

Phylogeography of Seychelles' endemic skink genera *Pamelaescincus* and *Janetaescincus*

Joana Valente

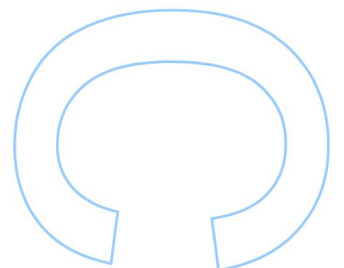
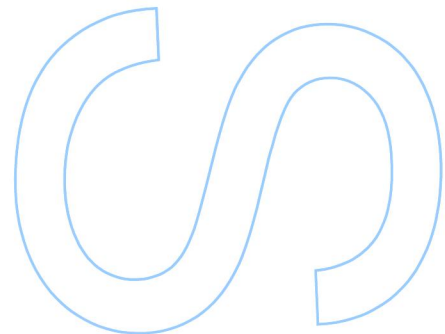
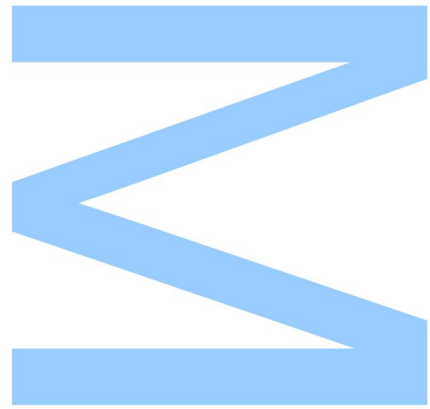
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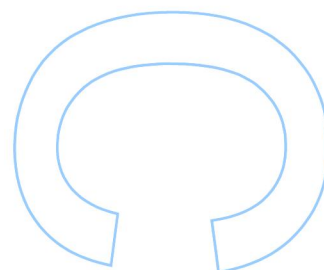
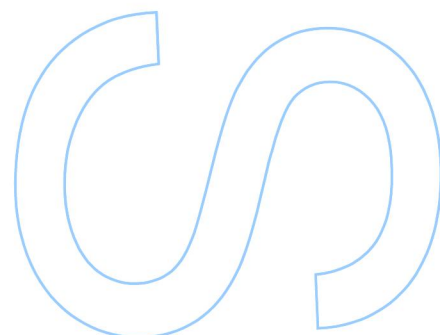
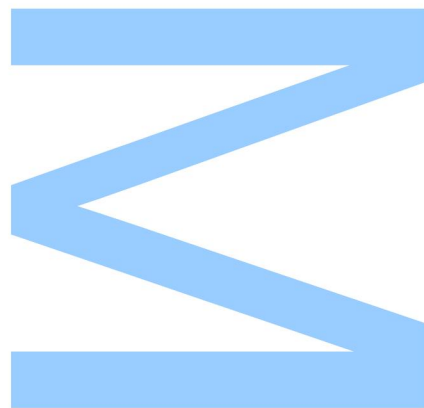




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Summary

The Seychelles is a diverse group of islands with a spectacular endemic fauna, particularly of amphibians and reptiles. Although the granitic islands are 65 million years old, and have been isolated several times for long periods in its history, many endemic species are currently considered to be widespread – there are many archipelago endemics, but few island endemics. However, recent studies on some groups (reptiles, amphibians, arthropods) have uncovered structured geographic patterns and considerable cryptic diversity within some species. There is thus a need to further reassess the molecular, morphological and ecological diversity within other endemic groups.

The burrowing skinks (genera *Pamelaescincus* and *Janetaescincus*) are particularly interesting, as they are an ancient endemic lineage but are poorly known due to their secretive lifestyles. In this study, evolutionary history and phylogeography of these skinks are assessed through mitochondrial (Cyt-b) and nuclear molecular markers (*c-mos* and MC1R).

Deep and cryptic differentiation was found in both groups: two highly divergent clades within *Pamelaescincus* genus, with a northern-southern geographic structure and four highly divergent clades within *Janetaescincus*, where the occurrence of hybridization and introgression was also detected. *Janetaescincus* was also notable in that highly divergent lineages were sometimes found in the same small islands.

A preliminary assessment of morphologic variation was conducted with *Pamelaescincus* and *Janetaescincus* specimens of the Natural History Museum, London's collection. However, due to the reduced sampling, conclusions were limited.

More data is needed to be collected for both groups, prior to a reassessment of their taxonomy.

Resumo

As Seychelles são um grupo de ilhas geológica e climaticamente diverso, cuja fauna tem uma alta proporção de endemismos. Apesar de suas as ilhas graníticas terem 65 milhões de anos, e terem estado isoladas por longos períodos, várias vezes na sua história, muitas espécies endémicas hoje consideram-se amplamente distribuídas pelo arquipélago – i.e., existem muitas espécies endémicas no arquipélago, mas poucas endémicas entre ilhas. No entanto, estudos recentes em alguns grupos (répteis, anfíbios, artrópodes) mostraram padrões geográficos estruturados e uma considerável diversidade críptica em algumas espécies. Há portanto, a necessidade de averiguar a diversidade molecular, morfológica e ecológica noutros grupos endémicos para reavaliar a sua taxonomia.

Os escincídeos dos géneros *Pamelaescincus* e *Janetaescincus* são particularmente interessantes por serem uma linhagem endémica antiga, mas pouco conhecidos devido a serem espécies escavadoras, de comportamento bastante críptico. Neste estudo, a história evolutiva e filogeografia destes escincídeos foi estudada através de marcadores mitocondriais (Cyt-b) e nucleares (*c-mos* e MC1R).

Diferenciação críptica profunda foi encontrada nos dois grupos: duas linhagens consideravelmente divergentes no género *Pamelaescincus*, com uma estrutura geográfica norte-sul e quatro linhagens também muito divergentes género *Janetaescincus*, onde foi detectada a ocorrência de hibridização e introgressão. Também de notar que linhagens altamente divergentes dentro do género *Janetaescincus* se encontram em simpatria em algumas ilhas. Foi conduzido uma avaliação preliminar sobre as variações morfológicas dos espécimes de *Pamelaescincus* e *Janetaescincus* da coleção do Museu de História Natural de Londres. No entanto, devido à reduzida amostragem, as conclusões são limitadas.

A recolha de mais dados para ambos os grupos é necessária, antes de qualquer revisão taxonómica.

Introduction

. Island Biogeography

Islands, being discrete, numerous and varied units are considered as natural laboratories for biologists, and ideal areas to study a wide range of organisms in a location with controlled conditions where theories and hypothesis can be more easily explored.

Continental fragments are islands that have a continental geological origin and differ from oceanic islands, that are characterized by never been connected to the mainland since its origin. In terms of biota, continental islands are generally species-poor but harbour a great number of endemic species. For this reason, many of the continental islands contribute considerably to global biodiversity and are considered biodiversity 'hotspots'. The faster rate of abrasion of islands biotas by human action is an important concern and most of these islands are now qualified also as 'threatspots' (Whittaker & Fernández-Palacios 2007).

Islands geological origins are a critical feature to consider when studying insular biota. The extant fauna and flora in a given island depends on its geological origins and on the natural events that occurred in it over time. For example, in continental islands, such as Madagascar, New Caledonia, and the Seychelles, when the tectonic drift led to separation from the mainland, existing species accompanied this process and moved as well. The extant fauna and flora of a "continental fragment" is thus defined by a mixture of ancient lineages, recent lineages resulting of their diversification into new groups of species, and also other recent lineages resulting from post-vicariant colonisations (Yoder & Nowak 2006; Agnarsson & Kuntner 2012).

The goal of phylogeography is to understand species distribution and diversity (Avice 2000). This is essential information to understand the diversity and evolutionary history of any species, and is particularly important in island taxa with high conservation status and small and fragmented distributions.

. The Seychelles Islands

The Western Indian Ocean archipelagos of Madagascar, Mauritius, Comoros and Seychelles, harbour a great number of endemic organisms (Mittermeier *et al.* 2005). The Seychelles, which are composed by islands of diverse geological origins, from coral to continental, offer an ideal setting for studying organisms' evolution. The islands with a continental origin, usually referred to as the granitic group, are approximately 40 and are situated on a vast undersea shallow shelf (Fig. 1). Initially located between the Madagascar and India platforms, these islands became completely isolated approximately 65 million years ago (Mya) (Plummer & Belle 1995).



Figure 1. Map of the Granitic Seychelles Islands. Different shadings show areas that would have emerged at -30m (dark grey) and -50m (light grey) below present sea-level stands.

Sea level changes (Fig. 2), particularly during the Pleistocene, should have had a profound effect on these islands, as lower sea levels would have greatly enlarged terrestrial areas and linked the currently isolated islands (Siddall *et al.* 2003) (Fig. 1).

Particularly between the Seychelles and the Mascarene Islands, other now submerged regions would have been extensive landmasses, possibly acting as "stepping stones" for faunal interchange (Warren *et al.* 2010). Like most islands, especially those that are both old and geographically isolated, the Seychelles are rich in endemics.

The Seychelles biota derives from Afro-Malagasy and Oriental species (Warren *et al.* 2010). The reptiles show similar patterns: the endemic skink genera (*Pamelaescincus* and *Janetaescincus*) are sister-taxa to all remaining Afro-Malagasy "scincines", and possibly related to Indian and/or Sri Lankan groups (Brandley *et al.* 2005); the Seychelles' wolf snake is related to Ethiopian and Oriental natricines (Dowling 1990; Vidal *et al.* 2008); and the endemic *Ailuronyx* genus and *Urocotyledon inexpectata* are sister-taxa respectively to Afro-Malagasy and Afro-Malagasy-Asian clades, without close relatives back almost to the origin of Gekkonidae *sensu stricto*, and thus with origins possibly going back to the Cretaceous (Aaron Bauer, personal communication). Remaining taxa are almost all closely related to other Western Indian Ocean ones, in great majority Malagasy and African.

The diversification patterns within the granitic islands led to the consideration of biogeographical groups as: the islands of Mahé, Silhouette and surrounding islets versus the northern islands of Praslin and La Digue plus the surrounding islands; and Frégate is usually taken as an intermediate or isolated biogeographic unit (Cheke 1984; Radtkey 1996; Rocha 2010).

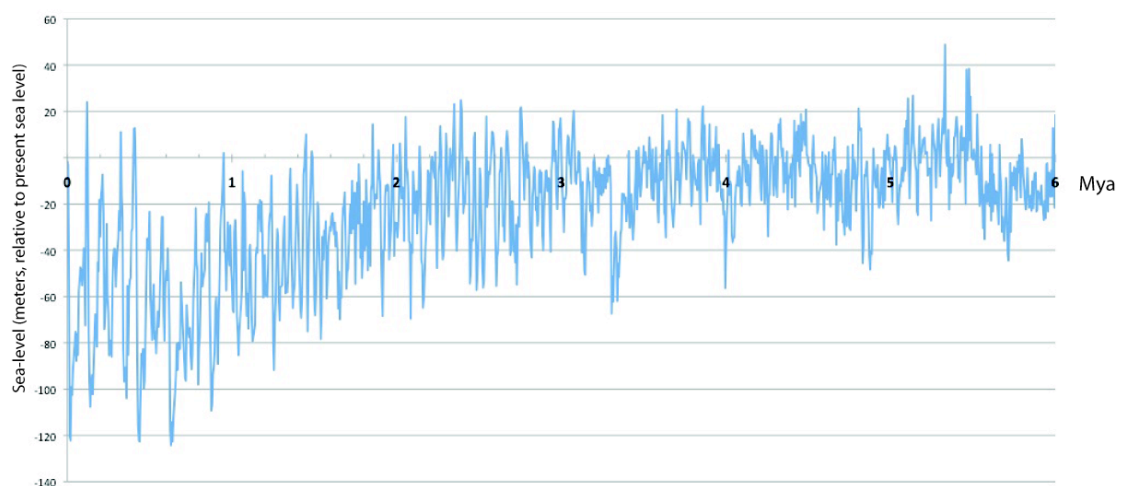


Figure 2. Global sea level estimate derived from $\delta^{18}\text{O}$ for the last 6 Myr – from Miller *et al.* (2005) supplementary Table S1.

