

# Weak divergence among African, Malagasy and Seychellois hinged terrapins (*Pelusios castanoides*, *P. subniger*) and evidence for human-mediated oversea dispersal

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**Abstract** Using phylogenetic and haplotype network analyses of 2036 bp of mitochondrial DNA, we compare samples of the two hinged terrapin species *Pelusios castanoides* and *P. subniger* from continental Africa, Madagascar and the Seychelles to infer their biogeography. Owing to the long independent history of Madagascar and the Seychelles, the populations from those islands should be deeply divergent from their African conspecifics. Seychellois populations of the two species are currently recognized as Critically Endangered endemic subspecies. However, even though we found within *P. subniger* evidence for a cryptic species from the

Democratic Republic of the Congo, all other samples assigned to this species were undifferentiated. This suggests that Malagasy and Seychellois populations of *P. subniger* were introduced by humans and that the Seychellois subspecies *P. s. parietalis* is invalid. This has implications for current conservation strategies for the Critically Endangered Seychellois populations and suggests that measures should rather focus on endemic species. The situation of *P. castanoides* could be different. Samples from Madagascar and the Seychelles are weakly, but consistently, differentiated from continental African samples, and Malagasy and Seychellois samples are

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reciprocally monophyletic in maximum likelihood analyses. However, due to a lack of samples from central and northern Mozambique and Tanzania, we cannot exclude that identical continental haplotypes exist there.

**Keywords** Reptilia · Testudines · Pelomedusidae · Phylogeography · Africa · Madagascar · Seychelles

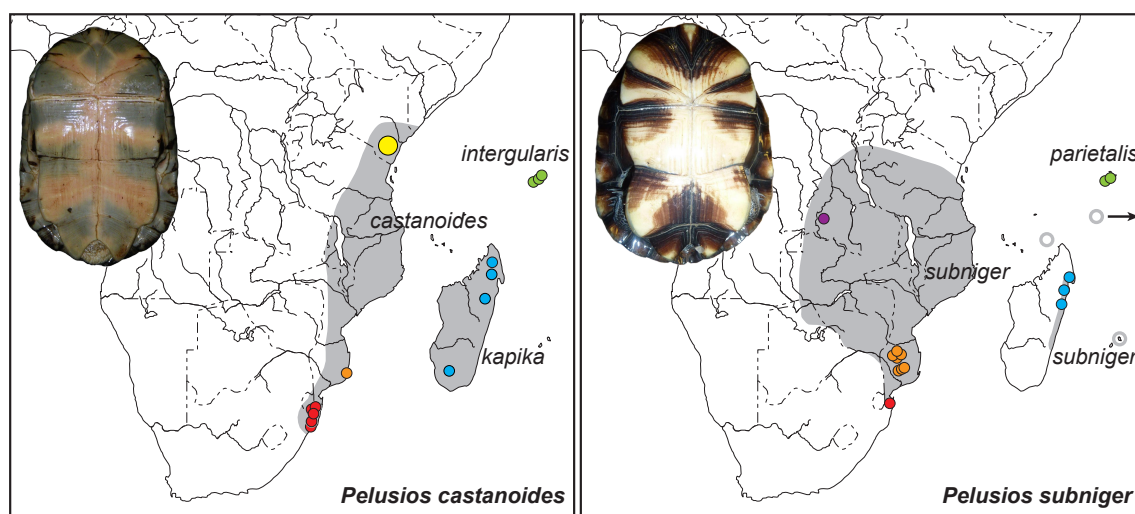
## Introduction

Hinged terrapins (*Pelusios* Wagler 1830) are a species-rich genus of side-necked turtles (Pleurodira: Pelomedusidae) widely distributed in freshwater habitats of sub-Saharan Africa, Madagascar and the Seychelles (Bour 1983; Ernst et al. 2000). These terrapins are characterized by a movable plastral forelobe, allowing the more or less complete closure of the anterior shell opening (Ernst et al. 2000). Among the 17 or 18 currently recognized species (Fritz and Havaš 2007; Fritz et al. 2011; van Dijk et al. 2011), *Pelusios castanoides* Hewitt 1931 and *P. subniger* (Bonnaterre 1789) have the most disjunct distribution ranges (Fig. 1), with populations occurring in continental East Africa, Madagascar and the Seychelles (Bour 1983; Iverson 1992; Ernst et al. 2000). In addition, records of introduced *P. subniger* exist for the islands of Diego Garcia (Chagos Archipelago), Grande Glorieuse and Mauritius (Bour 1983, 1984). Malagasy and Seychellois populations of *P. castanoides* have been described as the distinct subspecies *P. c. kapika* Bour 1978 and *P. c. interguarialis* Bour 1983, respectively. The Seychellois population of *P. subniger* has

