) and nuclear (right) haplotypes of *Pelomedusa subrufa* based on alignments of 1503 bp of mtDNA, 593 bp of the Rag1 and 644 bp of the Rag2 genes, and 700 bp of intron 1 of the nuclear R35 gene (*n* = number of individuals; nuclear sequences were not heterozygous). Colour code as in Figs. 1 and 2. Circle size reflects haplotype frequency; missing haplotypes are represented by small solid circles. Each line connecting haplotypes corresponds to one mutational step. Mitochondrial haplotypes or haplotype clusters shown in boxes remain unconnected under the 90–95% thresholds or when the maximum connection limit is set to 50 steps; numbers near lines linking clusters indicate minimum fixed differences to the most similar haplotype or cluster. Numbers inside boxes are mutation steps between individual haplotypes or from one haplotype to the next node haplotype. Connection limit for nuclear haplotypes 95%. Colour of slices indicates percentages corresponding to respective mitochondrial haplotypes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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from Somalia). Haplotypes VIa, VIb and VIc were placed with high support in the same clade by all phylogenetic analyses (Fig. 2). When a cluster in the network (Fig. 4) was represented by more than one haplotype, the maximum number of mutational steps within each cluster ranged from 1 (between the two Somali haplotypes VIa and VIb) to 48 (within the cluster of six South African haplotypes IXa–IXf). Fixed differences between individual clusters ranged from 63 between the two Somali clusters to 170 between cluster V from Kenya and cluster VIII from Botswana, the Democratic Republic of Congo, Madagascar, and Malawi.

Network analyses of nuclear sequences revealed a similar general pattern, but a distinctly lower level of differentiation compared to the mitochondrial network, suggestive of slower evolutionary rates and incomplete sorting. For the Rag1 fragment, four distinct haplotypes were identified; for the Rag2 fragment, eight haplotypes; and for the R35 fragment, 12 haplotypes. Within Rag1 haplotypes a maximum of three mutational steps were observed; within Rag2 haplotypes, a maximum of six steps; and within R35 haplotypes, a maximum of 16 steps. All networks suggested an incipient or complete differentiation of the haplotypes from the southern part of the range (Fig. 4). Compared to the Rag1 and Rag2 networks, the best resolution was obtained from the R35 sequences, where every mitochondrial clade corresponded to at least one distinct nuclear haplotype.

3.4. Impact of isolation-by-distance on mitochondrial haplotypes

The Mantel tests revealed a significant correlation both between genetic and geographical distances ($Z = 19098.7110$, $r = 0.6716$, $p = 0.0005$) and between genetic distance and phylogenetic association ($Z = 3.7510$, $r = 0.8716$, $p = 0.002$) of the mitochondrial haplotypes. This pattern was also supported by the partial Mantel tests calculating the partial correlation coefficients for genetic similarity as a function of geographical distance while controlling for the effect of ancestry ($r = 0.3149$, $p = 0.0461$), or for genetic similarity as a function of ancestry while controlling for the effect of geography ($r = 0.7783$, $p = 0.0019$). The higher correlation coefficients of genetic distances and ancestry indicate that the deep genetic divergence within *P. subrufa* was shaped mainly by long-term historical processes, but was also impacted by geography. This finding is in accord with the clustering of geographically neighbouring clades in three major inclusive groups.

4. Discussion

4.1. Genealogical lineages in Pelomedusa do not agree with traditionally defined subspecies

P. subrufa, as currently understood, is a widely distributed terrapin species occurring in many different habitats ranging from temperate fynbos and grassland of South Africa to subtropical savannahs and semi-deserts of eastern central and western Africa. Compared to other aquatic African chelonians (*Pelusios*, *Cycloderma*, *Cyclanorbis*, *Trionyx*), the helmeted terrapin is the most terrestrially adapted species, with the ability to move great distances overland and to survive even in very small temporary water bodies. It is a medium-sized species of typically 20–30 cm shell length with an overall brownish colouration and inconspicuous appearance (Boycott and Bourquin, 2008). Based on differences in plastral scutation and colouration, two or three subspecies were recognized for many years, but Gasperetti et al. (1993) have shown that the allegedly diagnostic characters are not consistent throughout the range. Since then, the species has been treated as monotypic (e.g., Boycott and Bourquin, 2008; Ernst et al., 2000; Fritz and Havaš, 2007).