. *Pamelaescincus* and *Janetaescincus*



Figure 3. *Pamelaescincus gardineri* and *Janetaescincus* spp. respectively (Rocha *et al.* 2009).

The two genera of burrowing skinks, *Pamelaescincus* and *Janetaescincus* belong to the family *Scincidae* Gray, 1825 that is present in a variety of habitats worldwide. These are sister-genera, endemic to the Seychelles Islands, and thought to be sister taxa to all Afro-Malagasy skinks (Pyron *et al.* 2013; Brandley *et al.* 2005).

Pamelaescincus is a monospecific genus (*P. gardineri*) and *Janetaescincus*' taxonomy is still uncertain, with either one or two species recognized (*J. braueri* and *J. veseyfitzgeraldi*). Until Greer's elevation of these two genera, these skinks were allocated to the *Scelotes* genus, designated as *Scelotes gardineri* and *Scelotes braueri* (Greer 1970). However, in 1984, Cheke was still referring to both genera as *Scelotes*, due to the new taxonomy not being fully established (Cheke 1984). Meanwhile, some inconsistencies were detected regarding the differences between *Janetaescincus*' two recognized species, which led to them being synonymised by some authors (Bowler 2006). The differences between the two *Janetaescincus* species are found in the general size (smaller in *J. veseyfitzgeraldi*) and in the rearrangement of head scales and colouration, being difficult to identify in the field (Gerlach 2007). According to the Gerlach 2007, *J. braueri* is restricted to Mahé and Silhouette, while *J. veseyfitzgeraldi* is found in most of the granitic islands: Mahé, Silhouette, Praslin, La Digue, Curieuse, Félicité and Frégate.

Little is known about the ecology of these two genera, particularly about *Janetaescincus*, probably due to its secretively lifestyle and small size. According to Gerlach (2007), this genus is restricted to the larger islands, usually at altitudes over 350 m altitude. This species is found under leaf litter and root mats, feeding on small invertebrates.

Pamelaescincus gardineri is known to occur in the islands of Mahé, La Digue, Praslin and Frégate, Cerf, Silhouette, Curieuse, Cousin, Aride, Round, Grande Soeur, reaching altitudes from sea level to 600 m (Gerlach 2007). Their habits are similar to those from

Janetaescincus, living in forests' leaf litter, feeding on small invertebrates (Gerlach 2007). Also according to this author, populations might be locally abundant. Diurnal activity of *P. gardineri* in the islands of Praslin, Mahé, La Digue and Frégate was suggested by Cheke (1984) to be due to nocturnal predators. On the other hand, high densities of the skink *Trachylepis seychellensis* on small seabird islands may push *P. gardineri* to a nocturnal niche (Evans & Evans 1980).

Only two previous studies provided molecular information about *P. gardineri*, *J. braueri* and *J. veseyfitzgeraldi*. These studies analysed both mitochondrial and nuclear fragments, in a broader phylogenetic context, positioning them as sister-genera to all other Afro-Malagasy scincines (Pyron *et al.* 2013; Brandley *et al.* 2005). Prior to this thesis, nothing was known about their intraspecific genetic variability.

. Molecular phylogeny

The use of molecular tools, particularly DNA, allows the analysis of high sample sizes, since sampling can be non-lethal (Beja-Pereira *et al.* 2009) and most importantly a large number of characters. This is particularly important when species are listed as endangered by the IUCN, as is the case of *Janetaescincus*.

In molecular phylogeny, the relationships between organisms or genes are studied by comparing homologous DNA or protein sequences. Dissimilarities among the sequences indicate genetic divergence as a result of molecular evolution during the course of time.

Phylogenetic analysis has the goal of reconstructing a phylogenetic tree (gene-tree or species-tree), which reflects the evolutionary history of the gene or species (often the first is equated to the second). Phylogenetic reconstruction include the parsimony method, various distance methods, maximum likelihood and Bayesian inference (Huelsenbeck & Ronquist 2001). The advantage of using maximum likelihood methods and Bayesian inference over distance and parsimony is the ability to use predefined models of evolution (Avice 2004). Yet, when one deals with biological data the exact tree is realistically impossible to get, only an approximate. To minimise precision errors, it is necessary to be cautious with the parameters or models that are applied in the different steps of the analysis. A good sample size with more than one individual from each morphotype is also essential for the accuracy of the resulting tree. To define the direction of the evolution, an outgroup is added to the data set, where the closest related group is the best choice (Graybeal 1998). Networks are the graphical representation of the different haplotypes present in the studied sequence and

the number of mutations separating them. These are particularly informative when there is minimal divergence between haplotypes, and can for example be used to visualise variation within clades identified from the previous phylogenetic analyses.

Mitochondrial DNA, specifically cytochrome-b gene (Cyt-b), that displays a set of useful properties, have highly contributed to phylogenetic and phylogeographic studies (Avice 2000; Kocher *et al.* 1989). However, since this only reflects the maternal lineage, it is recommended to also analyse variation within nuclear markers. In this thesis two nuclear markers were analysed. These were chosen due to the expected level of variability, availability of primers, and since they have been used in studies of other reptiles from the Seychelles (e.g. Rocha *et al.* 2011).

The *c-mos* gene is single-copy, without introns and is just over 1000 base pairs (Saint *et al.* 1998). The absence of repetitive elements in the sequence makes it a very liable gene to PCR amplification from genomic DNA. In Saint (1998), *c-mos*' primers for four reptile orders were described.

Melano-cortin 1 receptor gene (MC1R) is responsible for intraspecific colour variation in mammals and birds. Like *c-mos*, introns are absent which makes it a widely used nuclear marker. Pinho and colleagues (2009) described suitable primers to the amplification of this marker in Squamates.

Thesis aims

The primary aim of this study is to assess the genetic diversity within two endemic genera of burrowing skinks endemic from the Seychelles Islands (*Pamelaescincus* and *Janetaescincus*) using molecular tools including both mitochondrial and nuclear DNA sequence data.

Specifically, given that recent studies unveiled substantial geographic structure in co-distributed taxa, the study aims at: (1) exploring if *Pamelaescincus gardineri* demonstrates a geographical structure similar to other Seychellois taxa; (2) testing if the actual taxonomic categorization of *Janetaescincus* is appropriate and further investigate its intraspecific geographic structure; and (3) ascertaining age estimates for the *Janetaescincus* species divergence.

This thesis is composed by two articles: the first (in press) addresses the objective (1); and the second (in preparation), the objectives (2) and (3).

Article I

Differentiation within the endemic burrowing skink *Pamelaescincus gardineri*, across the Seychelles islands, assessed by mitochondrial and nuclear markers

Differentiation within *Pamelaescincus gardineri*

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Keywords

Seychelles; Phylogeography; Scincidae; *Pamelaescincus*; Cyt-b; MC1R; *c-mos*

Abstract

Unveiling patterns of genetic differentiation across insular distributions is relevant for biogeographic and conservation reasons. In the Indian Ocean, surprisingly, little is known regarding the genetic structure of many taxa across the Seychelles Islands, despite their importance as old Gondwanic islands, part of the Western Indian Ocean biodiversity hotspot. In recent molecular studies, a northeastern-southwestern subdivision pattern across the granitic islands has been uncovered within some species. *Pamelaescincus gardineri*, a Seychelles endemic skink and possibly one of the deepest lineages of Afro-Malagasy “scincines”, is another species widespread across these islands within which undescribed variation may occur. Both nuclear (*c-mos* and MC1R) and mitochondrial (Cyt-b) DNA data were used to address this issue. Mitochondrial DNA shows a marked

northeastern-southwestern structure of two highly divergent clades, similar to the pattern previously described in other reptile species. Nuclear DNA seems to corroborate this pattern, although these markers were much less informative. Migration between the two island groups was identified, and gene flow between the two mtDNA lineages is likely, the extent of which remains to be fully explored. This will require more variable nuclear markers and more detailed sampling across the island of Mahé. A suitable assessment of morphological variation is also needed prior to any taxonomic revision of this species. From a conservation point of view, however, these lineages should already be treated as two distinct evolutionary units.

Introduction

The Seychelles is a diverse group of islands located in the western Indian Ocean. They have different geological origins and are typically classified as granitic versus coralline islands. The granitic group consists of approximately 40 islands, situated on a vast undersea shallow shelf. Initially located between the Madagascar and India platforms, the islands finally became completely isolated approximately 65 million years ago (Mya) (Plummer & Belle 1995). Sea level changes, particularly during the Pleistocene, should have had a profound effect on these islands, as lower sea levels would have greatly enlarged terrestrial areas and linked the currently isolated islands (Fig. 1). Furthermore, other now submerged regions, particularly between the Seychelles and the Mascarene islands, would have been extensive landmasses, possibly acting as "stepping stones" for faunal interchange (Warren *et al.* 2010).

Like most islands, especially those that are both old and geographically isolated, the Seychelles are rich in endemics. Indeed, the Seychelles together with Madagascar and the other adjacent islands are one of the 34 "hotspots" defined by Conservation International in 2005 (Mittermeier *et al.* 2005). One of these endemics is the skink genus *Pamelaescincus* Greer, 1970 that together with its sister genus *Janetaescincus* Greer, 1970 are an endemic clade that may be sister taxa to all other Afro-Malagasy "scincines" (Brandley *et al.* 2005; Pyron *et al.* 2013), and thus constitute a particularly interesting lineage from biogeographical point of view.

Little is known about the species belonging to *Pamelaescincus* and *Janetaescincus*, probably due to their secretive lifestyle. *Pamelaescincus gardineri*, the lone species in this genus, lives under dead or rotting leaf-litter. It is known from many of the granitic islands, including the largest islands of Mahé, Praslin, La Digue, and also Silhouette, Grand Soeur, Frégate and Aride (Rocha *et al.* 2009). This species is classified as Least Concern in the

IUCN Red List, although there have been population reductions reported on Mahé and Praslin islands due to invasive species (Gerlach & Ineich 2006); its main predator seeming to be *Tenrec ecaudatus*, a hedgehog-like mammal that has been introduced from Mauritius. Other than the use of a couple of individuals to place the genus in higher level phylogenies (Brandley *et al.* 2005; Austin & Arnold 2006), there have been no studies assessing variation within the Seychelles, and particularly between the now isolated island populations. However, other studies of Seychelles fauna keep indicating strong phylogeographic structure (Rocha *et al.* 2013) and in some cases deep cryptic divergences (e.g. Rocha *et al.* 2011; Daniels 2011). Given the apparently old age of this genus (Pyron *et al.* 2013), such phylogeographic studies are clearly warranted.

Therefore, the aim of this study is to assess the phylogeographic history of *Pamelaescincus gardineri* throughout the Seychelles Archipelago using genetic data (nuclear and mitochondrial markers). We test whether the deep phylogeographic separation between the northeastern and the southwestern granitic islands, as found in the gecko *Urocotyledon inexpectata* (Rocha *et al.* 2011), also is recovered in this skink species. By using some of the same molecular markers, we can also determine if the degree of divergence between lineages is similar between these different groups.

Materials and Methods

We analysed 89 tissue samples (tail tips) that were collected in different field trips to the Seychelles from 2006 to 2011, covering approximately the whole of the species distribution range (Fig. 1), and stored in 100% ethanol. DNA extraction followed standard salt protocols (Kocher *et al.* 1989; Sambrook *et al.* 1989). All individuals were genotyped for a 670bp fragment of the mitochondrial cytochrome-b gene (Cyt-b), using the primers CBL14841 (Austin *et al.* 2004) and Cb3H (Palumbi *et al.* 1991). Standard polymerase chain reaction (PCR) conditions were carried out in total reaction volumes of 25 µl, following Rocha *et al.* (2011).

Subsets of these samples, selected based on the mtDNA haplotypes, were genotyped for two nuclear gene regions, melano-cortin 1 receptor (MC1R) and oocyte maturation factor MOS (*c-mos*) fragments (37 and 31 individuals respectively). The primers used to amplify MC1R were: MC1R F and MC1R R (Pinho *et al.* 2009), and the primers for *c-mos* were G74 and G73 (Saint *et al.* 1998). Amplifications were carried out as in Rocha *et al.* (2011) with minor adjustments in annealing temperatures when needed. PCR products were purified

and sequenced by a commercial facility (Macrogen, the Netherlands). To ensure that double peaks in the nuclear fragments were identified, they were sequenced for both strands. Alignment of sequences was conducted in Geneious Pro 5.6.3 (Drummond *et al.* 2011) and trimmed to 670bp in Cyt-b, 643bp in MC1R and 398bp in *c-mos*. Translation of protein coding regions was carried out to ensure that there were no stop codons for all analysed sequences including outgroups. Sequences were deposited in Genbank under accession numbers KF528161-KF528319. All samples, localities and accession numbers are given in Table 1.