also been described as a distinct subspecies (*P. s. parietalis* Bour 1983), whereas Malagasy *P. subniger* are thought to be taxonomically not distinct from their continental African conspecifics (Bour 1978, 1983). Besides *P. c. interguarialis* and *P. s. parietalis*, a third, possibly extinct, *Pelusios* species, *P. seychellensis* (Siebenrock 1906), has been recognized for the Seychelles, which is known from only three specimens collected in the late nineteenth century (Bour 1983, 1984; Bour and Gerlach 2008).

On Madagascar, *P. castanoides* is widespread throughout the island, whereas *P. subniger* occurs only in a narrow strip along the northeastern coast (Fig. 1). The two Seychellois taxa *P. c. interguarialis* and *P. s. parietalis* have highly restricted ranges of less than 11 ha for each species, which have decreased by more than 50% in recent years (Gerlach and Canning 2001). These subspecies were the only two reptiles of the Seychelles listed as Critically Endangered by the International Union for Conservation of Nature (IUCN 2007) and are protected by law. For several years, conservation measures have been implemented to protect the remaining populations (Gerlach 2008a, b; Silva et al. 2010).

From a biogeographic point of view, it should be expected that native terrapin populations in Madagascar and the Seychelles are genetically clearly distinct. The vast majority of the non-flying native terrestrial and freshwater faunas of Madagascar and the Seychelles are deeply divergent from their most closely related East African species owing to the long independent history of those islands (Harmon et al. 2008; Yoder and Nowak 2009; Daniels 2011; Rowson et al. 2011; Crottini et al. 2012; Samonds et al. 2012). Yet, three recent molecular investigations have suggested that the Malagasy and



**Fig. 1** Distribution ranges (shaded; Bour 1983, 1984; Iverson 1992) and sampling sites (coloured circles) for *Pelusios castanoides* and *P. subniger*; described subspecies for each species are indicated. Colour code of sites corresponds to Fig. 3. The large circle for *P. castanoides* represents an imprecise locality. Records of evidently introduced *P.*

*subniger* on Diego Garcia (Chagos Archipelago), Grande Glorieuse and Mauritius encircled in grey; it is unclear whether established populations exist or ever have existed on these islands (Bour 1983, 1984). Insets show plastral aspects of the two species

Seychellois populations of *P. castanoides* are only weakly differentiated and challenged their taxonomic distinctness (Silva et al. 2010; Fritz et al. 2011, 2012). However, these studies were based on limited sampling, which is why the authors deferred nomenclatural readjustments and refrained from biogeographic conclusions. Using the mitochondrial cytochrome *b* (cyt *b*) gene as a marker, Silva et al. (2010) studied many samples from all Seychelles islands with terrapin populations and found for each species just one haplotype. From outside the Seychelles, these authors had only two samples of *P. castanoides* from Madagascar available, which differed by four mutational steps from their Seychellois conspecifics (Silva et al. 2010 report five steps difference, but this is a typing error for four as is obvious from their GenBank sequences). Using three mitochondrial and nuclear DNA fragments (2054 bp mtDNA, 2025 bp nDNA), Fritz et al. (2011, 2012) studied one South African, one Kenyan, two Seychellois and three Malagasy samples of *P. castanoides*, and confirmed shallow divergence. In all three studies, the status of the island populations of *P. subniger* could not be addressed as only Malagasy specimens were available.

Based on an expanded sampling of terrapins from continental Africa (Democratic Republic of the Congo, Kenya, Mozambique, South Africa), Madagascar and the Seychelles (Fig. 1), we re-investigate in the present article the phylogeography of *P. castanoides* and examine for the first time the relationships of African, Malagasy and Seychellois *P. subniger*. For these purposes, we use sequence information of three mitochondrial genes (12S rRNA, ND4, cyt *b*) that were found in two previous studies (Fritz et al. 2011, 2012) to represent relatively rapidly evolving markers, while all studied samples of *P. castanoides* and *P. subniger* were virtually undifferentiated in three nuclear loci (coding: C-mos, Rag2; non-coding: R35). Therefore, we abstain from sequencing nuclear genes in the present study.

