

Phylogenetic relationships of African green snakes (genera *Philothamnus* and *Hapsidophrys*) from São Tomé, Príncipe and Annobon islands based on mtDNA sequences, and comments on their colonization and taxonomy

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Mitochondrial sequences (16S rRNA and cytochrome *b*) of the colubrine snake genera *Philothamnus* and *Hapsidophrys* were analysed. Samples were obtained from three volcanic islands in the Gulf of Guinea. The main objective was to infer phylogenetic relationships between the taxa and to trace back the colonization patterns of the group. Both insular species, *Philothamnus girardi* and *Philothamnus thomensis*, form a monophyletic unit indicating a single colonization event of one island (probably São Tomé) followed by dispersal to Annobon. Genetic divergence was found to be relatively low when compared with other *Philothamnus* species from the African mainland, but sufficient to consider the two taxa as distinct sister species. Here we also present evidence on the distinct phylogenetic position of *Hapsidophrys* sp. from the island of Príncipe, which should be considered as a distinct species, *Hapsidophrys principis*, a sister taxon of *H. smaragdina*.

Key words: 16S rRNA, cytochrome *b*, Gulf of Guinea islands, *Hapsidophrys principis*, *Philothamnus girardi*, *Philothamnus thomensis*

INTRODUCTION

The islands of the Gulf of Guinea form part of a volcanic chain that originated from the middle to late Tertiary. The islands São Tomé, Príncipe and Annobon are situated on the oceanic sector of a straight axis, the Cameroon volcanic line, which is a flaw or hotline (Meyers et al., 1998) in the African tectonic plate, about 1500 to 1600 km long (Déruelle et al., 1991; Burke, 2001; Caldeira & Munhá, 2002). In this volcanic line, the continental-shelf island Bioko is the largest and closest one to the African mainland; it is situated approximately 32 km from Cameroon and was formerly connected with the African mainland. The other three islands are truly oceanic ones; they are smaller than Bioko and were never connected with the mainland or with each other. Príncipe is situated about 220 km southwest from Bioko and 146 km northeast from São Tomé. The island of São Tomé is located about 275 km westwards from Gabon, while Annobon is about 180 km southwest of São Tomé. Príncipe (with an area of approximately 128 km²) has an estimated age of 31 million years, while the other islands are significantly younger: São Tomé (836 km²) is about 13 millions years old, and Annobon (17 km²) is apparently the youngest island with an age of about 4.9 million years (Lee et al., 1994).

The islands of the Gulf of Guinea belong biogeographically to the West African rainforest zone. They are situated between two large regions (the Guinea forests and the Congo basin) that have recently received increased attention due to their exceptional biodiversity. Based on their isolation, the islands presently harbour several endemic species. Moreover, the region could be considered as one of the world's hotspots for primates (Oates et al., 2004). As far as the herpetofauna are concerned, endemisms on the islands are known among amphibians (Measey et al., 2007) and some reptiles: *Hemidactylus* geckos (Jesus et al., 2003, 2005a), *Lygodactylus* geckos (Jesus et al., 2006) *Mabuya* skinks (Jesus et al., 2003, 2005b,c), *Afroablepharus* skinks (Jesus et al., 2007) and various snakes (*Philothamnus thomensis*, *Philothamnus girardi* and *Hapsidophrys* spp.).

According to Chippaux (2001), about 19 snake species belong to the genus *Philothamnus*, distributed in Africa and mainly living in forests and riparian vegetation in sub-Saharan Africa. Despite the comments made on a proposed revision (Hughes, 1985), the taxonomy of *Philothamnus* remains problematic (Trape & Roux-Estève, 1990).

Philothamnus and *Hapsidophrys* seem to constitute two closely related genera according to DNA sequence

Table 1. Samples used in the present study. Museum acronyms: PEM = Port Elizabeth Museum, Republic of South Africa; HLMD = Hessisches Landesmuseum Darmstadt, Germany.