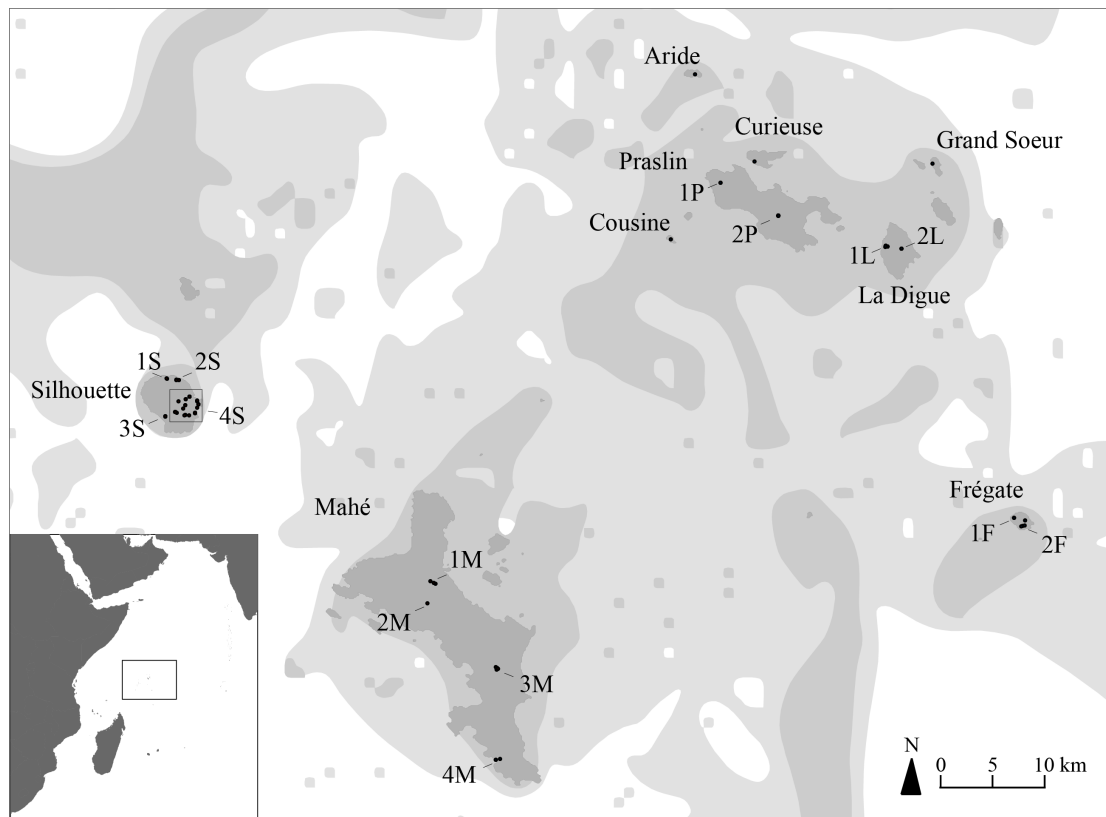


Figure 1. Map of the granitic Seychelles with sample localities. Black dots represent sampled individuals, while sampling localities are identified in Table 1. Different shadings show areas that would have emerged at -30m (dark grey) and -50m (light grey) below present sea-level stands.

The mitochondrial dataset was collapsed into haplotypes using ALTER (Glez-Peña *et al.* 2010) and jModeltest (Posada 2008) was used to select the best model of nucleotide substitution using the corrected Akaike Information Criteria (Posada & Buckley 2004) for the unpartitioned fragment, resulting in the use of the HKY+I model. The phylogenetic relationship between the haplotypes was estimated with MrBayes 3.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) under the best-fit model. Two runs of 11 million generations were performed and AWTY (Nylander *et al.* 2008) was used to assess

convergence and congruence across runs and to determine the adequate burnin. A maximum likelihood (ML) tree was also constructed in PhyML (Guindon *et al.* 2010), with support estimated using 10 000 bootstraps. As outgroup sequences we used data from two individuals of *Janetaescincus* spp. (Table 1), the closest known relative of *Pamelaescincus* (Brandley *et al.* 2005).

Median-joining (MJ) networks (Bandelt *et al.* 1999) with maximum-parsimony (MP) optimization (Polzin & Daneschmand 2003) were constructed with NETWORK v 4.6.1.1 (www.fluxus-engineering.com). Given the results from the phylogeny reconstruction (see below), we constructed two separate MJ networks for the main clades of the mitochondrial marker. Distances (uncorrected *p*-distance) were estimated using MEGA (Kumar *et al.* 2008).

Multiple runs of PHASE (Stephens *et al.* 2001) were conducted within DNAsp (Rozas *et al.* 2003) in order to determine the nuclear haplotypes. Results were congruent across runs and all the positions were resolved with posterior probabilities higher than 0.9 except for one single position in one sample (6561 in MC1R dataset) that had a posterior probability of 0.72; this position was coded as missing data (N) for the haplotype network. Alignment files, inferred haplotypes and phylogenetic estimates of relationships can be found in the dryad repository (<http://dx.doi.org/10.5061/dryad.9k19f>).

Results

Bayesian (BI) and ML trees were identical regarding major clades (Fig. 2). The mtDNA tree reveals two distinct clades within *Pamelaescincus gardineri*, highly distinct from *Janetaescincus* spp. (*p*-dist = 18%). Uncorrected *p*-distance between the two clades is 8.2%. One of the clades comprises all samples from the northeastern islands of Praslin, La Digue, Aride, Grand Soeur, Cousine, Curieuse plus Frégate – herein the "northeastern clade" – and also five samples from the island of Mahé. In the other clade there are only samples from the southwestern islands of Mahé and Silhouette – the "southwestern clade".

The northeastern Cyt-b clade has a roughly star-shaped network where La Digue and Cousine share the central haplotype with all samples from Frégate and Mahé (Fig. 2). In all cases, samples from Mahé belonging to this mtDNA clade (two haplotypes – five individuals) share haplotypes with Praslin and La Digue. These Mahé samples are widespread across the island, coming from the three different sampled areas. The highest number of differences detected in this network between haplotypes is seven, between samples from

Praslin and La Digue. The island of Aride shares its haplotype with Praslin, while Curieuse and Grand Soeur have haplotypes one mutation step away from the central one.

In the southwestern clade, Mahé and Silhouette do not share haplotypes and there are at least four substitutions between haplotypes from the two islands (Fig. 2). The highest number of mutations separating individuals within this group is 13.

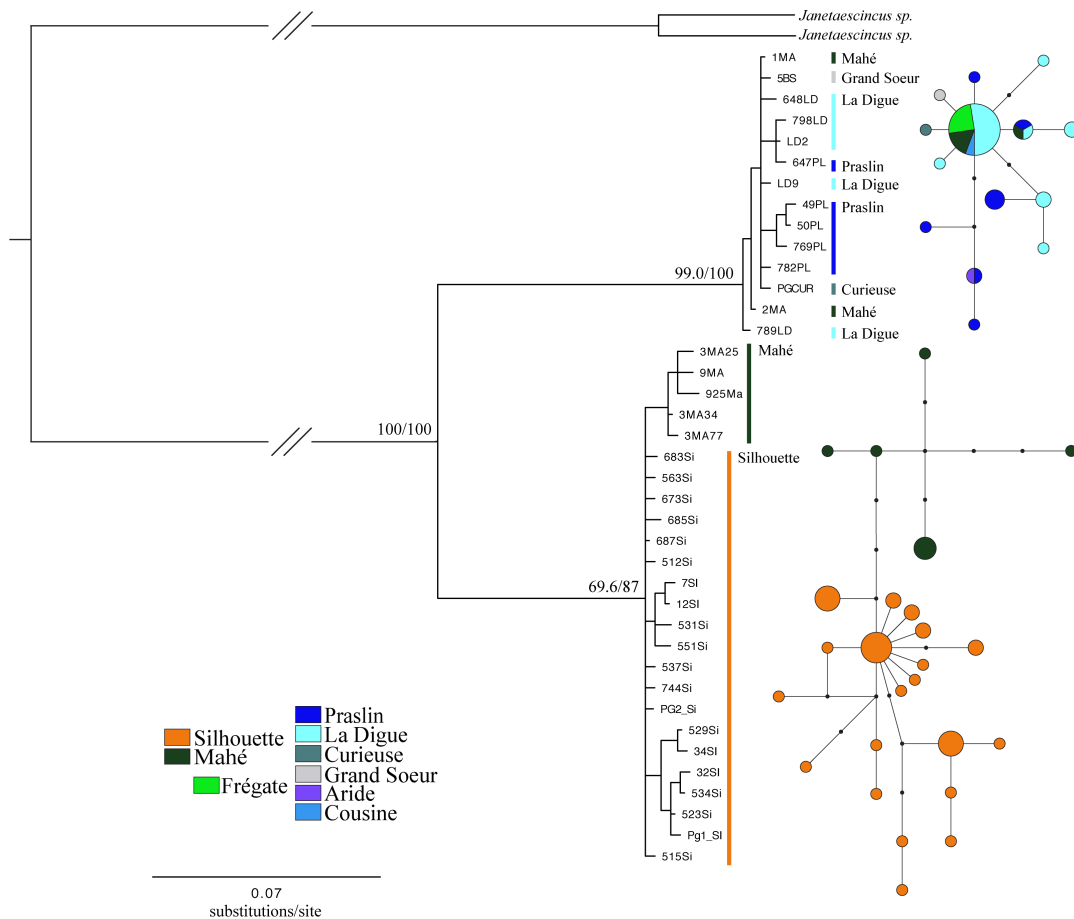


Figure 2. Bayesian Inference Cyt-b haplotype tree. Bootstraps support values from ML inference (BS) and posterior probabilities (PP) are shown only for main branches (above; BS/PP). The branch between the outgroup and the ingroup was shortened for visual representation. MJ networks for both clades (all individuals) are shown in front of each clade. Circle size is proportional to the number of individuals and full black circles represent missing haplotypes. Islands are color-coded. Scale bar represents nucleotide substitutions/site.

The networks derived from the nuclear fragments show less diversity, with MC1R being more variable than *c-mos* (Fig. 3). In the MC1R network a distinction between most of the haplotypes from the southwestern (Mahé and Silhouette) and northeastern islands is observed. Of the four genotyped individuals from Mahé, that for mtDNA grouped with the northeastern islands clade, three also exhibit MC1R haplotypes that otherwise are found

only in the northeastern islands. However, the two haplotypes from the remaining individuals are shared or closely related to other individuals from Mahé belonging to the southwestern mtDNA clade.

C-mos shows very little diversity, presenting only three haplotypes that are connected by one mutation steps respectively. Individuals from both mtDNA clades share the two most frequent haplotypes. The individuals from Mahé belonging to the northeastern mtDNA clade exhibit either the most frequent haplotype (shared with many northeastern islands individuals and almost all southwestern islands individuals), or a haplotype one mutation away from it.