## Materials and methods

### Sampling

A total of 31 samples of *Pelusios castanoides* and 47 samples of *P. subniger* were examined (Fig. 1; Supplementary Table S1). One sample each of *P. castanoides* came from Kenya and Mozambique, six samples from South Africa (subspecies *castanoides*) and seven samples from Madagascar (subspecies *kapika*); samples from the Seychelles (subspecies *intergrularis*) included five from Cerf, three from La Digue, and eight from Mahé. Regarding *P. subniger*, 2 samples came from the Democratic Republic of the Congo, 19 from Madagascar, 8 from Mozambique and 4 from South Africa (subspecies *subniger*). The Seychellois subspecies *P. s. parietalis* was represented by 13 samples from Mahé and

1 sample from Cerf. Cytochrome *b* sequences of many of these Seychellois samples were published previously by Silva et al. (2010) and downloaded from GenBank (Supplementary Table S1). Sequences of the 12S rRNA, ND4 and cyt *b* genes of seven *P. castanoides* and three *P. subniger* had been previously used in the articles by Fritz et al. (2011, 2012). The remaining samples and DNA extractions are maintained at  $-80^{\circ}\text{C}$  in the tissue collection of the Museum of Zoology, Senckenberg Dresden.

### Laboratory procedures and data analyses

For the majority of samples, for which mtDNA sequence data were not available, sequences of the 12S rRNA, ND4 and cyt *b* genes were generated according to Fritz et al. (2011) and using the same polymerase chain reaction and sequencing primers as in Fritz et al. (2012). The resulting 12S rRNA sequences were 384 bp long and the cyt *b* sequences were 795 bp long. The mtDNA fragment containing the second half of the ND4 gene (668 bp) also embraced 189 bp of adjacent DNA, in part coding for tRNAs (see Results section for details). Owing to small sample size or low DNA quality, not all DNA fragments could be amplified and sequenced for all samples. Accession numbers of newly generated sequences and previously published sequences used in the present study are listed in Supplementary Table S1.

For phylogenetic analyses the three mtDNA fragments were concatenated, resulting in an alignment of 2036 bp. Homologous sequences of *Pelusios bechuanicus*, *P. upembae*, *P. williamsi* and *Pelomedusa* lineage I from Vargas-Ramírez et al. (2010) and Fritz et al. (2011, 2012) were included as outgroups (Supplementary Table S1), and aligned using BIOEDIT 7.0.5.3 (Hall 1999). *Pelusios bechuanicus* and *P. upembae* together are the sister group of *P. subniger*, while *P. williamsi* is the sister species of *P. castanoides* (Fritz et al. 2011, 2012). The genus *Pelomedusa*, comprising about ten deeply divergent lineages most probably corresponding to distinct species (Vargas-Ramírez et al. 2010; Wong et al. 2010), constitutes the sister group of *Pelusios* (Fritz et al. 2011). *Pelomedusa* lineage I (sensu Vargas-Ramírez et al. 2010) was used for tree rooting. Phylogenetic relationships were inferred with RAXML 7.2.6 (Stamatakis 2006) using raxmlGUI 1.1 (Silvestro and Michalak 2011) and MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). For both approaches, the alignment was partitioned by gene. For RAXML calculations, the GTR+G model was applied across all partitions. For calculations with MRBAYES, evolutionary models were determined using the Akaike information criterion of MODELTEST 2.3 (Posada and Crandall 1998), with the following results: 12S rRNA – GTR+G, ND4 – GTR+I, tRNA-His – HKY, non-annotated DNA between tRNA-His and tRNA-Leu – HKY (for identification of

this fragment, see also Fritz et al. 2011, 2012 and the Results section), tRNA-Leu – K80, and cyt *b* – HKY+G.

Using RAXML, 10 independent Maximum Likelihood (ML) searches using different starting conditions were conducted and 1000 non-parametric thorough bootstrap replicates were run and plotted against the tree with the best likelihood value. Using MRBAYES, two parallel runs, each with 1 cold and 3 heated chains, were computed with  $10^7$  generations and every 100th generation sampled. For generating the final 50% majority rule consensus tree, a conservative burn-in of  $4 \times 10^4$  was used to sample only the most likely trees.

In addition, genealogical relationships of each of the three mtDNA fragments of *P. castanoides* and *P. subniger* were examined by parsimony networks. For this purpose, alignments of the sequences of either species were analysed independently with TCS 1.21 (Clement et al. 2000), with gaps coded as fifth character state. Under the default 95% criterion, the 12S rRNA haplotypes of each species were connected (connection limit for each species: 8 steps). However, with respect to the ND4 and cyt *b* sequences, haplotypes of both species or of *P. subniger*, respectively, remained unconnected under the 95% criterion (ND4 and cyt *b*: 12 steps connection limit). Therefore, connection of haplotypes was enforced by setting the limit of mutational steps manually to 50.