Species	Locality	Voucher specimen	Lab or specimen ID	Accession no.	
				16S rRNA	cyt <i>b</i>
<i>Philothamnus girardi</i> 2	Annobon island, Gulf of Guinea	621	621	FJ913475	FJ913495
<i>P. thomensis</i> 1	Vale do Contador, São Tomé, Gulf of Guinea	631	631	FJ913474	FJ913486
<i>P. thomensis</i> 2	Ponta Furada, São Tomé, Gulf of Guinea	573	573	FJ913480	FJ913487
<i>P. thomensis</i> 3	Vale do Contador, São Tomé, Gulf of Guinea	632	632	FJ913479	FJ913488
<i>P. thomensis</i> 4	Vale do Contador, São Tomé, Gulf of Guinea	568	568	FJ913473	FJ913489
<i>P. thomensis</i> 5	Vale do Contador, São Tomé, Gulf of Guinea	567	567	FJ913477	FJ913490
<i>P. thomensis</i> 6	Ponta Furada, São Tomé, Gulf of Guinea	570	570	FJ913478	FJ913491
<i>Hapsidophrys principis</i> 1	Terra Velha, Príncipe, Gulf of Guinea	592	592	FJ913476	FJ913492
<i>H. principis</i> 2	Ponta do Sol, Príncipe, Gulf of Guinea	769	769	FJ913482	FJ913493
<i>H. principis</i> 3	Montalegre, Príncipe, Gulf of Guinea	606	606	FJ913481	FJ913494
<i>H. lineatus</i>	Haute Dodo, Cote d'Ivoire	PEMR2307	J345	AY611873	AY612055
<i>H. smaragdina</i>	Rabi complex, Gabon	PEMR5383	J349	AY611875	AY612057
<i>Philothamnus angolensis</i>	Namagure village (16°58'12.5"S, 38°40'15"E), Zambezia Province, Mozambique	PEMR13207	J382	AY611886	AY612068
<i>P. natalensis</i>	Roteniqua Pass, Western Cape, South Africa	PEM FN441	J383	AY611887	AY612069
<i>P. carinatus</i> 1	Loango National Park, Gabon	PEMR5441	J335	AY611870	AY612052
<i>P. nitidus</i>	Rabi complex, Gabon	PEMR5397	J337	AY611871	AY612053
<i>P. hoplogaster</i>	Moebase Camp, Zambezia Province, Mozambique	PEMR13214	J389	FJ913484	FJ913496
<i>P. semivariiegatus</i> 1	Moebase Camp, Zambezia Province, Mozambique	PEMR13189	J390	AY611889	AY612071
<i>P. semivariiegatus</i>	Moebase Village, Zambezia Province, Mozambique	PEMR13189	J391	FJ913485	FJ913497
<i>P. heterodermus</i>	?	JLC 762	J238	AY611856	AY612038
<i>P. carinatus</i>	Rabi complex, Gabon	PEMR5938	J354	FJ913483	FJ913498
<i>Macroprotodon cucullatus</i>	Tunisia, Bou Hedma	HLMD RA-2974	J79	AY188065	AY188026

data (Lawson et al., 2005). They are similar in appearance ("green snakes"), but differ in that *Hapsidophrys* has strongly keeled dorsal scales.

Philothamnus thomensis Bocage, 1882 is endemic to São Tomé. It was treated by some authors (e.g. Mertens, 1934; Bogert, 1940; Loveridge, 1958) as a subspecies of *P. semivariiegatus* (Smith, 1840), and has been confused with *P. nitidus* (Günther, 1863). However, Hughes (1985) considered it as a distinct species in his revision of the genus *Philothamnus*. Similarly, *Philothamnus girardi* Bocage, 1893 is endemic to Annobon and was also treated as a subspecies of *P. semivariiegatus* by some authors, such as Mertens (1934) and Loveridge (1958). Furthermore, according to Hughes (1985) it shows some morphological resemblance to *P. nitidus*.

