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Assessment of mtDNA genetic diversity within the terrapins *Pelusios subniger* and *Pelusios castanoides* across the Seychelles islands

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Abstract. Two terrapin subspecies are currently considered Seychelles endemisms and Critically Endangered according to IUCN criteria, with several conservation measures having been proposed and actively initiated in the recent years. We use molecular data to examine their population diversity and structure across the archipelago and find a complete lack of variation at the mtDNA level. This can be explained by the strong founder effect associated with island colonisation, allied to the low evolution rate described for turtle mtDNA. However, a very recent arrival of Seychellois terrapins to the archipelago by human action cannot be discarded. The use of highly variable markers such as microsatellites and the clarification of their (native or not) status is therefore essential and should be implemented as an urgent priority in species management plans. Some differentiation between the Seychellois and Malagasy *P. castanoides* is observed but should be taken carefully until geographically broad level sampling across Madagascar is available.

Keywords: Conservation, cyt-b, islands, mtDNA, *Pelusios*, Seychelles, terrapins.

Pelusios is a genus of side necked turtles, found throughout tropical Africa, Madagascar and the granitic Seychelles (Mortimer and Bour, 2002). In the Seychelles islands, the genus is currently represented by two endemic subspecies: *Pelusios subniger parietalis* and *Pelusios castanoides intergularis*, both listed as Critically Endangered by the IUCN red list categories (IUCN, 2008). In fact, these are the only two reptile species from the Seychelles with this conservation status. A third species, *Pelusios seychellensis*, is now considered extinct (IUCN, 2008). Both extant subspecies have highly restricted ranges (less than 11 ha for each species) that have decreased by over 50 per cent in recent years (Gerlach and Canning, 2001). These species live in freshwater systems, occupying lowland marshes and surrounding areas, and also rivers and streams in the case of *P. c. intergularis*. Amongst major factors considered responsible for their population decline are pollution, drainage of marshes and building development around them, marsh invasion by exotic species and predation by domestic cats, dogs and introduced tenrecs (Gerlach and Canning, 2001).

The Seychelles archipelago lies in the western Indian Ocean (4°-10°S; 40°-56°E) and comprises about 155 islands divided in three main groups with different origins (granitic, raised coralline and low coralline islands) (Braithwaite, 1984). The former, where *Pelusios* can be found, is a group of 40 islands, remnants of the Seychelles microcontinent which became isolated following the Gondwana breakup, around 65 million years ago (Plummer and Belle, 1995). Although the granitic Seychelles were not affected directly by glaciations during the Pleistocene, the oscillations in sea-level associated to these glacial periods must have had a large effect on the distributions of fauna and

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flora in the region (Gardner, 1986). During periods of low sea level most of the Seychelles Bank has been exposed as a single large island, but even when global sea level was at its highest, most of the granitic islands remained above sea level, allowing the survival of unique endemic species (Stoddart, 1971; Geyth, Kudrass and Streiff, 1979; Braithwaite, 1984; Montaggioni and Hoang, 1988).

The archipelago was not permanently inhabited until 1770, but since human occupation started, habitats have been altered for agriculture purposes, some animal species were pushed to extinction and some invasive plant and animal species were introduced (Benedict, 1984; Stoddart, 1984). Humans have been responsible for the introduction of several reptile species, including the Stumped-Toed Gecko *Gehyra mutilata* (Rocha, Ineich and Harris, 2009), the Oriental Garden Lizard *Calotes versicolor* and, recently, the Red-eared Terrapin *Trachemys scripta elegans* (Gerlach, 2007). It is not known yet at which level this last species (*Trachemys scripta elegans*) may represent a threat to the *Pelusios* from Seychelles, as they may compete for the same resources.