## Results

All sequences could be easily aligned and used in the analyses. With respect to the mtDNA fragment containing the second half of the ND4 gene and adjacent DNA, all South African samples of *Pelusios castanoides* had an insertion of 18 bp within what is annotated as one of the two DNA sections coding for tRNA-Ser in the complete mitochondrial genome of a *Pelomedusa* in GenBank (accession number AF039066; Zardoya and Meyer 1998a, b). As explained in Fritz et al. (2011), the respective DNA portion does not correspond to the secondary structure of a tRNA, regardless of whether sequences with or without the insertion are concerned. Therefore, Fritz et al. (2011) did not annotate this piece of DNA, leaving open the possibility that it is non-functional. This 18-bp-long insertion was not found in any other terrapin, also not in the geographically closest specimen from Tofo, Mozambique (Fig. 1; Supplementary Table S1). However, the only Kenyan sample possessed at the same position a nine-bp-long insertion, identical with the first half of the insertion motif of the South African terrapins.

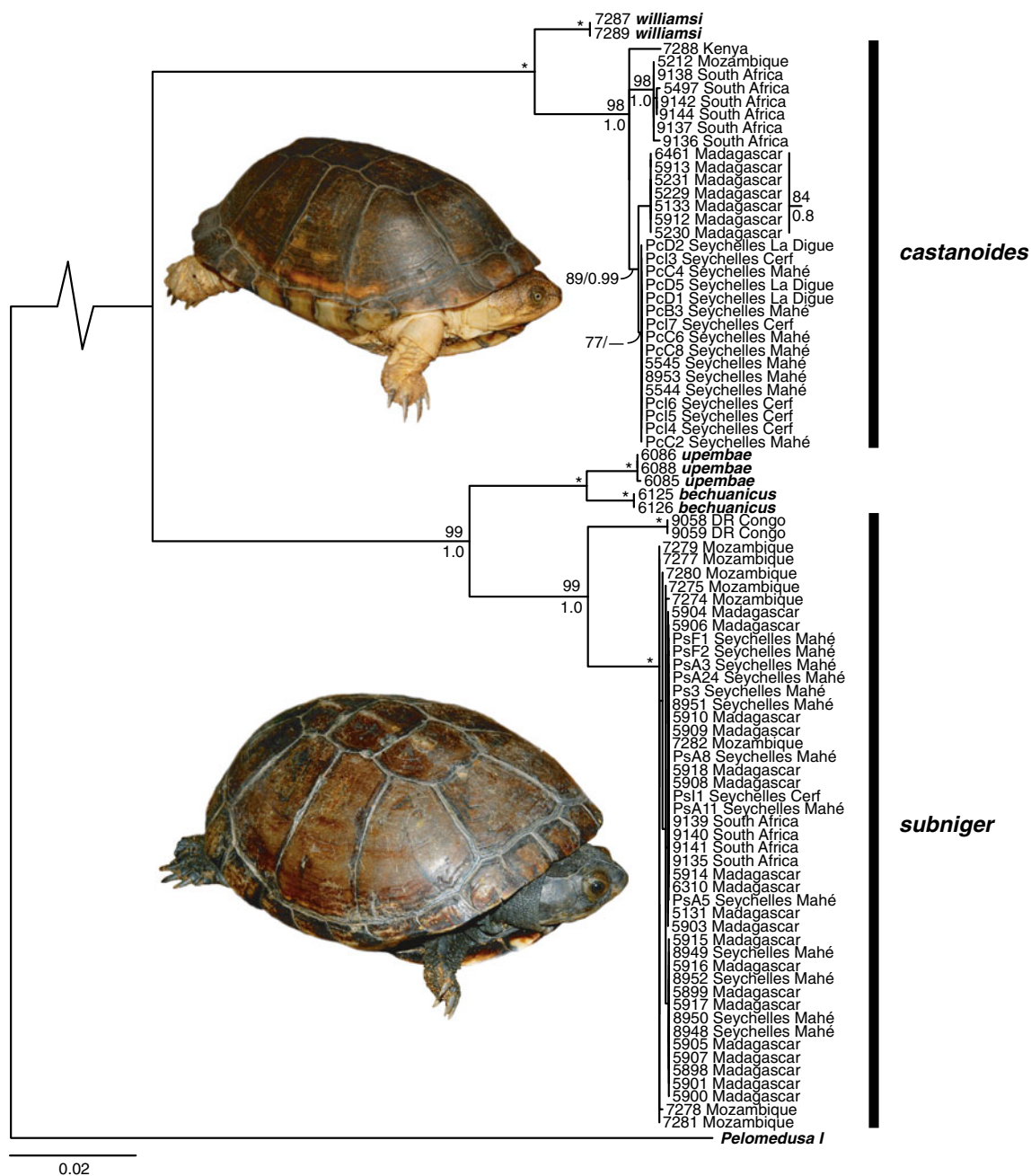
The topologies of all obtained ML and Bayesian trees were consistent, and the placement of the other included *Pelusios* species was in line with expectations (Fig. 2). *Pelusios williamsi* was sister to *P. castanoides* with maximum support, and *P. upembae* and *P. bechuanicus* together