Hapsidophrys Fischer, 1856 is a small genus, and with the transfer of *smaragdina* from *Gastropxyxis* it contains only two African species: *H. lineatus* Fischer, 1856 and *H. smaragdina* (Schlegel, 1837) (Broadley, 1966; Williams & Wallach, 1989; Chippaux, 2001). *Gastropxyxis principis* was described by Boulenger (1906), and although sometimes still accepted as a valid species (e.g. Manaças, 1956), it has also been treated as a synonym of *H. smaragdina* (see, for example, Lawson et al., 2005). According to Meirte (1992), another taxon, *Hapsidophrys coeruleus* described by Fischer, 1856, from Ghana, has also been treated as a synonym of *H. smaragdina*.

Despite recently published phylogenetic studies on the herpetofauna of the islands of the Gulf of Guinea (e.g. endemic amphibians: Drewes & Stoelting, 2004;

Nesionixalus treefrogs: Drewes & Wilkinson, 2004; *Ptychadena newtoni* frog: Measey et al., 2007; *Hemidactylus* geckos: Jesus et al., 2005a; *Lygodactylus* geckos: Jesus et al., 2006; *Mabuya* skinks: Jesus et al., 2005b,c; and *Afroablepharus* skinks: Jesus et al., 2007), little is known about the snake fauna of the region. Whilst some studies indicate the monophyly of some reptile genera and probably a single colonization event – for example *Lygodactylus* (Jesus et al., 2006) and probably *Afroablepharus* (Jesus et al., 2007) – other studies indicate multiple colonization events, e.g. *Mabuya* (Jesus et al., 2005b) and *Hemidactylus* (Jesus et al., 2005a). These results show remarkable variation between the relationships of taxa from different islands, indicating a complex pattern of colonization and dispersal.

In the present study, partial sequences from two mitochondrial genes, 16S rRNA and cytochrome *b*, were used to 1) study the phylogenetic relationships of green snakes from islands of the Gulf of Guinea; 2) examine the level of variation between populations and taxa on the islands; 3) infer the possible colonization events and patterns; and 4) infer the relationship of the insular species to other mainland species.

MATERIALS AND METHODS

Sampling and molecular methods

Locality and collection data on the specimens used in this study are given in Table 1 and Figure 1. Voucher specimens from São Tomé, Príncipe and Annobon are deposited in the herpetological/zoological collections of the University of Madeira. Further samples of the genera *Philothamnus* and *Hapsidophrys* are deposited in the Port Elizabeth Museum, South Africa.

Total genomic DNA was extracted using standard protocols (Sambrook et al., 1989). We used the following primers: 16SL and 16SH (Simon et al., 1990), and 16SA and 16SB (Palumbi et al., 1991) for amplification of 16S rRNA, and cytB1 and CB3H (Palumbi et al., 1991), L14910, L14919

and H16064 (Burbrink et al., 2000; modified by de Queiroz et al., 2002) for amplification of cytochrome *b*. The primers cytochrome b2 from Kocher et al. (1989), P1 (see Jesus et al., 2007), L14903 (a 5' end of L14910), L-410 and H-391 (Nagy et al., 2003) were used for sequencing of cytochrome *b* only. PCR protocols have been described elsewhere (Nagy et al., 2003; Jesus et al., 2007). PCR products were sequenced on ABI Prism 310 (Applied Biosystem) and MegaBACE 1000 (Amersham) capillary DNA sequencers.

DNA sequences were aligned using Clustal W (Thompson et al., 1994) followed by visual corrections.

The combined dataset used for phylogenetic analyses consisted of 1084 bp (447 bp for 16S rRNA and 637 bp for cytochrome *b*). However, only cytochrome *b* sequences were used to estimate genetic divergence since this is comparable to many other studies.

Because phylogenetic reconstruction is based on positional homologies, the regions that could not be unambiguously aligned were excluded from further analysis (about 48 bp of 16S rRNA). The alignment is available on request from the corresponding author.

Six *P. thomensis* from São Tomé, one *P. girardi* from Annobon and three *Hapsidophrys* from Príncipe were compared with representatives of various *Philothamnus* species and both *H. lineatus* and *H. smaragdina* from the mainland (Table 1).