Our study provides evidence for strikingly deep genetic differentiation within *P. subrufa*. Nine well-supported mitochondrial clades in three more inclusive phylogenetic units were identified. The separation of a southern from a northern + northeastern major group was concordantly suggested also by the phylogenetic analysis of the nuclear Rag1, Rag2 and R35 genes (Fig. 3). In a haplotype network analysis, separately carried out for each of the nuclear genes, the southern vs. northern/northeastern separation was supported by each of the genes, and the most variable of them (R35) analyzed for 33 samples furthermore showed no haplotype sharing among those specimens that had been assigned to the nine lineages by mtDNA analysis, thus largely supporting the hypothesis of genetic isolation between these. The geographical distribution of none of these lineages matches the ranges of the formerly recognized subspecies, confirming that the described races do not represent evolutionary lineages. *Pelomedusa subrufa subrufa* (Lacépède, 1788) was thought to occur from Ghana east to Somalia, and southward to the Cape and in Madagascar, while terrapins from the northern portion of the range (from Senegal eastwards to Ethiopia) were assigned to *P. subrufa olivacea* (Schweigger, 1812). Some authors (Bour, 1986; Branch, 1988) recognized *P. subrufa nigra* Gray, 1863 from the southeast of South Africa as a third subspecies (Boycott and Bourquin, 2008; Ernst et al., 2000; Fritz and Havaš, 2007; Gasperetti et al., 1993). Although our sampling is somewhat patchy, the clades revealed in the present study generally have more restricted ranges than the traditionally recognized subspecies.

4.2. The relationships between Pelusios and Pelomedusa require further research

An unexpected finding of our analyses was the mitochondrial paraphyly of *P. subrufa* with respect to *Pelusios* in some phylogenetic analyses (Figs. 2 and 3). The two genera *Pelomedusa* and *Pelusios* are morphologically well-defined. While *Pelusios* species are generally characterized, among other features, by a plastral hinge, *Pelomedusa* has a rigid plastron of completely different shape (Ernst et al., 2000). Early Miocene *Pelomedusa* fossils and approximately contemporary *Pelusios* remains suggest that both genera diverged in, or before, the lower Miocene (*Pelomedusa senutpickfordina*, Namibia, about 19 million years [Ma]; *Pelomedusa* sp., Namibia, about 17 Ma; *Pelusios* sp., Uganda, 19–20 Ma; *Pelusios rusingae*, Kenya, about 18 Ma; de Lapparent de Broin, 2000, 2003, 2008; Williams, 1954).

Since all tree-building methods revealed a well-supported monophyletic *Pelomedusa* for the nuclear data set and the same was true (albeit with low support) for the partitioned BA of the mitochondrial data set, we hypothesize that the mitochondrial paraphyly of *Pelomedusa* in some analyses is due to saturation, causing phylogenetic noise. This view is corroborated by plotting transitions and transversions against corrected pairwise divergences, which indicates mutational saturation mainly at the third codon positions. Yet, explorative unpartitioned analyses of cyt *b* and ND4 sequences placed *P. sinuatus* as sister to the two southern *Pelomedusa* lineages, also when then the third codon position was excluded (not shown). Alternatively to saturation, the observed paraphyly could be the result of ancient hybridization with introgression, as recently suggested for North American and Asian terrapins (*Actinemys*, *Emydoidea*: Spinks and Shaffer, 2009, but see Wiens et al., 2010; *Cuora*: Spinks and Shaffer, 2007). It is obvious that future efforts should be undertaken to analyze the phylogenetic relationships of all *Pelusios* species and *Pelomedusa* to gain a better understanding of the relation-

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ships and of the evolutionary history of both pelomedusid genera.