were the sister group of *P. subniger* with high support. Within *P. castanoides* only weak differentiation was found. The single sample from Kenya was clearly distinct from all others; the remaining samples corresponded to three weakly to well-supported clades whose basal branching pattern was not well resolved. One well-supported clade contained all samples from South Africa and the only sample from Mozambique. A weakly supported clade comprised all Malagasy samples. This Malagasy clade was sister to a weakly supported clade embracing all samples from the Seychelles; however, this clade was not recovered by Bayesian analyses. Divergence within most samples of *P. subniger* was even lower than within *P. castanoides*, except for the two samples from the Democratic Republic of the Congo. These two samples were deeply divergent from all other *P. subniger*, with branch lengths slightly exceeding the divergence between *P. upembae* and *P. bechuanicus*.

The parsimony networks (Fig. 3) based on the individual DNA fragments of *P. castanoides* and *P. subniger* confirmed limited divergence for the latter species, except for the two samples from the Democratic Republic of the Congo. Sequences of *P. subniger* from South Africa, Mozambique, Madagascar and the Seychelles were either assigned to the same haplotype or to haplotypes differing by only one or two mutational steps. However, the haplotypes of the two terrapins from the Democratic Republic of the Congo were always highly distinct, differing by a minimum of 6 (12S rRNA), 15 (ND4) or 23 steps (cyt *b*) from the next-similar haplotype.

Differentiation within *P. castanoides* was more pronounced (Fig. 3), even though 12S rRNA sequences were assigned to only two haplotypes being distinct by just one mutational step. One of these haplotypes corresponded to terrapins from Kenya, Madagascar and the Seychelles, and the other to terrapins from Mozambique and South Africa. ND4 and cyt *b* sequences represented eight and five haplotypes, respectively, that were in part highly distinct. There were no shared ND4 and cyt *b* haplotypes among terrapins from different geographic origins. Owing to the above mentioned 9-bp or 18-bp insertion, ND4 haplotypes of the Kenyan (one haplotype) and South African samples (four haplotypes) were highly distinct from all others, although most probably only a single insertion event had occurred. The ND4 haplotype of Malagasy terrapins differed from the Seychelles haplotype by one mutation, whereas the only sequence from Mozambique differed from the Madagascar haplotype by three steps and from the Seychelles haplotype by four steps. With respect to cyt *b* sequences, the Kenyan and Malagasy haplotypes differed from the Seychelles haplotype by six or four steps, and the two South African haplotypes were different by a minimum of seven steps from the most similar other haplotype (from the Seychelles).





