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Phylogenetic relationships of *Hemidactylus* geckos from the Gulf of Guinea islands: patterns of natural colonizations and anthropogenic introductions estimated from mitochondrial and nuclear DNA sequences

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Abstract

Mitochondrial DNA (12S rRNA, 16S rRNA, and cytochrome *b*) sequences and nuclear sequences (C-*mos* and α-Enolase) were analyzed within all known *Hemidactylus* species from all three volcanic islands in the Gulf of Guinea that have never been connected to the continent. These comprise both endemic and widespread species. Our aim was to determine if the widespread species was introduced anthropogenically, to determine the number of distinct genetic lineages within the islands, and to determine if the endemic forms constituted a monophyletic group. Our results suggest that a previously undescribed species on São Tomé is the sister taxon to *Hemidactylus newtoni*, endemic to Annobon. Genetic variation between populations of *Hemidactylus greefii* from São Tomé and Principe is very high based on mtDNA sequences, but the forms cannot be distinguished using the nuclear DNA sequences. *Hemidactylus mabouia* appears to have been anthropogenically introduced to all three islands. The island endemics do not form a monophyletic group, suggesting multiple independent colonizations of the islands.

Keywords: 12S rRNA; 16S rRNA; C-*mos*; Enolase; *Hemidactylus*; São Tomé; Principe; Annobon

1. Introduction

The forests of West Africa, including the islands of the Gulf of Guinea (Fig. 1) comprise one of the world's biodiversity hotspots (Myers et al., 2000). The volcanic chain was formed during the middle to late Tertiary. Bioko (formerly Fernando Po) is the largest and closest to Africa, only about 32 km from Cameroon. Smaller and more geographically isolated are São Tomé and Principe (1001 km^2 combined), that include a number of small islets, and 160 km southwest of São Tomé, Annobon (17 km^2). While Bioko was connected to the continent during sea-level fluctuations in the last glacial periods, the other islands have never been connected and are separated by deep-sea trenches. Thus while the herpetofauna of Bioko is essentially continental in nature, the remaining islands harbor far fewer species but far more endemics. Oldest geological dates for Principe, São Tomé, and Annobon are 31, 14, and 4.8 my, respectively (Lee et al., 1994).

Although many phylogenetic studies have been performed on the herpetofauna of the more northern Atlantic volcanic islands, such as the Cape Verdes (Carranza et al., 2001; Jesus et al., 2001, 2002a), Canary

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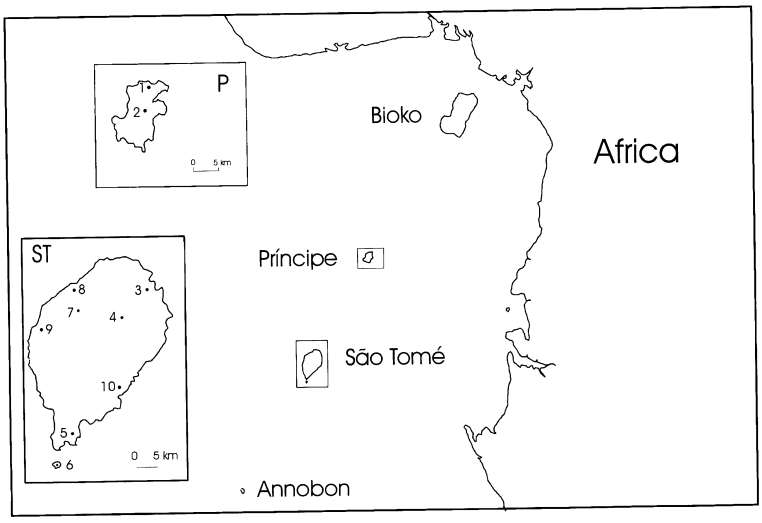

Fig. 1. Map showing the sampling localities of *Hemidactylus* from the Gulf of Guinea. The Cape Verde islands are also located off the West Coast of Africa, but over 2000 km to the North. Localities of *H. bouvieri* and *H. brookii*, for which 12S rRNA sequences were already published but additional sequences were generated, are given in Jesus et al. (2001).