Given the vulnerability of both terrapin species in the granitic Seychelles, conservation measures were proposed as part of a "Seychelles Terrapin Action Plan", and include site protection, captive breeding and reintroduction or introduction of *Pelusios* species across several islands (Gerlach, 2002). Some of these activities have already taken place such as (re)introductions to Silhouette and North Islands (Gerlach et al., 2007; Rocamora et al., 2009), due to limited resources on time to undertake such activities. Nevertheless, no mixing of individuals from different islands has been done during these reintroductions, with both North and Silhouette individuals being originally from Mahé (and Cerf). Thus, it is particularly important to know the genetic structure of these species across the islands. The aim of this study is to assess and characterize genetic variation of both terrapin species across the archipelago, which can help in the adoption of appropriate management and conservation measures. We thus used mitochondrial DNA sequence data to assess: (a) genetic variation of the populations of both species within and between islands, and (b) the degree of differentiation of the Seychellean subspecies *Pelusios castanoides intergularis* with respect to *Pelusios castanoides castanoides* from Madagascar.

A total of 62 individuals were sampled from six of the granitic islands of the Seychelles archipelago (24 *P. s. parietalis* and 38 *P. c. intergularis*, fig. 1 and table 1). Many of these samples were collected during preparatory surveys to assess status and abundance of potential source populations for future transfers (Trouvilliez, 2007; Mocq and Rocamora, 2008). Additionally, tissue samples from two individuals of *P. c. castanoides* from Madagascar were obtained. Genomic DNA was extracted from blood, epidermis, muscle or nails samples with E.Z.N.A® Tissue DNA Kit (OMEGA Bio-Tek) according to the manufacturers' instructions. Nail samples were previously smashed with liquid nitrogen and digested overnight with Holmes-Bonner lysis buffer and proteinase-k.

A fragment with approximately 900 bp of the cytochrome *b* (Cyt-*b*) mitochondrial gene was initially amplified via PCR for some individuals with forward primer CB1 (5′-CCATCCAACATCTCAGCATGATGAAA-3′), modified from the original CBL14841 from Kocher et al. (1989), and reverse primer L (Shaffer, Meylan and McKnight, 1997). For some (more degraded) samples for which amplification of the entire Cyt-*b* fragment was difficult we used a combination of previous primers with internal ones (CB2F and CB2R, Palumbi et al., 1991). Specific primers for these species were also designed; CBPcastF (5′-CATTGCACCTAAAACAGAATCC-3′) and CBPsubF (5′-CTCAGCATGATGAAACTTTGG-3′), both forward, which, when combined with L, amplify an internal region with 980 and 906 bp, respectively. The primer CBPcastF worked well for both subspecies, while CBPsubF just amplified *P. s. parietalis* samples. Amplifications were conducted in 15 μl reactions using standard conditions with magnesium concentration of 2.8 mM. The PCR cycling procedure was performed as follows: initial denaturation for 10 min at 95°C; 40 cycles of denaturation for 30 s at 95°C, primer annealing for 40 s at 50°C-53°C, extension for 50 s at 72°C, and stop reaction for 10 min at 72°C. PCR products were purified using SureClean (Bioline), and sequenced in an automated DNA sequencing ABI3130XL Genetic Analyzer apparatus. Sequences obtained for this study have been deposited in GenBank under the accession numbers HQ230244-HQ230307. Sequences were aligned manually using BioEdit (Hall, 1999), against a published sequence of *P. williamsi*.

Although each species is completely distinct at molecular level (∼12.4% uncorrected