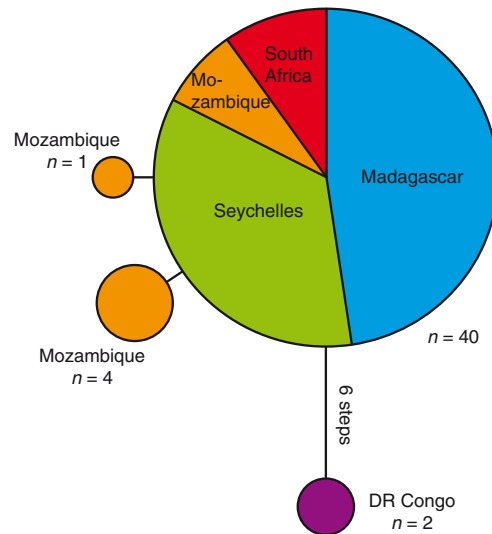
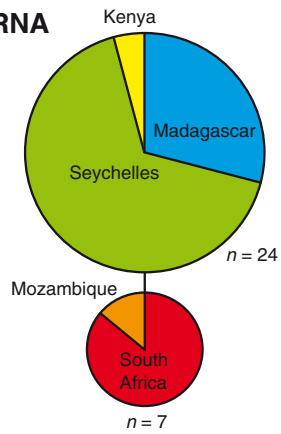
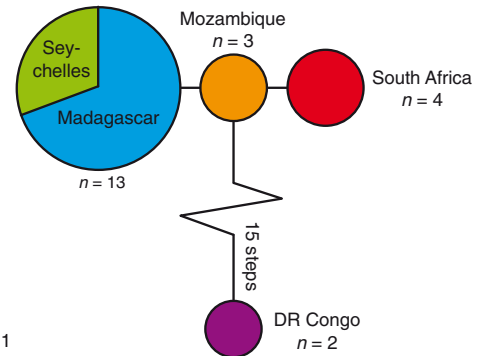
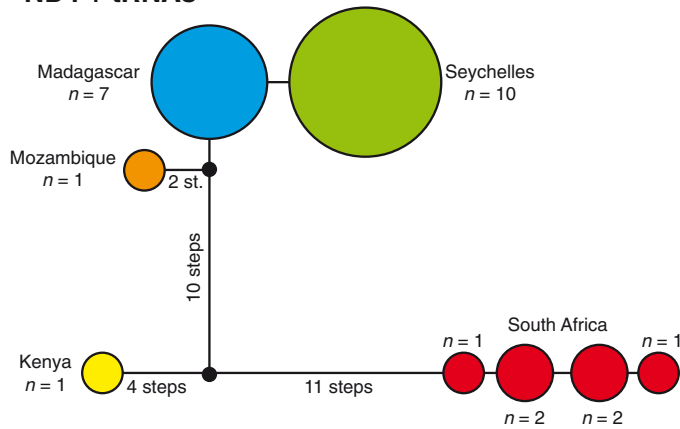
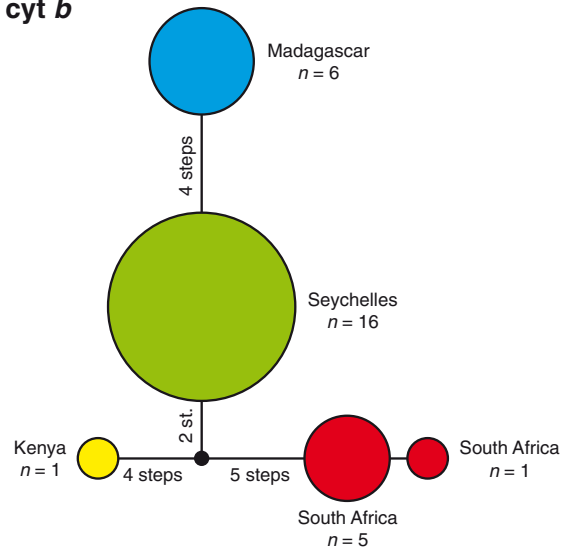
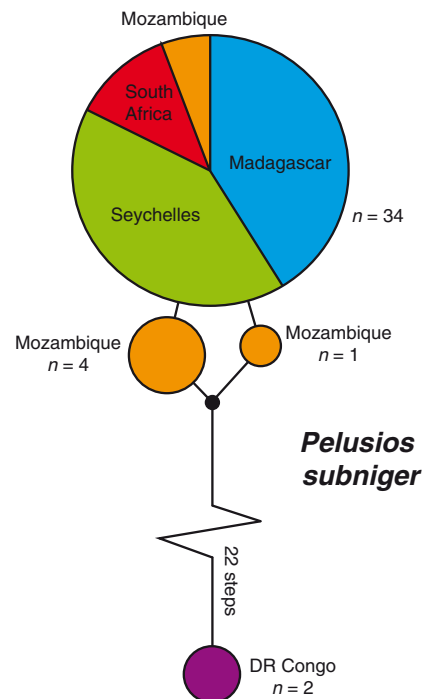
**Fig. 2** Maximum likelihood tree for *Pelusios castanoides*, *P. subniger* and their sister taxa, rooted with *Pelomedusa* lineage I. Support values along branches are thorough bootstrap values (1000 replicates) and Bayesian posterior probabilities (not shown for terminal clades with

short branch lengths). Root length shortened by 80%. For explanation of sample codes preceding taxon or locality names, see Supplementary Table S1