Islands (Carranza et al., 2000; Thrope et al., 1994), and Madeiran archipelago (Brehm et al., 2003), very little is known about the herpetofauna of the islands of the Gulf of Guinea. This is especially true of the geckos *Hemidactylus*, a genus of over 80 morphologically similar species found across Africa, Asia, and South America. Often commensal, they have been repeatedly translocated by humans, as shown by the recent report of an introduced population of *Hemidactylus mabouia* on Madeira (Jesus et al., 2002b). An extensive revision of *Hemidactylus* from Madagascar and the Indian Ocean islands indicated a complex pattern of anthropogenic introductions and natural colonizations (Vences et al., 2004). These introductions have serious conservation implications—in the Mascarene islands *Nactus* geckos have possibly been eliminated from some islands by introduced *Hemidactylus frenatus* (Arnold, 2000). However, no broad-scale phylogeny for *Hemidactylus* is available. In this paper, we attempt to unravel relationships of *Hemidactylus* from the Gulf of Guinea islands, including both endemic (*Hemidactylus newtoni* and *Hemidactylus greefii*) and the widespread species (*H. mabouia*). Using both mitochondrial and nuclear DNA sequences we aim to (a) distinguish natural island colonizations from recent anthropogenic introductions, (b) determine the number of genetically distinct lineages on the islands, and (c) determine the relationship of all known island species to other *Hemidactylus* species.

2. Materials and methods

The number and geographic locations of the specimens used in this study are given in Table 1 and Fig. 1. Total genomic DNA was extracted from small pieces of tail using standard methods (Sambrook et al., 1989). Primers used in both amplification and sequencing of mitochondrial DNA were 16SL and 16SH, 12Sa and 12Sb, and cytochrome *b*1 and 3 from Kocher et al. (1989). Amplification conditions were the same as described by Harris et al. (1998). Primers used to amplify a fragment of the nuclear gene C-*mos* were G73 and G74, and were used following the conditions given in Saint et al. (1998). C-*mos* sequences have been widely used to infer relationships at many levels within geckos (e.g. Austin et al., 2004; Carranza et al., 2002; Harris et al., 2004a,b). α-Enolase is an enzyme involved in glycosis. The primers used (Enol L731 and H912; Friesen et al., 1997) amplify intron eight, and small parts of exons eight and nine. In a recent study this region was more variable than C-*mos* within skinks, and within a single genus, *Scelotes* (Whiting et al., 2003). Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus. Sequences were aligned using Clustal W (Thompson et al, 1994). Two loop regions of the 16S rRNA fragment (totaling 53 bp) could not be unambiguously aligned, and were excluded from further analyses. Initially we sequenced all 53 *Hemidactylus* samples from the Gulf of Guinea for the fragment of 12S rRNA and compared this to eight published sequences—*H. frenatus* (Whiting et al., 2003), *H. mabouia* from Madeira (Jesus et al., 2002b) and the Cape Verdes (Jesus et al., 2001), *H. brooki* from Guinea and the Cape Verdes (Jesus et al., 2001), and three *H. bouvieri* from the Cape Verdes (Jesus et al., 2001). All *H. mabouia* were identical for this marker. To confirm that the *H. mabouia* showed very low genetic variation we sequenced three individuals from each island for 700 bp of the faster evolving gene cytochrome *b*. We then