4.3. Vicariance of the Arabian clade and possible introduction of Pelomedusa to Madagascar

While the pronounced mitochondrial divergence of the Arabian clade VII and the Somali clade VI (Fig. 2) is suggestive of a vicariance caused by the Early to Late Pliocene submersion of the Afar-Yemen land bridge (Bosworth et al., 2005; Redfield et al., 2003), the shallow mitochondrial differentiation of continental African and Malagasy helmeted terrapins (clade VIII, Fig. 2) indicates a fundamentally different pattern. Eleven samples corresponding to three distinct haplotypes were available from Madagascar, whereas continental haplotypes of clade VIII were represented by only three samples from Botswana, the Democratic Republic of Congo and Malawi, respectively. Each of the continental samples contained a distinct haplotype (Fig. 4). Despite this small sample size, some conclusions may be drawn. The three Malagasy haplotypes (VIIIa, VIIIb, VIIId) differ from one another by only one mutation step, and from the most similar continental African haplotype VIIIc by two steps. By contrast, a maximum of 28 mutational steps occurs between continental haplotypes of clade VIII. Moreover, according to network analyses, the Malagasy haplotype VIIIb is ancestral both to the two other Malagasy haplotypes (VIIIa, VIIId) and to all continental haplotypes of clade VIII, suggesting that the Malagasy haplotypes are part of the variation occurring on the African continent. This is supported also by the phylogenetic placement of the Malagasy haplotypes that are nested within continental haplotypes (Fig. 2). In the nuclear sequences, no differences occur among clade VIII terrapins. This situation indicates *P. subrufa* recently invaded Madagascar, and probably was introduced by man. A denser sampling is necessary to elucidate the possible impact of the African rift valley on the genetic structure of continental helmeted terrapins.

4.4. The African helmeted terrapin might represent a species complex

In previous investigations, the degree of sequence divergence of the mitochondrial cyt *b* gene has often been used as a yardstick to assess the species status of chelonians (e.g., Fritz et al., 2005, 2008; McGaugh et al., 2008; Praschag et al., 2007a,b, 2009; Spinks et al., 2004). Although we are convinced that species delineations should not be based on rigid thresholds of sequence divergence alone, we argue that considerable genetic divergences are a strong argument for specific distinctness (see also Vieites et al., 2009). Spinks et al. (2004) reported maximum uncorrected *p* distances of 5.0-10.7% between species of the genera *Cuora*, *Geoemyda*, *Heosemys*, *Mauremys*, *Pangshura*, and *Rhinoclemmys* (Testudines: Geoemydidae), and Fritz et al. (2008) found species of another geoemydid genus, *Cyclemys*, different by average values of 2.8–11.7% (one species with introgressed mitochondrial genome not considered). Also in this range fall values of *Batagur* species (Geoemydidae; Praschag et al., 2007a, 2009). Average values for species of the Western Palaearctic tortoise genus *Testudo* (Testudinidae) vary between 6.8% and 12.6% (Fritz et al., 2005), and those for South and Southeast Asian softshell turtles of the genus *Nilssonia*, between 4.9% and 10.1% (Praschag et al., 2007b). Species of the North American softshell turtle genus *Apalone* differ by 6.8–13.9% (McGaugh et al., 2008). Average uncorrected *p* distances between clades of *Pelomedusa* (8.6–18.3%; Table 3) therefore match and, in part, exceed the levels of genetic divergence occurring between all of these species. This suggests that more than one species of African helmeted terrapin could be involved.

Although species delimitation has recently been flagged as a Renaissance issue in systematics (Sites and Marshall, 2003), this topic is still plagued by competing species concepts (e.g., see the reviews or papers in Coyne and Orr, 2004; de Queiroz 2007; Ereshefsky, 1992; Wheeler and Meier, 2000; Wilson, 1999). Generally, we adhere to the Evolutionary Species Concept that emphasizes that lineages are recognizable as species when they have a unique evolutionary role, tendency, and historical fate (Simpson, 1951; Wiley, 1978). In agreement with de Queiroz (1999, 2007), we understand distinct species furthermore as separately evolving metapopulation segments. After a decades-long fierce debate on what species are, the insight that species delimitation has long been confused with the issue of their conceptualization (de Queiroz, 1999, 2007) constituted a major step forward. Nonetheless, until today dissent remains when evolutionary lineages are distinct and independent enough to considered as species, and which criteria should be used for their delimitation in practice (see Dayrat, 2005).