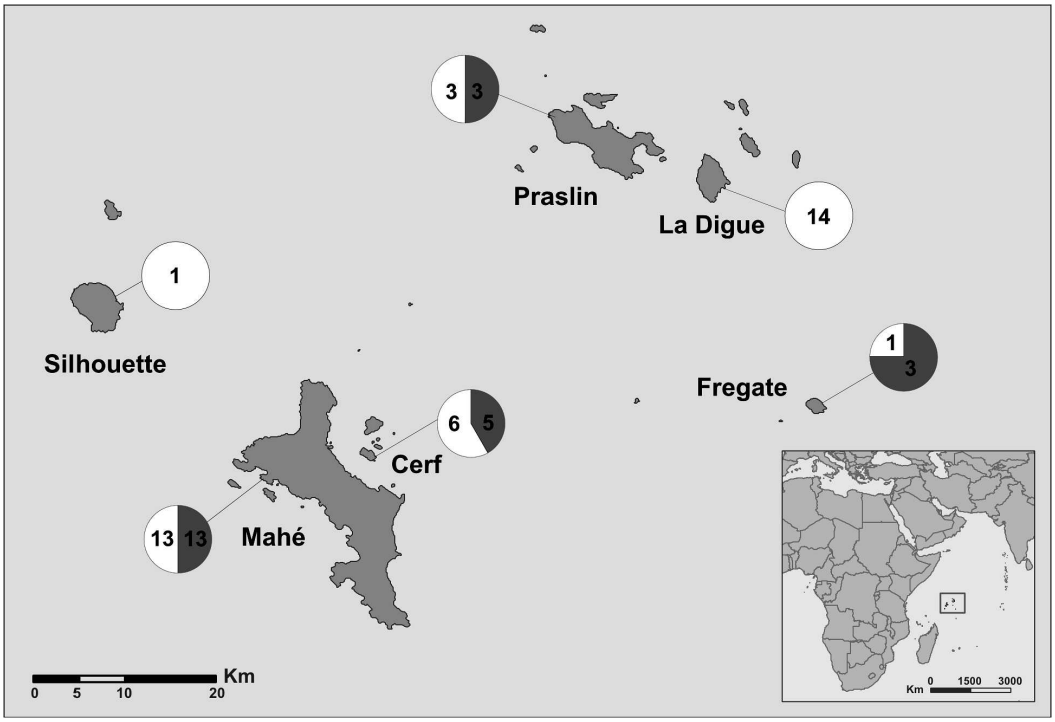

Figure 1. Map of the granitic Seychelles islands. The circles represent the number of individuals sampled per island; the grey slice represents the *P. s. parietalis* samples, and the white one represents *P. c. intergularis* samples.

Table 1. Specimens used in this study and respective locations.

Species	Location	Sample code
P. s. parietalis	Mahé	PsubSil2
	Anse Forbans, Mahé	Ps3, PsF1, PsF2
	Banyan Tree, Mahé	PsA1, PsA2, PsA3, PsA5, PsA7, PsA8, PsA11, PsA23, PsA24
	Praslin	PsP1, PcP1, PcP2
	Fregate	Ps2, PsFreg2, PsFreg3
	Cerf	PsI1, PsI2, PsI12, PsI13, PsI11
P. c. intergularis	Praslin	PsP3, PsP2, Pc11
	Silhouette	PcastSil1
	Mahé	12/02-2
	Rivière Mare Anglaise, Mahé	7.06, Pc1
	Banyan Tree, Mahé	PcA6, PcA15
	North East Point, Mahé	PcC1, PcC2, PcC4, PcC6, PcC8, PcC10
	Rivière Mare Anglaise, Mahé	PcB1, PcB3
	Fregate	PcFreg1
	La Digue	LD25
	Mare Soupape, La Digue	PcD1, PcD2, PcD3, PcD5, PcD9, PcD11, PcD12, PcD13, PcD14, PcD19, PcD20, PcD21, PcD26
	Cerf	PcI3, PcI7, PcI8, PcI5, PcI4, PcI6
P. c. castanoides	Lac Alaotra, Madagascar	DRV 5735, DRV 5736

p-distance), within each Seychelles subspecies there was no variation at the mitochondrial DNA level (Cyt-*b*). All 38 individuals of *P. c. intergularis* shared the same haplotype, the same happening with *P. s. parietalis* (24 individuals).

Differentiation between *P. castanoides intergularis* and the *P. c. castanoides* representative from Madagascar was of five mutational steps.