Table 1
Specimens used in this study

Species	Locality	Code
Hemidactylus mabouia	São Nicolau—ST4	726
Hemidactylus mabouia	São Nicolau—ST4	554
Hemidactylus mabouia	São Nicolau—ST4	555
Hemidactylus mabouia	São Nicolau—ST4	556
Hemidactylus mabouia	São Nicolau—ST4	728
Hemidactylus mabouia	São Nicolau—ST4	732
Hemidactylus mabouia	São Nicolau—ST4	737
Hemidactylus mabouia	São Nicolau—ST4	739
Hemidactylus mabouia	São Nicolau—ST4	740
Hemidactylus mabouia	São Nicolau—ST4	741
Hemidactylus mabouia	São Nicolau—ST4	742
Hemidactylus mabouia	São Nicolau—ST4	743
Hemidactylus mabouia	São Nicolau—ST4	744
Hemidactylus mabouia	São Nicolau—ST4	745
Hemidactylus mabouia	São Nicolau—ST4	746
Hemidactylus mabouia	São Nicolau—ST4	748
Hemidactylus mabouia	Santa Catarina—ST9	747
Hemidactylus mabouia	Monte Mário—ST5	533
Hemidactylus mabouia	Monte Mário—ST5	534
Hemidactylus mabouia	Monte Mário—ST5	735
Hemidactylus mabouia	Neves—ST8	549
Hemidactylus mabouia	Neves—ST8	550
Hemidactylus mabouia	Neves—ST8	551
Hemidactylus mabouia	Neves—ST8	544
Hemidactylus mabouia	Neves—ST8	545
Hemidactylus mabouia	Cavalete—ST10	709
Hemidactylus mabouia	Cavalete—ST10	773
Hemidactylus mabouia	São Tomé—ST3	723
Hemidactylus mabouia	São Tomé—ST3	730
Hemidactylus mabouia	Ilhéu das Rolas—ST6	557
Hemidactylus mabouia	Ilhéu das Rolas—ST6	558
Hemidactylus mabouia	Ponta do Sol—P1	753
Hemidactylus mabouia	Ponta do Sol—P1	754
Hemidactylus mabouia	Ponta do Sol—P1	755
Hemidactylus mabouia	Annobon	668
Hemidactylus mabouia	Annobon	669
Hemidactylus mabouia	Annobon	670
Hemidactylus newtoni	Annobon	667
Undescribed species	São Nicolau—ST4	722
Hemidactylus greefii	Vale do Contador—ST7	569
Hemidactylus greefii	Vale do Contador—ST7	571
Hemidactylus greefii	Nova Estrela—P2	590
Hemidactylus greefii	Nova Estrela—P2	591
Hemidactylus greefii	Nova Estrela—P2	597
Hemidactylus greefii	Nova Estrela—P2	598
Hemidactylus greefii	Nova Estrela—P2	701
Hemidactylus greefii	Nova Estrela—P2	702
Hemidactylus greefii	Nova Estrela—P2	703
Hemidactylus greefii	Nova Estrela—P2	704
Hemidactylus mabouia	Nova Estrela—P2	705
Hemidactylus greefii	Nova Estrela—P2	706
Hemidactylus greefii	Nova Estrela—P2	717
Hemidactylus greefii	Nova Estrela—P2	718
Hemidactylu bouvieri	Boavista—Sal Rei—Cabo Verde Islands	CV125
Hemidactylu bouvieri	Boavista—Sal Rei—Cabo Verde Islands	CV38
Hemidactylus bouvieri	Sal—Cabo Verde	
Hemidactylus brookii	Bissau—Guiné	782
Hemidactylus brookii	Santo Antão—Cabo Verde	HB38

Localities refer to Fig. 1. Codes refer to voucher specimens and to Fig. 2.

sequenced all the endemic *Hemidactylus* species, five individuals from two outgroup species (*H. brookii* and *H. bouvieri*) and at least three *H. mabouia* from each of the islands for a 500 bp fragment of the 16S rRNA. We used these combined 12S rRNA and 16S rRNA sequences for 37 taxa for our phylogenetic analyses. C-*mos* sequences were collected from specimens from all of the genetically distinct mtDNA lineages, five outgroups (two *H. brookii* and three *H. bouvieri*), and aligned against a published sequence of *H. frenatus* (Whiting et al., 2003). In total 20 sequences of 338 bp were included in the analyses. Since the intron of α-Enolase has been shown to evolve faster than C-*mos* in many reptiles (Whiting et al., 2003) we also sequenced nine *H. greefii* and seven *H. mabouia* for this marker. We failed to amplify *H. newtoni*.