One delimitation method, exclusively relying on molecular markers, is the Genealogic Concordance Method of Phylogenetic Species Recognition, proposed by Avise and Ball (1990). This method seeks for congruent differentiation patterns of several unlinked genetic loci, and concludes that such concordance qualifies for recognition for distinct species. The rationale behind is that only in distinct evolutionary lineages will the coalescent histories of the different markers agree. Applying this method to our data set would lead to the conclusion that at least the southern vs. the northern + northeastern lineages represent two distinct species, because, based on independent markers, they concordantly represent well-supported distinct clades in phylogenetic analyses and do not share haplotypes for the mitochon-

Table 3
Uncorrected *p* distances of the cyt *b*-coding part (664 bp) of the mtDNA fragment between and within haplotypes of the nine mitochondrial clades of *Pelomedusa subrufa*. Below diagonal, average percentages; above diagonal, standard error (500 bootstrap replicates) between clades. On diagonal, within clade variation (mean ± SE) in bold. *n* = number of haplotypes.

	n	I	II	III	IV	V	VI	VII	VIII	IX
I	1	—	1.10	1.19	1.16	1.14	1.22	1.23	1.40	1.37
II	6	11.12	**0.68 ± 0.20**	1.21	1.23	1.24	1.30	1.27	1.38	1.35
III	5	10.66	12.38	**0.44 ± 0.16**	1.17	1.30	1.31	1.33	1.47	1.37
IV	1	10.24	11.85	10.37	—	1.13	1.29	1.29	1.44	1.34
V	4	9.90	13.39	12.52	10.44	**0***	1.28	1.31	1.44	1.37
VI	3	13.93	14.58	15.25	13.88	13.90	**4.00 ± 0.62**	1.00	1.35	1.37
VII	3	13.66	13.40	14.41	13.30	14.39	8.64	**0.50 ± 0.22**	1.36	1.36
VIII	6	17.62	17.71	17.04	17.48	18.29	16.51	15.39	**1.16 ± 0.28**	1.03
IX	6	17.34	17.11	16.08	16.64	17.29	17.24	16.92	10.34	**1.12 ± 0.26**

* mtDNA differs in fragment containing the partial ND4 gene.

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drial genes, Rag2 and R35 (Figs. 2–4). However, this argument could, for instance, be also extended to lineage III which, too, shows no haplotype sharing in two of the nuclear markers with any other lineage (Fig. 4: Rag2, R35). It is obvious that this rationale will eventually depend on the used marker systems and sample sizes. Slowly evolving markers are expected to yield less information than rapidly evolving ones (cf. the patterns of mitochondrial and the three nuclear markers in Fig. 4), without, however, necessarily corroborating the null hypothesis that all lineages are conspecific. Moreover, with increasing sample size the probability to detect shared rare alleles will rise. For the time being, we conclude that our sampling, especially of the nuclear markers, is too restricted for allowing definite conclusions, although there is some evidence that *Pelomedusa* consists of more than one species.

In several groups of organisms claims for possible taxonomic inflation, an exaggerated recognition of species-level taxa, have recently been voiced (Isaac et al., 2004; but see Padial and De la Riva, 2006; Sangster, 2009). This also extends to chelonians, where several species and subspecies turned out to be ecotypes or geographical variants not representing independent evolutionary lineages (e.g., Daniels et al., 2010; Fritz et al., 2005, 2007). We therefore endorse proposals of approaching chelonian taxonomy carefully (Turtle Taxonomy Working Group, 2007) and to restrict taxonomic changes to cases supported by strong and unambiguous evidence. Within this framework, and given the current evidence, we cannot reject that *P. subrufa* represents a widespread species with several deep, but conspecific lineages, and that admixture between these lineages may occur in contact zones. Hence, we argue that a taxonomic revision of the African helmeted terrapin should be carried out in an integrative taxonomy approach (Dayrat, 2005), i.e., making use of a maximum number of lines of evidence, including morphology, ecology, and behaviour. Instead trying to infer the number of distinct species in *Pelomedusa* based on what we consider an insufficient data set, we follow the approach of Vieites et al. (2009) and treat the mitochondrial lineages I–IX of *Pelomedusa* as unconfirmed candidate species that require further study. In particular, we identify the following priorities for future research that are expected to contribute to a better understanding of the genetic patterns within *Pelomedusa*:

(1) *Study of contact zones*: The most conclusive test for the possible existence of multiple species in *Pelomedusa* is expected from the study of contact zones of distinct genealogical lineages. Sympatric occurrence of terrapins representing distinct lineages without hybridization would constitute an unequivocal argument for recognizing distinct species. Should hybridization be detected, it will be important to investigate whether geographically extensive admixture occurs, or whether gene flow is restricted to narrow hybrid zones, without wide-reaching introgression; the latter situation being in better accordance with the recognition of more than one species.
(2) *Extending the sampling*: Also beyond possible contact zones, the sampling should be increased by expanding the geographical coverage and the number of sampled terrapins. Ideally, high-quality samples of 10–20 individuals per site should be obtained, to allow for nuclear DNA sequencing and study of gene flow using microsatellite loci. This will improve the understanding of the pattern of genetic variation within each candidate species, and could lead to the discovery of additional deep genealogical lineages of *Pelomedusa*.
(3) *Morphological analyses*: A reassessment of morphological variation within *Pelomedusa* should be undertaken in order to find out whether morphological differentiation parallels the various genealogical lineages. Congruence of morphology and molecular differentiation would favour the recognition of more than one species. The same is true for clear behavioural differences.
(4) *Reappraisal of synonyms of P. subrufa*: Currently, 14 species-group names are nomenclaturally available and considered junior synonyms of *P. subrufa* (Fritz and Havaš, 2007). Recognizing any additional species within *Pelomedusa* would necessitate a detailed revision of all of these historical taxa, including a morphological and molecular comparison of their name-bearing type specimens or topotypic material with the newly identified species.

On the basis of such additional efforts, a re-examination of the conservation status of each candidate species will be an important further step. The current IUCN threat assessment of *P. subrufa* as "Least Concern" is based on the assumption of a single species inhabiting the whole range. If some of the geographically more limited candidate species might be accepted as distinct species in the future, their conservational situation could differ significantly from what is now considered to be the most widely spread sub-Saharan chelonian species by the current IUCN threat assessment (IUCN, 2009).

Note added in Proof

Simultaneously with our work, Wong et al. (submitted for publication) have studied *Pelomedusa* phylogeography based on sequences of *cytb* and R35 plus three additional mt genes (12S, 16S and COI). These authors find the same major geographic clades (named North, Northeast and South by us) and provide additional information on the occurrence of lineages II (Togo) and VIII (Namibia), and reveal probably a further lineage related to II and III in Nigeria and Senegal. The fact that independent research groups using partly different samples and genes obtained similar results reinforces the major conclusions of both studies.

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Appendix 1

Pelomedusa subrufa samples and outgroups used in the present study.