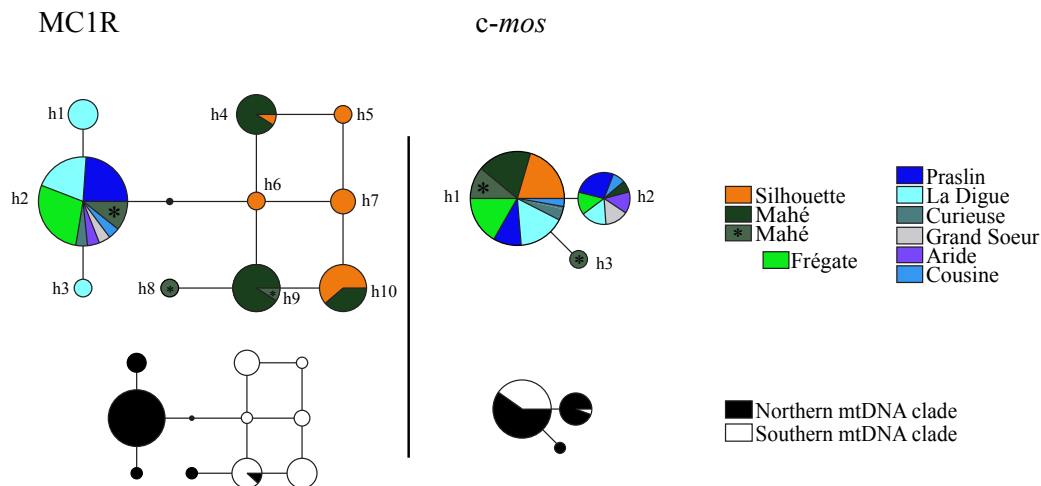


Figure 3. MJ networks from nuclear fragments (left; MC1R and right; *c-mos*). Haplotypes from individuals from Mahé that belong to mtDNA northern clade are marked with an asterisk. Circle size is proportional to the number of haplotypes and full black circles represent missing haplotypes. Islands are color-coded. Smaller (black and white) networks refer to the same data but with colour coding corresponding to mtDNA clades. Haplotype codes (h1-13) correspond to Table 1.

Discussion

Once again, the assessment of genetic diversity across the Seychelles archipelago reveals deep structure and diversity within a species. Similar to other reptile species (Rocha *et al.* 2011, 2013), *Pamelaescincus gardineri* comprises two highly divergent clades (p -distance 8.2%) that have broadly a northeastern and southwestern distribution within the studied islands. The level of differentiation between the two lineages is similar to that observed between the two main *Urocytyledon inexpectata* lineages at the same marker (9%;

Rocha *et al.* 2011), thus a similar Plio-Miocenic divergence between these is probable. As also is the case with *Urocotyledon inexpectata*, it appears that geographical distance was more important than depths between islands in shaping the distribution of lineages. Thus although Silhouette is the most isolated island in terms of sea depths, the distribution of the mtDNA diversity does not reflect this, but rather is associated with the geographical distance between the islands instead. The only exception is the situation in the southwestern island of Mahé where five samples collected (out of 13) group with the “northeastern” ones. This could be attributed to recent introductions (from the northeastern islands to Mahé), or to past connectivity and gene flow at times of lower sea levels, or to two distinct forms occurring on this island naturally, but based on the current data these alternative hypotheses cannot be distinguished.

In either scenario, the non-concordance between mtDNA and nuclear data, especially evident at MC1R (i.e., the fact that some individuals from Mahé harbouring “northeastern” mtDNA haplotypes now cluster within MC1R haplotypes from the southwestern islands) seems to indicate the existence of gene flow between the two mtDNA lineages in Mahé. Also, the fact that the individuals harbouring the “northeastern” mtDNA lineage in Mahé are widespread across the island does not seem to favour the hypothesis of punctual introductions.

In our study the samples from Frégate group with the “northeastern clade” whereas in most other taxa for which there is molecular data (Daniels 2011; Rocha *et al.* 2011, 2013), the Frégate samples group with those from Mahé. Based on sea levels between islands (Fig. 1), most of the northeastern islands would become connected by relatively limited drops in sea level. However, Frégate is both geographically distant in relative terms, and requires a greater reduction in sea levels before it is connected to other current islands. It may be therefore that while the other northeastern islands form a natural group, Frégate has been alternatively colonized by individuals from either the southeastern or northwestern island groups with no consistent pattern. Assessment of phylogenetic affinities of other organisms from Frégate may be useful to confirm this hypothesis.

Other than these two major clades, *Pamelaescincus* does not show deep inter-islands structure: most mtDNA haplotypes are shared within the northeastern islands (Fig. 2), although differentiation between Silhouette and Mahé is slightly higher, with the two islands not sharing haplotypes. This pattern is similar again to the one seen in *U. inexpectata* and is probably influenced by the fact that these two islands were not connected whenever minor sea level oscillations occurred, as there is a deep, although narrow, marine channel of more than 150m separating them. In the freshwater crab *S. alluaudi* for example it seems to have been a major barrier for dispersal, with the result that the deepest differentiation is actually

seen between Silhouette and remaining populations (Daniels 2011). Thus, although this barrier does not appear to have been associated with the primary subdivision within *Pamelaescincus*, it apparently still played some role in the phylogeographic partitioning of the species. Levels of variation within MC1R are similar to variation observed between for example subspecies of the day gecko *Phelsuma sundbergi* (Rocha *et al.* 2013). Between divergent mtDNA lineages of *U. inexpectata*, variation of levels within MC1R and within *c-mos* were similar, although in this case fixed differences occurred in *c-mos*, and only one haplotype was shared between lineages in MC1R. Thus, while *U. inexpectata* may well represent at least two distinct species, evidence is weaker for a species-level distinction within *P. gardineri*, despite the high degree of mtDNA divergence. Indeed the 8.2% between the two main clades is higher than between many recognized reptile species, although still less than the average between congeneric species (Harris 2002). Perhaps the greater dispersal ability of *P. gardineri* relative to *U. inexpectata*, which is extremely philopatric, meant that some, even if limited, gene flow occurred between island groups during periods of lower sea level.

Molecular assessments of fossorial skinks often identify “cryptic” forms, possibly because symplesiomorphic morphological characters in these taxa may be an obstacle to studies based on morphology (Daniels *et al.* 2006). Integrative studies of South African fossorial skinks have revealed various cryptic species (e.g. Daniels *et al.* 2009; Heideman *et al.* 2011), and such a study is clearly warranted for *Pamelaescincus*. To do this it would be necessary to analyse more variable nuclear markers as well as more samples, particularly from Mahé Island, in order to better understand the possibility and extent of gene flow between the two island groups. Furthermore, information about these two clades needs to be gathered from a morphological perspective, to determine if any differences exist between the northern and southern island groups, and, again, particularly to assess the situation on Mahé, from where both forms are known. This is important prior to any possible changes in the taxonomy of the species, although in terms of conservation management it is already clear that the two major clades should be treated as distinct evolutionary units. Given the high mtDNA diversity observed within both *U. inexpectata* and *P. gardineri*, it is also imperative to assess variation within other Seychellois endemic reptiles to determine if further undescribed diversity occurs on these unique islands.

Table 1. Samples used in this study, locations and accession numbers. Individuals from Mahé that belong to mtDNA northern clade are marked with an asterisk.

Code	Haplotype		Province	State	Locality code	Locality (GPS)		Cyt-b	c-mos	MC1R
	c-mos	MC1R				Latitude	Longitude			
1MA *		h2	Mahé		4M	-4,785728	55,501625	KF528163		KF528318
2MA *	h1/h3	h2	Mahé		4M	-4,785728	55,501625	KF528164	KF528282	KF528319
3MA80 *	h1	h8/h9	Mahé		1M	-4,638611	55,451158	KF528165	KF528281	KF528317
6919 *	h1		Mahé		3M	-4,708048	55,501543	KF528166	KF528280	
6924 *		h2	Mahé		4M	-4,784865	55,505238	KF528167		KF528316
6630	h1		La Digue		1L	-4,356753	55,827412	KF528169	KF528273	
6633	h1/h2		La Digue		1L	-4,356613	55,827508	KF528185	KF528272	
6646	h1/h2	h2	La Digue		1L	-4,356663	55,827495	KF528172	KF528275	KF528300
6648		h2	La Digue		1L	-4,356523	55,82751	KF528173		KF528301
6660		h1/h2	La Digue		1L	-4,357087	55,827522	KF528184		KF528302
6779			La Digue		1L	-4,3565	55,827522	KF528174		
6781	h1		La Digue		1L	-4,35733	55,827168	KF528175	KF528271	
6789		h2	La Digue		1L	-4,356732	55,827393	KF528182		KF528303
6795	h1	h1	La Digue		1L	-4,3567	55,827585	KF528176	KF528274	KF528304
LD5			La Digue		1L	-4,357142	55,828915	KF528179		
LD6			La Digue		1L	-4,357142	55,828915	KF528180		
LD9			La Digue		1L	-4,357142	55,828915	KF528187		
LD12			La Digue		1L	-4,357142	55,828915	KF528181		
5LD			La Digue		1L	-4,357142	55,828915	KF528170		
60LD			La Digue		2L	-4,358718	55,840623	KF528171		
78LD			La Digue		2L	-4,358718	55,840623	KF528177		
80LD			La Digue		2L	-4,358718	55,840623	KF528178		
LD2			La Digue		1L	-4,357142	55,828915	KF528186		
6666			La Digue		1L	-4,356628	55,827543	KF528188		
6798		h2	La Digue		1L	-4,35671	55,827453	KF528183		KF528305
5BS	h2	h2	Grand Soeur		-	-4,28773	55,866485	KF528168	KF528279	KF528314
6842	h1/h2	h2	Cousine		-	-4,351152	55,647827	KF528204	KF528278	KF528315
PGCUR	h1	h2	Curieuse		-	-4,286222	55,7177	KF528205	KF528276	KF528312
15ARD	h2	h2	Aride		-	-4,213183	55,667958	KF528197	KF528277	KF528313
6874	h1	h2	Frégate		1F	-4,583527	55,934632	KF528201	KF528264	KF528307
6891		h2	Frégate		1F	-4,583568	55,9346	KF528200		KF528308
6894	h1	h2	Frégate		2F	-4,590377	55,941905	KF528199	KF528266	KF528309
6895	h1/h2	h2	Frégate		2F	-4,590592	55,940863	KF528202	KF528262	KF528310
6896	h1/h2	h2	Frégate		2F	-4,590058	55,94363	KF528203	KF528265	KF528311
44FG	h1	h2	Frégate		1F	-4,585808	55,943872	KF528198	KF528263	KF528306
6659	h1		Praslin		2P	-4,331317	55,737718	KF528190	KF528267	
6782			Praslin		2P	-4,331232	55,737568	KF528195		
6647	h2	h2	Praslin		2P	-4,33138	55,737817	KF528189	KF528270	KF528295
6769			Praslin		2P	-4,331265	55,737653	KF528196		
49PL	h1	h2	Praslin		1P	-4,30394	55,68944	KF528192	KF528268	KF528296
50PL	h1/h2	h2	Praslin		1P	-4,30394	55,68944	KF528193	KF528269	KF528297
53PL		h2/h3	Praslin		2P	-4,331433	55,737617	KF528191		KF528298
54PL		h2	Praslin		2P	-4,331433	55,737617	KF528194		KF528299
6915	h1	h9	Mahé		3M	-4,71029	55,502258	KF528211	KF528259	KF528288
6917	h1	h4/h10	Mahé		3M	-4,709007	55,502783	KF528212	KF528260	KF528289
6925	h1	h9	Mahé		1M	-4,637935	55,449628	KF528213	KF528261	KF528290
9MA	h1/h2	h4	Mahé		1M	-4,636489	55,446989	KF528210	KF528257	KF528294
3MA25		h4/h9	Mahé		3M	-4,709614	55,5035	KF528206		KF528291
3MA34		h10	Mahé		3M	-4,709614	55,5035	KF528207		KF528292
3MA35	h1	h4/h9	Mahé		3M	-4,709614	55,5035	KF528208	KF528258	KF528293
3MA77			Mahé		2M	-4,65487	55,44441	KF528209		
6523			Silhouette		4S	-4,489428	55,242148	KF528244		
6529			Silhouette		4S	-4,482387	55,245582	KF528241		
6534	h1	h7/h10	Silhouette		2S	-4,468557	55,234663	KF528246	KF528253	KF528284
6543			Silhouette		1S	-4,46745	55,226948	KF528245		