Mitochondrial DNA sequences were imported into PAUP* 4.0b10 (Swofford, 2003) for phylogenetic analysis. For the phylogenetic analysis of the combined data, we used maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference. When estimating phylogenetic relationships among sequences, one assumes a model of evolution. We used the approach outlined by Hulsenbeck and Crandall, 1997 to test 56 alternative models of evolution, employing PAUP* 4.0b10 and Modeltest (Posada and Crandall (1998) described in detail in Posada and Crandall (2001)). Once a model of evolution was chosen, it was used to estimate a tree using ML (Felsenstein, 1981) with random sequence addition (10 replicate heuristic search). The MP analysis was also performed with random sequence addition (100 replicate heuristic search), and support for nodes was estimated using the nonparametric bootstrap technique (Felsenstein, 1985) with 1000 replicates. The Bayesian analysis was implemented using MrBayes (Huelsenbeck and Ronquist, 2001), which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) sampling approach. Bayesian analyses were conducted with random starting trees, run 0.5×10^6 generations, and sampled every 100 generations using a general-time-reversible model of evolution with a gamma model of among-site rate variation. In both searches, stationarity of the Markov chain was determined as the point when sampled ln-likelihood values plotted against generation time reached a stable mean equilibrium value; "burn-in" data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a summary phylogeny. Two independent replicates were conducted and inspected for consistency to check for local optima (Huelsenbeck and Bollback, 2001). New sequences from C-*mos* were aligned against *H. frenatus*. There were no indels. Because variation is low, the sequences were joined in a median network (Bandelt et al., 2000). Similarly for the sequences of α-Enolase, variation was low

within *Hemidactylus*, so sequences were joined in a network. A single base pair insertion was needed to align the sequences.

3. Results

For the combined 12S rRNA and 16S rRNA gene fragments, 37 taxa were included for a total of 930 base pairs; ML, MP, and Bayesian analyses gave identical estimates of relationships (Fig. 2). *H. frenatus* was used to root the trees. The most appropriate model for the combined data was the GTR model with an estimate of invariable sites (0.50) and a discreet approximation of the gamma distribution (0.70). The ML heuristic search using this model found a single tree of −Ln 3151. Bayesian analysis produced an identical estimate of relationships. For MP 201 characters were informative, and the MP search found one tree of 434 steps (Fig. 2). In all analyses five clades, all with 100% Bayesian support, can be identified. The species *H. bouvieri*, *H. brookii*, and *H. mabouia* are all monophyletic units. *H. bouvieri* shows differentiation between samples from the two Cape Verde islands, Sal and Boavista. *H. greefii* is monophyletic, and specimens from São Tomé and Principe are also reciprocally monophyletic. These two islands show a considerable degree of genetic distinctiveness, with an average of 3.3% genetic divergence between them. The single sample of *H. newtoni* from Annobon is very distinct from any other samples, but it is clearly the sister taxon of an individual from an undescribed form from São Tomé. This form was noticeably darker and more robust than other specimens from São Tomé (unpublished data), but unfortunately it was the only sample of this kind that we collected. In all analyses *H. greefii* is the sister taxon to *H. bouvieri* from the Cape Verde islands and not to the other genetic lineages from the Gulf of Guinea islands.

In the combined analysis of 12S and 16S rRNA sequences, all *H. mabouia* from São Tomé (including the islet Rolas), Principe and Annobon are identical. Our sequences of 12S rRNA from additional samples of *H. mabouia* (Table 1) confirm that all of the samples from the islands had an identical haplotype that was also shared by individuals on Madeira and the Cape Verde islands. Sequences from the faster-evolving cytochrome *b* gene similarly showed no differences.