The false smooth snake *Macroprotodon cucullatus* was used as outgroup taxon (AY188026 for cytochrome *b*, AY188065 for 16S rRNA; see Table 1).

Data analysis

True evolutionary relationships may be obscured in DNA sequence data sets if sites have become saturated by multiple substitutions (Swofford et al., 1996). To test for saturation, observed pairwise proportions of transitions and transversions in the separate 16S and cytochrome *b* data sets were plotted against sequence divergence using DAMBE version 4.2.13 (Xia & Xie, 2001).

Furthermore, differences in substitution rates between gene regions can produce conflicting signals. Thus, before proceeding with the analysis, a partition homogeneity test was applied to our data (Farris et al., 1994) using PAUP* 4.0b10 (Swofford, 2002) to evaluate whether the two gene regions show significantly different phylogenetic signals. This test indicated no significant incongruence between regions ($P=0.692$), so they were combined in all subsequent phylogenetic analyses.

Phylogenetic analyses were carried out using PAUP* 4.0b10 (Swofford, 2002) and MEGA version 3.1 (Kumar et al., 2004). We used maximum likelihood (ML) analyses and Bayesian inference of phylogeny. The most appropriate model of nucleotide substitution was inferred by Modeltest 3.7 (Posada & Crandall, 1998) and selected according to the Akaike information criterion (see Posada & Buckley, 2004). Non-parametric bootstrap support for nodes was estimated using the “fast” option with 100 heuristic bootstrap replicates as implemented in PAUP* 4.0b10. The Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001). Bayesian analysis was conducted with random starting

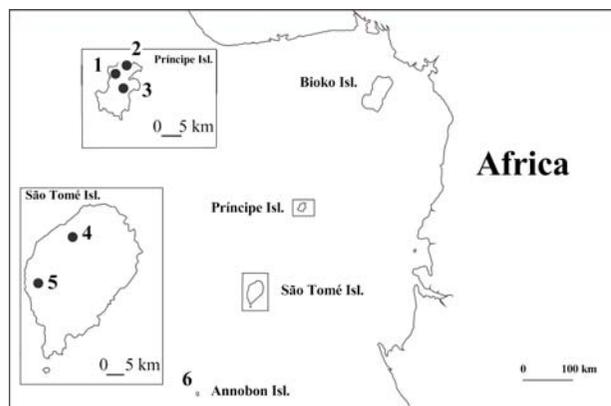


Fig. 1. Sampling sites of *Philothamnus* and *Hapsidophrys* specimens from the islands of São Tomé, Príncipe and Annobon used in this study. Numbers are as follows: 1) Montalegre, 2) Ponta do Sol, 3) Terreiro Velho, 4) Vale do Contador, 5) Ponta Furada, 6) Annobon.

Table 2. Cytochrome *b* K2P pairwise divergences for the *Philothamnus* and *Hapsidophrys* samples used in this study. Coding/abbreviations: Ptho = *Philothamnus thomensis*, Pgir = *Philothamnus girardi*, Pang = *Philothamnus angolensis*, Pnat = *Philothamnus natalensis*, Pcar = *Philothamnus carinatus*, Pnit = *Philothamnus nitidus*, Phop = *Philothamnus hoplogaster*, Psem = *Philothamnus semivariegatus*, Phet = *Philothamnus heterodermus*, Haps = *Hapsidophrys principis*, Hlin = *Hapsidophrys lineatus*, Hsma = *Hapsidophrys smaragdina*.