6706		Silhouette	3S	-4,499018	55,225258	KF528247		
6763	h1	Silhouette	3S	-4,498913	55,225373	KF528256	KF528256	
Pg1SILH		Silhouette	4S	-4,482387	55,245582	KF528248		
32SILH		Silhouette	4S	-4,492272	55,240319	KF528243		
34SILH		Silhouette	4S	-4,4845	55,2426	KF528242		
38SILH		Silhouette	4S	-4,4845	55,2426	KF528250		
6512		Silhouette	4S	-4,496092	55,250205	KF528222		
6515		Silhouette	4S	-4,489548	55,24221	KF528251		
6526	h1 h7/h10	Silhouette	4S	-4,495203	55,233623	KF528227	KF528252	KF528283
6531		Silhouette	2S	-4,468112	55,234797	KF528225		
6537		Silhouette	2S	-4,4685	55,236922	KF528229		
6544		Silhouette	1S	-4,467237	55,226643	KF528215		
6551		Silhouette	2S	-4,468728	55,234708	KF528226		
6560		Silhouette	4S	-4,486195	55,236272	KF528231		
6561	h1 h5/h10	Silhouette	4S	-4,497903	55,245178	KF528216	KF528254	KF528285
6563	h1 h10	Silhouette	4S	-4,496142	55,250245	KF528218	KF528255	KF528286
6568		Silhouette	4S	-4,495873	55,2503	KF528233		
6673		Silhouette	4S	-4,497802	55,242202	KF528219		
6683		Silhouette	4S	-4,495792	55,250373	KF528214		
6684		Silhouette	4S	-4,495818	55,250158	KF528228		
6685		Silhouette	4S	-4,497828	55,24204	KF528220		
6687		Silhouette	4S	-4,497948	55,241338	KF528221		
6712		Silhouette	4S	-4,495938	55,235052	KF528235		
6718	h4/h6	Silhouette	4S	-4,49529	55,233417	KF528239		KF528287
6729		Silhouette	3S	-4,498818	55,225332	KF528240		
6744		Silhouette	4S	-4,495138	55,234083	KF528230		
7SILH		Silhouette	4S	-4,486283	55,251897	KF528223		
12SILH		Silhouette	4S	-4,491681	55,251931	KF528224		
PamGar1		Silhouette	4S	-4,482387	55,245582	KF528217		
PG2_Si		Silhouette	4S	-4,482387	55,245582	KF528232		
PgSilh		Silhouette	4S	-4,482387	55,245582	KF528234		
38709		Silhouette	4S	-4,48556	55,25222	KF528236		
38719		Silhouette	4S	-4,48889	55,25306	KF528237		
38720		Silhouette	4S	-4,48889	55,25306	KF528238		
6554		Silhouette	2S	-4,46853	55,23702	KF528161		
6821		La Digue	1L	-4,357035	55,82899	KF528162		

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Article II

Deep genetic differentiation within *Janetaescincus* spp. from the Seychelles Islands

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Keywords

Seychelles; Phylogeography; Scincidae; *Janetaescincus*; Cyt-b; MC1R, c-mos

Abstract

Genetic diversity within the burrowing skink genus *Janetaescincus*, endemic to the granitic Seychelles Islands, was assessed using mitochondrial and nuclear DNA sequences. Considerable diversity was recovered, with at least three groups distinguishable at a level of differentiation more typically observed between species. Even within small islands such as Silhouette and Frégate, multiple clades co-occur, and within Silhouette this seems at least partially related to altitude. Comparisons between markers indicate some apparent hybridization between clades. Clearly, more data, particularly regarding morphological variation, is needed to reassess the taxonomy of this group, which we recommend to be referred to as a species complex pending a thorough revision.

Introduction

Janetaescincus Greer 1970, is a genus of burrowing skinks (Reptilia; Scincidae) endemic to the Seychelles Islands (Fig. 1). They are leaf-litter burrowing species, found in forest habitats, apparently intolerant of much habitat disturbance (Gerlach 2008). Probably due to this secretive lifestyle, their distribution and ecology are still poorly known. In addition to skull morphological differences, this genus is easily distinguished from its sister-taxon *Pamelaescincus* by possessing only four fingers instead of five and a lower midbody scale number (Greer 1970). The main predators of *Janetaescincus* are the introduced tenrecs (*Tenrec ecaudatus*) and the endemic magpies (*Copsychus sechellarum*) (Cheke 1984).

The genus has an unstable taxonomic history, and although two species are currently recognized, these have often been synonymised and resurrected (Bowler 2006) and in the field they are very difficult to distinguish (Gerlach 2008). Both species, *Janetaescincus veseifitzgeraldi* (Parker, 1947) and *Janetaescincus braueri* (Boettger, 1896), are listed as endangered in the IUCN redlist (Gerlach and Ineich 2006). Gerlach (2008) considers *J. braueri* as apparently restricted to the high forests of the islands of Mahé and Silhouette, and *J. veseifitzgeraldi* as a lowland species that occurs below 500m on these and several other islands (Curieuse, Félicité, La Digue and Frégate). However, this author also states that the two species are indistinguishable without a detailed examination and that their distributions have not been clearly elucidated.

Janetaescincus, together with its sister genus *Pamelaescincus* Greer, 1970 form possibly one of the oldest endemic vertebrate clades existing in the Seychelles, as they are sister taxa to all other Afro-Malagasy “scincines” (Pyron *et al.* 2013). Furthermore, some recent molecular studies unveiled greater diversity within Seychelles' fauna than it was previously expected, with many taxa exhibiting deeply divergent lineages, whose ecology and morphology are not properly studied, but that may eventually correspond to different species (Daniels *et al.* 2006; Rocha *et al.* 2011; van der Meijden *et al.* 2007; Valente *et al.* 2013), and that are certainly evolutionary lineages with conservation interest, and important to be characterized (Taylor *et al.* 2012). Additionally, skinks, particularly burrowing ones, are one of the reptile groups with the mostly poorly-known taxonomy, and where the use of molecular tools have often revealed the existence of cryptic species and unexpected estimates of phylogenetic relationships (Crottini *et al.* 2009; Daniels *et al.* 2009).

The aim of this study was therefore to assess the phylogeographic structure of *Janetaescincus* spp. across the Seychelles archipelago using multiple molecular markers.

Specifically, we address how many distinct genetic lineages constitute this genus and how their genetic diversity is distributed throughout the Seychelles archipelago.



Figure 1. Map of the granitic Seychelles with sample localities. Different shadings show areas that would have emerged at -30m (dark grey) and -50m (light grey) below present sea-level stands.

Material and Methods

Tail tips from 75 individuals were collected between 2008 and 2011 during several field trips to the Seychelles. Samples approximately covered the whole species range and included the islands of Silhouette, Mahé, Frégate, La Digue, Praslin and Curieuse (Fig. 1). Samples were stored in 100% ethanol. DNA extraction followed standard salt or phenol-chlorophorm protocols (Kocher *et al.* 1989; Sambrook *et al.* 1989). It became evident during fieldwork that identification to species level would not be possible without sacrificing animals, and since our collecting permits did not allow this, we did not identify individuals beyond the generic level. All individuals were genotyped for a 715 bp fragment of the mitochondrial cytochrome-b gene (Cyt-b), using the primers CBL14841 (Austin *et al.* 2004) and Cb3H

(Palumbi *et al.* 2002). Standard polymerase chain reaction (PCR) conditions were carried out in total reaction volumes of 25µl, following Rocha *et al.* (2011).

Based on the mtDNA haplotypes we genotyped a subset of the collected samples for two nuclear gene regions, melanocortin 1 receptor (MC1R) and oocyte maturation factor MOS (*c-mos*) fragments (36 and 33 individuals respectively). The primers used were MC1RF and MC1RR (Pinho *et al.* 2009), and G74 and G73 (Saint *et al.* 1998) for MC1R and *c-mos* respectively. Amplifications were carried out as in Rocha *et al.* (2011) with minor adjustments in annealing temperatures when needed. A commercial facility (Macrogen, the Netherlands) purified and sequenced all PCR products. The PCR products from the nuclear fragments were sequenced in both directions to ensure that double peaks were identified. Manual alignment of sequences was made using Geneious Pro 5.6.3 (Drummond *et al.* 2011) and fragments trimmed to 679bp for Cyt-b, 646bp for MC1R and 348bp for *c-mos*. To ensure that there were no stop codons, we translated all sequences of protein coding regions. Sequences were deposited in Genbank. All samples, localities, nuclear haplotypes and accession numbers are given in Table 1.

The mitochondrial dataset was collapsed into haplotypes using ALTER (Glez-Peña *et al.* 2010). The selection of the best-fit model of nucleotide substitution was conducted in jModeltest (Posada 2008) using the corrected Akaike Information Criteria (Posada and Buckley 2004) for the unpartitioned mtDNA gene fragment.

The phylogenetic relationships between the mtDNA haplotypes was estimated with MrBayes 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) under the selected model. Two runs of 11 million generations were performed and AWTY (Nylander *et al.* 2008) was used to assess convergence and congruence across runs and to determine the adequate burnin. We also constructed a maximum likelihood (ML) tree using PhyML (Guindon *et al.* 2010), with support estimated using 1 000 bootstraps. As outgroup we used an individual of *Pamelaescincus gardineri* (GenBank accession number KF528251), the closest known relative of *Janetaescincus* (Pyron *et al.* 2013).

Median-joining (MJ) networks (Bandelt *et al.* 1999) with maximum-parsimony (MP) optimization (Polzin & Daneshmand 2003) were constructed with NETWORK v 4.6.1.1 (www.fluxus-engineering.com). Given the results from the mtDNA phylogeny reconstruction (see below), we constructed three separate MJ networks for the main clades of the mitochondrial marker, except for one that only had two different haplotypes (and three individuals). Distances between lineages (uncorrected *p*-distance) were estimated using MEGA 5 (Tamura *et al.* 2011).

In order to determine the nuclear haplotypes, we ran PHASE (Stephens *et al.* 2001) four times for each dataset using DNAsp (Rozas *et al.* 2003). Results were congruent across

runs and all polymorphic positions were resolved with posterior probabilities higher than 0.9. Only three positions (each one in a different individual) did not meet this condition, and were thus coded as missing data (N) for the haplotype networks and with ambiguity codes for *BEAST analyses. Alignment files of the inferred haplotypes (nuclear) and phylogenetic trees can be found in Figshare (access codes to be added upon submission).

We used BEASTv1.7.5 (Drummond & Rambaut 2007) to employ *BEAST, the bayesian multispecies coalescent species-tree method (Heled & Drummond 2010) to obtain a multilocus perspective of the diversification within the whole group. Two *Pamelaescincus* spp. sequences were used as outgroup (GenBank accession numbers KF528251 and KF528163). MtDNA clades were used to define the “species-tree” tips, with some individuals, considered to be evidence of hybridization between different *Janetaescincus* clades being removed for this analysis (see Results). The substitution rate of the mitochondrial locus was set to a normal distribution prior of mean of 0.01 (~1% per lineage per Myr) and a standard deviation of 0.0027 (Paulo *et al.* 2008). Substitution rates of nuclear fragments were co-estimated along the run, relative to the mitochondrial one. An uncorrelated relaxed clock model was assumed for the Cyt-b dataset, whereas for the nuclear gene fragments a strict clock was assumed given their low variability. Two runs were performed and checked for convergence and congruence using Tracer v1.5 (Rambaut and Drummond 2007). Tree distribution was summarized in a maximum clade credibility (MCC) tree after appropriate burnin, with median values used for node heights.

Results

Bayesian (BI) and maximum likelihood (ML) trees were identical regarding major clades (Fig. 2). The selected model was GTR+I. The uncorrected *p*-distance between all individuals of *Janetaescincus* spp. and the outgroup (*Pamelaescincus* spp.) was 18.3%. The mtDNA tree reveals four distinct clades within *Janetaescincus* (Fig. 2), with uncorrected *p*-distances from clade 1 to other clades being 15.9% (to clade 2), 13.1% (to clade 3) and 13.7% (to clade 4). The distance between clades 2 and 3 is 10.1%, and between clades 2 and 4 is 10.6%, while the distance between clades 3 and 4 is 4.1%.