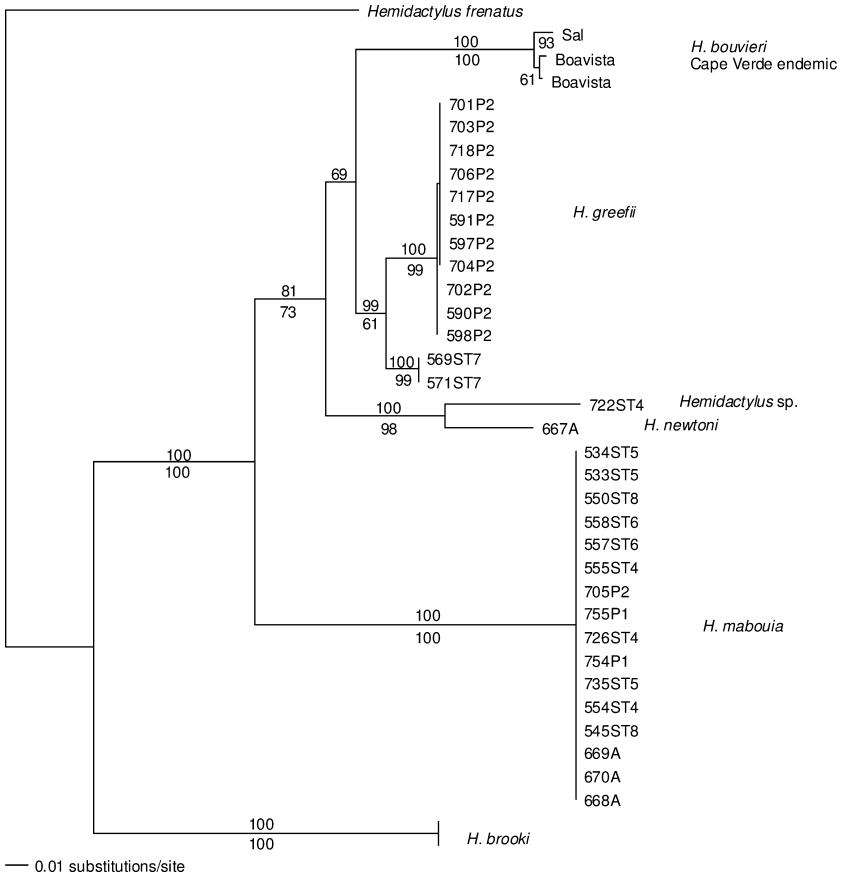

Fig. 2. Tree derived from a ML analysis of combined 12S and 16S rRNA fragments using the model described in the text. MP and Bayesian analyses gave identical estimates of relationships. Bootstrap values (>50%) for MP are given below the nodes, and Bayesian probabilities are given above the nodes. The tree was rooted using *H. frenatus*.

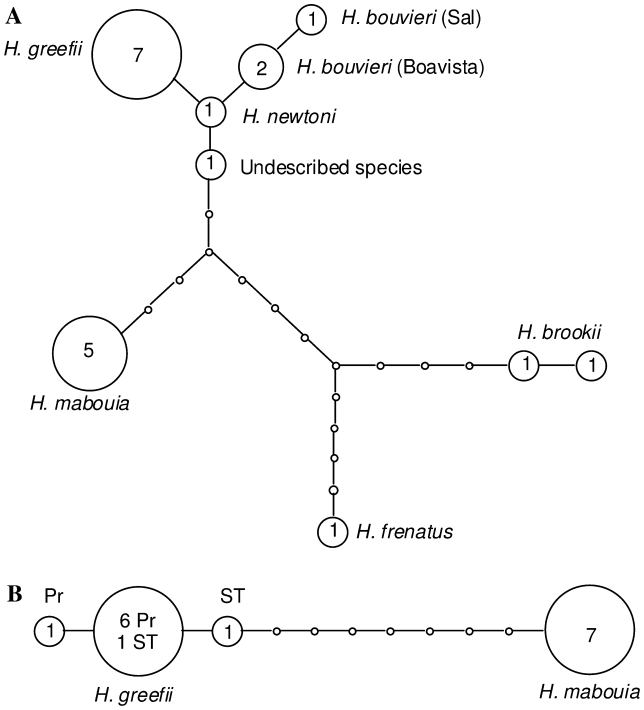

Fig. 3. Median networks showing relationships derived from partial sequences of C-*mos* (A) and α-Enolase (B).