	Ptho1	Ptho3	Pgir2	Pang	Pnat	Pcar1	Pnit	Phop	Psem1	Psem	Phet	Pcar	Haps1	Haps3	Hlin
Ptho3	0.002														
Pgir2	0.086	0.088													
Pang	0.181	0.183	0.163												
Pnat	0.162	0.165	0.169	0.159											
Pcar1	0.169	0.171	0.184	0.172	0.169										
Pnit	0.155	0.157	0.154	0.111	0.155	0.170									
Phop	0.183	0.185	0.167	0.172	0.165	0.182	0.162								
Psem1	0.159	0.161	0.157	0.124	0.157	0.159	0.109	0.164							
Psem	0.161	0.164	0.155	0.126	0.155	0.157	0.111	0.166	0.002						
Phet	0.157	0.159	0.171	0.190	0.179	0.138	0.163	0.178	0.172	0.170					
Pcar	0.169	0.171	0.189	0.165	0.173	0.013	0.172	0.180	0.166	0.164	0.140				
Haps1	0.161	0.163	0.165	0.188	0.171	0.194	0.158	0.175	0.173	0.175	0.173	0.196			
Haps3	0.163	0.166	0.168	0.190	0.173	0.196	0.161	0.177	0.175	0.178	0.175	0.198	0.002		
Hlin	0.180	0.178	0.197	0.186	0.175	0.194	0.163	0.177	0.158	0.161	0.188	0.198	0.125	0.128	
Hsma	0.166	0.168	0.170	0.191	0.186	0.175	0.165	0.168	0.144	0.146	0.193	0.177	0.120	0.122	0.160

trees, four Markov chains (one cold, three heated), run for 5×10^6 generations, and sampled every 100 generations using a GTR+I+G model of nucleotide substitution. One additional analysis with 7.5×10^6 generations was performed, leading to the same results. Two independent replicates were conducted and inspected for consistency (see Huelsenbeck & Bollback, 2001). Convergence between runs and posterior probabilities of the estimates were determined using the software Tracer (Rambaut & Drummond, 2005).

RESULTS

The dataset of the combined and aligned mtDNA sequences is comprised of 1084 bp from 22 specimens. Plots of observed pairwise divergences of haplotypes for transitions and transversions in the separate 16S rRNA and cytochrome *b* data against total sequence divergence revealed negligible saturation (not shown), so our analyses included all sites. ML and Bayesian analyses gave identical topologies (Fig. 2). For the ML analysis we concluded that the GTR model (Rodríguez et al., 1990) with an estimate of invariable sites (0.62) and a discrete approximation of the gamma distribution (0.7497) was the most appropriate model. A heuristic search incorporating this model found one tree with a value of $-\ln L=4658$.

In all analyses, *Philothamnus thomensis* and *Philothamnus girardi* formed a monophyletic group supported by a posterior probability of 100% and a bootstrap value of 92%. This group is related to the group including *P. natalensis*, *P. carinatus*, *P. heterodermus* and *P. hoplogaster*. *Philothamnus* remains monophyletic with respect to *Hapsidophrys*, although only nine of the 19 recognized species of *Philothamnus* were used in our analysis.

All *Hapsidophrys*, including samples from Príncipe in the Gulf of Guinea and from the African mainland, form a monophyletic group supported by an average posterior probability of 100% and a bootstrap value of 86%.

Average levels of sequence divergence between congeneric reptile species are known to be approximately 12% for cytochrome *b* (Harris, 2002). We found a sequence divergence of 8.5% for cytochrome *b* between populations of *Philothamnus girardi* and *Philothamnus thomensis*. Sequence divergence between *Hapsidophrys* from Príncipe and *H. smaragdina* from the mainland was approximately 12%, and between *Hapsidophrys* from Príncipe and *H. lineatus* was approximately 12.5% (Table 2).

DISCUSSION

Colonization patterns

Our results clearly indicate that snakes belonging to the genus *Philothamnus* from Annobon and São Tomé are sister taxa. Despite the relatively low sequence divergence (8.5% for cytochrome *b*), they should be considered as distinct species, as proposed by Hughes (1985). Although monophyly of any group is dependent on outgroup sampling, the relatively low divergence between *P. thomensis* and *P. girardi* makes it less likely that any unsampled continental species will alter their status as sister species. Further, although sampling within islands is limited, genetic variation within both *P. thomensis* and *Hapsidophrys* from Príncipe is extremely low. Combined with the small size of the islands and the considerable dispersal ability of relatively large organisms such as these snakes, it seems unlikely that additional genetically distinct units remain unsampled