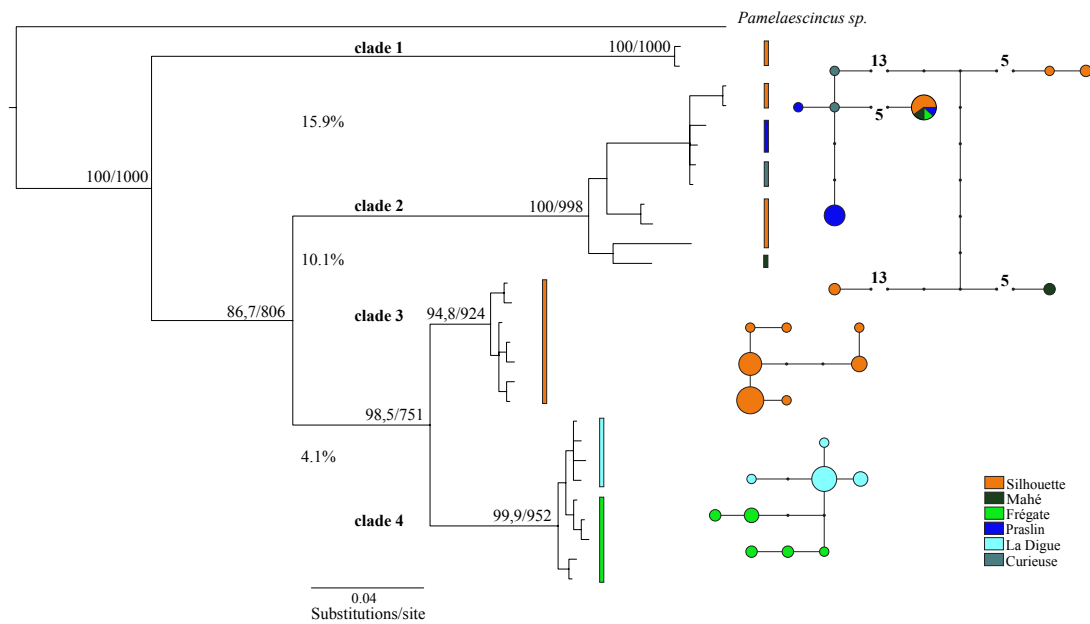


Figure 2. Estimate of relationships using Bayesian inference on Cyt-b haplotypes. The tree was rooted using *Pamelaescincus*. Bootstrap support values from ML inference (BS) and posterior probabilities (PP) are shown only for main branches (above; BS/PP). Scale bar represents branch lengths (substitutions/site). Percentages between clades refer to p -distances. MJ networks for three clades (all individuals) are shown in front of each clade. Circle size is proportional to the number of individuals and full black dots represent missing haplotypes. Islands are color-coded. Numbers in the network of clade 2 represent the number of mutations, shortened for schematic purposes.

Clade 1 is formed by three individuals from Silhouette island, with two haplotypes differing by one mutation. Clade 2 comprises samples from all sampled islands except La Digue. Its haplotype network shows a total of nine haplotypes, with a maximum of 46 differences between them. The most common haplotype is present in individuals from four of the sampled islands (Silhouette, Praslin, Mahé and Frégate). Seven mutation steps separate this from its closest one, which belongs to a single individual from Curieuse island. The second most abundant haplotype belongs to five individuals from Praslin. Only considering within this clade, individuals from Mahé and Silhouette exhibit quite divergent haplotypes. Clade 3 is composed only of individuals from Silhouette (26), where the maximum number of differences between haplotypes is six. There are seven different haplotypes and the two most frequent ones (11 and seven individuals) differ only by one mutation. Clade 4 comprises 23 individuals from Frégate (10) and La Digue (13). Haplotypes are not shared across islands. A minimum of two mutation steps separates haplotypes from each island.

From the two nuclear fragments, MC1R shows considerably greater haplotype diversity than *c-mos* (Fig. 3). At *c-mos* there are only four different haplotypes across all mtDNA lineages, and the highest number of differences between haplotypes is six. Some haplotypes are shared between individuals from different islands, and different mtDNA

clades. The MC1R network shows a total of 23 different haplotypes. MtDNA lineages can also be roughly distinguished in the MC1R haplotype network (Fig. 3). Some individuals have thus haplotypes clustering within different clades in mtDNA and nuclear markers, which suggests the existence of hybridization and introgression. Specifically, individual 6510 (Table 1), from Silhouette, belongs to the mtDNA clade 3 but for *c-mos* exhibits one haplotype otherwise only found in individuals from mtDNA clade 2 (h4, Fig. 3). Similar cases occur with individuals 6714 (h3, *c-mos* / h22 and h23, MC1R) and 6710 (h2, *c-mos*/h18 MC1R), both in *c-mos* and MC1R. These individuals were removed for the *BEAST analyses, which assumes no hybridization between the “species” from the “species-tree” - “species” here not necessarily referring to any taxonomic ranking.

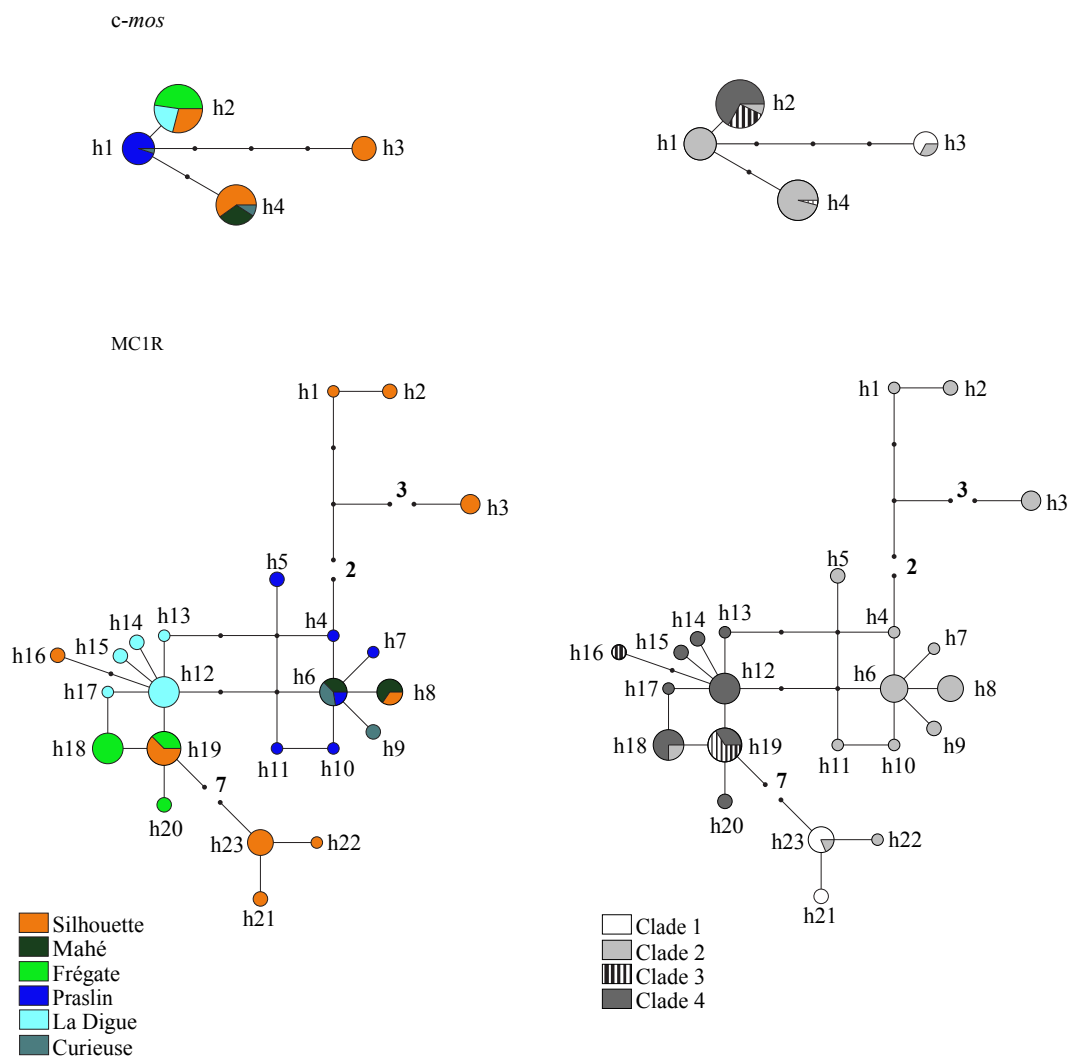


Figure 3. MJ networks of nuclear fragments (MC1R and *c-mos*) Circle size is proportional to the number of haplotypes and black dots represent missing haplotypes. Numbers in bold represent the number of mutations along the respective branch, shortened for graphical representation. Islands are color-coded. Grey-scale networks refer to the same data but with colour coding corresponding to mtDNA clades.

Regarding the relationship between altitude and the distribution of genetic variation, our results are not congruent with the low/high altitude distribution previously reported for *J. veseyfitzgeraldi* / *J. braueri*, respectively (limit at around 500m according to Gerlach, 2008), although in Silhouette the distribution of the different lineages does appear to be related with altitude. Clade 1 and 2 are exclusively distributed at higher altitudes, whereas clade 3 seems restricted to lower ones (although possibly higher than 500m) (Fig. 4).

The topology of the “species-tree” recovered by *BEAST is identical to the mtDNA tree. Divergence time estimates reflect a possible divergence of *Janetaescincus* spp. and *Pamelaescincus* spp. around 38.38 Mya (median = 38.37; 95HPD = 16.37 - 67.08). Within *Janetaescincus* divergence between mtDNA clade 1 and remaining is also clearly pre-Pleistocenic (95HPD = 5.47 - 19.05 Mya), as well as the one from clade 2 from 3 and 4 (Fig. 5). Divergence between clades 3 and 4 is more recent, possibly Pleistocenic.

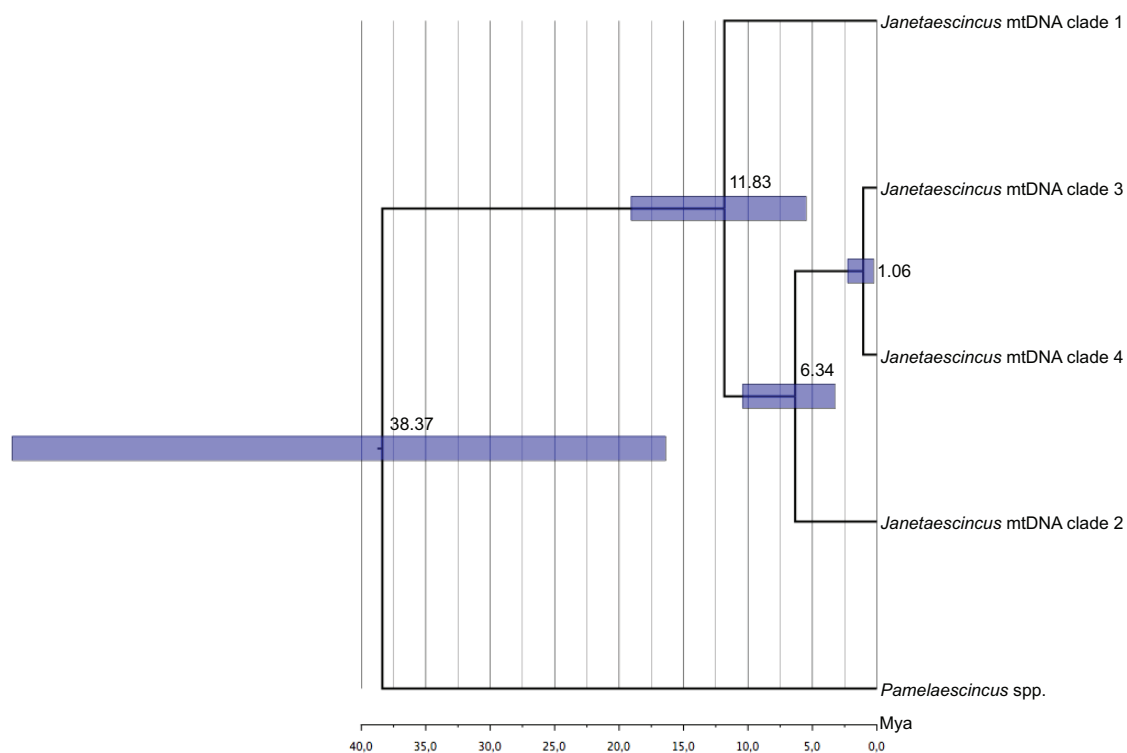


Figure 5. “Species-tree” of *Janetaescincus* The tree shown corresponds to the maximum clade credibility tree estimates used for node heights and it is based on the multispecies coalescent analysis of three molecular markers. Node bars correspond to the 95% high posterior credibility intervals for node height (age). Horizontal axis corresponds to time in million years before present.