Our analyses of variation in C-*mos* nuclear DNA sequences is quite similar to our estimate of relationships derived from mtDNA (Fig. 3). *H. greefii*, *H. newtoni*, and the undescribed lineage from São Tomé all have unique haplotypes. *H. bouvieri* is closely related to these, while *H. mabouia*, *H. brookii*, and *H. frenatus* are more genetically differentiated. Despite the high mtDNA differentiation between *H. greefii* populations from São Tomé and Principe, they all share a single haplotype for C-*mos*. A similar case has been shown in *Phelsuma* geckos in the Mascarene islands (Austin et al., 2004). This result could be due to the slowly evolving nature of this region of the C-*mos* gene. However, we obtained the same result with the faster evolving α-Enolase sequences (Fig. 3).

4. Discussion

Due to the known ease with which many *Hemidactylus* species are anthropogenically transported, it is often difficult to distinguish natural populations from introductions (Vences et al., 2004). The morphological conservativeness of some widespread forms further challenges taxonomists, such that *H. brookii*, *H. mabouia*, and *H. frenatus* are often confused (Vences et al., 2004). The molecular data presented here clearly separate these forms. The complete lack of genetic variation with these markers within *H. mabouia* from islands as geographically separate as São Tomé, the Cape Verdes and Madeira, however, strongly indicate a recent anthropogenic introduction to all these islands. This conclusion is reinforced by the considerable genetic diversity revealed within island endemics, such as *H. greefii* and *H. bouvieri*.

Hemidactylus greefii populations from Principe and São Tomé are apparently monophyletic groups with respect to the mtDNA sequences. Differentiation between them is higher that that reported between *Phelsuma* lineages that appear to be distinct species (Austin et al., 2004). However, since we did not obtain any differentiation in two nuclear genes, we recommend maintaining the current taxonomy pending a more detailed morphological analysis. Sequences from α-Enolase showed variation within *H. greefii*, while those from C-*mos* did not. Unfortunately we could not amplify this part of α-Enolase for *H. newtoni*, so we could not use this marker in a more detailed phylogenetic analysis. Similarly Whiting et al. (2004) failed to amplify *H. frenatus*. However, it has been shown to be useful within *Scelotes* skinks (Whiting et al., 2004), and it may be a useful nuclear marker at lower taxonomic levels where C-mos is often uninformative.

Hemidactylus newtoni from Annobon is clearly a distinct species, endemic to this tiny island. Its sister taxon appears to be an undescribed species from São Tomé, from which it can be distinguished by mtDNA and nuclear C-*mos* sequences. The degree of divergence between these groups (21% for the region of cytochrome *b* sequenced) far exceeds that typically observed between reptile species (Harris, 2002). Our observations on Annobon suggest that introduced *H. mabouia* is now much more common than *H. newtonii*, and that both species share similar habitats (Jesus et al., 2003). This situation deserves careful monitoring.

None of our analyses suggest that the endemic *Hemidactylus* from these islands form a monophyletic unit. This result implies that the islands were colonized independently at least twice. One lineage from São Tomé presumably then colonized Annobon to give rise to *H. newtoni*.

5. Conclusions

Our study again highlights the extraordinarily high genetic diversity revealed within morphologically conservative gecko species (e.g., Austin et al., 2004; Harris et al., 2004a,b). Similar, widespread species such as *H. brookii*, *H. frenatus*, and *H. mabouia*, which are often mistaken for each other in the field, can be clearly differentiated. *H. mabouia* has been introduced to all three islands of the Gulf of Guinea, which has important conservation implications. The island endemics *H. greefii* and *H. newtoni* are genetically distinct lineages, suggesting a long evolutionary history on the islands. An additional undescribed species exists on São Tomé.

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