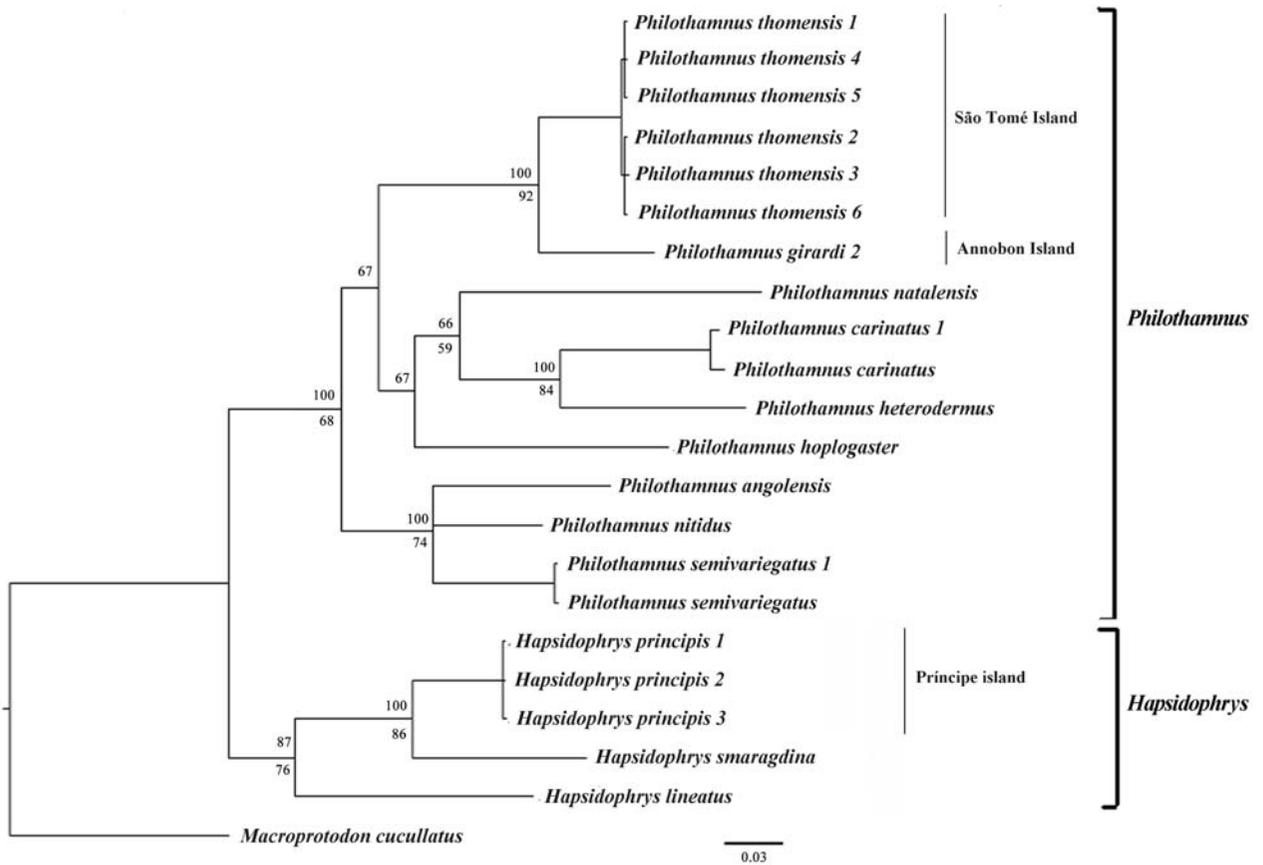


Fig. 2. Bayesian tree based on combined 16S rRNA and cytochrome *b* fragments. Posterior probabilities are shown above branches. The tree was rooted using *Macroprotodon cucullatus*. The maximum likelihood tree, obtained by PAUP and using the GTR+I+G model of sequence evolution, shows identical topology. Bootstrap values (>50%) for ML are given below the branches.

that might alter our colonization hypotheses. The ancestor of these species probably colonized one of these islands and then colonized the other. According to Emerson (2002), there are methods available to infer the colonization sequence. One uses tree topology and geography under the premise that an island is more likely to be colonized by a neighbouring island than by a more distant one. The other method infers the direction of colonization using information from tree topology and branch length. This method is based on the assumption that there is a rapid molecular diversification caused by a founding event. So, taking into account the bigger branch length and longer distance of Annobon from the continent, the most parsimonious scenario seems to be that the first island that was colonized was São Tomé, followed by a radiation to Annobon. Measey et al. (2007) also suggest that São Tomé is a more probable target island to be first colonized, prior to Annobon, due to predicted rafting routes after river flooding on the continent.