Discussion

The genetic diversity within the *Janetaescincus* genus identified in this work is considerably more than expected for one or two species. Levels of divergence between mtDNA clades 1, 2 and (3,4) are all at levels higher than typically seen between species for Cyt-b (Harris 2002), with the current taxonomy of this genus being clearly inappropriate. Overall, four differentiated clades form this genus, at least 3 of which are likely to correspond to distinct species, while variation between clades 3 and 4, and even within clade 2, indicate that possibly even more could potentially be recognized.

Interestingly, Silhouette island harbours three very distant clades, with two of them being endemic from this island (1, 2 and 3, with 1 and 3 being endemic). Although this does not match the hypothesis of one high-altitude and one low-altitude species on this island, altitude does seem to play a role on diversification/differentiation, with clades 1 and 2 found at higher levels, and clade 3 elsewhere (Fig. 4). Further, within clade 2, haplotypes within Silhouette are highly differentiated, which may indicate an old age of this lineage in the island, with multiple colonisations of other islands, possibly at different times. Except for the divergence between clades 3 and 4, the differentiation between main lineages seems to be relatively old, certainly pre-Pleistocenic. On the other hand, the geographic distribution and structure within each lineage can possibly be explained by different migration events during Pleistocenic ice ages, when lower sea level enabled islands to be connected multiple times (Miller *et al.* 2005). Interestingly, clade 4 comprises only samples from Frégate and La Digue, and is sister taxa to clade 3, present only in Silhouette. While this may be the outcome of stochastic colonization processes, it may also be the case that each lineage previously had a wider geographic distribution, but have been highly affected by extinction. Limited sampling in the largest island of Mahé, where the species was very difficult to find, may also affect our estimates of diversity.

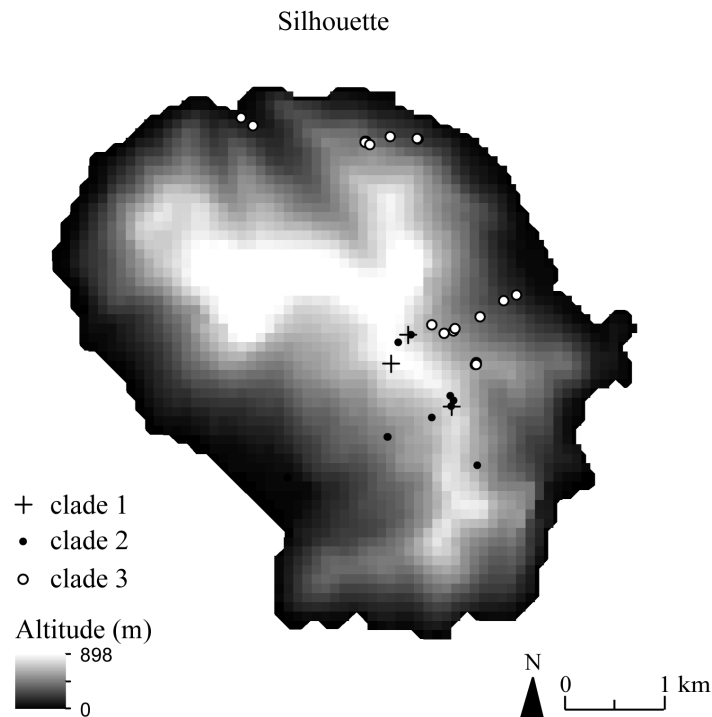


Figure 4. Detail of Silhouette Island's altitude and distribution of mtDNA lineages.

The nuclear fragments, although less variable, are essentially congruent with the patterns observed at mtDNA level. Particularly in MC1R, mtDNA clades can be distinguished based on haplotype frequency and the observed haplotype sharing may be interpreted in terms of hybridization and introgression (except between clades 3 and 4, which are much more closely related, and thus probably still share haplotypes due to incomplete lineage sorting). From this, we argue for evidence of hybridization and introgression of mtDNA from clade 2 into individuals from clades 3 or 4 (*c-mos*, h2; MC1R, h18), from clade 3 into clade 2 (*c-mos*, h4), and from individuals from clade 2 into individuals from clade 1 (*c-mos*, h3; MC1R, h22 and h23). The patterns observed could also be due to nuclear introgression, in which case it would be in the opposite direction. This means that both in Silhouette and Frégate, where different lineages currently meet and hybridization is possible, it does seem to occur. This does not necessarily mean that none of these lineages merit species-level recognition. Hybridization between well-recognized species often occurs (e.g. Placyk *et al.* 2012; Leaché & Cole 2007) and the fact that, in Silhouette, at least one of the mtDNA lineages (clade 3 relative to clades 1 and 2) has a clear altitudinal segregation from the others argues for their distinctiveness, as well as the fact that nuclear gene fragments also seem to corroborate these clades' distinctiveness. Further, the genetic distance between the four mtDNA clades is higher than between many recognized species, although around the average between

congeneric species (uncorrected p -distance 13.6%) (Harris 2002), except for distance between clades 3 and 4 (4.1%) that is considerably lower.

Various recent studies have uncovered unexpected deep genetic differentiation within the herpetofauna of the Seychelles (e.g. Rocha *et al.* 2011; Taylor *et al.* 2012). What is particularly unusual in the case of *Janetaescincus* is not only the high level of diversity – with up to 15.9% Cyt-b divergence between clades - but also that multiple forms are found on very small islands such as Frégate (around three square kilometres). This emphasises the need for extensive within-island sampling for phylogeographic studies of the fauna of these islands. It also makes *Janetaescincus* an ideal model for studying factors leading to isolation versus gene flow within some islands, particularly Frégate and Silhouette. It is also interesting to compare it to *Pamelaescincus gardineri*, which also demonstrated considerable diversity between islands (Valente *et al.* 2013). However, in this species only one lineage occurred per island except on Mahé, and even there, it was not clear if this was due to anthropogenic introductions. Clearly more assessments are needed for both groups on Mahé. It will also be interesting to compare these burrowing skinks with caecilians, another old endemic group from the Seychelles which are also in general poorly studied (Emel & Storfer 2012).

To conclude, complementary studies are urgently needed on both morphological variation and the ecology of these different lineages, and should be performed in different islands in order to better understand the diversity within this genus and the distinctiveness of these different lineages. Taking into account that the described forms are considered endangered, the status of the actual lineages is likely to be of higher concern, even if much of the diversity occurs in the protected area of Silhouette. More detailed sampling on other islands, especially on those where multiple lineages are found, and molecular as well as ecological characterization of the populations would also be valuable to further understand their degree of isolation and possible evolutionary history. Until then we suggest that these species are referred to as a species complex, pending a revision of their taxonomy and the likely description of at least one new species.

Table 1. Samples used in this study, locations and accession codes (to be added upon submission).

Code	Haplotype		Province State	Locality (GPS)		Cyt-b	c-mos	MC1R
	c-mos	MC1R		Latitude	Longitude			
6764		h23	Silhouette	-4,492515	55,240033	√		√
39SILH	h3	h23	Silhouette	-4,486061	55,236111	√	√	√
JbSilh	h3	h21	Silhouette	-4,488639	55,234567	√	√	√
6695	h4		Silhouette	-4,486793	55,235260	√	√	
6708	h4		Silhouette	-4,492515	55,240033	√	√	
6714	h3	h23/h22	Silhouette	-4,491587	55,239932	√	√	√
6743		h2/h22	Silhouette	-4,495248	55,234320	√		√
6740	h4		Silhouette	-4,495272	55,234288	√	√	
6547			Silhouette	-4,486095	55,236417	√		
6483			Silhouette	-4,492017	55,240243	√		
6566	h4	h2/h1	Silhouette	-4,497817	55,242342	√	√	√
6733	h4	h3	Silhouette	-4,498928	55,225345	√	√	√
6766		h8	Silhouette	-4,493537	55,238275	√		√
6628	h1	h4	Praslin	-4,331248	55,737597	√	√	√
6631	h1	h10/h11	Praslin	-4,331387	55,737623	√	√	√
6649			Praslin	-4,331317	55,737718	√		
6656	h1	h6/h7	Praslin	-4,331532	55,737730	√	√	√
6786			Praslin	-4,331428	55,737812	√		
PL21	h1	h5	Praslin	-4,331433	55,737617	√	√	√
6655	h1		Praslin	-4,331502	55,737597	√	√	
6710	h2	h18	Frégate	-4,584133	55,934318	√	√	√
JV CUR	h1/h4	h6	Curieuse	-4,286222	55,717700	√	√	√
CUR11		h9	Curieuse	-4,286222	55,717700	√		√
6760	h4	h6/h8	Mahé	-4,707213	55,500833	√	√	√
2MA55	h4	h8	Mahé	-4,706583	55,500694	√	√	√
6913	h4	h6/h8	Mahé	-4,707617	55,500810	√	√	√
6503			Silhouette	-4,488743	55,242278	√		
6504			Silhouette	-4,488790	55,242258	√		
6510	h2/h4		Silhouette	-4,488797	55,242193	√	√	
6517			Silhouette	-4,488625	55,242270	√		
6564			Silhouette	-4,485752	55,240220	√		
6470			Silhouette	-4,468343	55,234555	√		
6500			Silhouette	-4,488778	55,242267	√		
6501			Silhouette	-4,468777	55,232360	√		
6508	h2	h16	Silhouette	-4,482535	55,245860	√	√	√
6511	h2	h19	Silhouette	-4,485562	55,240340	√	√	√
6513		h19	Silhouette	-4,468907	55,232440	√		√
6532			Silhouette	-4,468763	55,232287	√		
6536			Silhouette	-4,467365	55,222223	√		
6545			Silhouette	-4,468823	55,232300	√		
6548			Silhouette	-4,469040	55,232708	√		
6554			Silhouette	-4,468533	55,237023	√		
6556			Silhouette	-4,468497	55,236942	√		
6669	h2		Silhouette	-4,485968	55,239368	√	√	
6713			Silhouette	-4,485223	55,238263	√		
25SILH		h19	Silhouette	-4,484500	55,242600	√		√
36SILH			Silhouette	-4,484500	55,242600	√		
41SILH	h2	h19	Silhouette	-4,484500	55,242600	√	√	√
43SILH			Silhouette	-4,484500	55,242600	√		
38724			Silhouette	-4,483056	55,244722	√		
Jabr1			Silhouette	-4,483056	55,244722	√		
JVAM SIL			Silhouette	-4,466634	55,221201	√		
63LD			La Digue	-4,358718	55,840623	√		
79LD	h2		La Digue	-4,358718	55,840623	√	√	
81LD	h2	h15	La Digue	-4,358718	55,840623	√	√	√
LD4			La Digue	-4,357142	55,828915	√		
LD7		h17/h12	La Digue	-4,357142	55,828915	√		√
2LD			La Digue	-4,357142	55,828915	√		
7LD			La Digue	-4,357142	55,828915	√		
6810	h2	h14/h12	La Digue	-4,356953	55,828828	√	√	√
6811	h2	h12	La Digue	-4,356943	55,829033	√	√	√

6818		h13/h12	La Digue	-4,357110	55,829067	√		√
6819			La Digue	-4,356958	55,828900	√		
6821		h12	La Digue	-4,357035	55,828998	√		√
6826		h14/h12	La Digue	-4,356960	55,828850	√		√
42FG	h2	h19	Frégate	-4,286222	55,717700	√	√	√
40FG	h2	h19	Frégate	-4,286222	55,717700	√	√	√
43FG			Frégate	-4,286222	55,717700	√		
48FG			Frégate	-4,286222	55,717700	√		
6889	h2	h20	Frégate	-4,590543	55,940920	√	√	√
Jave1			Frégate	unknown	unknown	√		
6880	h2	h18	Frégate	-4,584133	55,934318	√	√	√
6890	h2	h18	Frégate	-4,583565	55,934533	√	√	√
6893			Frégate	-4,584133	55,934318	√		
6892	h2	h18	Frégate	-4,584133	55,934318	√	√	√

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Final Considerations

The present work provided important information about *Janetaescincus* and *Pamelaescincus* phylogeography and phylogeny. We unveiled the existence of deep cryptic genetic divergence within the two studied groups, contradicting the current taxonomy of both genera.

