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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