## Discussion

Phylogeographic structuring is present within both studied *Pelusios* species. Yet, as anticipated for *Pelusios castanoides* by Silva et al. (2010) and Fritz et al. (2011), it does not conform to expectations. Madagascar and the Seychelles are well known for their high degree of endemism, corresponding to a long and independent history of the

respective local faunas and floras. Most of the non-flying and non-marine vertebrate fauna colonized Madagascar in the Early Cenozoic, favoured by ocean currents different from those prevailing today. Only few terrestrial or fresh-water clades arrived after the change of sea currents in the Miocene, but even these are, in many cases, differentiated from their closest African relatives by levels of genetic divergence typically characterizing distinct species (e.g.,

**12S rRNA****ND4 + tRNAs****cyt b*****Pelusios castanoides******Pelusios subniger***

◀ **Fig. 3** Parsimony networks for the three DNA fragments of *Pelusios castanoides* and *P. subniger*. Geographic origins of samples are colour-coded. Circle size corresponds to haplotype frequency. Slices indicate percentages of terrapins of different geographic origins having the respective haplotype. If not otherwise noted, lines connecting two haplotypes represent one mutational step. Missing node haplotypes are shown as small black circles. The high level of divergence of the ND4 haplotypes of Kenyan and South African *P. castanoides* is caused by a 9-bp or 18-bp insertion, respectively (see Results section)

the frog *Ptychadena mascareniensis*; Measey et al. 2007) or are at least representing endemic haplotype lineages (e.g., the gecko *Hemidactylus mercatorius*; Vences et al. 2004). The granitic Seychelles, where the terrapin species occur, are remnants of the supercontinent Gondwana and were ‘lost in the sea’ during the north-eastward rafting of India after its detachment from Africa, approximately 63.4 million years ago (Collier et al. 2008). Besides many paleo-endemic species as a legacy of the breakup of Gondwana, the Seychelles also harbour a number of younger endemics that arrived later by oversea dispersal (Nagy et al. 2003; Vences et al. 2003; Klaus et al. 2010; Daniels 2011; Townsend et al. 2011).

Therefore, it should be expected that the populations of *Pelusios castanoides* and *P. subniger* from Madagascar and the Seychelles are well-differentiated and genetically deeply divergent from their continental African conspecifics. Indeed, Bour (1983) described the Seychellois populations of each species as a distinct subspecies (*P. c. intergularis*, *P. s. parietalis*), and Bour (1978) described Malagasy *P. castanoides* as another distinct subspecies (*P. c. kapika*; Fig. 1), which was later synonymized with *P. c. castanoides* (Bour 1983), however.

Bour (1983) diagnosed the two subspecies of *P. subniger* by minute morphological differences, referring to head scalation and size differences of the intergular scute of the plastron. However, among 11 *P. s. subniger* Fritz et al. (1994) found five terrapins that displayed characters of *P. s. parietalis*. Therefore, these authors concluded that the morphology-based subspecies delineation of *P. subniger* is insufficient. This is supported by our current genetic results. According to our mitochondrial DNA sequences, phylogeographic structuring within *P. subniger* is decidedly weak, with the exception of the deeply divergent samples from the Democratic Republic of the Congo. Their differentiation from the remaining *P. subniger* resembles the divergence between *P. upembae* and *P. bechuanicus* (Fig. 2), two species that together constitute the sister group of *P. subniger*. This suggests that the continental African populations currently assigned to *P. subniger* consist of at least two distinct species. This finding warrants a denser sampling of continental African populations and further research. By contrast, samples of *P. subniger* from South Africa, Mozambique, Madagascar and the Seychelles were not only phylogenetically undifferentiated (Fig. 2), they also shared the same 12S

rRNA and cyt *b* haplotypes, and ND4 haplotypes from Mozambique and South Africa were differentiated by only one or two mutational steps from the common haplotype of terrapins from Madagascar and the Seychelles (Fig. 3). This provides evidence for a very recent origin of both the populations in Madagascar and the Seychelles.