Within *Pamelaescincus*, two highly divergent clades exist, exhibiting the northern-southern geographic structure already observed in other species (Rocha *et al.* 2013; Daniels *et al.* 2006). The two clades occur on Mahé Island, but the interaction between the lineages – if admixture is occurring widely or is limited – remains unclear without further sampling. It is hoped that the morphological variation, preliminarily assessed in this thesis (see additional information), should also be useful in the future in determining if these lineages maintain morphological differences in Mahé. The distribution of these lineages in general seems to have been more influenced by the geographical distances between islands than the inter-island depths, which is in agreement with other reptile studies in Seychelles archipelago (Rocha *et al.* 2011).

Concerning *Janetaescincus* genus, for which there was a debate regarding if one or two species formed the genus, actually four highly divergent mtDNA lineages were found, revealing an unexpected amount of cryptic differentiation. In both *Janetaescincus* and *Pamelaescincus* we recovered higher distance values between clades than between many recognized reptile species. Although these were lower than the average between congeneric species (Harris 2002) for *Pamelaescincus*, at least three clades in *Janetaescincus* are more divergent than this which, combined with the nuclear variation indicates that at least one new species will likely need to be described. These three seem to have relatively old divergence times, possibly pre-Pleistocene. Although they likely correspond to distinct species, hybridization and introgression were found, but limited sampling within some islands makes the extent of this difficult to assess.

There is an urgent need therefore for a wider sampling in some of the islands like Frégate and Mahé that had some interesting results, to better understand these organisms' phylogeographic history. An extensive analysis of morphological and ecological characteristics will be essential to the taxonomic revision of these two genera. A larger study including more individuals from each lineage and from across all distribution range will be needed in order to confirm the existence of morphological differences among those species.

While taxonomic revision is not definitive, both *Pamelaescincus* clades and all *Janetaescincus* clades should be treated as different evolutionarily significant units for

conservation management purposes within Seychelles archipelago. We propose *Janetaescincus* sp. to be treated as a species complex.

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Additional Information

During March 26th to April 5th 2012 a visit to the Natural History Museum (NHM, UK) was done to analyze the voucher specimens of the two genera (*Pamelaescincus* spp. and *Janetaescincus* spp.) studied in this thesis and to collect some morphological data. This study was funded by the Synthesys project (GB-TAF-1993).

A series of significant morphometric measurements were taken to the next 0.01 mm using a digital caliber (Table 1 and 2). Data were always taken from the same side of the specimens (left side of the side up specimen). Additionally to the body measurements, we also counted the midbody scales (NSM) at mid-point between the forelimbs and the hind limbs, in two parts: dorsal plus lateral scales and ventral scales (Table 2).

In addition, detailed photographs of the body and head were taken. These pictures were then used for counting additional pholidotic characters: number of superior labial scales (SLS), inferior labial scales (ILS), superior eye scales (SES), inferior eye scales (IES), superior ocular scales right (SOSR), superior ocular scales left (SOSL), and number of lamellae scales (LAM) (Table 3). An exploratory analysis of the data was conducted. The islands of La Digue and Silhouette for *Pamelaescincus* spp., and Mahé for *J. veseyfitzgeraldi* were represented by only one sample. Due to this reduced sampling, it was unreasonably to take any definite conclusions.

Table 1. Detailed description of the body measurements included in the morphological assessment.

Variable	Abbreviation	Description
snout-vent length	SVL	from the tip of the snout until the cloaca opening
trunk-length	TrL	from posterior edge of forelimb to anterior edge of the hindlimb
tail width	TW	at the base of the tail (widest point)
forelimb length	FLL	from the nail tip of the longest toe to the axil
partial forelimb length	pFLL	length from the tip of the toe to the elbow
hindlimb length	HLL	from the nail tip of the longest toe (the 4 th) to the tail insertion
partial hindlimb length	pHLL	length from the tip of the fourth toe to the knee
head width	HW	maximum head width
head height	HH	maximum head height from occiput to jaws
snout-eye distance	SED	distance between tip of the snout and the anterior side of the eye
orbital diameter	OD	maximum eye diameter
eye-ear length	EEL	distance between the posterior side of the eye and the anterior side of the ear
ear diameter	ED	measured in the widest point

Table 2. Morphometric measurements of all specimens analyzed in NHM. ID numbers correspond to the museum specimen codes.

ID No	Species	Locality	SVL	TrL	TW	FLL	pFLL	HLL	pHLL	HW	HH	OD	EL	SED	EED	NSM	
																1	2
1946.8.3.10	<i>P. gardineri</i>	Mahé	46.83	24.52	6.63	9.52	6.18	12.92	9.45	7.78	6.70	2.52	1.50	4.18	4.16	-	17
1907.10.15.82	<i>P. gardineri</i>	Mahé	68.67	33.17	10.86	11.93	7.12	14.93	10.33	12.01	9.70	3.24	2.06	5.56	6.06	16	17
1946.8.2.81	<i>P. gardineri</i>	Mahé	43.37	24.12	6.80	9.83	5.87	12.28	8.58	8.19	6.21	2.69	1.34	4.00	4.23	18	22
1946.8.2.82	<i>P. gardineri</i>	Mahé	51.31	27.94	7.35	9.82	6.51	12.38	10.53	9.02	6.83	3.55	1.42	4.36	4.19	16	17
1907.10.15.83	<i>P. gardineri</i>	Praslin	48.41	24.45	8.21	10.17	6.50	12.14	9.80	8.60	7.03	2.79	1.23	4.28	4.20	20	18
1907.10.15.84	<i>P. gardineri</i>	Praslin	38.33	18.61	5.67	8.51	5.23	11.10	8.60	6.85	5.65	2.75	1.10	3.63	3.41	19	-
1976.2245	<i>P. gardineri</i>	Praslin	41.86	18.40	7.07	9.88	6.64	10.72	8.27	7.98	5.82	2.98	1.71	4.50	4.51	20	19
1976.1947	<i>P. gardineri</i>	Praslin	58.75	32.19	9.73	9.65	6.75	11.78	9.39	9.99	7.35	2.44	1.77	5.01	4.74	19	15
1976.1237	<i>P. gardineri</i>	Cousin	77.17	39.07	12.74	9.37	8.18	14.80	11.01	12.83	9.63	3.73	1.78	5.52	6.31	18	18
1976.1238	<i>P. gardineri</i>	Cousin	76.67	39.33	11.99	10.64	7.33	14.93	10.75	12.81	9.40	3.72	2.18	5.71	6.51	19	16
1976.1239	<i>P. gardineri</i>	Cousin	77.54	41.28	12.22	9.84	7.15	12.06	8.74	12.58	9.61	3.62	1.50	5.86	6.82	18	20
1976.1240	<i>P. gardineri</i>	Cousin	66.37	33.97	10.69	7.70	6.47	12.99	10.09	11.04	7.95	3.44	1.98	4.90	5.45	16	15
1976.1246	<i>P. gardineri</i>	Cousin	68.75	33.72	11.43	10.39	7.48	14.08	9.51	11.49	8.52	3.15	1.34	5.60	5.51	18	20
1976.2244	<i>P. gardineri</i>	Cousin	65.22	29.58	11.82	7.42	6.29	11.24	9.07	12.23	8.73	3.24	1.50	5.14	5.42	20	18
1976.2246	<i>P. gardineri</i>	Frégate	50.58	27.86	7.30	6.96	5.19	11.29	8.76	8.45	6.21	2.61	1.83	3.68	3.79	19	16
1938.8.3.48	<i>P. gardineri</i>	Frégate	46.44	25.60	7.98	8.72	5.30	11.63	7.89	7.88	6.59	2.58	1.32	3.99	3.93	17	19
1938.8.3.49	<i>P. gardineri</i>	Frégate	53.65	31.09	8.73	7.61	5.26	11.40	7.88	9.38	7.57	2.48	1.54	4.46	4.64	-	-
1938.8.3.49 (little)	<i>P. gardineri</i>	Frégate	28.94	15.70	4.02	5.67	3.79	8.61	6.23	5.44	4.10	1.86	0.89	3.09	2.67	16	14
1976.1236	<i>P. gardineri</i>	La Digue	57.41	33.15	7.03	10.68	7.29	15.17	11.04	8.79	6.61	2.74	1.44	4.14	4.48	15	16
1910.3.18.87	<i>P. gardineri</i>	Silhouette	69.34	38.61	11.91	9.72	7.45	13.91	10.46	12.34	10.43	3.64	2.03	5.33	6.52	18	17
1905.4.25.4	<i>J. braueri</i>	Mahé	46.45	25.86	4.80	5.87	3.79	9.04	6.49	5.15	3.66	1.93	0.44	3.00	3.62	14	13
1905.4.25.5	<i>J. braueri</i>	Mahé	37.90	19.87	3.10	5.82	3.38	8.37	6.25	4.61	3.26	2.99	0.37	2.43	3.47	16	12
1907.10.15.81	<i>J. braueri</i>	Mahé	47.05	25.61	4.68	6.10	3.67	7.64	6.93	5.78	4.11	2.43	0.38	2.66	3.06	14	13
1910.3.18.35	<i>J. braueri</i>	Mahé	50.23	25.94	5.43	6.62	4.01	10.07	7.34	6.34	4.13	2.72	0.61	2.94	4.12	12	13
1910.3.18.31	<i>J. braueri</i>	Silhouette	45.09	25.89	4.89	5.68	3.88	9.91	7.34	5.91	3.99	2.48	0.45	2.48	3.68	15	11
1947.2.18.33	<i>J. vesityfzgeraldi</i>	Frégate	37.46	23.90	4.30	5.26	3.06	7.40	4.87	5.02	3.28	2.13	-	2.29	2.75	19	13
1973.481	<i>J. vesityfzgeraldi</i>	Mahé	28.71	15.71	2.65	3.64	2.47	5.14	4.26	3.49	2.55	2.02	-	1.93	2.29	-	-
1947.2.18.36	<i>J. vesityfzgeraldi</i>	Frégate	34.94	19.92	4.18	4.94	2.59	8.17	5.05	4.51	3.31	2.05	-	2.55	2.78	13	10
8.9.99	<i>Janetaescincus sp</i>	-	35.40	19.66	3.78	4.54	3.49	6.71	5.70	4.31	2.99	1.87	-	1.96	2.90	-	-
13.9.99	<i>Janetaescincus sp</i>	Frégate	35.55	20.76	4.03	5.49	3.09	7.23	5.90	4.40	3.21	2.21	-	1.96	3.63	12	10
8.8.2006	<i>Janetaescincus sp</i>	Frégate	51.22	29.73	4.73	5.38	3.77	6.98	6.44	5.29	3.78	2.32	0.48	3.22	3.55	13	-
1947.2.18.37	<i>J. vesityfzgeraldi</i>	Frégate	35.06	20.44	4.00	3.69	2.84	7.42	5.43	4.60	3.31	1.65	-	1.98	2.37	-	-
20.6.2001	<i>J. braueri</i>	Silhouette	29.34	16.83	2.60	3.41	2.26	5.98	4.17	3.49	2.83	1.96	-	1.92	2.28	-	-

Table 3. *Pamelaescincus* scalation variables retrieved from photographs.

ID No	SLS	ILS	SES	IES	SOSR	SOSL	LAM
1946.8.3.10	7	5	18	19	5	5	20
1907.10.15.82	6	6	18	15	-	-	19
1946.8.2.81	5	5	18	12	-	-	19
1946.8.2.82	6	5	15	13	-	-	19
1907.10.15.83	6	6	18	14	-	5	19
1907.10.15.84	6	6	18	14	-	-	17
1976.2245	6	6	-	17	-	-	18
1976.1947	6	6	16	16	-	-	19
1976.1237	6	6	-	15	-	-	17
1976.1238	6	6	16	16	-	-	16
1976.1239	7	6	-	16	-	-	-
1976.1240	5	6	19	16	-	-	18
1976.1246	6	7	16	15	-	-	16
1976.2244	6	6	16	14	-	-	17

1976.2246	6	6	16	13	-	-	18
1938.8.3.48	6	6	16	15	-	-	20
1938.8.3.49	6	6	16	15	-	-	18
1938.8.3.49 (little)	7	6	14	12	-	-	15
1976.1236	6	6	-	15	-	-	18
1910.3.18.87	6	6	17	18	-	-	-