Although the number of samples from some of the islands is low, samples were taken from individuals from all the islands from where *P. c. intergularis* is recorded and from all but one island (La Digue) in the case of *P. s. parietalis* (given they are considered re-introduced – from Mahé – on Silhouette). In fact, sampling could well be complete because the Fregate population of *P. subniger* is possibly introduced from La Digue, and would therefore represent its initial stock. Thus, sampling should represent much of the existent genetic variation across the archipelago. However, no variability was detected in this fragment of mitochondrial DNA within each subspecies across the whole archipelago. It was hypothesized that Malagasy populations of *Pelusios*, and probably the Seychellois also, originated in the Pliocene or Pleistocene in a major dispersal event including also other Malagasy vertebrates conspecific with African populations (Vences et al., 2004). Also Taylor et al. (1979) argued that their arrival to the Seychelles islands probably occurred during the Pleistocene, at the last glacial maximum (LGM), around 17 000 years before present (BP) when sea levels were about 120 m lower than today (Camoin, Montaggioni and Braithwaite, 2004). In fact, both along the Pleistocene and the Holocene several instances of lower sea levels occurred (Colona et al., 1996; Rohling et al., 1998; Siddall et al., 2003; Camoin, Montaggioni and Braithwaite, 2004; Miller et al., 2005), that, although not completely providing any land passage between Africa, Madagascar and the Seychelles, would considerably reduce the water distance to be crossed by any organism when colonising the Seychelles from either Africa or Madagascar. The fact that no variation was detected at mtDNA level is still compatible with two hypotheses. *Pelusios* spp. may have naturally colonised the Seychelles very recently, at least by the middle or end of the Pleistocene. Colonisation by a very small number of individuals possibly harbouring only one haplotype (founder effect), together with the low evolution rate consistently reported for turtle mtDNA (Lamb et al., 1989; Avise et al., 1992; Bowen et al., 1993; Sommer et al., 2009) may explain the current lack of variation observed. Recent results obtained by Sommer et al. (2009) for *Emys orbicularis* further support this idea, showing – using both extant distribution of this terrapin species and ancient DNA data – that 10 000 year old samples and extant ones harbour the same cytochrome-*b* mitochondrial haplotype.

The second hypothesis would be a human mediated introduction of these terrapins in the archipelago, maybe as a food resource, such as reported for other islands including Mauritius and Glorieuse (Bour, 1984). However, given these results, based only on mtDNA markers it is not possible to determine if their presence is due to a natural colonisation or human-aided introduction(s), after the peopling of the Seychelles. To further resolve this question the characterization of the populations of these species for more variable markers, such as microsatellites, should be performed. These have shown to be useful and even reveal high levels of genetic variability in other terrapin species, such as *Emys orbicularis* (Velo-Antón, García-París and Rivera, 2008) at the Iberian Peninsula.

Regarding their morphological differentiation, Fritz, Obst and Gunther (1994), examining *P. subniger* specimens from Madagascar (the nominotypical subspecies *Pelusios subniger subniger*), report specimens with the characteristic head scalation of *P. s. parietalis* and even some "intermediate" forms, pointing out that the subspecific structuring of *P. subniger* does not seems to be sufficiently resolved. The distinctive intergular proportions however have not been reported for any non-Seychellois *P.*

subniger specimen. The origins of the Seychelles *P. subniger* population cannot be determined without further studies incorporating samples from Madagascar and Africa to determine whether the described subspecific differences have a genetic basis and taxonomic value or represent local frequency variation in characters, possibly attributable to a founder effect following a relatively recent colonisation, natural or not.

Concerning *P. castanoides*, the Seychellois subspecies *P. c. intergularis* seems to be very similar also to the nominate form *P. c. castanoides*, being distinguished mainly by the shape of their intergular scute but also in cranial osteology (Hewitt, 1931; Bour, 1983). Five mutational steps at the mtDNA marker cytochrome-*b* differentiate *P. c. intergularis* from *P. c. castanoides* haplotype. The interpretation of these results requires some prudence as only two samples from Madagascar (collected at the same location; Lac Alaotra in NE Madagascar) were available and thus this value certainly does not reflect the overall real differentiation between Seychellois and Malagasy individuals. Proper estimation of origin of island taxa, colonisation patterns and ages, always requires an extensive assessment of the diversity within the taxa in its colonised and putative areas of origin. In this particular case, given the absence of genetic variation within the Seychellois subspecies, further hypotheses about the process and time of colonisation of the Seychelles by *Pelusios* sp. can only be advanced following a deeper knowledge of the genetic variability of *Pelusios* species in Madagascar and Africa.

In conclusion, our results depict intraspecific differentiation in *P. castanoides* and suggest that both *Pelusios* subspecies arrived to the Seychelles archipelago very recently, being at this point impossible to distinguish between natural or human-aided colonisation. We recommend further detailed analyses using higher variable markers, such as microsatellites, as a high priority component of management plans for these taxa considered Critically Endangered under their current taxonomic identification.

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