Taxon	Locality/Origin	mtDNA haplotype	Accession number					Voucher[a]
			cyt b	ND4	Rag1	Rag2	R35	
Pelomedusa subrufa	Cameroon: Maroua (between Mora and Kaélé); 10.5960°N 14.3240°E	Ia	FN645215	FN645274	FN645333	FN645350	FN645377	MTD T 5183
Pelomedusa subrufa	Ivory Coast: Bouaké; 7.6870°N 5.0320°W	IIa	FN645216	FN645275	—	—	—	MTD T 5134
Pelomedusa subrufa	Ghana: Shai Hills Production Preserve, pond 0.5 km W of camp; 5.8803°N 0.0378°W	IIb	FN645217	FN645276	—	FN645351	FN645378	MVZ 245229
Pelomedusa subrufa	Ghana: Shai Hills Production Preserve, pond 0.5 km W of camp; 5.8803°N 0.0378°W	IIb	FN645218	FN645277	FN645334	—	FN645379	MVZ 245230
Pelomedusa subrufa	Ghana: Shai Hills Production Preserve, pond 0.5 km W of camp; 5.8803°N 0.0378°W	IIb	FN645219	FN645278	—	—	—	MVZ 245429
Pelomedusa subrufa	—	IIb	FN645220	FN645279	—	—	—	MVZ 230517
Pelomedusa subrufa	Ghana: approx. 10 mi N of Accra; 5.6519°N 0.1694°W	IIc	FN645221	FN645280	—	—	FN645380	MVZ 245428
Pelomedusa subrufa	GenBank	IId	AF039066	AF039066	—	—	—	—
Pelomedusa subrufa	Ghana: approx. 10 mi N of Accra; 5.6519°N 0.1694°W	IIe	FN645222	FN645281	—	—	FN645381	MVZ 245226
Pelomedusa subrufa	Ghana: Shai Hills Production Preserve, dam 1 km E of camp; 5.8746°N 0.05258°W	IIf	FN645223	FN645282	FN645335	FN645352	FN645382	MVZ 245228
Pelomedusa subrufa	Burkina Faso: Bobo-Dioulasso; 11.1780°N 4.2900°W	IIIa	FN645224	FN645283	FN645336	FN645353	FN645383	MTD T 5139
Pelomedusa subrufa	Niger: Guesselbodi, 30 km SE Niamey on road to Dosso; 13.4132°N 2.3535°E	IIIb	FN645225	FN645284	—	—	FN645384	MVZ 238887
Pelomedusa subrufa	Niger: Moli Haoussa, 15 km NW (by Tamou Rd.) Tapoa; 12.5320°N 2.3338°E	IIIb	FN645226	FN645285	—	—	FN645385	MVZ 238880
Pelomedusa subrufa	Niger: Moli Haoussa, 15 km NW (by Tamou Rd.) Tapoa; 12.5320°N 2.3338°E	IIIb	FN645227	FN645286	—	FN645354	FN645386	MVZ 238881
Pelomedusa subrufa	Niger: Moli Haoussa, 15 km NW (by Tamou Rd.) Tapoa; 12.5320°N 2.3338°E	IIIb	FN645228	FN645287	—	—	—	MVZ 238879
Pelomedusa subrufa	Niger: Moli Haoussa, 15 km NW (by Tamou Rd.) Tapoa; 12.5320°N 2.3338°E	IIIc	FN645229	FN645288	—	—	—	MVZ 238882
Pelomedusa subrufa	Niger: Tafadek, ca. 40 km (by air) N Agadez; 17.3868°N 7.9563°E	IIId	FN645230	FN645289	—	—	—	MVZ 238885
Pelomedusa subrufa	Niger: Tafadek, ca. 40 km (by air) N Agadez; 17.3868°N 7.9563°E	IIId	FN645231	FN645290	—	FN645355	—	MVZ 238883
Pelomedusa subrufa	Benin: Pendjari National Park, near Batia; 10.9126°N 1.4852°E	IIIe	FN645232	FN645291	—	—	—	MTD T 5601
Pelomedusa subrufa	Niger: 57 km SSW (by Niamey Rd.) Dogondoutchi; 13.2372°N 3.8160°E	IIIe	FN645233	FN645292	—	—	—	MVZ 238878
Pelomedusa subrufa	Central African Republic: Zémio; 6.6040°N 18.2510°E	IVa	FN645234	FN645293	FN645337	FN645356	FN645387	MTD T 4243
Pelomedusa subrufa	Kenya: South Horr; 2.1020°N 36.7885°E	Va	FN645235	FN645294	—	—	—	MTD T 5521
Pelomedusa subrufa	Kenya: South Horr; 2.1020°N	Va	FN645236	FN645295	—	—	FN645388	MTD T 5522

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Appendix 1 (*continued*)