This pattern seems to be similar to the case of *Afroablepharus* (Jesus et al., 2007), but differs from what was found in *Mabuya* skins (Jesus et al., 2005b) and *Hemidactylus* geckos (Jesus et al., 2005a), which might have independently colonized each island.

When simply assuming a constant molecular clock for cytochrome *b* showing about 2% substitution rate per million years (see Carranza et al., 2000), the lineage inhabiting Annobon diverged from the lineage of São Tomé about 4.3 million years ago. This estimate is rather close to the genesis of Annobon. In fact, Annobon, the youngest and smallest of the Gulf of Guinea islands, has an estimated age of 4.9 million years (Lee et al., 1994).

Considering a similar molecular clock for *Hapsidophrys*, and assuming that the sister species of *H. principis* is *H. smaragdina*, the colonization might have been occurred about 6.1 million years ago from the mainland, or later from São Tomé from a lineage that has gone extinct meanwhile. However, the latter situation is less probable according to the hypothesis of Measey et al. (2007) about the direction of colonization on the Gulf of Guinea islands.

One additional aspect to be considered for the green snakes of these islands is that no island has multiple species. This is an observation that differs from the patterns found in *Mabuya* and *Hemidactylus*, where São Tomé harbours at least two species of each genus (Jesus et al., 2005a,b). It has been argued in other island systems that a filled ecological niche may reduce the success of further

colonizations (Gillespie & Roderick, 2002), and this could explain the non-overlapping distribution of green snakes on these islands. As pointed out by Jesus et al. (2007), these differences in colonization patterns highlight the difficulties in drawing general conclusions regarding how islands are colonized by only a few species – stochastic processes obviously play an important role.

Taxonomic comments

Broadley's (1966) action of synonymizing *Gastropyxis* Cope, 1860 (type species: *Dendrophis smaragdina* Schlegel, 1837) with *Hapsidophrys* Fischer, 1856 (type species: *Hapsidophrys lineatus*) is supported.

The taxonomic status of *Hapsidophrys* snakes from Príncipe was controversially discussed in the past, including most recent literature referring to them as *Hapsidophrys smaragdina* (e.g. Lawson et al., 2005). During most of the twentieth century, and even before 1906, these taxa were considered as synonyms. However, our results indicate high genetic divergences (based on partial cytochrome *b* sequences) between *H. smaragdina* and *Hapsidophrys* sp. from Príncipe, thus supporting the distinct status of the Príncipe population at species level. *Hapsidophrys principis* should therefore be considered as a valid species and not a synonym of *H. smaragdina*. Described by Boulenger (1906) as *Gastropyxis principis*, it is represented by two syntypes stored in the Museo Civico di Storia Naturale, Genova, Italy (MSNG 28144a and MSNG 28144b) and one specimen in BMNH (according to the TIGR Reptile Database; Uetz et al., 2007).

Our molecular data also support specific status for *Philothamnus thomensis* and *P. girardi*, but do not indicate a close relationship with *P. semivariiegatus* as proposed by Loveridge (1951, 1958), but questioned by Hughes (1985).

Our data indicate a close relationship between *P. semivariiegatus* and *P. nitidus* (sequence divergence is approximately 11% – K2P distance), and between *P. carinatus* and *P. heterodermus* (sequence divergence is approximately 14%), as also found by Hughes (1985). Further studies, incorporating a wide range of continental samples of *Philothamnus* snakes, are needed to evaluate this topic.

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