In several other cases it has been shown, or suggested, that terrapins and land tortoises have been introduced to islands by humans. There is genetic evidence for the introduction, probably by early settlers from south-east Africa, of two other species occurring in Madagascar, the hinge-back tortoise *Kinixys zombensis* and the helmeted terrapin *Pelomedusa subrufa* (Vargas-Ramírez et al. 2010; Wong et al. 2010; Kindler et al. 2012). The hinged terrapin *Pelusios castaneus* has even been introduced to Guadeloupe (Lesser Antilles) from West Africa, most probably during the time of the Triangular Trade (sixteenth to early nineteenth century; Lescure 1983; Fritz et al. 2011), and records of *P. subniger* on Diego Garcia (Chagos Archipelago), Grande Glorieuse and Mauritius are considered to also represent introduced terrapins (Bour 1983, 1984). Three species of land tortoises (*Testudo graeca*, *T. hermanni*, *T. marginata*) and the European pond turtle *Emys orbicularis* have been introduced to several Mediterranean islands, sometimes evidently in ancient or prehistoric times (Bringsøe et al. 2001; Fritz 2001; Fritz et al. 2006, 2009; Giacalone et al. 2009; Pedall et al. 2011; Vamberger et al. 2011), and the large-scale translocations of giant tortoises by seventeenth- to nineteenth-century sailors are well-known (Townsend 1925; MacFarland et al. 1974; Stoddart and Peake 1979; Chambers 2004) and led to naturalized non-native populations and admixture with native tortoises (Poulakakis et al. 2011; Garrick et al. 2012). In all of these cases, terrapins and tortoises seem to have served as live provision, as ‘living cans’, during sea voyages (Townsend 1925; MacFarland et al. 1974; Stoddart and Peake 1979; Chambers 2004; Vamberger et al. 2011) and surplus specimens were either abandoned after arriving at the final destination or intentionally released somewhere else for later victualling.

Considering this recurrent pattern of translocation of terrapins and tortoises and the lack of genetic differentiation among Mozambican, South African, Malagasy and Seychellois populations of *P. subniger*, we suggest that the populations on Madagascar and the Seychelles represent just another case of an introduced and naturalized terrapin species. Consequently, we relegate the nominal subspecies *P. s. parietalis* Bour 1983 into the synonymy of *P. subniger* (Bonnaterre 1789). This has implications for conservation strategies for the dwindling Seychellois populations, currently considered to represent a Critically Endangered taxon (Gerlach 2008b), and suggests that measures should rather focus on endemic species on this archipelago.

It remains unclear when *P. subniger* was introduced to Madagascar and the Seychelles. However, we assume that

early settlers and sailors arriving from Africa were responsible, as in the case of *K. zombensis*, which is thought to be introduced to Madagascar between 2300 and 1000 years before present (Kindler et al. 2012).

The situation of *P. castanoides* could be different. Phylogeographic structuring within this species is somewhat more pronounced than within *P. subniger* (if the Congolese samples of *P. subniger* are not considered). *Pelusios castanoides* from Madagascar and the Seychelles are at least weakly differentiated from continental African samples (Figs. 2 and 3). However, the degree of differentiation of Malagasy and Seychellois *P. castanoides* from the only available sample from Mozambique is weaker than the differentiation among continental African samples from Kenya and South Africa when these are compared with the Mozambican specimen (Fig. 3). In view of this weak differentiation, we cannot exclude that an expanded continental African sampling would lead to the discovery of the same haplotypes as in Madagascar and the Seychelles. Therefore, we refrain from explicit taxonomic and biogeographic conclusions. Nevertheless, the observed sequence variation allows speculating that, when the Malagasy and Seychellois populations of *P. castanoides* were introduced from the African continent, they must have originated from two distinct source regions and, consequently, from two distinct introduction events. If these populations result from natural transoceanic dispersal, they must either derive also from two distinct source regions or, via a stepping stone model, from an initial colonization of Madagascar and subsequent dispersal to the granitic Seychelles. All of these scenarios are in accordance with the finding that haplotypes of the ND4 and cyt *b* genes (two genes faster evolving than the more conserved 12S rRNA gene; e.g., Caccone et al. 1999; Jiang et al. 2007) from the Seychelles and Madagascar are consistently different and reciprocally monophyletic in maximum likelihood analyses (Figs. 2 and 3). The southernmost and northernmost parts of the continental range of *P. castanoides* (South Africa, southern Mozambique and Kenya, respectively) harbour clearly distinct haplotypes, so that these regions can be clearly ruled out as possible sources for the terrapins in Madagascar and the Seychelles. However, we had no samples available from much of the African range of *P. castanoides*, corresponding to approximately 2,500 km along the Mozambican and Tanzanian coast. Further phylogeographic studies on *P. castanoides* should focus on this large region to examine whether the populations in Madagascar and the Seychelles could have been introduced from there.

Our findings with respect to *P. subniger* underline that a good understanding of the phylogeography and ‘correct taxonomy’ are the necessary prerequisites for designing any well-founded conservation strategy and that further research is warranted to assess the status of Malagasy and Seychellois *P. castanoides*.

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