Taxon	Locality/Origin	mtDNA haplotype	Accession number					Voucher[a]
	36.7885°E							
Pelomedusa subrufa	Kenya: South Horr; 2.1020°N 36.7885°E	Va	FN645237	FN645296	—	—	—	MTD T 5523
Pelomedusa subrufa	Kenya: South Horr; 2.1020°N 36.7885°E	Vb	FN645238	FN645297	FN645338	FN645357	FN645389	MTD T 5524
Pelomedusa subrufa	Kenya: South Horr; 2.1020°N 36.7885°E	Vc	FN645239	FN645298	—	—	—	MTD T 5525
Pelomedusa subrufa	Kenya: Kakamega; 0.2840°N 34.7420°E	Vd	FN645240	FN645299	FN645339	FN645358	FN645390	MTD T 5526
Pelomedusa subrufa	Kenya: Kakamega; 0.2840°N 34.7420°E	Vd	FN645241	FN645300	—	—	—	MTD T 5527
Pelomedusa subrufa	Somalia: Awdal Region: 4 km N (by road) Borama; 9.9705°N 43.1460°E	VIa	FN645242	FN645301	FN645340	FN645359	FN645391	MVZ 241329
Pelomedusa subrufa	Somalia: Awdal Region: 4 km N (by road) Borama; 9.9705°N 43.1460°E	VIb	FN645243	FN645302	FN645341	FN645360	FN645392	MVZ 241330
Pelomedusa subrufa	Somalia: Awdal Region: 4 km N (by road) Borama; 9.9705°N 43.1460°E	VIb	FN645244	FN645303	—	FN645361	FN645393	MVZ 241331
Pelomedusa subrufa	Somalia: Awdal Region: Rugi, 30 km NE (by road) Borama; 9.9698°N 43.4325°E	VIc	FN645245	FN645304	—	FN645362	FN645394	MVZ 241332
Pelomedusa subrufa	Saudi Arabia: close to Yemeni border; 16.9296°N 42.9046°E	VIIa	FN645246	FN645305	—	FN645363	FN645395	MTD T 592
Pelomedusa subrufa	Saudi Arabia: close to Yemeni border; 16.9296°N 42.9046°E	VIIa	FN645247	FN645306	—	FN645364	FN645396	MTD T 593
Pelomedusa subrufa	Saudi Arabia: close to Yemeni border; 16.9296°N 42.9046°E	VIIb	FN645248	FN645307	—	FN645365	FN645397	MTD T 594
Pelomedusa subrufa	Saudi Arabia: Wadi Turaba near Tai'if; 20.7040°N 41.3810°E (questionable locality)	VIIb	FN645249	FN645308	FN645342	—	FN645398	MTD T 595
Pelomedusa subrufa	Yemen: Gwol Madram, Wadi Tuban; 13.3318°N 44.6942°E	VIIc	FN645250	FN645309	—	FN645366	—	MVZ 236628
Pelomedusa subrufa	Madagascar: Analaiva (at main road east of Morondava); 20.3333°S 44.4999°E	VIIIa	FN645251	FN645310	—	—	—	MTD T 5224
Pelomedusa subrufa	Madagascar: between Ambondro and Tsiombe; 25.3833°S 45.6707°E	VIIIa	FN645252	FN645311	—	—	—	MTD T 5060[*]
Pelomedusa subrufa	Madagascar: between Ambondro and Tsiombe; 25.3833°S 45.7095°E	VIIIa	FN645253	FN645312	—	—	—	MTD T 5061
Pelomedusa subrufa	Madagascar: just north of Andranomena; 20.1705°S 44.4370°E	VIIIa	FN645254	FN645313	—	—	—	MTD T 5227
Pelomedusa subrufa	Madagascar: just south of Andranomena; 20.1915°S 44.4240°E	VIIIa	FN645255	FN645314	—	—	—	MTD T 5228
Pelomedusa subrufa	Madagascar: Kirindy creek near Alan Amborompotsy; 20.0475°S 44.6485°E	VIIIa	FN645256	FN645315	—	—	—	MTD T 5225
Pelomedusa subrufa	Madagascar: Ifaty near Toliara; 23.1500°S 43.6167°E	VIIIb	FN645257	FN645316	FN645343	FN645367	FN645399	MTD T 6018
Pelomedusa subrufa	Madagascar: Ifaty near Toliara; 23.1500°S 43.6167°E	VIIIb	FN645258	FN645317	—	FN645368	FN645400	MTD T 6019
Pelomedusa subrufa	Madagascar: Ifaty near Toliara; 23.1500°S 43.6167°E	VIIIb	FN645259	FN645318	—	FN645369	FN645401	MTD T 6020

(*continued on next page*)

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Appendix 1 (*continued*)

Taxon	Locality/Origin	mtDNA haplotype	Accession number					Voucher[a]
Pelomedusa subrufa	Madagascar: Kirindy creek near Alan Amborompotsy; 20.0475°S 44.6485°E	VIIIb	FN645260	FN645319	—	—	—	MTD T 5226
Pelomedusa subrufa	Botswana: Mashatu Game Reserve; 22.2630°S 28.6840°E	VIIIc	FN645261	FN645320	—	—	—	MTD T 5562
Pelomedusa subrufa	Madagascar: between Ambondro and Tsiombe; 25.2598°S 45.6352°E	VIIId	FN645262	FN645321	—	—	FN645402	MTD T 5062
Pelomedusa subrufa	Malawi: Mt Mulanje; 15.9230°S 35.7150°E	VIIIe	FN645263	FN645322	FN645344	FN645370	FN645403	MTD T 5221
Pelomedusa subrufa	Democratic Republic of Congo: Haut Katanga Province: Kalakundi Mine; 11.2080°S 27°3820°E	VIIIf	FN645264	FN645323	FN645345	FN645371	FN645404	MTD T 5220
Pelomedusa subrufa	South Africa: Port Elizabeth; 33.8510°S 25.5810°E	IXa	FN645265	FN645324	FN645346	FN645372	—	MTD T 5218
Pelomedusa subrufa	South Africa: Port Elizabeth; 33.8510°S 25.5810°E	IXa	FN645266	FN645325	—	—	FN645405	MTD T 5219
Pelomedusa subrufa	South Africa: Swellendam District; 33.9999°S 20.3680°E	IXb	FN645267	FN645326	FN645347	FN645373	FN645406	MTD T 5484
Pelomedusa subrufa	South Africa: Sterkstroom; 31.5733°S 26.5006°E	IXc	FN645268	FN645327	—	FN645374	FN645407	MTD T 5505
Pelomedusa subrufa	South Africa: Ndumo; 26.8764°S 32.0781°E	IXd	FN645269	FN645328	—	—	FN645408	MTD T 5508
Pelomedusa subrufa	South Africa: Ndumo; 26.8764°S 32.0781°E	IXd	FN645270	FN645329	—	—	—	MTD T 5509
Pelomedusa subrufa	South Africa: Bothnithong; 27.0167°S 23.7514°E	IXe	FN645271	FN645330	FN645348	FN645375	FN645409	MTD T 5510
Pelomedusa subrufa	South Africa: Gauteng Area; 25.9140°S 28.1310°E	IXf	FN645272	FN645331	—	—	—	MTD T 5563
Pelusios sinuatus	South Africa: Phalaborwa; 24.0360°S 31.1931°E	—	FN645273	FN645332	FN645349	FN645376	FN645410	MTD T 5506
Erymnochelys madagascariensis	GenBank	—	AM943834	FM165619	AY988099	AM943835	AM943849	—
Peltocephalus dumerilianus	GenBank	—	AM943833	FM165622	AY988101	AM943837	AM943848	—
Podocnemis expansa	GenBank	—	AM943830	FM165620	AY988100	AM943839	AM943843	—

[a] MTD T = Museum of Zoology Dresden, Tissue Collection; MVZ = Museum of Vertebrate Zoology, Berkeley.
[*] Complete voucher specimen in Zoologische Staatssammlung München, ZSM 177/2004.

Appendix 2. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.03.019.

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